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Structure-activity relationships, pharmacokinetics, and pharmacodynamics of the Kir6.2/SUR1-specific channel opener, VU0071063

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Supplemental Methods:

DMPK Methods

Plasma protein binding

The protein binding of VU0071063 was determined in plasma via equilibrium dialysis employing RED Plates (ThermoFisher Scientific, Rochester, NY). Plasma was added to the 96 well plate containing VU0071063 and mixed thoroughly for a final concentration of 5 µM. Subsequently, an aliquot of the plasma-compound mixture was transferred to the *cis* chamber (red) of the RED plate, with an phosphate buffer (25 mM, pH 7.4) in the *trans* chamber. The RED plate was sealed and incubated for 4 hours at 37°C with shaking. At completion, aliquots from each chamber were diluted 1:1 with either plasma (*cis*) or buffer (*trans*) and transferred to a new 96 well plate, at which time ice-cold acetonitrile containing internal standard (50 ng/mL carbamazepine) (2 volumes) was added to extract the matrices. The plate was centrifuged (3000 rcf, 10 min) and supernatants transferred and diluted 1:1 (supernatant: water) into a new 96 well plate, which was then sealed in preparation for LC/MS/MS analysis. Each compound was assayed in triplicate within the same 96-well plate. Fraction unbound was determined using the following equation

$$F_u = \frac{Conc_{buffer}}{Conc_{plasma}}$$

Intrinsic clearance

Mouse or rat hepatic microsomes (0.5 mg/mL) and 1 μM VU0071063 were incubated in 100 mM potassium phosphate pH 7.4 buffer with 3 mM $MgCl_2$ at 37°C with constant shaking. After a 5 min preincubation, the reaction was initiated by addition of NADPH (1 mM). At selected time intervals (0, 3, 7, 15, 25, and 45 min), aliquots were taken and subsequently placed into a 96-well plate containing cold acetonitrile with internal standard (50 ng/mL carbamazepine). Plates were then centrifuged at 3000 rcf (4° C) for 10 min, and the supernatant was transferred to a separate 96-well plate and diluted 1:1 with water for LC/MS/MS analysis. The *in vitro* half-life ($T_{1/2}$, min, Eq. 1), intrinsic clearance (CL_{int}, mL/min/kg, Eq. 2) and subsequent predicted hepatic clearance (CL_{hep}, mL/min/kg, Eq. 3) was determined employing the following equations:

(1) $$T_{1/2} = \frac{Ln(2)}{k}$$

where k represents the slope from linear regression analysis of the natural log percent remaining of test compound as a function of incubation time

(2) $$CL_{\text{int}} = \frac{0.693}{in\,vitro\,T_{1/2}} x \frac{mL\ incubation}{mg\ microsomes} x \frac{45\,mg\ microsomes}{gram\ liver} x \frac{20^{a}\ gram\ liver}{kg\ body\ wt}$$

[a]scale-up factors: of 20 (human) or 45 (rat)

(3) $$CL_{hep} = \frac{Q_h \cdot CL\,\text{int}}{Q_h + CL\,\text{int}}$$

where Q_h (hepatic blood flow, mL/min/kg) is 21 (human) or 70 (rat){Obach, 1999 #5744}{Houston, 1994 #5745}.

LC/MS/MS Bioanalysis of Samples from Plasma Protein Binding and Intrinsic Clearance Assays

Samples were analyzed on a Thermo Electron TSQ Quantum Ultra triple quad mass spectrometer (San Jose, CA) via electrospray ionization (ESI) with two Themo Electron Accella pumps (San Jose, CA), and a Leap Technologies CTC PAL autosampler (Carrboro, NC). Analytes were separated by gradient

elution on a dual column system with two Thermo Hypersil Gold (2.1 x 30 mm, 1.9 µm) columns (San Jose, CA) thermostated at 40°C. HPLC mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The gradient started at 10% B after a 0.2 min hold and was linearly increased to 95% B over 0.8 min; hold at 95% B for 0.2 min; returned to 10% B in 0.1 min. The total run time was 1.3 min and the HPLC flow rate was 0.8 mL/min. While pump 1 ran the gradient method, pump 2 equilibrated the alternate column isocratically at 10% B. Compound optimization, data collection and processing was performed using Thermo Electron's QuickQuan software (v2.3) and Xcalibur (v2.0.7 SP1).

