Discovery and Characterization of VU0542270, the First Selective Inhibitor of Vascular Kir6.1/SUR2B K$_{\text{ATP}}$ Channels

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ABSTRACT

Vascular smooth muscle K$_{\text{ATP}}$ channels critically regulate blood flow and blood pressure by modulating vascular tone and therefore represent attractive drug targets for treating several cardiovascular disorders. However, the lack of potent inhibitors that can selectively inhibit Kir6.1/SUR2B (vascular K$_{\text{ATP}}$) over Kir6.2/SUR1 (pancreatic K$_{\text{ATP}}$) has eluded discovery despite decades of intensive research. We therefore screened 47,872 chemically diverse compounds for novel inhibitors of heterologously expressed Kir6.1/SUR2B channels. The most potent inhibitor identified in the screen was an N-aryl-N'-benzyl urea compound termed VU0542270. VU0542270 inhibits Kir6.1/SUR2B with an IC$_{50}$ of approximately 100 nM but has no apparent activity toward Kir6.2/SUR1 or several other members of the Kir channel family at doses up to 30 μM (>300-fold selectivity). By expressing different combinations of Kir6.1 or Kir6.2 with SUR1, SUR2A, or SUR2B, the VU0542270 binding site was localized to SUR2. Initial structure-activity relationship exploration around VU0542270 revealed basic texture related to structural elements that are required for Kir6.1/SUR2B inhibition. Analysis of the pharmacokinetic properties of VU0542270 showed that it has a short in vivo half-life due to extensive metabolism. In pressure myography experiments on isolated mouse ductus arteriosus vessels, VU0542270 induced ductus arteriosus constriction in a dose-dependent manner similar to that of the nonspecific K$_{\text{ATP}}$ channel inhibitor glibenclamide. The discovery of VU0542270 provides conceptual proof that SUR2-specific K$_{\text{ATP}}$ channel inhibitors can be developed using a molecular target-based approach and offers hope for developing cardiovascular therapeutics targeting Kir6.1/SUR2B.

SIGNIFICANCE STATEMENT

Small-molecule inhibitors of vascular smooth muscle K$_{\text{ATP}}$ channels might represent novel therapeutics for patent ductus arteriosus, migraine headache, and sepsis; however, the lack of selective channel inhibitors has slowed progress in these therapeutic areas. Here, this study describes the discovery and characterization of the first vascular-specific K$_{\text{ATP}}$ channel inhibitor, VU0542270.

Introduction

ATP-sensitive potassium channels (K$_{\text{ATP}}$) are regulated by intracellular nucleotide concentrations and thus serve to couple metabolic state to membrane excitability in diverse cell types (Nichols, 2006; Davis et al., 2022; McClanahan and Nichols, 2022). K$_{\text{ATP}}$ channels are hetero-octameric complexes composed of four pore-forming inward rectifier potassium (Kir) channel subunits, Kir6.1 or Kir6.2, and four regulatory sulfonylurea receptor (SUR) subunits, SUR1, SUR2A, or SUR2B (Inagaki et al., 1997; Shyng and Nichols, 1997; Aguilar-Bryan and Bryan, 1999; Martin et al., 2017). Kir6.1 and Kir6.2 are encoded by KCNJ8 and KCNJ11, respectively. SUR1 is encoded by ABCC8, whereas SUR2A and SUR2B are carboxyl-terminus splice variants of ABCC9. Different combinations of Kir and SUR subunits give rise to functionally and pharmacologically distinct K$_{\text{ATP}}$ channel subtypes that are expressed in a cell type–specific manner, creating opportunities for developing selective therapeutics targeting different organ systems (Gribble et al., 1997, 1998; Inagaki et al., 1995, 1996; Nichols, 2023).

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ABBREVIATIONS: CL$_{\text{hep}}$, hepatic clearance; CL$_{\text{int}}$, in vitro intrinsic clearance; CL$_{\text{pu}}$, plasma clearance; CRC, concentration-response curve; DA, ductus arteriosus; DPBS, Delbecco’s phosphate buffered saline; FDA, Food and Drug Administration; f$_{\text{ub}}$, fraction unbound; HTD, HTDialysis; K$_{\text{ATP}}$, ATP-regulated potassium channel; Kir, inward rectifier potassium; LC-MS/MS, liquid chromatography tandem mass spectrometry; MS, mass spectrometry; PDA, patent ductus arteriosus; PK, pharmacokinetic; Q$_{\text{hep}}$, hepatic blood flow; rcf, relative centrifugal force; SAR, structure-activity-relationship; SUR, sulfonylurea receptor; t$_{\text{1/2}}$, half-life; T-Rex-HEK293, tetracycline-regulated expression human embryonic kidney-293 cell; VICB, Vanderbilt Institute of Chemical Biology.

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The best-known example of this is the Kir6.2/SUR1 channel subtype expressed in insulin-secreting β cells of the pancreas (Inagaki et al., 1995). In the fasting state, Kir6.2/SUR1 channels are kept open by the low concentration of intracellular ATP relative to ADP. However, the influx of glucose following a meal and ensuing stimulation of ATP production leads to inhibition of Kir6.2/SUR1, membrane potential depolarization, opening of voltage-gated calcium channel and calcium influx, exocytotic release of insulin into the circulation, and lowering of blood glucose levels back toward preprandial levels (Nichols, 2006). Sulfonylurea drugs, such as glibenclamide, that inhibit Kir6.2/SUR1 have been used clinically for decades to treat type 2 diabetes due to their ability to stimulate insulin secretion and lower blood glucose (Kharade et al., 2016; Nichols, 2023).

