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Discovery, characterization, and effects on renal fluid and electrolyte excretion of the Kir4.1 potassium channel pore blocker, VU0134992

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Abbreviations: CCD, cortical collecting duct; DCT, distal convoluted tubule; EAST, Epilepsy Ataxia Sensorineural deafness Tubulopathy; MECP2, methyl CpG binding protein 2; SIDS, sudden infant death syndrome; TAL, thick ascending loop; SeSAME, Seizures Sensorineural deafness Mental retardation Electrolyte imbalances; SSRI, Selective Serotonin Reuptake Inhibitor; TCA, tricyclic antidepressant; VU0134992, 2-(2-Bromo-4-iso-propylphenoxy)-N-(2,2,6,6-tetramethylpiperidin-4-yl)acetamide

ABSTRACT

The inward rectifier potassium (Kir) channel Kir4.1 (*KCNJ10*) carries out important physiological roles in epithelial cells of the kidney, astrocytes in the central nervous system, and stria vascularis of the inner ear. Loss-of-function mutations in *KCNJ10* lead to EAST/SeSAME syndrome, which is characterized by epilepsy, ataxia, renal salt wasting, and sensorineural deafness. While genetic approaches have been indispensable for establishing the importance of Kir4.1 in the normal function of these tissues, the availability of pharmacological tools for acutely manipulating the activity of Kir4.1 in genetically normal animals has been lacking. We therefore carried out a high-throughput screen of 76,575 compounds from the Vanderbilt Institute of Chemical Biology library for small-molecule modulators of Kir4.1. The most potent inhibitor identified was 2-(2-Bromo-4-iso-propylphenoxy)-*N*-(2,2,6,6-tetramethylpiperidin-4-yl)acetamide (VU0134992). In whole-cell patch clamp electrophysiology experiments, VU0134992 inhibits Kir4.1 with an IC_{50} of 0.97 μ M and is 9-fold selective for homomeric Kir4.1 over Kir4.1/5.1 concatemeric channels (IC_{50} =9 μ M) at -120 mV. In thallium (Tl^+) flux assays, VU0134992 is greater than 30-fold selective for Kir4.1 over Kir1.1, Kir2.1, and Kir2.2, is weakly active toward Kir2.3, Kir6.2/SUR1, and Kir7.1, and is equally active toward Kir3.1/3.2, Kir3.1/3.4, and Kir4.2. This potency and selectivity profile is superior to Kir4.1 inhibitors amitriptyline, nortriptyline, and fluoxetine. Medicinal chemistry identified components of VU0134992 that are critical for inhibiting Kir4.1. Patch clamp electrophysiology, molecular modeling, and site-directed mutagenesis identified pore-lining glutamate 158 and isoleucine 159 as critical residues for block of the channel. VU0134992 displayed a large free fraction in rat plasma (f_u = 0.213). Consistent with the known role of Kir4.1 in renal function, oral dosing of VU0134992 led to a dose-dependent diuresis, natriuresis, and kaliuresis in rats. Thus, VU0134992 represents the first *in vivo*-active tool compound for probing the therapeutic potential of Kir4.1 as a novel diuretic target for the treatment of hypertension.

INTRODUCTION

Kir4.1 inward rectifier potassium (Kir) channels (encoded by *KCNJ10*) are critical for regulating potassium (K^+) transport and electrolyte homeostasis in the central nervous system (CNS), retina, inner ear, and nephrons of the kidney (Hibino et al., 2010; Welling, 2016). In the brain and spinal cord, homotetrameric Kir4.1 channels are expressed almost exclusively in glia, oligodendrocytes, and astrocytes, although some evidence for neuronal expression has been reported (Zhang et al., 2011) (see below). Kir4.1 expression levels are variable between different brain and spinal cord regions, indicating the channel's contributions to CNS physiology are complex and anatomically heterogeneous (Nwaobi et al., 2016). Astroglial Kir4.1 channels are best known for their role in clearance, or “spatial buffering,” of extracellular K^+ and glutamate in the CNS. By virtue of their prominent membrane expression and high open probability, Kir4.1 channels drive the membrane potential (V_m) of glial cells to negative voltages. When K^+ is released from active neurons, thereby locally depolarizing the Nernst equilibrium potential for K^+ with respect to the astrocyte V_m , an inwardly directed electrochemical driving force that promotes K^+ and glutamate uptake into astrocytes is generated (Djukic et al., 2007; Kofuji et al., 2002; Kucheryavykh et al., 2007; Olsen and Sontheimer, 2008).

Alterations in Kir4.1 expression and function are associated with a number of human diseases (Nwaobi et al., 2016). Autosomal recessive mutations in *KCNJ10* cause EAST (Epilepsy Ataxia Sensorineural deafness Tubulopathy) or SeSAME (Seizures Sensorineural deafness Ataxia Mental retardation Electrolyte imbalances) syndrome (Bockenhauer et al., 2009; Reichold et al., 2010; Scholl et al., 2009). The clinical presentation of EAST/SeSAME syndrome is readily explained by the loss of Kir4.1 function in the CNS, inner ear, and kidney. In two separate mouse models of Huntington's disease (i.e. R6/2 and Q175 mice), the functional expression of Kir4.1 in astrocytes is reduced, leading to elevated extracellular K^+ concentration and increase in neuronal excitability. Viral delivery of Kir4.1 channels to

striatal astrocytes prolongs the lifespan and attenuates some of the motor deficits in R6/2 mice (Tong et al., 2014). Epigenetic changes in Kir4.1 expression have been linked to Rett syndrome, a neurological disorder that affects cognitive, sensory, motor, and autonomic functions (e.g. cardiac function, digestion, and breathing). In most cases, Rett syndrome is caused by mutations in the methyl CpG binding protein 2 (MECP2) gene located on the X chromosome, which is responsible for the transcriptional regulation of dozens of genes, including *KCNJ10* (Nwaobi et al., 2016). Zhang and colleagues proposed that the overexpression of Kir4.1 homotetrameric channels in respiratory-related neurons from MecP2 mice leads to a reduction in CO₂/pH chemosensitivity and disruption of normal breathing (Zhang et al., 2011). This overexpression could be restricted to respiratory neurons since recent data from Olsen and colleagues suggest that there is a reduction in glial cell Kir4.1 from MeCP2 mice (Kahanovitch et al., 2018). An emerging body of literature has implicated Kir4.1 in autism spectrum disorder, SIDS, epilepsy, pain, and multiple sclerosis (Sicca et al., 2016; Sicca et al., 2011), although in most cases a clear mechanistic link between the channel and these diseases has not yet been established.

In polarized epithelial cells of the distal convoluted tubule (DCT) and cortical collecting duct (CCD), Kir4.1 is expressed on the basolateral (i.e. blood-facing) membrane, predominantly in a heteromeric complex with Kir5.1 (encoded by *KCNJ16*) (Lourdel et al., 2002; Welling, 2016; Zhang et al., 2014). Kir5.1 does not produce functional K⁺ channels on its own, but serves to modify the functional (e.g. unitary conductance, activation kinetics, rectification) and regulatory (e.g. pH sensitivity) properties of Kir4.1-containing channels (Paulais et al., 2011; Pessia et al., 2001; Tucker et al., 2000). In the DCT, Kir4.1/5.1 channels 1) recycle K⁺ ions across the basolateral membrane to sustain the activity of the basolateral Na⁺/K⁺/ATPase, which maintains a favorable chemical driving force for Na⁺ reabsorption across the luminal (urine-facing) membrane by the Na⁺/Cl⁻ co-transporter NCC, and 2) create a negative membrane potential that promotes Cl⁻ exit across the basolateral membrane. In the CCD, basolateral

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Kir4.1/5.1 and Kir4.1 channels hyperpolarize the basolateral membrane potential and electrically coupled luminal membrane to promote Na^+ reabsorption via the Epithelial Na^+ Channel (ENaC). Thus, Kir4.1-containing channels play key roles in promoting NaCl reabsorption in the DCT and CCD of the nephron (Welling, 2016).

The lack of specific pharmacological tool compounds has slowed progress in exploring the function, integrative physiology, and in some cases therapeutic potential of Kir4.1, leading us to carry out a high-throughput screen (HTS) of 76,575 compounds for pharmacological modulators of Kir4.1. Here we report the discovery and characterization of VU0134992, the first *in vivo*-active inhibitor of renal Kir4.1 channels.

MATERIALS AND METHODS

Stable Cell Lines and Transient Transfection

Stably transfected monoclonal T-Rex-HEK-293 cell lines expressing Kir1.1, Kir2.2, Kir2.3, Kir4.1, Kir4.2, Kir6.2/SUR1, or Kir7.1-M125R from a tetracycline-inducible promoter were generated as described previously (Lewis et al., 2009; Raphemot et al., 2013; Raphemot et al., 2011; Swale et al., 2016). To promote cell surface expression and function of Kir4.2, lysine 110 was mutated to asparagine and the most distal 22 amino acids were deleted from the carboxyl-terminus (Kir4.2-K110N-Δ22), as described by Nichols and colleagues (Pearson et al., 2006). The day before TI^+ flux assays, cells were plated at a density of 20,000 cells per well in black-walled, clear-bottomed, amine-coated 384-well plates (PurecoatTM, Corning) in the presence of 1 $\mu\text{g}/\text{ml}$ tetracycline to induce Kir channel expression. Constitutively expressing Kir3.1/3.2, Kir3.1/3.4 cells were used without any induction. Kir6.2/SUR1 was activated with 30 μM VU063 (Raphemot et al., 2014) for testing inhibitors. Transient transfections were performed using Lipofectamine-LTX according the manufacturer's instructions. The pcDNA3.1-Kir4.1-5.1 concatemer plasmid was obtained from Chung Jiang (Georgia State University) with permission from John Adelman (Oregon Health and Science University).

