Discovery of a Selective Small Molecule ROMK Inhibitor for the Treatment of Hypertension

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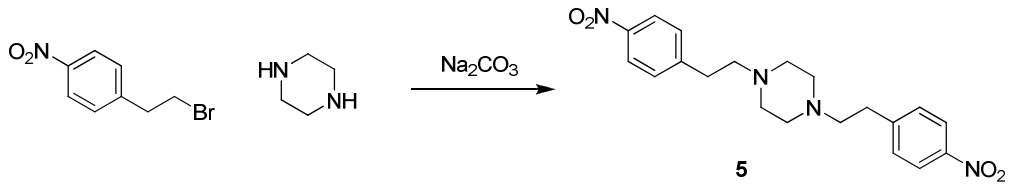
Experimental Section

Part I. Synthesis of Key Compounds

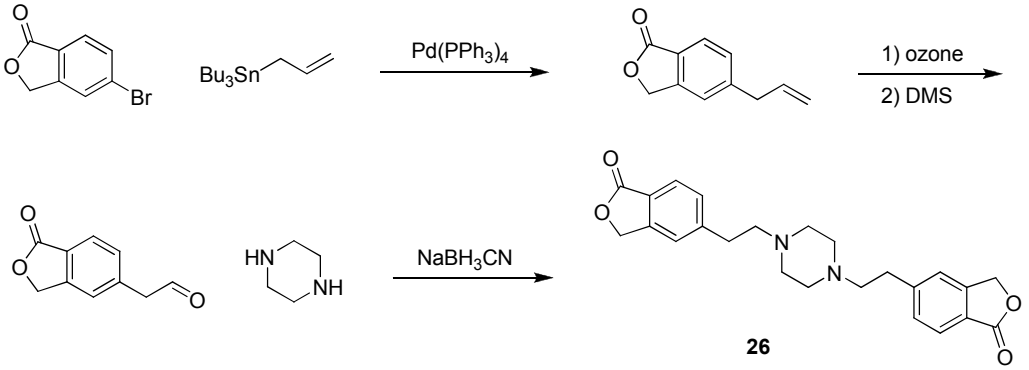
Unless otherwise noted, all materials were obtained from commercial suppliers and used without purification. Dry organic solvents were purchased from Sigma-Aldrich and packaged under nitrogen in Sure Seal bottles. Reactions were monitored using thin-layer chromatography on 250 μm plates or using Agilent 1100 series LCMS with UV detection at 254 nm and a low resonance electrospray mode (ESI). Purification of title compounds was accomplished by flash column chromatography using silica gel 60 (particle size 0.04-0.063 mm, 230-400 mesh) or medium pressure liquid chromatography on a CombiFlash Companion (Teledyne Isco) with RediSep normal phase silica gel. ^{1}H NMR spectra were recorded on a Varian spectrometer (500 MHz) at ambient temperature. Chemical shifts are reported in ppm relative to $CDCl_3$ or DMSO and coupling constants (J) are reported in hertz (Hz). Purity of final compounds was ≥95% based on analytical HPLC and NMR analysis.

Synthetic procedure for key compounds **5**, **26**, and **30**.



1,4-Bis(4-nitrophenethyl)piperazine (**5**):

To a flask charged with stir bar was added 1-(2-bromoethyl)-4-nitrobenzene (2.1 g, 9.3 mmol), piperazine (0.20 g, 2.3 mmol), sodium carbonate (0.98 g, 9.3 mmol), and DMF (20 mL). The mixture was allowed to stir at 55 °C for 16 hours. The reaction was evaporated to dryness, and the residue was diluted with water, extracted with DCM, dried over sodium sulfate, and purified by flash chromatography with DCM-MeOH gradient system. About 0.50 g orange solid (**5**, 56% yield) was collected after removal of solvent. **^{1}H-NMR** ($CDCl_3$, 500 MHz), δ 8.17 (d, *J* = 8.5 Hz, 4H), 7.39 (d, *J* = 8.5 Hz, 4H), 2.96 (t, *J* = 8.0 Hz, 4H), 2.69 (t, *J* = 8.0 Hz, 4H), 2.64 (broad s, 8H); **^{13}C-NMR** ($CDCl_3$, 500 MHz), δ 148.31, 146.81, 129.74, 124.00, 59.54, 53.16, 33.55; HRMS calculated for $C_{20}H_{25}N_4O_4$ $[M+H]^+$: 385.1870, Found: 385.1867.