Kir6.1/SUR2B channels are expressed primarily in vascular smooth muscle cells, where they play critical roles in regulating vascular tone, blood pressure, and blood flow (Quayle et al., 1997; Yamada et al., 1997; Cui et al., 2002; Li et al., 2003; Aziz et al., 2014; McClenaghan and Nichols, 2022). Like pancreatic KATP channels, vascular KATP channels also couple metabolic energy status to cell excitability; however, Kir6.1/SUR2B opening and closing lead to vasodilation and vasoconstriction of the vasculature, respectively. Consequently, Kir6.1/SUR2B is an attractive drug target for therapeutics designed to work by modulating vascular tone (Kharade et al., 2016; Nichols, 2023). One potential therapeutic application of vascular-specific KATP channel inhibitors is the treatment of patent ductus arteriosus (PDA) in newborns (Shelton et al., 2018). The ductus arteriosus (DA) is a fetal artery that diverts blood supply away from the fluid-filled lungs and toward the placental circulation for gas exchange. Following the first breath at birth, an increase in blood oxygen and reduction in prostaglandin levels promotes DA closure, which, in turn, redirects the blood supply to the newly inflated lungs for gas exchange. PDA, resulting from the failure of the DA to contract and close, is one of the most common congenital heart conditions, affecting approximately 1 in 2000 births and up to 10% of all congenital heart diseases (Dice and Bhatia, 2007). Microarray analysis identified KCNJ8 and ABC9 transcripts as being enriched in DA tissues relative to other vessels, suggesting that Kir6.1/SUR2B channels play important roles in regulating DA tone (Shelton et al., 2014; Yarboro et al., 2018). Gain-of-function mutations in KCNJ8 or ABC9 result in Cantu syndrome, a rare genetic disorder characterized by excessive hair growth, distinctive facial features, and an enlarged heart (Harakalova et al., 2012; van Bon et al., 2012; Brownstein et al., 2013; Li et al., 2013; Nichols et al., 2013; Cooper et al., 2014). More than 50% of Cantu patients are born with symptomatic PDA (McClenaghan and Nichols, 2022). Taken together, these observations indicate that KATP channels are functionally expressed in DA tissues and represent potential drug targets for treating PDA. However, the lack of vascular-specific KATP channel inhibitors represents a critical barrier to testing this model, prompting us to take a molecular target-based approach to developing novel Kir6.1/SUR2B inhibitors. Here, we report the discovery and characterization of the first potent and selective vascular KATP channel inhibitor, VU0542270, and demonstrate that it induces vasoconstriction in mouse DA vessels.

**Materials and Methods**

**Plasmids and Cell Lines.** The pcDNA3.1-SUR2B plasmid was generously provided by Dr. Colin Nichols (Washington University). The pcDNA3 TO-Kir6.1 plasmid was synthesized by GenScript. Stably transfected tetracycline-regulated expression human embryonic kidney-293 (T-Rex-HEK293) cells expressing both plasmids were generated by cotransfecting cells with Lipofectamine LTX followed by antibiotic selection and clonal selection using the thallium flux assay described below. Monoclonal lines expressing robust pinacidil-activated thallium flux were selected for high KATP channel activity. T-Rex-HEK293 cells expressing Kir6.1/SUR1 were created as described previously (Raphemot et al., 2014). The cell culture media contained Dulbecco’s modified Eagle’s medium ( Gibco, 11965-092) supplemented with heat-inactivated FBS (bio-technue, Minneapolis, MN S11150H,10%), Blastocidin HCl (Gibco, A11319-03, 10 μg/mL), Penicillin-Streptomycin (Gibco, 15104-227, 2 mM), G418 Sulfate (CORNING, Corning, NY, 10-234-CSR, 1 mg/mL) and Hygromycin B (Invitrogen, Carlsbad, CA, 10687-010, 250 μg/mL). For selectivity assays, HEK293T cells were cotransfected with Kir6.x and SURx with Lipofectamine LTX and incubated for 48 hours before thallium flux assays.

**Thallium Flux Assays.** Quantitative thallium flux assays of Kir6.1/SUR2B activity were performed essentially as described previously for Kir6.2/SUR1 (Kharade et al., 2014; Kharade et al., 2019). Stable T-Rex-HEK293-Kir6.1/SUR2B cells or transiently transfected HEK293T cells were plated in polyamine-coated, clear-bottomed, black-walled 384-well plates and were cultured (37°C/5% CO2) overnight in Dulbecco’s modified Eagle’s medium (Gibco, Carlsbad, CA, 11995-065) containing 10% FBS (2 mM Penicillin-Streptomycin). The following day, cells were washed with assay buffer (i.e., Hank’s balanced salt solution/20 mM HEPES) and loaded for 1 hour with thallium sensor dye Brilliant Thallium (Ion Bioscience, San Marcos, TX) at room temperature. Dye-loaded cells were washed with assay buffer and transferred to a Panoptic Kinetic Imaging Plate Reader (Wavefront Bioscience, Franklin, TN). Control and test compound treatments were for 4 minutes prior to adding 0.5 mM chloride-free thallium stimulus buffer (Ion Bioscience) to initiate thallium flux. Live-cell measurements were collected at 1 Hz (482/35 nm excitation and 536/40 nm emission) for 6 minutes. The following control activators and inhibitor were used for assay development and library screening: pinacidil (10 μM; SUR2-specific opener), VU0071063 (30 μM; SUR1-specific opener) (Raphemot et al., 2014), and glibenclamide (10 μM; SUR1/SUR2 inhibitor). Selectivity assays against other Kir channels were performed essentially as described previously (Kharade et al., 2018; McClenaghan et al., 2022).

**High-Throughput Screening.** Test compounds from the Vanderbilt Institute of Chemical Biology (VICB) Discovery Collection were screened against Kir6.1/SUR2B in singlicate at a nominal concentration of 10 μM. Data were acquired using Panoptic Software, Waveguide (Wavefront Biosciences). Raw fluorescence values were imported for analysis into custom software (VICB, Vanderbilt University). Fluorescence versus time values for each well were normalized to the initial fluorescence value [F/F0]. Slopes of the normalized data were calculated for each well between 5 and 12 seconds following thallium stimulation and normalized to the percentage of maximal activator (pinacidil or VU0071063) response for each 384-well plate. Screening hits were defined as compounds that meet both Z-score and Robust Z-score criteria (i.e., 3 standard deviations from the mean and 3 mean absolute deviations of the median) and do not have activity prior to thallium addition (e.g., no fluorescent tags). Hits that retested positive and were negative against nontransfected T-Rex-HEK293 cells and Kir6.2/SUR1 cells were tested in triplicate in nine-point, threefold dilution concentration-response curves (CRCs) ranging between 30 μM and 5 nM. CRC data were plotted in GraphPad Prism (GraphPad Software, San Diego, CA) and fitted with a four-parameter logistic model to determine IC50 values for rank-ordering hit potency.

**Whole-Cell Patch Clamp Electrophysiology.** T-Rex-HEK293-Kir6.1/SUR2B cells were plated into tissue culture-treated 35-mm
activity relationship (SAR) are described in Supplemental Fig. 1.