Kir4.1 Primary Screen

TI^+ flux assays were performed as described previously (Lewis et al., 2009; Raphemot et al., 2013; Raphemot et al., 2011; Swale et al., 2016) with modifications to reagents optimized per cell line as noted below. Briefly, cells cultured overnight 37 °C and 5% CO_2 in 384-well plates were loaded with the thallium-sensitive dye Thallos-AM (TEFLabs, Austin, TX) in ambient conditions, washing before and after dye using Hank's Balanced Salt Solution/20mM HEPES (assay buffer). Media and buffer exchange was performed on the ELx405 plate washer (BioTek). Dye loaded cells were then transferred to the Hamamatsu Functional Drug Screening System 6000 (FDSS6000; Hamamatsu, Tokyo, Japan) or

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Panoptic kinetic imaging plate reader (Wavefront Bioscience, Franklin, TN, USA). Both instruments collect live measurements at 1 Hz (480/40 nm excitation and 540/40 nm emission) during simultaneous 384-well pipetting of 10 μ M small-molecules (0.1% DMSO assay buffer) or control (100 μ M fluoxetine). Cells are treated 20 minutes, continuing ambient conditions, before adding 5X Ti^+ stimulus buffer (125 mM NaHCO_3 , 1 mM MgSO_4 , 1.8 mM CaSO_4 , 5 mM glucose, 20 mM HEPES, and 1.8 – 6 mM Ti_2SO_4) to each well to initiate Ti^+ flux. Screening data were collected into a custom database and data reduction software application to merge individual compounds with the associated values on a well-to-well, then plate-by-plate basis. Fluorescence values for individual wells were normalized by dividing each data point for that well by the initial data point (F/F_0). After normalization of all wells, the fluorescence data (i.e. fluorescence vs. time) from each of the designated vehicle-control wells was averaged and this averaged control wave was subtracted from all wells on the plate to yield normalized, vehicle control-subtracted data. Fluorescence amplitudes (max-min) were extracted from the control-subtracted wells over a range from 0-200 seconds. Alternatively, the slope 5-20 sec after Ti^+ addition was calculated. Hit selections were made by analysis on a per plate basis evaluating 3 standard deviations from the mean of the compound population. Compounds that were tested in dose-response were calculated as above, and reduced values were then plotted with GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA) to generate estimated potencies of concentration response curves (CRC) fit using a four-parameter logistic model of non-linear regression analysis.

Site-Directed Mutagenesis

Mutations in Kir4.1 were introduced using a QuickChange II Site-Directed Mutagenesis kit (Agilent Technologies) according to the manufacturer's instructions. The complete cDNA was sequenced to verify introduction of the intended codon changes.

Patch Clamp Electrophysiology

HEK-293T cells were transfected with wild type or mutant pcDNA5-Kir4.1 (1 μ g) and pcDNA3.1-EGFP (0.5 μ g; 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 1h in a 37 °C 5% CO₂/95% air incubator before starting experiments. Patch clamp experiments were performed essentially as described previously (Raphemot et al., 2013). Briefly, patch electrodes (2-3M Ω) 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), pH 7.3, 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. 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 s to -120 mV for 200 msec before ramping to 120 mV at a rate of 1.2 mV/msec. 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, Sunnyvale, CA). Pharmacology experiments were terminated by applying 2 mM barium (Ba²⁺) chloride to measure leak current. Cells exhibiting <90% block by Ba²⁺ were excluded from analysis. The mean current amplitude recorded over five successive steps to -120 mV in WT control or mutants at single concentration were expressed as mean \pm SD. Statistical analysis was performed using one-way ANOVA with Bonferroni's multiple comparisons test with statistical significance defined at $P < 0.05$. IC₅₀ values were determined by fitting the Hill equation to concentration-response curves (CRCs) using variable-slope nonlinear regression analyses. All the analysis was performed with GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA).

Homology Modeling of Kir4.1

The Kir4.1 sequence (residues 28-360) was threaded onto the Kir2.2 crystal structure (PDB 3JYC) based on a sequence alignment generated by ClustalW. The sequence identity between Kir4.1 and Kir2.2 is 43.3%. Transmembrane segments for Kir4.1 were predicted using the OCTOPUS topology prediction web server (<http://octopus.cbr.su.se/>). Missing coordinates in the threaded Kir4.1 model were reconstructed using Rosetta with fragment insertion from the fragment libraries generated by the Rosetta server (Leaver-Fay et al., 2007). The modeling pipeline utilized RosettaMembrane (Barth et al., 2007) and RosettaSymmetry (King et al., 2012) in the Rosetta revision 58019. Loops were closed using the cyclic coordinate descent (CCD) algorithm and refined using Kinematic loop closure (KLC) from the Rosetta Loop Modeling application (Mandell et al., 2009). 1000 models were generated and the top eight models by score and RMSD to Kir2.2 (models 1-8) were further relaxed using FastRelax in the Rosetta relax application producing 100 models each. The top 3 models from parent models 1, 2, 3, 5 and 7 were chosen for ligand docking studies.

Docking VU0134992 in the Kir4.1 Channel Pore

VU0134992 was manually placed in a coordinate frame that corresponds to the pore cavity below the selectivity filter of Kir4.1. VU0134992 conformers were generated using BCL::Conf (Kothiwale et al., 2015). The top 15 homology models described above were used for ligand binding studies with RosettaLigand (Meiler and Baker, 2006), producing 7500 VU0134992-Kir4.1 complexes. The top 10% of 750 models by Rosetta interface score were analyzed for favorable residue interactions (better than -1 REU) and highest frequency interaction between residues of Kir4.1 and VU0134992.

Chemical Synthesis

Synthesis and Characterization of VU0134992

Synthetic scheme of VU0134992 is shown in Supplemental Figure S1 as an example of general synthetic scheme. Experimental procedure for VU0134992 is described below. Specific synthetic schemes for selected compounds are also shown in supplemental information (Figures S2 and S3).

2-Bromo-4-iso-propylphenol (2): To a solution of 4-*iso*-propylphenol (2.00 g, 14.7 mmol) in CH₃CN (30 mL) was added *N*-bromosuccinimide (2.88 g, 16.2 mmol) at 0°C. After a resulting greenish solution was stirred at 0°C for 1.5 hours, ice/NaHCO₃-*aq* was added to the reaction mixture which was extracted with ethyl acetate twice. Combined organic extracts were washed with brine and dried over MgSO₄. The filtrate was evaporated under reduced pressure to give crude residue which was purified on silica gel chromatography (hexane/ethyl acetate) to yield 2-bromo-4-*iso*-propylphenol **2** (723 mg (content 618 mg), 20% yield) as a pale yellow oil. Crude fraction collected was purified by Gilson HPLC separation system using (0.1% TFA in water)/CH₃CN as an eluent. Extraction from the collected fraction with DCM gave another batch of **2** (1.06 g (content 1.02 g), 32% yield) as a pale reddish oil. Total yield was 52%.

2-Chloro-*N*-(2,2,6,6-tetramethylpiperidin-4-yl)acetamide hydrochloride (4): To a solution of 4-amino-2,2,6,6-tetramethylpiperidine **3** (2.345 g, 15.0 mmol) in DCM (30 mL) was added chloroacetyl chloride (1.19 mL, 15.0 mmol) at 0°C. After a resulting white suspension was stirred at 0°C for 40 min, precipitates were collected by filtration to yield 2-chloro-*N*-(2,2,6,6-tetramethylpiperidin-4-yl)acetamide hydrochloride **4** (4.052 g (content 3.908 g), 97% yield).

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2-(2-Bromo-4-iso-propylphenoxy)-N-(2,2,6,6-tetramethylpiperidin-4-yl)acetamide (VU0134992): To a solution of **2** (935 mg, 4.35 mmol) in DMF (30 mL) was added **4** (975 mg, 3.62 mmol), Cs₂CO₃ (2.95 g, 9.05 mmol) and NaI (179 mg, 1.19 mmol) at ambient temperature. After a resulting white suspension was stirred at 50°C for 18h hour, the reaction mixture was poured into cold 1 mol/L NaOH-*aq* (120 mL) and it was extracted with DCM three times (1st: 50 mL, 2nd: 50 mL, 3rd: 30 mL). Combined organic extracts were washed with brine (40 mL) and dried over MgSO₄. The filtrate was evaporated under reduced pressure. The residue was purified by Gilson HPLC separation system using (0.1% TFA in water)/CH₃CN as an eluent. Desired fractions were collected and concentrated up to about half amount. CH₃CN was added to the aqueous solution until precipitates were formed (ratio of water:CH₃CN was about 1:2). After the precipitate was collected by filtration, it was extracted with DCM from 1 mol/L NaOH-*aq* three times. Combined organic extracts were dried over MgSO₄. The filtrate was evaporated under reduced pressure to yield 2-(2-bromo-4-*iso*-propylphenoxy)-N-(2,2,6,6-tetramethylpiperidin-4-yl)acetamide **VU0134992** (1.16 g, 78% yield) as a white powder. ¹H NMR (400.1 MHz, CDCl₃): 7.42 (d, J = 1.7 Hz, 1H), 7.13 (dd, J = 8.4, 1.7 Hz, 1H), 6.80 (d, J = 8.4 Hz, 1H), 6.74 (d, J = 8.0 Hz, 1H), 4.48 (s, 2H), 4.39-4.30 (m, 1H), 2.85 (sept, J = 6.9 Hz, 1H), 1.96-1.92 (m, 2H), 1.27 (s, 6H), 1.22 (d, J = 6.9 Hz, 6H), 1.14 (s, 6H), 1.03-0.97 (m, 2H). ¹³C NMR (100.6 MHz, CDCl₃): 166.87, 151.81, 144.44, 131.41, 126.90, 113.87, 112.10, 68.51, 51.17, 45.27, 42.47, 35.10, 33.38, 28.73, 24.12. LCMS: R_T = 0.912 min, *m/z* = 411 [M + H]⁺. HRMS calculated for C₂₀H₃₁BrN₂O₂ [M⁺], 410.1569; found 410.1571.