Animal Care and Housing

All *in vivo* studies were carried out using adult male Sprague−Dawley rats (~300 g; 9-11 weeks old; Harlan, Indianapolis, IN), or age-matched adult male wild-type C57BL/6 mice (~40 g; 7-8 weeks old; Taconic Farms, Hudson, NY). Animals were group-housed under a 12/12 h light-dark cycle (lights on at 6 AM) with food and water available ad libitum unless stated elsewhere. All animal experiments were approved by the Vanderbilt University Animal Care and Use Committee, and experimental procedures conformed to guidelines established by the National Research Council Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and the number of animals used.

In vivo DMPK experimental

VU0071063 was formulated as 10% Tween 80 microsuspensions in sterile water at the concentration of 1 mg/ml and administered intraperitoneally to male Sprague- Dawley rats weighing 225 to 250 g (Harlan, Inc., Indianapolis, IN) at the dose of 10 mg/kg. The rat blood and brain were collected at 0.25 hr. Animals were euthanized and decapitated, and the brains were removed, thoroughly washed in cold phosphate buffered saline and immediately frozen on dry ice. Trunk blood was collected in EDTA Vacutainer tubes, and plasma was separated by centrifugation and stored at -80°C until analysis. Plasma

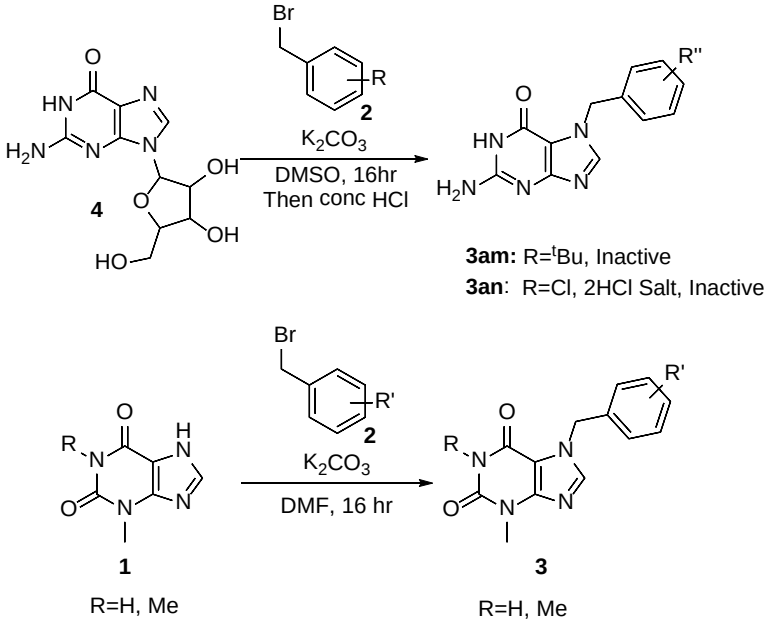
was separated by centrifugation (4000 rcf, 4°C) and stored at 80°C until analysis. On the day of analysis, frozen whole-rat brains were weighed and diluted with 1:3 (w/w) parts of 70:30 isopropanol:water. The mixture was then subjected to mechanical homogenation employing a Mini-Beadbeater™ and 1.0 mm Zirconia/Silica Beads (BioSpec Products) followed by centrifugation. The sample extraction of plasma (20 μL) or brain homogenate (20 μL) was performed by a method based on protein precipitation using three volumes of ice-cold acetonitrile containing an internal standard (50 ng/mL carbamazepine). The samples were centrifuged (3000 rcf, 5 min) and supernatants transferred and diluted 1:1 (supernatant: water) into a new 96 well plate, which was then sealed in preparation for LC/MS/MS analysis.