Dose-response studies, vessels were challenged with 50 mM KCl to test reactivity. Vessels that failed to contract were voltage-clamped at a holding potential of -75 mV, stepped to -120 mV for 200 milliseconds, ramped from -120 mV to 120 mV at a rate of 20-mV increments. A ramp protocol was used to generate the current-response curves. Cells were voltage-clamped at a holding potential of -75 mV, stepped to -120 mV for 200 milliseconds, ramped from -120 mV to 120 mV at a rate of 1.2 mV/msec, and held at 120 mV for 100 milliseconds. This voltage protocol was repeated every 5 seconds. Pharmacology experiments were terminated by applying the nonselective K\textsubscript{ATP} channel inhibitor glibenclamide (10 \mu M) to block the expressed currents and measure residual leak current. Cells exhibiting <90% block by glibenclamide were excluded from the analysis. Data acquisition and analysis were performed using the pClamp 9.2 software suite (Molecular Devices). For the step protocol, mean current amplitude during the last 50 milliseconds of the 150-milliseconds step was used for current-voltage (IV) calculations. For the ramp protocol, mean current amplitude of the cell at 120 milliseconds over 15 milliseconds was used. IC\textsubscript{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).

**Pharmacokinetics of VU0542270 in Rats after Intravenous Administration.** All animal housing and experimental procedures were approved by the Vanderbilt University Animal Care and Use Committee and followed the guidelines set forth by the Guide for the Care and Use of Laboratory Animals. VU0542270 was formulated as a solution in ethanol, PEG400, and saline (1:4.5 v/v, respectively) at a concentration of 1 mg/mL and administered as a single 1-mg/kg IV dose (1 mL/kg) to male Sprague Dawley rats (n = 2; between 366–418 g body weights) via injection into a surgically implanted jugular vein catheter. Blood samples were collected serially from a surgically implanted carotid artery catheter in each animal over multiple postadministration time points (0.033, 0.117, 0.25, 0.5, 1, 2, 4, 7, and 24 hours) into chilled K2EDTA anticoagulant-fortified tubes and immediately placed on wet ice. The blood samples were then centrifuged (1700 relative centrifugal force (rcf), 5 minutes, 4°C) to obtain plasma samples, which were stored at -80°C until analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS). The intravenous pharmacokinetic (PK) data were used for the calculation of relevant intravenous pharmacokinetic parameters (plasma clearance (CL\textsubscript{p}), volume of steady state, elimination half-life (t\textsubscript{1/2}), and mean residence time). In vivo pharmacokinetic parameters of VU0542270 were determined from the observed individual animal time-versus-concentration data using noncompartmental analysis via WinNonlin (WNL; v.5.3, Pharsight Corp., Mountain View, CA).

**Brain: Plasma Distribution Determination.** VU0542270 was formulated as a solution in ethanol, PEG400, and saline (1:4.5 v/v, respectively) and administered to male Sprague Dawley rats (n = 1) intravenously at 0.2 mg/kg. At 15 minutes postdose, a blood sample was collected terminally into chilled K2EDTA anticoagulant-fortified tubes and immediately placed on wet ice. The blood sample was then centrifuged (1700 rcf, 5 minutes, 4°C) to obtain a plasma sample. At the same postadministration time point(s), a whole-brain sample was obtained by rapid dissection, rinsing with PBS, and immediate freezing in individual tissue collection boxes (dry ice). All brain and plasma samples were stored at -80°C until analysis by LC-MS/MS. In vivo brain:plasma distribution partition coefficient of VU0542270 was determined from the observed animal brain versus plasma concentrations.

**Samples Preparation for Bioanalysis.** Plasma samples from the in-life phases of the studies were thawed at ambient temperature (benchtop) and then aliquots (20 \mu L per sample) were transferred to a 96-shallow-well (V-bottom) plate. Matrix-matched quality-control samples and a standard curve of VU0542270 (1 mg/mL DMSO stock solution) were prepared in blank rat plasma (K2EDTA treated) or blank brain homogenate via serial dilution and transferred (20 \mu L) to the plate along with multiple blank plasma and brain homogenate samples. Acetonitrile (120 \mu L) containing internal standard (50 nM carbamazepine) was added to each well of the plate to precipitate protein. The plate was then centrifuged (4000 rcf, 5 minutes, ambient temperature), and the resulting supernatants (60 \mu L each) were transferred to a new 96-shallow-well (V-bottom) plate containing an equal volume (60 \mu L per well) of water (Milli-Q purified). The plate was then sealed in preparation for LC-MS/MS analysis (see below).

Preparation of brain samples was identical to that of plasma samples except for the following modifications. While thawing, brains were weighed (inside their collection boxes using a universal empty collection box tare weight) and then subjected to mechanical homogenization (Mini-BeadBeater, BioSpec Products, Inc., Bartlesville, OK) in the presence of zirconia/silica beads (1.0 mm) and extraction buffer (isopropanol:water, 7:3, v/v; 3 mL per sample, prepared for postquantitation). Homogenized brain samples were then centrifuged (4000 rcf, 5 minutes, ambient temperature), and the resulting supernatants were diluted in three volumes of plasma (dilution factor of 4). An aliquot (20 \mu L) was transferred to a 96-shallow-well (V-bottom) plate.

**Binding of VU0542270 in Plasma from Mouse, Rat, And Human.** Determination of VU0542270 fraction unbound (fu) in plasma from mouse, rat, and human was conducted in vitro via
equilibrium dialysis using high-throughput dialysis (HTD) membrane plates. Dialysis membranes (four paired strips per HTD assay) were hydrated as described by the manufacturer and inserted into the HTD plate, which was assembled and prepared for sample addition by the dispensing of blank buffer (Delbecco’s phosphate buffered saline (DPBS), 100 μL/well) into the “top half” of each membrane-split well. VU0542270 was diluted into plasma from each species (5 μM final concentration), which was aliquoted in triplicate to the “bottom half” of the prepared HTD plate wells. The HTD plate was sealed and incubated for 6 hours at 37 °C. Following incubation, each well (both top and bottom halves) was transferred (20 μL) to the corresponding well of a 96-shallow-well (V-bottom) plate. The daughter plates were then matrix matched (buffer side wells received equal volume of plasma, and plasma side wells received equal volume of buffer), and extraction solution (120 μL; acetonitrile containing 50 mM carbamazepine as IS) was added to all wells of both daughter plates to precipitate protein and extract test article. The plates were then sealed and centrifuged (3500 rcf) for 10 minutes at ambient temperature. Supernatant (60 μL) from each well of the daughter plates was then transferred to the corresponding wells of new daughter plates (96-shallow-well, V bottom) containing water (Milli-Q, 60 μL/well), and the plates were sealed in preparation for LC-MS/MS analysis (see below).

\[ f_u = \frac{1}{4} \left( \frac{1}{2} \right) - 1 + \frac{1}{4} \]  

Mean values for each species were calculated from three replicates.