Drug Metabolism and Pharmacokinetics (DMPK)

Detailed methods for *in-vitro* and *in vivo* DMPK analyses are described in Supplemental Methods.

Metabolic Cage Studies

All studies involving animals were approved by Institutional Animal Care and Use Committee. Metabolic cage studies were done as described previously (Kharade et al., 2015). Briefly, male Sprague-Dawley rats (250-300 g) were allowed to acclimatize to single housing in metabolic cages for 2h before an experiment. Access to food and water was restricted during the entire experiment. Rats were given drug VU0134992 or vehicle (10% ethanol + 40% PEG400 + 50% saline) by oral gavage (PO). After 30 mins, voiding was induced by giving each animal a saline load (18 ml/kg by oral gavage), and urine was collected at 2h, 4h and 6h time points. Urine volumes were measured, centrifuged, aliquoted, and frozen at -80°C until analyzed. Urine Na⁺ and K⁺ concentrations were measured by flame photometry (Instrumentation Laboratories model 943, Lexington, MA). The total number of moles of Na⁺ or K⁺ excreted over the 4 hours was normalized to animal body weight (i.e. μmol/100 g/4h). The data are expressed as the mean ± SD and was analyzed using one-way ANOVA with Bonferroni's multiple comparisons test with statistical significance defined at $P < 0.05$.

RESULTS

Discovery of VU0134992

76,575 structurally diverse small-molecules from the Vanderbilt Institute of Chemical Biology library were screened against Kir4.1 using a Ti^+ flux assay that we have described previously (Raphemot et al., 2013). Briefly, the assay reports the inwardly directed movement of Ti^+ through the ion conduction pore of Kir4.1 using the intracellular, Ti^+ -sensitive dye Thallo. The mean Z' plate statistic for the primary screen was 0.78, indicating the assay's performance was robust and reproducible, and a hit rate of 0.8%. We selected 640 hit compounds resupplied from the vendor for confirmation and counter screening against induced and uninduced cells. 58 hits inhibited Ti^+ flux in tetracycline-induced T-Rex-HEK-293-Kir4.1 cells in duplicate testing at 10 μM . Screening against uninduced cells identified 42 compounds that inhibited endogenous Ti^+ flux pathways expressed in T-Rex-HEK293 cells. This left 16 authentic inhibitors of Kir4.1 (0.02% success rate). We focused on VU0134992 because of its superior potency, selectivity, and chemical tractability. In gold-standard patch clamp electrophysiology experiments, VU0134992 markedly inhibited Kir4.1 at 3 μM with an IC_{50} of 0.97 μM (95% CI, 0.5 μM to 1.7 μM) (Fig. 1). Because Kir4.1 forms heteromeric channels with Kir5.1 in the kidney (Lachheb et al., 2008; Tucker et al., 2000; Zhang et al., 2015; Zhang et al., 2014), we evaluated the sensitivity of Kir4.1-5.1 concatemeric channels to VU0134992. As shown in Fig. 1, VU0134992 inhibits Kir4.1-5.1 concatemeric channels with an IC_{50} of 9.05 μM (95% CI 7.4 μM to 11.1 μM) at -120 mV, providing approximately 9-fold selectivity toward Kir4.1 over Kir4.1-5.1 channels at this test potential. The IC_{50} values for inhibition of outward currents through Kir4.1 and Kir4.1-5.1 at +120 mV were 1.2 μM (95% CI – 0.97 μM to 1.6 μM) and 26.8 μM (95% CI 18.2 μM to 39.8 μM)(22-fold selectivity).

VU0134992 Selectivity

The selectivity of VU0134992 for Kir4.1 versus 9 other members of the Kir channel family was evaluated at concentrations ranging from 0.3 nM to 30 μ M in 11-point CRC experiments, using established TI^+ flux assays (Swale et al., 2016). The results are summarized in Table 1. VU0134992 exhibited no apparent activity toward Kir1.1, Kir2.1, or Kir2.2, and caused only partial inhibition of Kir2.3 (73% inhibition at 30 μ M), Kir6.2/SUR1 (12% inhibition at 30 μ M), and Kir7.1 (15% inhibition at 30 μ M). However, upon further evaluation, we found that VU0134992 inhibits Kir3.1/3.2 (92% inhibition at 30 μ M, IC_{50} =2.5 μ M), Kir3.1/3.4 (92% inhibition at 30 μ M, IC_{50} =3.1 μ M), and Kir4.2 (100% inhibition at 30 μ M, IC_{50} =8.1 μ M with approximately the same efficacy and potency that VU0134992 inhibits Kir4.1 (100% at 30 μ M, IC_{50} =5.2 μ M). The rightward shift in Kir4.1 IC_{50} derived from TI^+ flux experiments compared to electrophysiology experiments is commonly observed with other Kir channels (Bhave et al., 2011; Raphemot et al., 2013; Raphemot et al., 2011; Swale et al., 2016).

The antidepressants amitriptyline, nortriptyline, and fluoxetine have been used to inhibit Kir4.1 activity in heterologous expression and native cell systems (Furutani et al., 2009; Ohno et al., 2007; Su et al., 2007). However, these drugs have never been systematically evaluated for selectivity toward Kir4.1 over several closely related Kir channels in a single, systematic study. Toward this end, and to provide a comparison for VU0134992, we evaluated the potency and efficacy of amitriptyline, nortriptyline, and fluoxetine toward Kir1.1, Kir2.1, Kir2.3, Kir3.1/3.2, Kir3.1/3.4, Kir4.1, Kir4.2, Kir6.1/SUR1, and Kir7.1-M125R, in 11-point CRCs at concentrations up to 90 μ M. As summarized in Table 1, all 3 drugs exhibited weak and non-specific activity toward most members of the Kir channel family at concentrations that also inhibit Kir4.1. Thus, VU0134992 is more potent and selective than amitriptyline, nortriptyline, and fluoxetine toward Kir4.1 channels.

VU0134992 Interacts with E158 and I159 to Block the Kir4.1 Channel Pore

A common mechanism by which small-molecules inhibit Kir channels is through blockade of the ion-conduction pore (Furutani et al., 2009; Kharade et al., 2017; Swale et al., 2014; Swale et al., 2016). Some pore-blocker exhibit voltage-dependent “knock-off” behavior at test potentials more negative than the Nernst K^+ equilibrium potential difference. We therefore evaluated the voltage- and K^+ -dependence of VU0134992 inhibition of Kir4.1 to begin identifying the VU0134992 binding site. As shown in Fig. 2A, 30 μ M VU0134992 fully inhibited Kir4.1-mediated outward and inward currents across all test potentials when cells were bathed in a solution containing 5 mM K^+ and patch clamped using an internal solution containing 135 mM K^+ (Nernst equilibrium potential = -83 mV). However, when extracellular K^+ concentration was raised to 50 mM K^+ (Nernst equilibrium potential = -25 mV), we observed a time-dependent knock-off of VU0134992 at strong hyperpolarizing potentials (Fig. 2) and statistically significant reduction of block between -120 mV and -100 mV (Fig. 2). These data suggested that the VU0134992 binding site is indeed located within the Kir4.1 channel pore.

To identify pore-lining residues that participate in VU0134992 binding in the pore, we generated a comparative homology model of Kir4.1 based on the human Kir2.2 crystal structure and employed *in silico* docking to identify energetically favorable VU0134992 docking sites within the channel pore. Analysis of the top scoring VU0134992-Kir4.1 complexes by Rosetta interface score identified 4 pore-lining residues in Kir4.1 that 1) showed high frequency (>10% out of 750 models) interactions with VU0134992 and 2) were different between Kir4.1 and Kir1.1. These residues are in the rank order of glutamate (E)158 (39.5% of interactions) > threonine (T)162 (37.3% of interactions) > T155 (10.1 % of interactions) > isoleucine (I)159 (10.0 % of interactions). The interactions are a combination of hydrogen bonds and van der Waals interactions (Fig. 3A)The homology model in Fig. 3A shows the predicted location of the VU0134992 in the membrane-spanning pore (right), whereas the close-up view

(left) identifies close interactions of VU0134992 with E158, the top-scoring residue. Interactions with E158 are dominated by hydrogen bonds. However, several van der Waals contacts exist in addition.

We mutated these four amino acids to the corresponding residues in Kir1.1, which is virtually insensitive to VU0134992 (Fig. 3B-E), and measured the inhibition of the mutant channels to 3 μ M VU0134992 by whole-cell patch clamp electrophysiology. As shown in Figure 3B, 3 μ M VU0134992 inhibited inward current at -120 mV carried by WT Kir4.1 by 85.9 ± 11 % (n = 12). In striking contrast, mutation of E158 to asparagine (E158N) dramatically reduced the sensitivity of the channel to VU0134992 (0.4 ± 12 % inhibition, n = 8; $IC_{50} \sim 235$ μ M with only 38.2 ± 14.7 % inhibition at 30 μ M) at -120mV. To determine if this loss of sensitivity is caused by alteration of side chain charge or structure, we evaluated channels in which E158 was mutated to either glutamine (E158Q) or aspartate (E158D). Both mutant channels exhibited a complete loss of sensitivity to 3 μ M VU0134992 and a large rightward shift in IC_{50} (Kir4.1-E158Q ~ 28 mM; Kir4.1-E158D = no fit), suggesting that changes in side-chain structure at position 158 are sufficient to affect VU0134992 block. Mutation of I159 to serine (I159S) caused a complete loss of block by 3 μ M VU0134992 and large rightward shift in the CRC (9.5 ± 7.7 %, n=6; $IC_{50} \sim 199$ μ M and 77 ± 20 % inhibition at 30 μ M; Fig. 3B, D). Kir4.1-T155V channels were not functional (NF) and therefore could not be evaluated. We also mutated nearby residues T162 to cysteine (T162C), T164 to alanine (T164A) and F165 to isoleucine (F165I), however neither mutation affected VU0134992 sensitivity.