5,5'-(2,2'-(Piperazine-1,4-diyl)bis(ethane-2,1-diyl))diisobenzofuran-1(3H)-one (**26**):

Step A: 5-allylisobenzofuran-1(3H)-one

To a 500 mL flask was added 5-Bromoisobenzofuran-1(3H)-one (1.5 g, 7.0 mmol), allyltributylstannane (4.4 mL, 14 mmol), Palladium tetrakis (0.81 g, 0.70 mmol), lithium

chloride (0.36 g, 8.4 mmol), and toluene (100 mL). The resulting mixture was heated to 110 °C for 2 hours. The reaction was cooled, diluted with EtOAc (100 mL), washed with brine, and filtered. The filtrate was dried over sodium sulfate, evaporated to dryness, loaded onto silica gel, and purified by flash chromatography with Hexane-EtOAc gradient system. About 1.2 g pure product (82% yield) was collected as a white solids after removal of solvents. **^{1}H-NMR** ($CDCl_3$, 500 MHz), δ 7.82 (d, *J* = 8 Hz, 1H), 7.36 (d, *J* = 8 Hz, 1H), 7.32 (s, 1H), 5.96-5.93 (m, 1H), 5.28 (s, 2H), 5.16-5.11 (m, 2H), 3.52-3.51 (m, 2H).

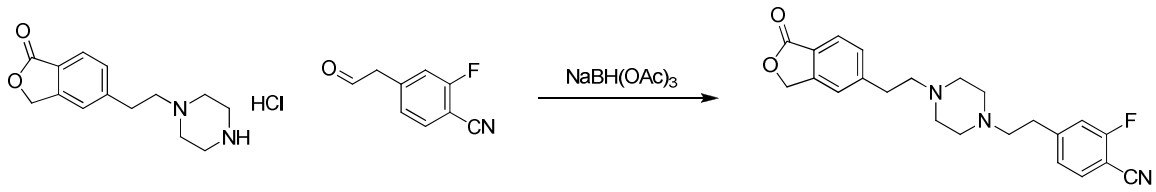
Step B: 2-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)acetaldehyde

A solution of 5-allylisobenzofuran-1(3H)-one (1.0 g, 5.7 mmol) in DCM (20 mL) and MeOH (20 mL) was cooled to -78 °C. Ozone was bubbled into the solution for 20 minutes. At that point, TLC indicated complete consumption of the starting material. After removing excess ozone by bubbling Nitrogen through the solution, dimethyl sulfide (2 mL) was added to the reaction, and the mixture was allowed to warm to RT for 1 hour. The reaction was evaporated to dryness, redissolved in DCM (100 mL), adsorbed onto silica gel, and purified by flash chromatography with Hexane and EtOAc (1 : 1). After removal of solvent, about 0.52 g of the pure product was collected (51% yield). **^{1}H-NMR** ($CDCl_3$, 500 MHz), δ 9.85 (s, 1H), 7.94 (d, *J* = 7.5 Hz, 1H), 7.41-7.38 (m, 2H), 5.35 (s, 2H), 3.92 (s, 2H).

Step C: 5,5'-(2,2'-(Piperazine-1,4-diyl)bis(ethane-2,1-diyl))diisobenzofuran-1(3H)-one

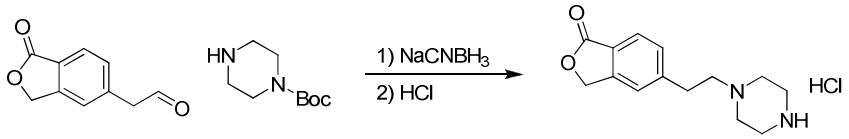
To a flask charged with 2-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)acetaldehyde (0.22 g, 1.2 mmol) and a stir bar was added piperazine (0.043 g, 0.50 mmol), Sodium cyanoborohydride (0.12 g, 2.0 mmol), Methanol (10 mL), and three drops of acetic acid. The mixture was allowed to stir at RT for 16 hours. LC showed formation of the desired product. The reaction was diluted with EtOAc, washed with brine, dried over sodium sulfate, and purified by flash chromatography with DCM-MeOH gradient system. About 0.13 g pure product (**26**, 64% yield) was collected after removal of solvent. **^{1}H-NMR** ($CDCl_3$, 500 MHz), δ 7.86 (d, *J* = 8.0 Hz, 2H), 7.41 (d, *J* = 8.0 Hz, 2H), 7.36 (s, 2H), 5.32 (s, 2H), 2.97 (t, *J* = 8.0 Hz, 4H), 2.68 (t, *J* = 8.0 Hz, 4H), 2.62 (broad s, 8H); **^{13}C-**