Binding of VU0542270 in Brain Homogenate from Mouse and Rat. Determination of VU0542270 \( f_u \) in plasma from mouse and rat was conducted using the same methodology and procedure as described for plasma protein binding assay with the following modifications: 1) a final compound concentration of 1 μM was used, and 2) naïve rat brains were homogenized in DPBS (1:3 composition of brain-DPBS, w/v) using a Mini-Bead Beater machine to obtain brain homogenate.

The diluted fraction unbound (\( f_u \)) in brain was calculated as (analyte to IS mass spectrometry (MS) peak area ratio from Trans-buffer side)/(analyte to IS MS peak area ratio from Cis-plasma side). Undiluted fraction unbound for the brain was calculated using the following equation:

\[ f_u = \frac{1}{2} \left( \frac{1}{2} \right) - 1 + \frac{1}{4} \]

Mean values for each species were calculated from three replicates.

**Intrinsic Clearance of VU0542270 in Rat, Mouse, and Human Liver Microsomes.** The in vitro intrinsic clearance (CL\(_{int}\)) of VU0542270 was investigated in commercially obtained hepatic microsomes from rat, mouse, and human donors using the substrate depletion (i.e., loss-of-parent versus time, or \( t_{1/2} \) method) approach with analyte detection via LC-MS/MS. For each species, mean per cent parent remaining values at each time point were calculated from raw data (analyte:IS peak area ratios) and used to determine in vitro \( t_{1/2} \) and CL\(_{int}\).

Experiments were carried out using a robot-assisted liquid handling system (TECAN model EVO 200). VU0542270 was incubated (1 μM final concentration) in buffer (100 mM potassium phosphate, pH 7.4, with 3 mM MgCl\(_2\) containing hepatic microsomes (0.5 mg/mL final concentration) from multiple species, discretely, at 37 °C under constant orbital shaking. After 5 minutes (preincubation), reactions were initiated by the addition of NADPH (1 mM final concentration). At selected time intervals (0, 3, 7, 15, 25, and 45 minutes) post addition of NADPH, aliquots (50 μL) were taken and placed into a 96-shallow-well plate containing ice-cold acetonitrile (150 μL) with carbamazepine (IS, 50 nM). The plates were then centrifuged (3000 ref at 4 °C) for 10 minutes. The supernatants were transferred to a new 96-shallow-well daughter plate and diluted (1:1 v/v) with water (Milli-Q filtered). The plates were then sealed in preparation for LC-MS/MS analysis (see below).

Raw LC-MS/MS peak area data generated from the assay samples were used to construct natural log-transformed per cent parent remaining versus time plots. In vitro VU0542270 \( t_{1/2} \) values were obtained using the following equation:

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

where \( k \) is the slope from linear regression analysis of the natural log-transformed data (using means from all replicates at each time point). Resulting \( t_{1/2} \) values were then used to calculate hepatic CL\(_{int}\) values according to the following equation and with the use of species-specific scale-up factors for liver weight (grams) per total body weight (kg):

\[ CL_{int} = \frac{0.693}{T_{1/2}} \times \frac{1 \text{ mL incubation}}{0.5 \text{ mg microsomes}} \times \frac{45 \text{ mg microsomes}}{1 \text{ gram liver}} \]

\[ x \frac{a \text{ gram liver}}{kg \text{ body wt}} \]

Predicted hepatic clearance (CL\(_{hep}\)) was calculated using the following equation:

\[ CL_{hep} = \frac{Q_b + CL_{int}}{Q_b + CL_{hep}} \]

\( Q_b \) represents hepatic blood flow (ml/min per kg): 21 for human, 70 for rat, and 90 for mouse.

**LC-MS/MS Analysis.** Prepared samples were injected (10 μL each) onto an AB Sciex triple quad-4500 mass spectrometer system with an Agilent 1290 Infinity Binary Pump and multisampler. Analytes were separated on a reverse phase column Phenomenex Kinetex C18 (50 × 2.1 mm, 5 μm) that was thermostated at 40 °C. High-pressure liquid chromatography mobile phase A was 0.5% formic acid in water (pH unadjusted); mobile phase B was 0.5% formic acid in acetonitrile (pH unadjusted). A 5% B gradient was held for 0.2 minutes and was linearly increased to 90% B over 0.8 minutes, with an isocratic hold for 0.5 minutes, before transitioning to 10% B over 0.05 minutes. The column was re-equilibrated (1 minute) before the next sample injection. The total run time was 2.55 minutes, and the high-pressure liquid chromatography 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 both analyte (m/z 335.7–236.7 at 35 eV) and internal standard (m/z 237.0–193.9 at 25 eV). Quantitation of VU0542270 was performed via AB Sciex Analyst software using the raw analyte:IS peak area ratios. The typical detection range for VU0542270 was 0.5 ng/mL to 5000 ng/mL, utilizing a quadratic equation regression with 1/x2 weighting.

Correction for dilution of all brain samples (in extraction buffer and subsequently in blank plasma as previously described) was performed postquantitation. The corrections for dilution in extraction buffer employed correction factors specific to brain weight (2.63x) and to dilution of brain extract in plasma (4x).