VU0134992 Structure-Activity Relationships

We employed medicinal chemistry in an effort to improve the potency of VU0134992 and establish its structure-activity relationships (SAR) for inhibition of Kir4.1. No modifications made to the scaffold of VU0134992 improved its activity toward Kir4.1. It was revealed that VU0134992 bears fundamental

components for inhibition of Kir4.1 channels (Supplemental Tables. S1-3). An overview of the SAR is shown in Fig. 4. While flat SAR was observed at the 4-position in the left-hand part for inhibitory activity of Kir4.1 with IC_{50} of 3.0 – 9.6 μ M in the thallium flux assay (methyl, ethyl, *iso*-propyl (VU0134992), *tert*-butyl, chloro and phenyl substituents), the mono-bromine atom at the 2-position was critical for the potency (Not only F, Cl, H substituents for Br were all inactive (No Fit) but also addition of Br and Cl at the 6-position resulted in notable decrease in potency) (Table. S1). While the linker modification was tolerated, such as incorporation of dimethyl substituents at α -position of the amide (Table. S3), all of modifications in the right-hand part made the compound noticeably less active (Table. S2).

VU0134992 Serum Binding, Clearance, and Blood-Brain-Barrier Permeability

VU0134992 displayed a large free fraction in rat plasma ($f_u = 0.213$), modest fraction unbound in rat brain ($f_u = 0.013$), moderate predicted hepatic clearance in human (13.4 mL/min/kg), and high predicted hepatic clearance in rat (60.5 mL/min/kg) liver microsomes. A good *in vitro:in vivo* correlation was noted, with clearance above cardiac output ($CL_p > 1000$ mL/min/kg), and with a short half-life ($t_{1/2} = 10.6$ min) driven by a large volume of distribution at steady state ($V_{ss} = 13.5$ L/kg). In a standard rat plasma:brain level (PBL) cassette, which is routinely used to rapidly screen the brain distribution potential of compounds, VU0134992 levels were below the limit of quantification (BLQ) in brain. We also measured plasma and brain levels following an oral dose of 50 mg/kg, a dosing regimen that was used in subsequent metabolic cage studies (see below). Total VU0134992 brain and plasma concentrations were 0.94 μ M and 0.76 μ M ($K_p = 1.2$), respectively, whereas unbound VU0134992 concentrations in brain and plasma were 0.012 μ M and 0.16 μ M ($K_{p,uu} = 0.08$).

Effects of VU0134992 on Renal Fluid and Electrolyte Excretion

Given the important role of Kir4.1 in renal Na⁺ reabsorption and K⁺ secretion, we have postulated that small-molecule inhibitors of Kir4.1 will induce diuresis, natriuresis, and kaliuresis (Denton et al., 2013). To test this hypothesis, we evaluated the effects of VU0134992 on renal function in volume-loaded Sprague-Dawley rats. The rats were administered with either vehicle or different doses of VU0134992 followed by volume-loading. The effects of VU0134992 on urine output are summarized in Fig. 5. Consistent with the hypothesis, oral administration of VU0134992 led to a statistically significant increase in urine output as compared to vehicle at doses of 50 mg/kg and 100 mg/kg. Urine Na⁺ and K⁺ concentrations were measured to determine how VU0134992 alters electrolyte transport along the nephron. As shown in Figs. 5B and 5C, VU0134992 statistically significantly ($P < 0.05$) increased urinary Na⁺ as well as K⁺ excretion at doses of 50 mg/kg and 100 mg/kg compared to vehicle-treated control animals.

DISCUSSION

Here we report the discovery and characterization of VU0134992, the first Kir4.1 K⁺ channel inhibitor to emerge from a high-throughput screening campaign. VU0134992 affords approximately 9-22 fold selectivity between homotetrameric Kir4.1 and heterotetrameric Kir4.1/5.1 channels when evaluated at -120 mV and +120 mV, respectively, a property that could be useful for sorting out the relative contribution of these two channel populations in native cells. To our knowledge, VU0134992 is the first subtype-preferring Kir4.1 inhibitor to be reported. Efforts to understand the molecular basis of this selectivity and to identify VU0134992 analogs with improved potency and selectivity toward Kir4.1 and Kir4.1/5.1 channels are underway.

The pharmacology of Kir4.1 has been primarily limited to inorganic cations (e.g. Ba²⁺, cesium) and antidepressants that have relatively weak off-target activity toward Kir4.1. A summary of published small-molecule Kir4.1 inhibitors, their intended drug targets, and IC₅₀ values for Kir4.1 are shown in Table 2. The tricyclic antidepressant (TCA) drugs nortriptyline, amitriptyline, desipramine, and imipramine were the first small-molecules shown to inhibit Kir4.1 (Su et al., 2007). The IC₅₀ for inhibition by nortriptyline is 16 μM and 38 μM at test potentials of +30 mV and -110 mV, respectively, and block is relieved upon elevation of extracellular K⁺ concentration. Kir4.1 is also inhibited by the Selective Serotonin Reuptake Inhibitor (SSRI) drugs fluoxetine, sertraline, and fluvoxamine (Ohno et al., 2007). Similar to nortriptyline, block by fluvoxamine is voltage-dependent and reduced by membrane hyperpolarization. Mutational analysis revealed that Kir4.1 inhibition by fluoxetine and nortriptyline requires T128 and E158 (Furutani et al., 2009). More recently, the anti-malarial drugs quinacrine and chloroquine were also shown to inhibit Kir4.1 in a voltage-dependent manner involving interactions with T128 and E158 (Marmolejo-Murillo et al., 2017a; Marmolejo-Murillo et al., 2017b). The most potent intracellular pore blocker of Kir4.1 is currently pentamidine (Arechiga-Figueroa et al., 2017).

When applied to the intracellular face of excised membrane patches, pentamidine inhibits Kir4.1 at +80 mV with an IC_{50} of 97 nM. Similar to the other inhibitors, Kir4.1 block by pentamidine requires interactions with T127, T128, and E158. Unfortunately, pentamidine is a poor inhibitor of Kir4.1 in the whole-cell configuration (i.e. ~25% inhibition by 1 μ M pentamidine after 20 mins) and presumably intact cells. Thus, pentamidine is a useful tool for probing the structure of the intracellular pore, but will be limited as an *in vitro* and *in vivo* tool compound for exploring the physiology and druggability of Kir4.1. In the present study, we found that inhibition of Kir4.1 by VU0134992 exhibited voltage-dependent knockoff behavior at hyperpolarized test potentials that is consistent with block of the inner pore cavity. We employed *in silico* docking calculations to guide mutagenic analysis of potential pore-lining binding sites in Kir4.1. Mutation of two of the top-scoring residues led to a severe (E158N/Q/D, I159S) loss of VU0134992 sensitivity, lending experimental validation for this computational approach to identifying small-molecule binding sites in the Kir4.1 channel pore. Interestingly, VU0134992 inhibited Kir3.1/3.2 and Kir3.1/3.4 channels with similar potencies. Because Kir3.2 and Kir3.4 have asparagine (N) residues at positions equivalent to E158 in Kir4.1, we postulate that this cross-activity is due to D173 in Kir3.1 (also equivalent to Kir4.1-E158) in heteromeric Kir3.1/3.2 and Kir3.1/3.4 channels.

Kir4.1 was the first K^+ channel to be immunolocalized to the basolateral membrane of renal epithelial cells (Ito et al., 1996). The “silent” subunit, Kir5.1, was subsequently co-localized with Kir4.1 in the nephron, which, taken together with heterologous expression experiments showing that Kir4.1 and Kir5.1 form heteromeric channel complexes, suggested that the native channel subtype in the nephron is a Kir4.1-5.1 heteromer. Patch clamp experiments on isolated nephron segments have identified a predominant ~40-picoSiemens (pS) basolateral K^+ current that exhibits biophysical and regulatory properties that resemble heterologously expressed Kir4.1-5.1 (Lachheb et al., 2008) and is absent in

KCNJ10-knockout mice (Zhang et al., 2015; Zhang et al., 2014), and a less common ~20 pS current that resembles homomeric Kir4.1 (Zaika et al., 2013a; Zhang et al., 2013). Both of these channels appear to play important physiological roles in the nephron (see below).

The basolateral K^+ conductance of the cortical TAL is carried by a 40-pS channel that is likely comprised of Kir4.1-5.1 heteromeric channels, and an 80-pS channel possibly carried by Na^+ -activated K^+ (K_{Na}) channels (Fan et al., 2015; Zhang et al., 2015)(Fig. 6). Genetic ablation of *KCNJ10* has no effect on the basolateral membrane potential in the TAL due to compensatory upregulation of the 80-pS channel (Zhang et al., 2015), suggesting that depolarization of the basolateral membrane potential in the TAL is unlikely to contribute to salt wasting in *KCNJ10*-knockout mice and EAST/SeSAME syndrome patients (Bockenhauer et al., 2009; Scholl et al., 2009). Interestingly, however, inhibition of the 40-pS channel with Ba^{2+} reduces the activity of basolateral ClC-Kb Cl^- channels through mechanisms that are currently unclear but do not appear to involve membrane depolarization (Fan et al., 2015). These data suggest that the natriuretic and diuretic effects of VU0134992 could result at least in part through inhibition of NaCl reabsorption in the TAL, as follows. NaCl reabsorption from the tubule fluid in the TAL is mediated by the electroneutral, loop diuretic-inhibitable, $Na^+-K^+-2Cl^-$ co-transporter, NKCC2 (Fig. 6). Na^+ is then pumped across the basolateral membrane through the $Na^+-K^+-ATPase$, and Cl^- is conducted out of the cell through ClC-Kb Cl^- channels. The indirect inhibition of ClC-Kb channels with VU0134992 should increase intracellular Cl^- concentrations and thereby reduce the inwardly directed driving force for Cl^- and coupled Na^+ and K^+ transport by NKCC2. In addition, basolateral K^+ secretion provides substrate K^+ ions that are needed to maintain the activity of the $Na^+-K^+-ATPase$. Provided that the 80-pS channel cannot compensate for the pharmacological inhibition of 40-pS Kir4.1-5.1 channels, VU0134992 should also slow the activity of the $Na^+-K^+-ATPase$, increase intracellular Na^+ , and further reduce the chemical driving force for NaCl reabsorption via NKCC2 (Fig. 6).