NMR ($CDCl_3$, 500 MHz), δ 171.27, 147.39, 130.16, 125.98, 124.14, 122.41, 115.29, 69.72, 59.95, 53.19, 34.04; HRMS calculated for $C_{20}H_{25}N_4O_4$ $[M+H]^+$: 385.1870, Found: 385.1867.HRMS calculated for $C_{24}H_{27}N_2O_4$ $[M+H]^+$: 407.1965, Found: 407.1961.



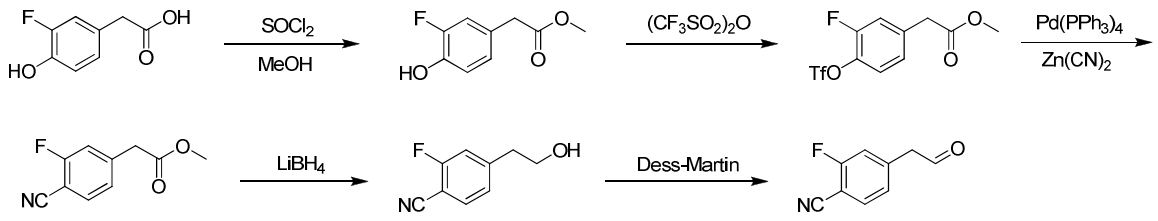
2-Fluoro-4-(2-(4-(2-(1-oxo-1,3-dihydroisobenzofuran-5-yl)ethyl)piperazin-1-yl)ethyl) benzonitrile (**30**):

Step A: 5-(2-(piperazin-1-yl)ethyl)isobenzofuran-1(3H)-one hydrochloride



To a flask charged with a stir bar was added 2-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)acetaldehyde (200 mg, 1.1 mmol), N-Boc piperazine (210 mg, 1.1 mmol), sodium cyanoborohydride (140 mg, 2.3 mmol), MeOH (10 mL), and 3 drops of acetic acid. The mixture was allowed to stir at RT for 16 hours. LC showed formation of the desired product. The reaction was quenched with sat. $NaHCO_3$, extracted with EtOAc, washed with brine, dried over sodium sulfate, adsorbed onto silica gel, and purified by flash chromatography. About 280 mg pure product (72% yield) was collected after removal of solvents. **^{1}H-NMR** ($CDCl_3$, 500 MHz), δ 7.87 (d, J = 8.0 Hz, 1H), 7.40 (d, J = 8.0 Hz, 1H), 7.37 (s, 2H), 5.32 (s, 2H), 3.53 (broad s, 4H), 3.01 (broad s, 2H), 2.72 (broad s, 2H), 2.55 (broad s, 4H), 1.49 (s, 9H). The above product was further treated with 4N HCl in dioxane to remove the Boc group. After removal of solvents, the resulting white solids were used without further purification. **LC-MS (IE, *m/z*):** 247 $[M+1]^+$.

Step B: 2-Fluoro-4-(2-oxoethyl)benzonitrile



Step B1: Methyl (3-fluoro-4-hydroxyphenyl) acetate:

(3-Fluoro-4-hydroxy-phenyl)-acetic acid (25 g, 150 mmol) was dissolved in methanol (100 mL), and thionyl chloride (5 mL) was added drop wise to the solution. The solution was heated to 85 °C for 16 hours. The reaction mixture was allowed to cool and evaporated to dryness in *vacuo*. The residue was partitioned between ethyl acetate and sat. $NaHCO_3$. The organic layer was dried over $MgSO_4$, filtered, and the filtrate was evaporated to dryness under reduced pressure to yield the crude title compound as an off white solid (25 g). **^{1}H NMR** (500 MHz, $CDCl_3$): δ 7.07 (d, J = 7.07 Hz, 1H), 7.00-6.97 (m, 2H), 3.74 (s, 3H), 3.73 (s, 2H). **LC-MS (IE, *m/z*):** 185 $[M+1]^+$.