**Results**

**Kir6.1/SUR2B Thallium Flux Assay.** The thallium flux assay measures the inward movement of thallium through the Kir6.1/SUR2B channel pore after opening with pinacidil (Fig. 1A). We confirmed the functional expression of Kir6.1/SUR2B in T-Rex-HEK293 cells by comparing their pharmacological responses to the SUR2-specific opener, pinacidil, and SUR1-specific opener, VU0071063. T-Rex-HEK293-Kir6.2/SUR1 cells were used as a comparison. As expected, pinacidil
Percent inhibition is calculated using the slope values during plots showing screening data for pinacidil, glibenclamide, from data analysis and repeated on a subsequent day. Violin Z
m 10 control wells that received either 10 Kir6.1/SUR2B channels. Screening plates contained quality from the VICB library for inhibitors of pinacidil-activated that the assay is robust and reproducible.

age Z
strating a clear separation of the two cell populations. The aver-
ge stimulation with an EC50 of 7.3 ± 2.4 μM (n = 5 wells/dose) but had no effect on Kir6.2/SUR1 (Fig. 1B). In contrast, VU0071063 opened Kir6.2/SUR1 (EC50 = 15.3 ± 0.7 μM; n = 5 wells/dose) but not Kir6.1/SUR2B (Fig. 1C). Thus, heterologous expression of Kir6.1/SUR2B in HEK293 cells recapitulates the pharmacological properties of vascular KATP.

Kir6.1/SUR2B-mediated thallium flux was tolerant to DMSO at doses up to 0.625% v/v (Fig. 1D), indicating that DMSO has no confounding effects at a screening concentration of 0.1% v/v. Assay uniformity was evaluated in fluorescent checkerboard assays where every other well of a 384-well plate was treated with 10 μM pinacidil or 10 μM pinacidil plus 10 μM glibenclamide (Fig. 1E) before stimulating thallium flux. A plot of thallium-induced fluorescence from activated (pinacidil) and inhibited (pinacidil + glibenclamide) wells is shown in Fig. 1F, demonstrating a clear separation of the two cell populations. The average Z' calculated from 150 plates over 10 days was 0.57, showing that the assay is robust and reproducible.

**Discovery of VU0542270.** We screened 47,872 compounds from the VICB library for inhibitors of pinacidil-activated Kir6.1/SUR2B channels. Screening plates contained quality control wells that received either 10 μM pinacidil alone or 10 μM pinacidil + 10 μM glibenclamide to allow calculation of Z' for each plate. Any plates with Z' below 0.5 were rejected from data analysis and repeated on a subsequent day. Violin plots showing screening data for pinacidil, glibenclamide, inhibitors, and inactive test compounds are shown in Fig. 2A. Percent inhibition is calculated using the slope values during thallium plus pinacidil stimulation and normalized to maximal inhibition values represented in the glibenclamide plus pinacidil controls. Test compounds that were associated with modulation of the pinacidil-stimulated response in real-time fluorescent measurements were designated as hits and selected for confirmation testing (see Materials and Methods). Hits were retested at 10 μM in duplicate in Kir6.1/SUR2B cells, nontransfected HEK293 cells (control cells), and Kir6.2/SUR2 cells. Of 639 compounds that retested positive in both replicate wells and did not have activity in parental cells or Kir6.2/SUR1 cells, 99 were selected for dose-response experiments based on their rank in inhibition. The most potent Kir6.1/SUR2B channel inhibitor identified from this screen was an N-aryl-N'-benzyl urea analog, which we termed VU0542270.

**VU0542270 Potency.** Voltage-clamp electrophysiology was used to confirm the inhibitory activity of VU0542270 toward Kir6.1/SUR2B. Whole-cell currents were small in control bath solution (Fig. 3A, top left panel) but increased dramatically following channel activation with 1 μM pinacidil (Fig. 3A, top right panel). Bath application of 10 μM VU0542270 in the continued presence of pinacidil led to a complete inhibition of KATP current (Fig. 3A, bottom left panel). No further inhibition was observed with the addition of 10 μM glibenclamide (Fig. 3A, bottom right panel). Mean ± S.D. current amplitude recorded at 120 mV under these conditions is summarized in Fig. 3B. A timecourse of a typical pharmacology experiment used to determine concentration-response relationships is shown in Fig. 3C. Kir6.1/SUR2B activation by 1 μM pinacidil was slow, often requiring greater than 30 minutes to reach a steady state. Once
whole-cell currents stabilized in the presence of pinacidil, escalating doses of VU0542270 in the continued presence of pinacidil were applied to establish dose responses. Experiments were terminated by bath addition of 10 μM glibenclamide to estimate leak current amplitude (Fig. 3C). A fit of individual data points normalized to maximal inhibition by 10 μM VU0542270 produced an IC50 concentration of ~279 nM (Fig. 3D).

**VU0542270 Is a SUR2-Specific Inhibitor.** VU0542270 could inhibit Kir6.1/SUR2B activity through interactions with the pore-forming Kir6.1 subunit, the regulatory SUR2B subunit, or both. As a first step toward determining VU0542270's mechanism of action, we exploited the ability of different Kir (i.e., Kir6.1, Kir6.2) and SUR (i.e., SUR1, SUR2A, SUR2B) subunits to form functional channels when expressed together. The six possible KATP channel subunit combinations, Kir6.1/SUR1, Kir6.2/SUR1, Kir6.1/SUR2A, Kir6.2/SUR2A, Kir6.1/SUR2B, and Kir6.1/SUR2B, were reconstituted in transfected HEK-293 cells and evaluated for VU0542270 sensitivity in 10-point

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Fig. 2. Discovery of VU0542270 in a screen of 47,872 compounds. (A) Summary of screening results. Wells were treated with 10 μM pinacidil (negative control; 0% inhibition), 10 μM pinacidil + 10 μM glibenclamide (positive control; 100% inhibition), or 10 μM pinacidil + test compounds. Each point represents the value of a single well of a 384-well plate using the slope normalized to maximal inhibition (i.e., pinacidil plus glibenclamide). Inhibitors are defined as those decreasing thallium-induced fluorescence by 3 S.D. below the mean pinacidil response and 3 mean absolute deviation below the median pinacidil plate response. Six hundred thirty-nine inhibitors (black) and 44,267 inactives are shown. Not shown are putative activators, fluorescent compounds, and retest negatives (2966). (B) Chemical structure of VU0542270. (C) Representative fluorescence traces from single wells treated with pinacidil (blue), pinacidil plus glibenclamide (red), or pinacidil plus VU0542270 (black).