NaCl reabsorption in the DCT is mediated by the luminal membrane, electroneutral, thiazide-sensitive $\text{Na}^+\text{-Cl}^-$ co-transporter, NCC (Fig. 6). A rapidly developing body of evidence indicates that heterotetrameric Kir4.1-5.1 channels, which dominate the basolateral K^+ conductance in the DCT, are critical regulators of NCC expression and activity (Penton et al., 2016; Zhang et al., 2014). Deletion of *KCNJ10* in mice depolarizes the basolateral membrane potential, inhibits the basolateral Cl^- conductance, and reduces the expression of NCC (Zhang et al., 2014). Furthermore, Penton and colleagues demonstrated that application of Ba^{2+} to kidney slices induced dephosphorylation and hence inhibition of NCC. Taken together, these data are consistent with a model in which inhibition of Kir4.1 activity increases intracellular Cl^- concentration, which, in turn, inhibits NCC function through the Cl^- -sensitive WNK/SPAK kinase pathway (reviewed in (Su and Wang, 2016). Ongoing studies are testing if VU0134992 administration induces the dephosphorylation of NCC in mice.

In the mouse CCD, Kir4.1 is expressed as a common 40-pS Kir4.1/5.1 current and a scarce 20-pS current carried by homomeric Kir4.1 channels (Zaika et al., 2013b)(Fig. 6). Although the 40 pS channel is more common, the 20 pS current has a higher open probability and thus likely plays an important role in setting the resting membrane potential. In principle cells of the CCD, activation of insulin receptors stimulates the activity of Kir4.1-containing channels, which, in turn, increases the electrochemical driving force for Na^+ reabsorption by the epithelial sodium channel (ENaC). In contrast, inhibition of Kir4.1 and Kir4.1/5.1 channels by dopamine acting on D2-like receptors leads to reduced Na^+ reabsorption via ENaC (Zaika et al., 2013a). These data suggest that VU0134992 induces natriuresis at least in part through inhibition of Kir4.1-containing channels expressed in the CCD (Fig. 6).

The diuretic and natriuretic responses induced by VU0134992 were accompanied by increases in urinary K^+ excretion. In this regard, the renal actions of VU0134992 are reminiscent of those caused by loop or thiazide diuretics. The simplest interpretation is that the increased volume and Na^+ load delivered to the distal nephron, secondary to inhibition of Na^+ reabsorption in the TAL and/or DCT, stimulates K^+ secretion via flow-stimulated large-conductance “BK” K^+ channels expressed in the CCD (Liu et al., 2007; Liu et al., 2009). These responses are different from those induced by inhibitors of the Renal Outer Medullary K^+ channel, ROMK (Kir1.1), another emerging diuretic target in the Kir channel family (Denton et al., 2013). We and others have shown that ROMK inhibition evokes diuresis and natriuresis without causing K^+ wasting (Garcia et al., 2014; Kharade et al., 2015; Zhou et al., 2017). The K^+ -sparing effect appears to result predominantly from inhibition of K^+ secretion in the TAL with a relatively minor effect on distal K^+ secretion (Kharade et al., 2015).

In conclusion, we have identified and characterized a moderately potent and selective inhibitor of Kir4.1 that exhibits preference for the homotetrameric channel over heteromeric Kir4.1-5.1 channels. Oral administration of VU0134992 induces dose-dependent increases in urine volume, Na^+ excretion, and K^+ excretion in rats. We propose that the observed changes in renal excretion of water and electrolytes results from inhibition of basolateral Kir4.1-containing channels somewhere along the nephron. The low unbound VU0134992 brain-to-plasma ratio ($K_{p,uu} = 0.08$) also supports the idea that the effects of VU0134992 on renal excretion is due to inhibition of Kir4.1 channels in the kidney and not in the brain. Future studies will be aimed at characterizing the activity of VU0134992 on electrolyte transport in the TAL, DCT, and CCD. The data presented herein raise the intriguing possibility that inhibition of basolateral Kir4.1-containing channels could be an effective way to lower blood pressure in hypertensive and heart failure patients. Targeting a basolateral membrane protein could provide therapeutic advantages over conventional diuretics. For example, loop (furosemide and bumetanide) and

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thiazide diuretics must be secreted into the tubule fluid before reaching their respective luminal target sites in the TAL and DCT (Hasannejad et al., 2004; Hasegawa et al., 2007; Uwai et al., 2000; Vallon et al., 2008). In clinical settings where diuretic secretion is compromised, such as chronic kidney disease or when other drugs compete for renal tubule secretion, the availability of a diuretic that acts basolaterally and independently of renal secretion would be beneficial over conventional diuretics. Furthermore, a recent study by Rao and colleagues demonstrated that a majority of loop diuretic resistance cases in their heart failure patient cohort resulted from enhanced distal tubule Na^+ reabsorption. The authors suggested that administration of thiazide diuretics or a combination of thiazide and K^+ -sparing diuretics should help overcome loop diuretic resistance in some patients (Rao et al., 2017). An inhibitor of Kir4.1-containing channels would be potentially superior to thiazide or K^+ -sparing diuretics because it would simultaneously inhibit Na^+ reabsorption all along the nephron without sparing K^+ . The development of VU0134992 provides the first *in vivo*-active tool compound to enable these and other hypotheses to be tested.

AUTHOR CONTRIBUTIONS

Research Design: SVK, HK, ALB, EF, AD, LMS, JM, CWL, CDW, CRH, JSD

Conducted experiments: SVK, HK, ALB, EF, AD, MK, ED, PV, DF

Performed data analysis: SVK, HK, ALB, EF, AD, MK, ED, DF

Contributed new reagents or analytical tools: HK, AMB, JM, CRH

Wrote manuscript: SVK, HK, ALB, EF, PV, AD, CDW, CWL, CRH, JSD

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FOOTNOTES

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FIGURE LEGENDS

Figure 1: VU0134992 exhibits preference for Kir4.1 over Kir4.1-5.1 channels. **A.** Chemical structure of VU0134992. **B.** Representative traces showing inhibition of Kir4.1 homomeric and Kir4.1-5.1 heteromeric currents recorded in absence (blue) or in presence of 3 μ M VU0134992 (red) and 2 mM BaCl₂ (black). **C.** Mean \pm SD CRCs of VU0134992 for homotetrameric Kir4.1 and heterotetrameric concatemer of Kir4.1/5.1 currents measured at -120 mV ($n \geq 5$). **D.** Mean \pm SD CRCs of VU0134992 for homotetrameric Kir4.1 and heterotetrameric concatemer of Kir4.1/5.1 currents measured at +120 mV ($n \geq 5$).

Figure 2: VU0134992 exhibits voltage-dependent block of Kir4.1 **A.** Representative whole-cell current responses to voltage steps (1 s) from a holding potentials (-80 mV for 5K⁺ or -25 mV for 50K⁺) in presence or absence of 30 μ M VU0134992. **B.** Percent VU0134992 inhibition was calculated at each voltage with 5 and 50 mM extracellular K⁺. The VU0134992 induced block of Kir4.1 was statistically significantly lower at -120 mV and -100 mV in 50 mM K⁺ as compared to 5 mM K⁺ containing bath solution. *P < 0.05, statistically significantly different from the 5 mM K⁺ extracellular solution control ($n \geq 5$).

Figure 3: E158 and I159 are required for effective block of Kir4.1 by VU0134992. **A.** (Left) Kir4.1 homology model showing the location of E158 (green), which is it predicted to interact with VU0134992. (Right) magnified section of the Kir4.1 homology model (red square in left) showing interaction of VU0134992 with the pore lining E158 residue in Kir4.1. **B.** Mutagenesis studies were carried out to test the prediction of homology model. Bar graph showing percent inhibition of Kir4.1-WT (solid grey bar), and various mutant channels (colored or open bars) at 3 μ M VU0134992. Kir4.1 carrying mutations at E158 and I159 residue showed statistically significantly reduced sensitivity to VU0134992. **C.** Representative traces showing inhibition of WT and E158N/Q/D and I159S mutants in

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absence (blue) or in presence of 3 μ M VU0134992 (red) and 2 mM BaCl₂ (black). **D.** VU0134992 CRCs comparing Kir4.1-WT, Kir1.1 and Kir4.1-E158N, E158D, E158Q and I159S mutants at -120 mV. **E.** CRCs for indicated channels measured at +120 mV. *P < 0.05, statistically significantly different from the WT control (n \geq 5).

Figure 4. Overview of VU0134992 SAR. Inhibition of TI⁺ flux toward Kir4.1 was evaluated for each compound synthesized according to Figures S1-S3. Specific figures of IC₅₀ are described in Tables S1-S3.

Figure 5. Dose-dependent effects of VU0134992 on renal excretion. Rats were given oral gavage of either vehicle alone or the indicated doses of VU0134992 followed by saline volume load and were placed in metabolic cages for collection of urine over a 4 h period. VU0134992 statistically significantly (P<0.05) increased **A.** urine volume, **B.** urine Na⁺, and **C.** urine K⁺ at doses of 50 mg/kg and 100 mg/kg as compared to the vehicle control. *P < 0.05, statistically significantly different from the vehicle control (n \geq 4).