Step B2: Methyl (3-fluoro-4-{[(trifluoromethyl)sulfonyl]oxy}phenyl)acetate:

The crude Methyl (3-fluoro-4-hydroxyphenyl)acetate (25 g) was dissolved in anhydrous dichloromethane (200 mL). 4-Dimethylaminopyridine (1.7 g) was added, followed by triethylamine (23 mL, 160 mmol). The solution was then cooled to in a dry ice and acetone bath while under nitrogen. Trifluoromethanesulfonic anhydride (28 mL, 160 mmol) was slowly added and the reaction mixture was allowed to warm to ambient temperature. The reaction mixture was then diluted with dichloromethane (200 mL) and washed with water (2 x 100 mL). The organic layer was dried over magnesium sulfate, filtered, and concentrated to dryness under reduced pressure and a cold water bath to yield the crude triflate (43 g, 99%). **LC-MS (IE, *m/z*):** 316 $[M+1]^+$.

Step B3: Methyl(4-cyano-3-fluorophenyl)acetate**:**

The crude triflate (43 g) was subsequently dissolved in anhydrous Dimethylformamide (100 mL). Zinc cyanide (7.2 g, 78 mmol) was added, and the solution was purged thoroughly with nitrogen. Tetrakis(triphenylphosphine)palladium(0) (12 g, 14 mmol) was then added and the reaction mixture was heated to 80 °C for 4 hours. After cooling to

ambient temperature and diluting with water (300 mL), ethyl acetate (500 mL) was added. The combined layers were filtered to remove any solids, the filtrate transferred to a separatory funnel, and the layers separated. The aqueous layer was re-extracted with ethyl acetate (1 x 100 mL), the organic portions were combined and dried over magnesium sulfate. The dry organics were then filtered and evaporated to dryness under reduced pressure and excess Dimethylformamide was removed by evaporation in *vacuo* at 65 °C for 1.5 hours to yield the crude title compound (42 g). The crude product was purified through silica gel chromatography (EtOAc : Hexanes = 2:3) to yield the title compound (24 g, 91%). **^{1}H NMR** (500 MHz, $CDCl_3$): δ 7.62 (t, *J* = 6.1 Hz, 1H), 7.23-7.21 (m, 2H), 3.76 (s, 3H), 3.73 (s, 2H). **LC-MS (IE, *m/z*):** 194 $[M+1]^+$.

Step B4: 2-Fluoro-4-(2-hydroxyethyl)benzonitrile:

$LiBH_4$ (1.9 mL, 3.9 mmol, 2 M in THF) was added to a stirred solution of Methyl(4-cyano-3-fluorophenyl)acetate (0.50 g, 2.6 mmol) in THF (25 ml) at 0 °C. The resulting solution was stirred for 12 hours. Water (10 mL) was added, and the resulting solution was extracted with dichloromethane (2 × 50 mL). The combined organic layers were dried over $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography eluting with EtOAc–Hexanes (7:3 → 1:1) to give the product as colorless oil (0.40 g, 94%); **^{1}H NMR** (500 MHz, $CDCl_3$) *δ* 7.60 (dd, *J* = 7.4, *J* = 7.1 Hz, 1H), 7.30 (s, 1H), 7.18 (dd, *J* = 7.3, *J* = 9.1 Hz, 1H), 3.95 (t, *J* = 6.2 Hz, 2H), 2.93 (t, *J* = 6.3 Hz, 2H); **LC-MS (IE, *m/z*):** 166 $[M+1]^+$.

Step B5: 2-Fluoro-4-(2-oxoethyl)benzonitrile:

To a stirred solution of 2-Fluoro-4-(2-hydroxyethyl)benzonitrile (0.40 g, 2.4 mmol) in dry CH_2Cl_2 (20 mL) at 0 °C was added Dess–Martin periodinane (1.5 g, 3.6 mmol) in one portion. The mixture was stirred for 12 hours at RT and quenched with a 1:1 mixture of saturated $Na_2S_2O_3$ (10 mL) and saturated $NaHCO_3$ (10 mL). The resulting mixture was diluted with CH_2Cl_2 (70 mL) and the layers were separated. The aqueous phase was extracted with CH_2Cl_2 (2 × 50 mL). The combined organic phases were washed with brine, dried (Na_2SO_4), and concentrated in *vacuo* to give crude aldehyde (0.40 g, 99%) as

colorless oil. The residue was used in the next step without further purification. **LC-MS (IE, *m/z*):** 164 $[M+1]^+$.