Fig. 3. Electrophysiological characterization of VU0542270-dependent inhibition of Kir6.1/SUR2B. (A) Representative whole-cell currents recorded from HEK-293-Kir6.1/SUR2B cells bathed in control buffer (top, left), 1 μM pinacidil (top, right), 1 μM pinacidil + 10 μM VU0542270 (bottom, left), or 1 μM pinacidil + 10 μM glibenclamide (bottom, right). Cells were voltage clamped at a holding potential of −75 mV and stepped between −120 mV and +120 mV in 20-mV increments. Current amplitude has been normalized to cell capacitance (pA/pF). (B) Current-voltage relationships measured under the indicated conditions (n = 4–6). (C) Representative time-course of Kir6.1/SUR2B channel activation by pinacidil (1 μM) and inhibition by the indicated dose of VU0542270 or glibenclamide (10 μM). (D) CRC data showing Kir6.1/SUR2B-dependent inhibition by VU0542270 (n = 4–6 at each dose). Fitting a four-parameter logistic function to the data yields an IC50 = 278 nM.
dose-response experiments ranging from 1 nM to 30 μM. The nonspecific $K_{ATP}$ channel inhibitor glibenclamide was used as a positive control. Dose-response data for VU0542270 and glibenclamide against the major pancreatic and brain $K_{ATP}$ channel subtype, Kir6.2/SUR1, activated with either VU0071063 (Raphemot et al., 2014) or metabolic inhibition (ATP depletion) are shown in Fig. 4A, circles) or ATP depletion (IC$_{50}$ = 12 nM, VU0542270 = no fit, B) glibenclamide = 10 nM, VU0542270 = no fit, C) glibenclamide = 115 nM, and VU0542270 = 129 nM. (D) Mean ± S.D. per cent inhibition of the indicated Kir6/SUR combination with 3 μM VU0542270 (n = 9 wells/dose), M, molar.

**TABLE 1**

Selectivity profile of VU0542270 within the Kir channel family

<table>
<thead>
<tr>
<th>Potassium Channel</th>
<th>IC$_{50}$ (μM)</th>
</tr>
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<tbody>
<tr>
<td>Kir6.1/SUR2B</td>
<td>0.11</td>
</tr>
<tr>
<td>Kir6.2/SUR1</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Kir1.1</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Kir2.0</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Kir2.1</td>
<td>&gt;30</td>
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<tr>
<td>Kir2.2</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Kir3.1</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Kir3.0/Kir3.2</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Kir3.1/Kir3.4</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Kir4</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Kir4.1</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Kir4.1/Kir5.1</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Kir4.2</td>
<td>&gt;30</td>
</tr>
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</table>
Discussion

Here, we describe the discovery and characterization of what, to our knowledge, is the first selective small-molecule inhibitor of vascular Kir6.1/SUR2B KATP channels reported to date. The most salient features of VU0542270 include its moderate potency (IC₅₀ ∼100 nM), greater than 300-fold selectivity for Kir6.1/SUR2 over Kir6.2/SUR1, clean ancillary pharmacology within the Kir channel family, and ability to inhibit native vascular KATP channels expressed in DA vessels. VU0542270 therefore represents the current state of the art in vascular KATP channel inhibitors and an attractive entry point for developing novel therapeutics for disorders of vascular hypo-activity.

Many seminal advances in KATP channel pharmacology came from serendipitous discoveries or chemical optimization of existing scaffolds with medicinal chemistry. French chemist Marcel Janbon first noted in 1942 that people being treated with sulfonamides for typhoid fever exhibited symptoms of severe hypoglycemia. This was later confirmed at Auguste Loubatieres by showing that sulfonylurea drugs stimulated insulin secretion and hypoglycemia in dogs (Loubatieres-Mariani, 2007). First-generation KATP channel inhibitors were relatively weak and poorly selective; however, extensive optimization efforts over the last several decades have led to the development of numerous different structural classes of highly potent and highly selective Kir6.2/SUR1 inhibitors (Kharade et al., 2016), many of which are Food and Drug Administration (FDA)-approved for treating type 2 diabetes.

In striking contrast, the pharmacology of vascular KATP is comprised primarily of potassium channel openers, such as

![Fig. 5. Synthesis of VU0542270 and initial SAR study plan. (A) The synthetic route for VU0542270. (Ai) LAH, THF, 0°C; (Aii) SOCl₂, CHCl₃, 0°C; (Aiii) NaN₃, K₂CO₃, DMF, 70°C; (Aiv) PPh₃, EtOAc, THF, 50°C. (B) Initial two-track SAR study approaches; iterative parallel synthesis and SAR by catalog. (C) Selected compounds from the initial SAR study.](image)

![Fig. 6. VU0542270 induces constriction of isolated mouse DA vessels. Isolated DA vessel before (A) and after (B) exposure to high extracellular potassium (i.e., 50 mM bath KCl), demonstrating vascular reactivity to membrane depolarization. (C) Vasoconstriction of isolated DA vessels in response to escalating doses of glibenclamide alone (black circles) or together with pinacidil (open circles). (D) Vasoconstriction of isolated DA vessels in response to escalating doses of VU0542270 alone (closed circles) or together with 10 μM pinacidil (open circles). Data are means ± S.D. from 4–6 vessels. M, molar.](image)

<table>
<thead>
<tr>
<th>Property</th>
<th>VU0542270</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir6.1/SUR2B IC₅₀ (nM)</td>
<td>104</td>
</tr>
<tr>
<td>MW</td>
<td>335.46</td>
</tr>
<tr>
<td>cLogP</td>
<td>3.51</td>
</tr>
<tr>
<td>TPSA</td>
<td>53.5</td>
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<td>Fₚ, plasma (mouse)</td>
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</tr>
<tr>
<td>Fₚ, plasma (rat)</td>
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</tr>
<tr>
<td>Fₚ, plasma (human)</td>
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</tr>
<tr>
<td>Fₚ, brain (mouse)</td>
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</tr>
<tr>
<td>Fₚ, brain (rat)</td>
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</tr>
<tr>
<td>Rₚ</td>
<td>0.11</td>
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<tr>
<td>CLₚₕ (mL/min per kg) (m, r, h)</td>
<td>3587, 600, 154</td>
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<tr>
<td>Predicted CLₚₕ (mL/min per kg) (m, r, h)</td>
<td>88, 63, 19</td>
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<tr>
<td>Predicted CLₚₕ,u (mL/min per kg) (m, r, h)</td>
<td>27, 6.0, 1.3</td>
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Rat intravenous PK