Figure 6. Regulation of renal ion transport by Kir4.1. In the TAL (left panel), heteromeric Kir4.1/5.1 channels recycle K⁺ across the basolateral membrane to maintain the activity of the Na⁺/K⁺-ATPase, which creates a favorable gradient for Na⁺ and K⁺ uptake via NKCC2. Kir4.1/5.1 is functionally coupled to basolateral ClC-Kb channels through unknown mechanisms. Inhibition of Kir4.1 should reduce Na⁺, Cl⁻, and K⁺ reabsorption in the TAL. In the DCT (middle panel), basolateral K⁺ secretion via Kir4.1/5.1 energizes the Na⁺/K⁺-ATPase to maintain a favorable chemical driving force for Na⁺ uptake by NCC. Kir4.1/5.1 also regulates the basolateral membrane potential and is functionally coupled to ClC-Kb. Inhibition of Kir4.1/5.1 should increase intracellular Cl⁻ concentration and inhibit NCC function via the WNK-SPAK kinase pathway (not shown; see Discussion). In the CCD (right panel), homomeric Kir4.1 and heteromeric Kir4.1/5.1 channels hyperpolarize the basolateral membrane potential and electrically

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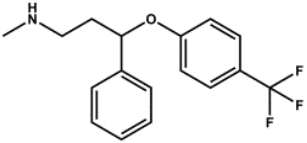
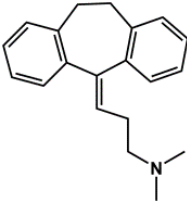
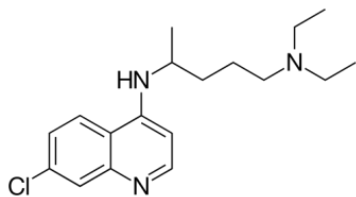
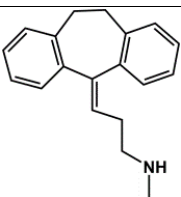
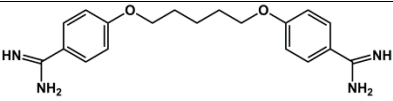
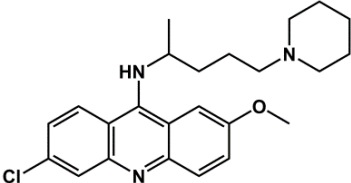
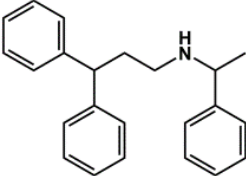
coupled apical membrane potential and promote luminal Na⁺ reabsorption by ENaC. Inhibition of Kir4.1 or Kir4.1/5.1 channels in the CCD should depolarize the membrane potential and reduce the electrochemical driving force for Na⁺ reabsorption.

TABLES

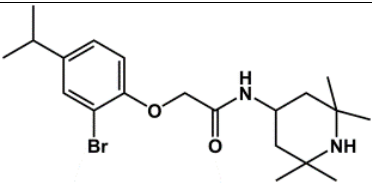
Table 1. Comparison of VU0134992 and antidepressant selectivity for Kir4.1 over other Kir channels. Data shown are mean IC₅₀ values in μ M and maximal % inhibition at 30 μ M and 90 μ M in parentheses derived from TI⁺ flux assays. Experiments were performed in triplicate on two separate days.

Channel	VU0134992	Amitriptyline	Nortriptyline	Fluoxetine
Kir1.1	Inactive	33.0 (16%, 58%)	31.0 (6%, 50%)	27.0 (6%, 30%)
Kir2.1	Inactive	35.0 (1%, 36%)	Inactive	10.1 (10%, 86%)
Kir2.2	Inactive	41.7 (2%, 41%)	Inactive	Inactive
Kir2.3	13.2 (73%)	21.3 (53%, 89%)	19.4 (60%, 91%)	7.1 (98%, 100%)
Kir3.1/3.2	2.5 (92%)	13.6 (73%, 91%)	16.1 (65%, 87%)	3.4 (97%, 100%)
Kir3.1/3.4	3.1 (92%)	12.0 (67%, 88%)	15.0 (56%, 80%)	2.4 (94%, 100%)
Kir4.1	5.2 (100%)	89.0 (0%, 66%)	41.7 (10%, 93%)	31.2 (35%, 95%)
Kir4.2	8.1 (100%)	42.7 (0%, 45%)	46.7 (0%, 65%)	20.2 (98%, 99%)
Kir6.2/SUR1	11.4 (12%)	32.2 (4%, 64%)	42.0 (0%, 52%)	64 (4%, 65%)
Kir7.1	34.2 (15%)	22.9 (65%, 89%)	26.0 (50%, 82%)	5.8 (75%, 92%)

Table 2. Published Kir4.1 inhibitors

Compound	Structure	Drug Class	Kir4.1 IC ₅₀ (μM)	Reference
Fluoxetine		SSRI	15	(Ohno et al., 2007)
Amitriptyline		TCA	89	This study
Chloroquine		Antimalarial	7	(Marmolejo-Murillo et al., 2017b)
Nortriptyline		TCA	16-38*	(Su et al., 2007)
Pentamidine		Antimicrobial	0.097**	(Arechiga-Figueroa et al., 2017; Takanari et al., 2013)
Quinacrine		Antimalarial	~3	(Marmolejo-Murillo et al., 2017a)
VU717		None	6	(Raphemot et al., 2013)

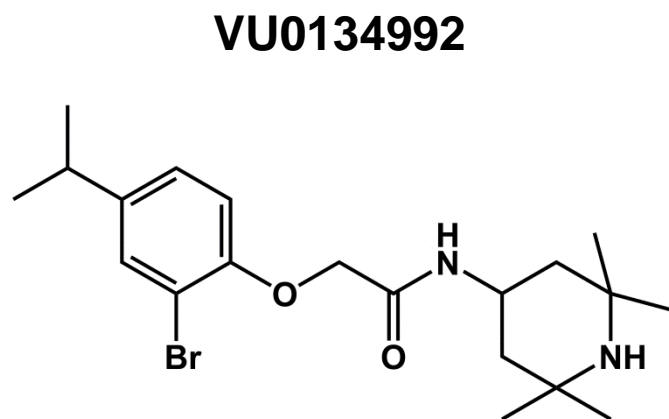
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VU0134992		None	0.97	This study
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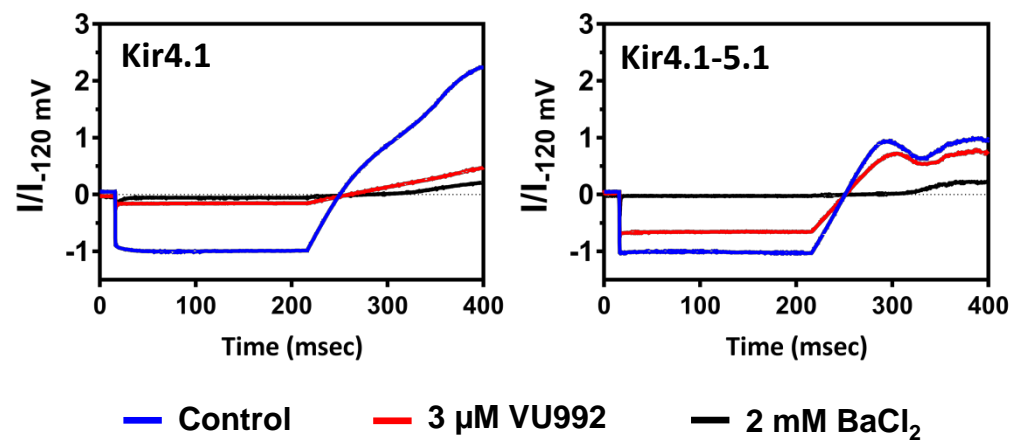
*Voltage-dependent block; calculated at +30 mV and -110 mV, respectively; **voltage-dependent block; calculated at +80 mV in inside-out patch clamp experiments.