VU60071063 was formulated as 10% Tween 80 microsuspensions in sterile water at a concentration of 3 mg/mL and administered intraperitoneally to adult male wild-type C57BL/6 mice (Taconic Farms, Hudson, NY) at a dose of 30 mg/kg. Plasma and brain samples were collected at 15 min, 30 min, 2 hr, 4 hr time points (n=3 animals per time point) and prepared for LC/MS/MS analysis as described above.

In vivo samples were analyzed via electrospray ionization (ESI) on an AB Sciex API-5500 QTrap (Foster City, CA) instrument that was coupled with Shimadzu LC-20AD pumps (Columbia, MD) and a Leap Technologies CTC PAL auto-sampler (Carrboro, NC). Analytes were separated by gradient elution using a Fortis C18 3.0 x 50 mm, 3 μm column (Fortis Technologies Ltd, Cheshire, UK) thermostated at 40°C. HPLC mobile phase A was 0.1% formic acid in water (pH unadjusted), mobile phase B was 0.1% formic acid in acetonitrile (pH unadjusted). The gradient started at 30% B after a 0.2 min hold and was linearly increased to 90% B over 0.8 min; held at 90% B for 0.5 min and returned to 30% B in 0.1 min followed by a re-equilibration (0.9 min). The total run time was 2.5 min and the HPLC flow rate was 0.5 mL/min. The source temperature was set at 500°C and mass spectral analyses were performed using multiple reaction monitoring (MRM), with transitions specific for each compound utilizing a Turbo-

Ionspray® source in positive ionization mode (5.0 kV spray voltage). The calibration curves were constructed in blank plasma. All data were analyzed using AB Sciex Analyst software v1.5.1.

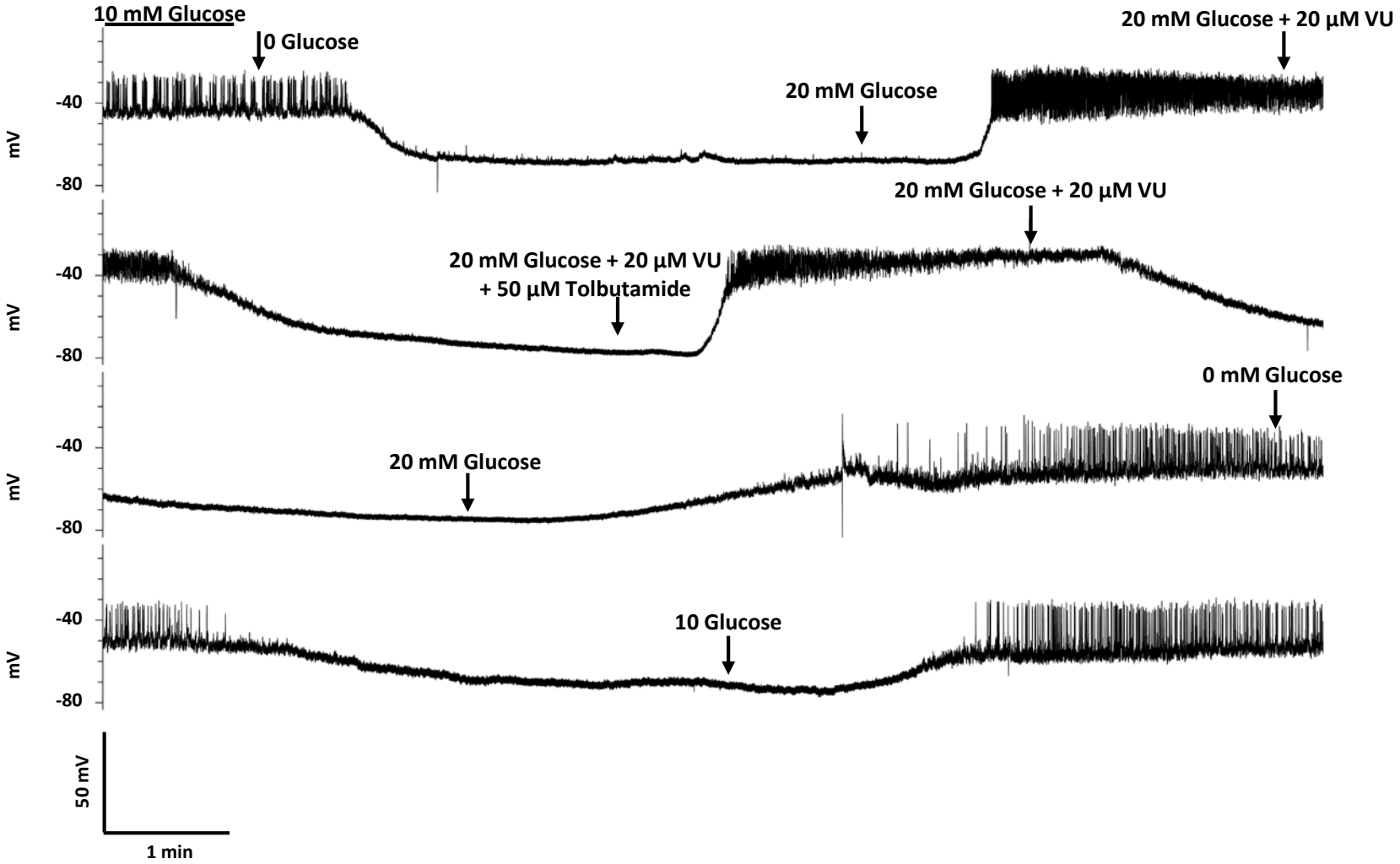
Supplemental Table S1. Summary of SAR for VU0071063