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
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</thead>
<tbody>
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<td>CLₚₕ (mL/min per kg)</td>
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<tr>
<td>t₁/₂ (h)</td>
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<tr>
<td>MRT (h)</td>
<td>0.19</td>
</tr>
<tr>
<td>Vₚₑ (L/kg)</td>
<td>0.20</td>
</tr>
</tbody>
</table>

CLₚₕ,u predicted hepatic clearance corrected to plasma unbound fraction; h, human; m, mouse; MW, molecular weight; r, rat; TPSA, topological polar surface area; Vₚₑ, volume of steady state.
pinacidil, P1075, minoxidil, and levromakalim. Levromakalim is an FDA-approved drug that has been used in clinical trials as a “headache trigger” related to its vasodilatory effects on intracranial arteries (Ploug et al., 2012; Clement et al., 2023). Minoxidil is the active ingredient in the topical treatment Rogaine and is believed to promote hair growth by activating SUR2-containing K<sub>ATP</sub> channels (Shorter et al., 2008). P1075 is a high-affinity analog of pinacidil that was shown recently in cryo-electron microscopy structures to bind between transmembrane domain (TMD)-1 and TMD2 of SUR2 to induce channel opening (Ding et al., 2022). Interestingly, the vascular-prefering K<sub>ATP</sub> channel inhibitor PNU-37883A (also known as U-37883A) is an analog of P1075 that exhibits “mode switching” to an inhibitor that blocks the pore of Kir6.1 independently of SUR2 (Kovalev et al., 2004). Perhaps not surprisingly given the strong structural conservation of potassium channel pores, PNU-37883A also inhibits Kir6.2 and Kir1.1 (ROMK, KCNJ1) (Wang et al., 1995; Kovalev et al., 2004), making it unsuitable for use as an in vivo tool compound.

The lack of vascular-specific K<sub>ATP</sub> channel inhibitors motivated us to take a molecular target-based approach to discovering novel SUR2-specific inhibitors. Several different structural classes of inhibitors that are selective for SUR2 over SUR1 and range in potencies from 100–500 nM were discovered (data not shown). We focused our initial attention on VU0542270 because of its potency and selectivity for Kir6.1/SUR2B over Kir6.2/SUR1 and other Kir channels, and several modifications to VU0542270 were made to identify key pharmacophores. Our initial scouting efforts failed to identify analogs with improved potency over VU0542270; however, they did reveal structural properties that are essential for high-affinity inhibition of Kir6.1/SUR2B. In general, modifications and/or decorations on the biaryl motif were tolerated as long as certain heteroatoms were in place. In addition, (thiophen-2-yl)urea-containing analogs tend to show better potencies than those having other heterocycles or alternative linkers. In-depth SAR trends will be reported as they become available.

An important outstanding question is: What makes VU0542270 specific for SUR2-containing K<sub>ATP</sub> channels? The VU0542270 binding site and/or mechanism of action does not appear to require the distal carboxyl terminus of SUR2 since VU0542270 inhibits channels containing both SUR2A and SUR2B splice variants. We hypothesize that VU0542270 inhibits the channel through distinct interactions with residues in or near the glibenclamide binding site. Recent cryo-EM structures of Kir6.2/SUR1 and Kir6.1/SUR2B with atomic-level resolution revealed the glibenclamide binding site (Martin et al., 2017; Sung et al., 2021). In both structures, glibenclamide interacts with the same binding site in SUR1 and SUR2B. In SUR1, two arginine residues, R1246 and R1300, form the primary anchor to interact with the two oxygens of the sulfonamyl group (Martin et al., 2017). In SUR2B, glibenclamide is anchored by R1213 and R1263 (Sung et al., 2021). However, glibenclamide shows higher potency against Kir6.2/SUR1 than Kir6.1/SUR2B. One key difference that may contribute to this observation is the presence of S1238 in SUR1 versus Y1205 in SUR2B. Studies have shown that SUR1-S1238Y enhances glibenclamide off rate and changes its interaction with SUR1 from irreversible to reversible as observed in SUR2B (Ashfield et al., 1999). Ongoing structural modeling and site-directed mutagenesis experiments are exploring VU0542270s molecular mechanism of action.

Gain-of-function mutations in Kir6.1 or SUR2B result in Cantu syndrome, an autosomal dominant disorder that affects multiple organ systems, including the cardiovascular system (Harakalova et al., 2012; van Bon et al., 2012; Brownstein et al., 2013; Li et al., 2013; Cooper et al., 2014) More than half of Cantu patients are born with symptomatic PDA (Nichols, 2023). We and others have shown that Kir6.1/SUR2B expression is enriched in DA vessels and may represent a druggable target for treating PDA (Shelton et al., 2014, 2018; Yarboro et al., 2018). Untreated PDA is associated with serious complications, including pulmonary edema, renal dysfunction, congestive heart failure, and bronchopulmonary dysplasia. There are currently limited pharmacotherapies available for treating PDA, leaving surgical resection as a commonly used alternative. The only two drugs approved by the FDA are indomethacin and ibuprofen, which are nonsteroidal anti-inflammatory drugs and reduce prostaglandin production by inhibiting cyclooxygenase (De Leon et al., 2023). Acetaminophen is used to lower prostaglandin production; however, its mechanism of action is unclear, and its efficacy is lower in premature infants for reasons that are unclear (Graham and Scott, 2005; El-Khuffash et al., 2014; Bardanzellu et al., 2017). Glibenclamide, when used at 100 times the clinically recommended dose, contracts and closes DA in premature mice (Nakanishi et al., 2020). This observation strongly suggests that a specific inhibitor of Kir6.1/SUR2B may offer new therapeutic opportunities for treating PDA and potentially other cardiovascular complications observed in Cantu syndrome.