Figure 1

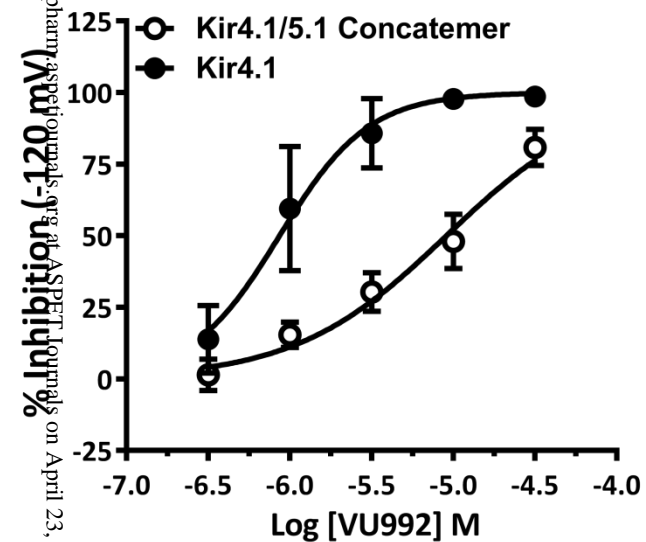
A



B



C



D

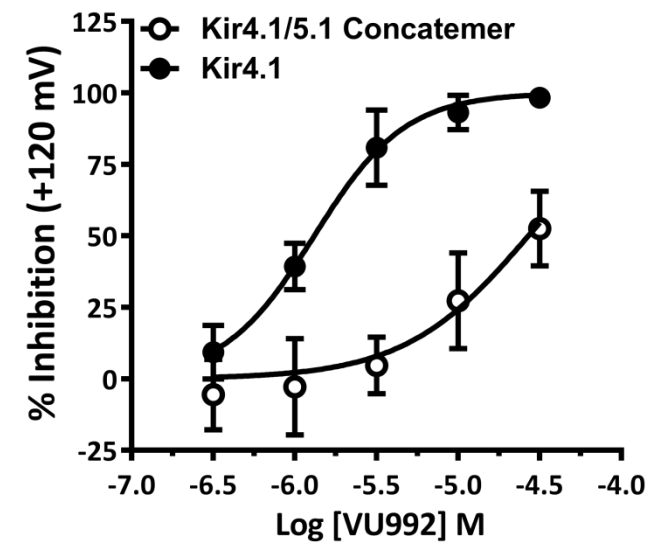
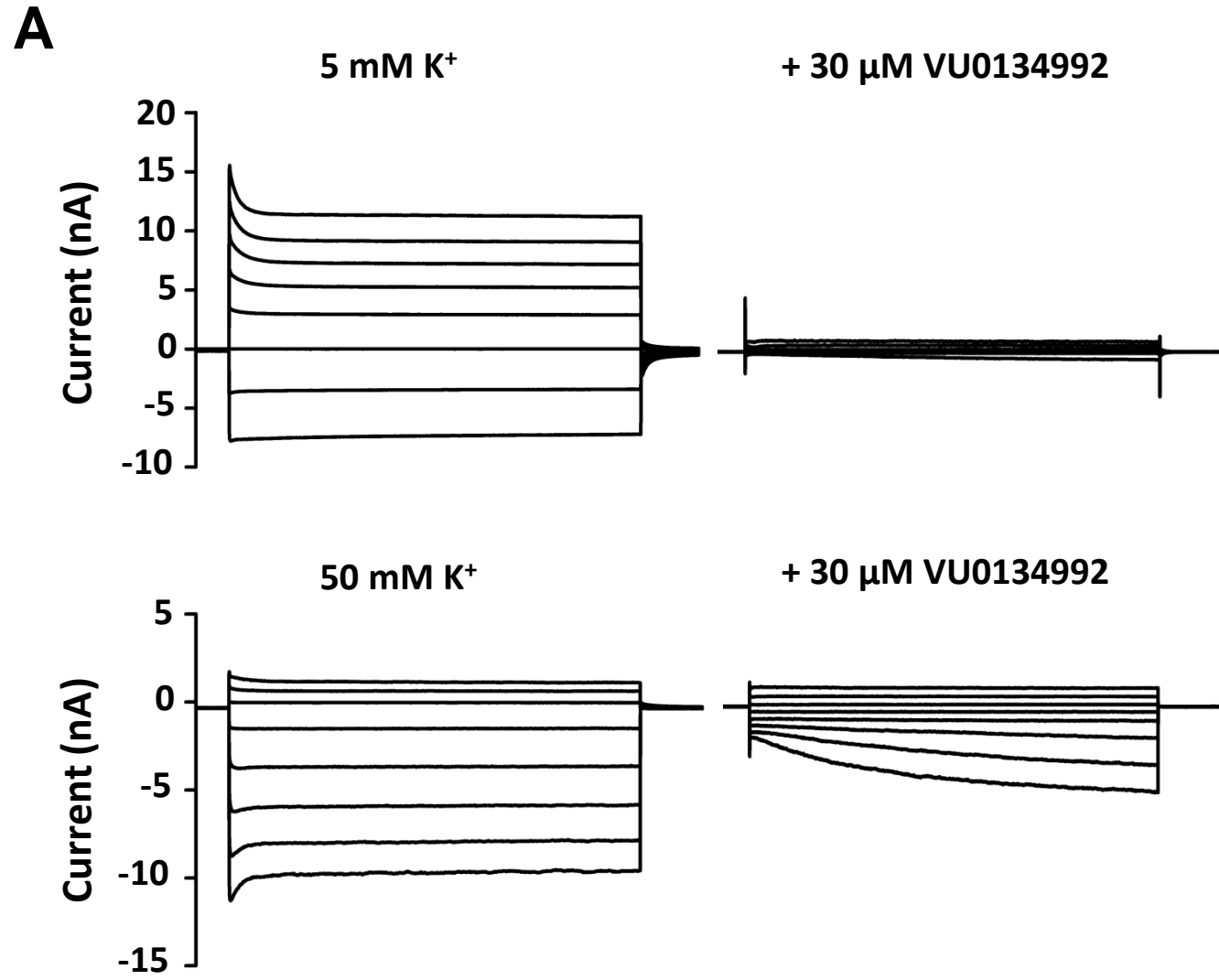


Figure 2



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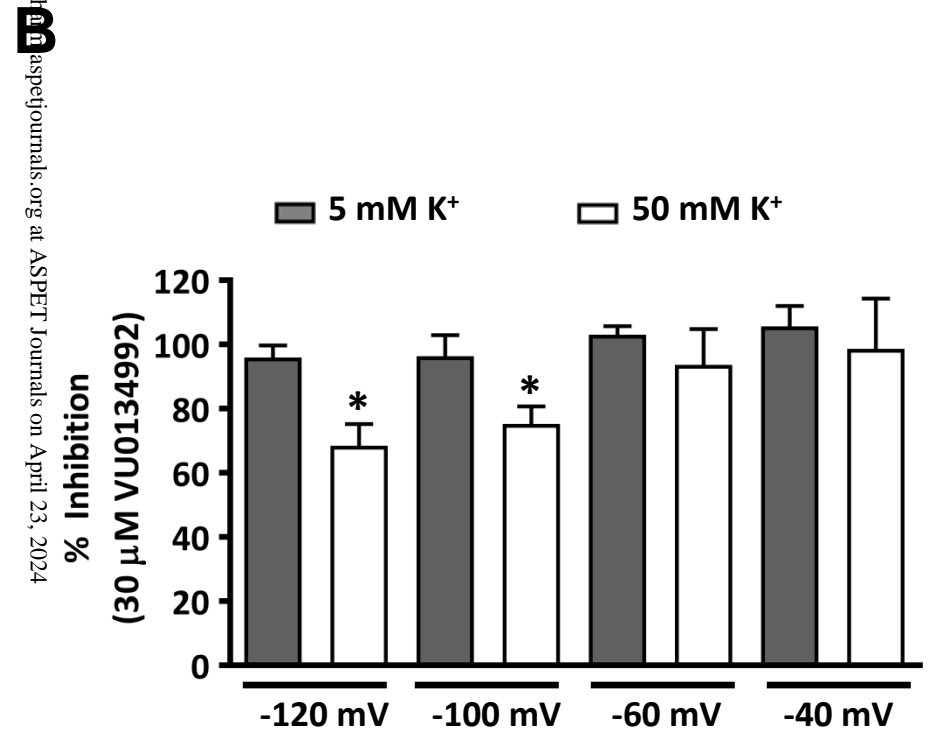
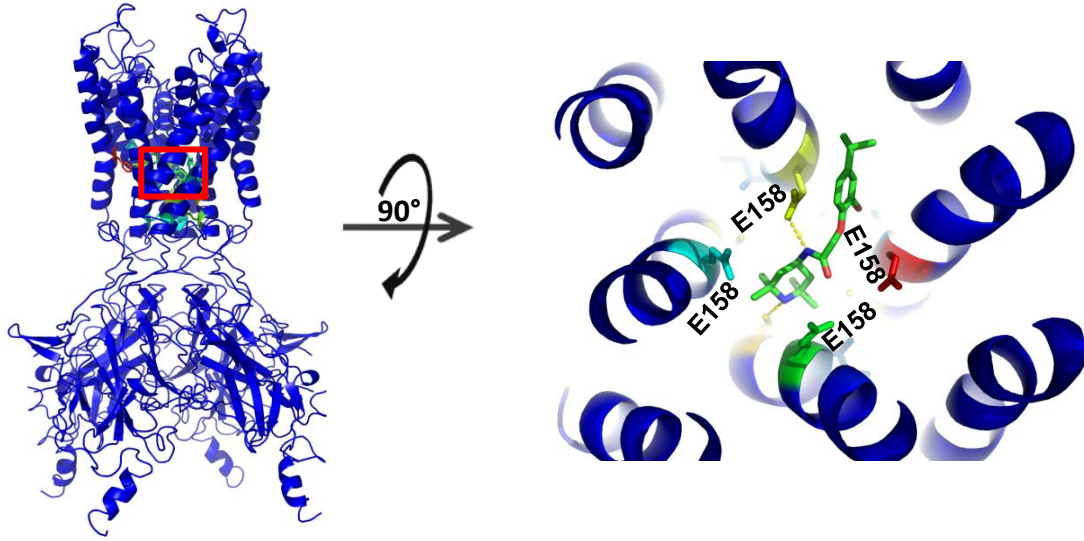
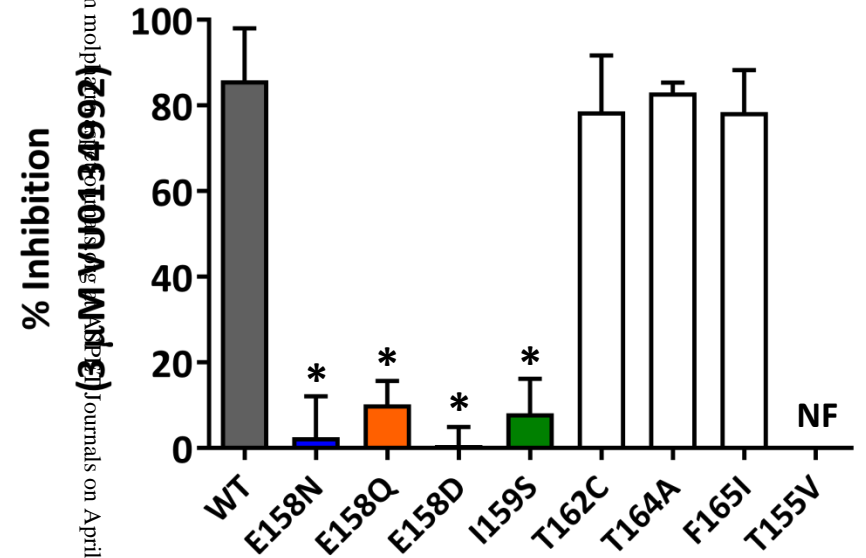