Compound	R	R’	$K_{ir}6.2/SUR1$ EC_{50} (μM)
VU0071063	Me	4-^{t}Bu	9.08
VU0071063·HCl		4-^{t}Bu	10.3
3a		3-^{t}Bu	34.7
3b		2-^{t}Bu	Inactive
3c		4-^{i}Pr	19.6
3d		4-^{i}Bu	Inactive
3e		4-OMe	Inactive
3f		3-OMe	Inactive
3g		2-OMe	Inactive
3h		4-CF_3	Inactive
3i		3-CF_3	Inactive
3j		2-CF_3	Inactive
3k		4-Cl	Inactive
3l		3-Cl	Inactive
3m		2-Cl	Inactive
3n		4-F	Inactive
3o		3-F	Inactive
3p		2-F	Inactive
3q		4-Me	Inactive
3r		3-Me	Inactive
3s		2-Me	Inactive
3t		4-OCF_3	14.9
3u		3-OCF_3	43.1
3v		2-OCF_3	Inactive

3w		4-CN	Inactive
3x		3-CN	Inactive
3y		2-CN	Inactive
3z		4-N	Inactive
3aa		3-N	Inactive
3ab		2-N	Inactive
3ac		4-$(CH_3)_2NH_2$	Inactive
3ad		4-$(CH_3)_2OH$	Inactive
3ae	H	4-^{t}Bu	Inactive
3af		4-CN	Inactive
3ag		4-F	Inactive
3ah		4-Cl	Inactive
3ai		4-Me	Inactive
3aj		4-OMe	Inactive
3ak		4-CF_3	Inactive
3al		4-OCF_3	Inactive

Figure S1



Supplemental Figure S1. Representative current clamp recording from a mouse beta-cell impaled with a sharp microelectrode. Bath perfusion with modified Krebs buffer containing no glucose (0G) causes membrane hyperpolarization that is reversed by 20 mM glucose. Application of 20 µM VU0071063 in the presence of 20 mM glucose induces membrane hyperpolarization, which is reversed by co-application of 50 µM Tolbutamide.