Vascular K<sub>ATP</sub> channels may be therapeutic targets for other diseases as well. As noted above, Levromakalim, a Kir6.1/SUR2B channel opener, induces migraine headaches through the dilation of extracerebral arteries, suggesting that vascular K<sub>ATP</sub> channel inhibitors might help treat migraines (Clement et al., 2023). K<sub>ATP</sub> channels might also represent therapeutic targets for treating life-threatening vascular collapse during sepsis. Both Kir6.1 and SUR2B expression levels are upregulated in rats treated with lipopolysaccharide, a commonly used preclinical model of sepsis (Shi et al., 2010). In vivo studies have shown that glibenclamide can rapidly restore blood pressure and vasopressor responsiveness in lipopolysaccharide-treated animals while exhibiting no vascular effects on healthy control animals (Landry and Oliver, 1992; Vanelli et al., 1995; Zhang et al., 2023). A clinical trial studying whether glibenclamide, when used at 100 times the clinically recommended dose, contracts and closes DA in premature mice (Nakanishi et al., 2020). This observation strongly suggests that a specific inhibitor of Kir6.1/SUR2B may offer new therapeutic opportunities for treating PDA and potentially other cardiovascular complications observed in Cantu syndrome.
migraine headache, and vascular collapse in sepsis without potentially confounding effects of inhibiting pancreatic and brain Kir2.2/SUR1 channels.

Data Availability
Primary screening data not included in the article may be made available upon request.

Authorship Contributions
Participated in research design: Li, McClenan, Han, Bungard, Rathnayake, Boutaud, Lindsley, Shelton, Denton.
Conducted experiments: Li, McClenan, Rathnayake, Boutaud, Bauer, Shelton.
New reagents or analytical tools: Han, Bungard, Rathnayake, Lindsley.
Performed data analysis: Li, McClenan, Rathnayake, Boutaud, Bauer, Days, Shelton.

Wrote or contributed to the writing of the manuscript: Li, McClenan, Han, Bungard, Days, Lindsley, Shelton, Denton.

References


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Discovery and characterization of VU0542270, the first selective inhibitor of vascular Kir6.1/SUR2B K_{ATP} channels

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Molecular Pharmacology

Supplemental Fig 1. Synthesis of VU0542270

Step 1. (4-methyl-2-(thiophen-2-yl)thiazol-5-yl)methanol To a solution of ethyl 4-methyl-2-(thiophen-2-yl)thiazole-5-carboxylate (1500 mg, 5.92 mmol, 1 eq) in THF (45 mL) at 0 °C, LAH (3.26 mL, 6.51 mmol, 1.1 eq) was added and stirred for 1 h at the same temperature. The reaction mixture was then warmed to rt and concentrated in vacuo. The crude residue was then partitioned between EtOAc (150 mL) and H$_2$O (40 mL). The aqueous phase is extracted with EtOAc (3 x 100 mL). The combined organic extracts were dried (Na$_2$SO$_4$), filtered, and concentrated in vacuo. The crude residue was purified using silica gel chromatography (0-100% EtOAc in hexanes to 0-20% MeOH in DCM) to afford the title compound. (1273.6 mg, assumed theoretical yield; 1251 mg). LCMS MS-ES [M+H]$^+$ = 212.

Step 2. 5-(chloromethyl)-4-methyl-2-(thiophen-2-yl)thiazole To a solution of (4-methyl-2-(thiophen-2-yl)thiazol-5-yl)methanol (300 mg, 1.42 mmol, 1 eq) in CHCl$_3$ (3.6 mL) at 0 °C, SOCl$_2$ (0.16 mL, 2.13 mmol, 1.5 eq) was added and stirred for 2 h at the same temperature. The
reaction mixture was then warmed to rt and concentrated in vacuo. The crude residue was used for the next step without further purification (326 mg, assumed theoretical yield). LCMS MS [M-Cl+MeOH]+ = 226.

**Step 3. 5-(azidomethyl)-4-methyl-2-(thiophen-2-yl)thiazole** To a solution of 5-(chloromethyl)-4-methyl-2-(thiophen-2-yl)thiazole (326 mg, 1.42 mmol, 1 eq) in DMF (4 mL), NaN₃ (111 mg, 1.7 mmol, 1.2 eq) and K₂CO₃ (597 mg, 4.26 mmol, 4.3 eq) were added and stirred at 70 °C for 6 h. After which time, H₂O (3 mL) was added to the reaction mixture and the mixture was extracted with EtOAc (3 x 20 mL). The organic layer was then dried (Na₂SO₄) and concentrated in vacuo. The crude residue was used for the next step without further purification (335 mg, assumed theoretical yield).

**Step 4. (4-methyl-2-(thiophen-2-yl)thiazol-5-yl)methanamine** To a solution of 5-(azidomethyl)-4-methyl-2-(thiophen-2-yl)thiazole (335 mg, 1.42 mmol, 1 eq) in EtOAc (2.5 mL), a solution of PPh₃ (447 mg, 1.7 mmol, 1.2 eq) in THF (1.5 mL) was added dropwise at 50 °C. After stirring for 4 h, H₂O (1 mL) was added to the reaction mixture, and the mixture was further stirred for 4 h at 50 °C. After which time, the reaction mixture was cooled to rt and concentrated in vacuo. The crude residue was then purified using silica gel chromatography (0-100% EtOAc in hexanes to 0-20% MeOH in DCM) to afford the title compound (278.4 mg, 93%). ¹H NMR (400 MHz, MeOD) δ 7.48 – 7.43 (m, 2H), 7.05 (dd, J = 5.1, 3.7 Hz, 1H), 3.87 (s, 2H), 2.31 (s, 3H).

**Step 5. 1-((4-methyl-2-(thiophen-2-yl)thiazol-5-yl)methyl)-3-(thiophen-2-yl)urea** To a solution of (4-methyl-2-(thiophen-2-yl)thiazol-5-yl)methanamine (10 mg, 0.05 mmol, 1 eq) in 1,4-dioxane (0.5 mL), 2-isocyanatothiophene (8 mg, 0.06 mmol, 1.3 eq) and 4-methylmorpholine (20 µL, 0.14 mmol, 3 eq) were added and stirred for 3 h at rt. After which time, the reaction mixture was filtered and concentrated. The crude product was then purified using reverse-phase HPLC (9-95% CH₃CN in H₂O containing 0.05% NH₄OH) to afford the title compound (8.5 mg,
53%). $^1$H NMR (400 MHz, MeOD) $\delta$ 7.56 – 7.47 (m, 2H), 7.09 (dd, $J = 5.1$, 3.7 Hz, 1H), 6.83 (dd, $J = 5.5$, 1.5 Hz, 1H), 6.78 (dd, $J = 5.6$, 3.7 Hz, 1H), 6.53 (dd, $J = 3.7$, 1.4 Hz, 1H), 4.51 (s, 2H), 2.41 (s, 3H). LCMS MS-ES [M+H]$^+$ = 336.