Figure 3

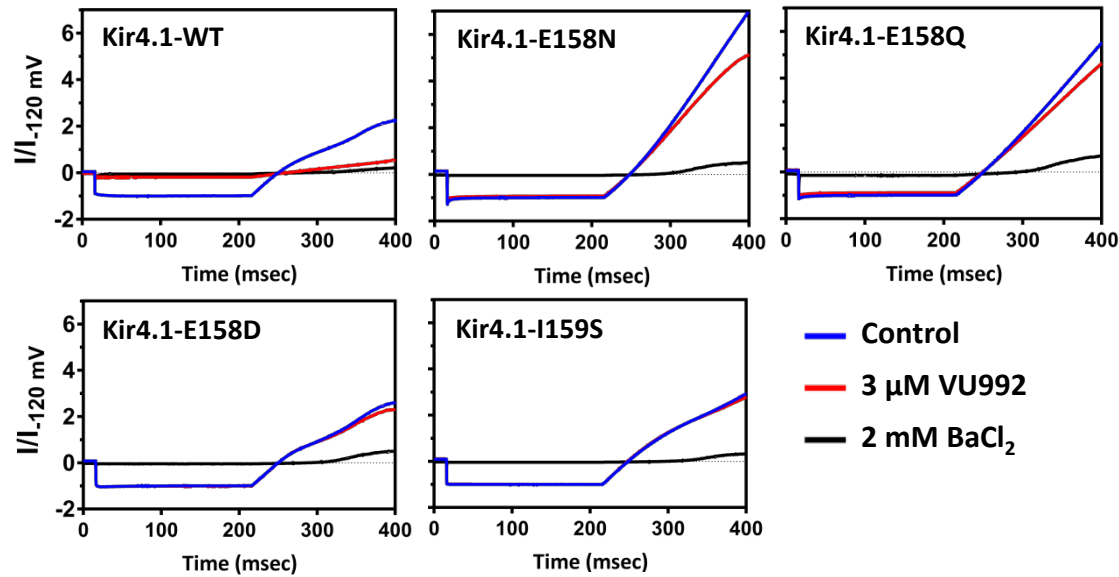
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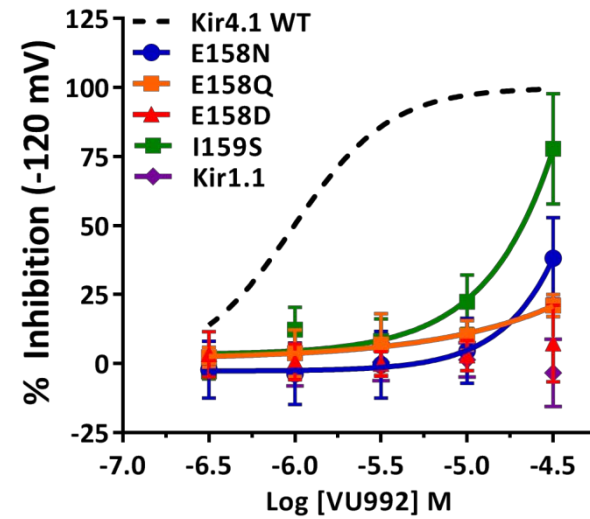
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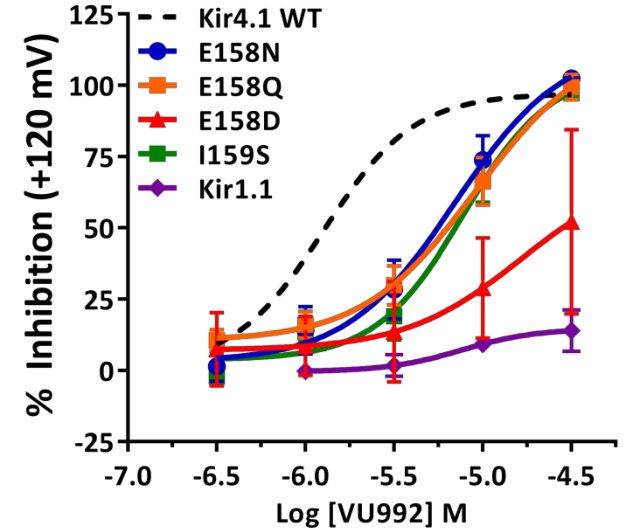


Figure 4

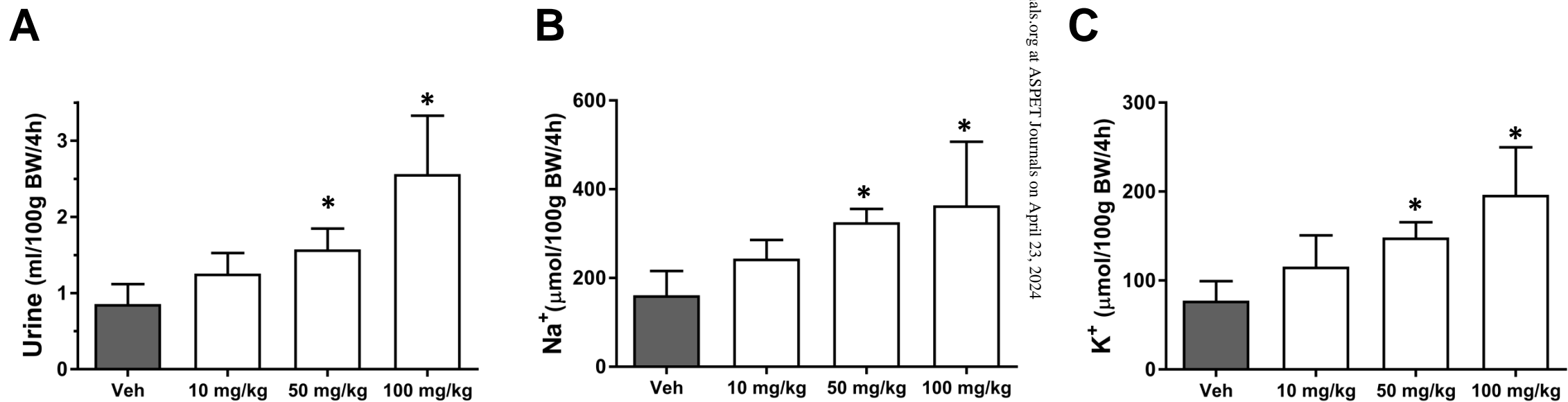


Figure 5

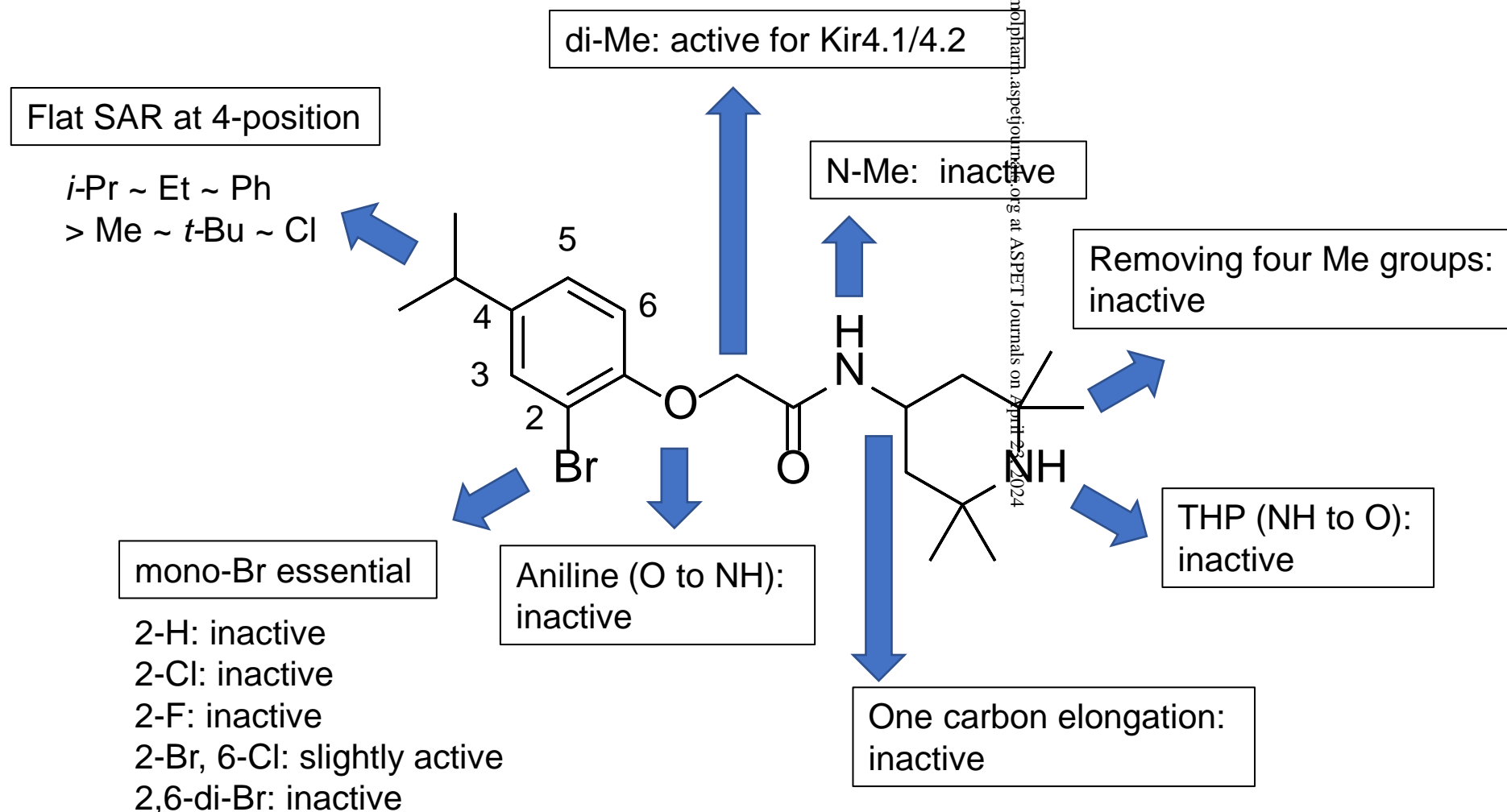


Figure 6