Step C: 2-Fluoro-4-[2-[4-[2-(1-oxo-3H-isobenzofuran-5-yl)ethyl]piperazin-1-yl]ethyl] benzonitrile (**30**)

To a flask charged with 2-fluoro-4-(2-oxoethyl)benzonitrile (0.39 g, 2.4 mmol) was added dichloromethane (40 mL) and 5-[2-(piperazin-1-yl)ethyl]-2-benzofuran-1(3H)-one hydrochloride [(0.68 g, 2.4 mmol), in dichloromethane (3 mL), and triethylamine (0.67 mL, 4.8 mmol)], and the mixture was stirred at room temperature for 0.5 hour. Sodium triacetoxyborohydride (2.5 g, 12 mmol) was added, and the reaction mixture was stirred at room temperature for 24 hours. The reaction was quenched with water (5 mL), and the organics were extracted with EtOAc (2 × 50 mL). The combined organic layers were washed with water (20 mL) and brine (20 mL) and dried ($MgSO_4$). Filtration followed by concentration afforded an oily residue, which was purified via mass-directed reverse-phase HPLC followed by evaporation and drying of the pure fraction obtained off white TFA salt foam which then converted to HCl salt by triturating in 1M HCl in diethyl ether (1 mL, 1 hour). Evaporation and dried under vacuum provided the final product as an off white solid (0.19 g, 20%). **^{1}H-NMR** ($CDCl_3$, 500 MHz), δ 7.85 (d, *J* = 7.5 Hz, 1H), 7.55 (dd, *J* = 8.0, 7.0 Hz, 1H), 7.39 (d, *J* = 8.0 Hz, 1H), 7.35 (s, 1H), 7.12 (m, 2H), 5.30 (s, 2H), 2.97 (t, *J* = 8.0 Hz, 2H), 2.88 (t, *J* = 7.0 Hz, 2H), 2.70-2.56 (m, 12H); **^{13}C-NMR** ($CDCl_3$, 500 MHz), δ 171.28, 164.41, 149.60, 147.70, 147.38, 133.49, 130.17, 125.97, 125.57, 124.12, 122.41, 116.99, 116.84, 114.41, 69.72, 59.96, 59.12, 53.24, 53.16, 34.06, 33.72; HRMS calculated for $C_{23}H_{25}FN_3O_2$ $[M+H]^+$: 394.1925, Found: 394.1921.

Part II. $^{86}Rb^+$ Efflux Assay Protocol

Cell Culture Conditions- CHO-DHFR- cells stably expressing hROMK1 ($K_{ir}1.1$) are grown at 37°C in a 10%CO_2 humidified incubator in Iscove's Modified Dulbecco's Medium (Gibco 12440) supplemented with HT Supplement, Penicillin/Streptomycin/Glutamine, G418 (500 µg/ml) and 10% FBS. Cells are seeded in Sterile and Tissue Culture Treated Packard CulturPlate White Opaque Microplates at a concentration of 5.0E5 – 7.0E5 cells/ml - PerkinElmer 6005680 (96-well); Corning 3707

(384 well) in complete media containing 1.5 µCi/ml Rubidium-86. Cells are incubated in 37°C-10% CO_2 incubator overnight. On the day of the experiment, the media is removed and cells are washed with low K assay buffer. $^{86}Rb^+$ efflux is initiated after addition of assay buffer ± test compound followed by 35 min incubation at room temperature. ROMK-sensitive component of efflux is defined in the presence of 10 mM $BaCl_2$. Assay buffer is removed and transferred to a plate and cells are solubilized in the presence of SDS. Radioactivity associated with assay and cell plate is determined.

Step Protocol

1. Remove cell media and wash cells with low K assay buffer (126.9 mM NaCl, 4.6 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes/NaOH; pH 7.4)
    - 200 µl for 96-well plate; 70 µl for 384-well plate
2. Add assay buffer (121.5 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes/NaOH; pH 7.4) ± test compound to cells
    - 100 µl for 96-well plate; 50 µl for 384-well plate
3. Incubate at ambient temperature (22-24°C) for 35 min
4. Remove assay buffer add it to a 96- or 384-well plate containing Microscint-20
    - 96-well Plate: 100 µl buffer, 170 µl MicroScint 20 (for TopCount)
    - 384-well plate: 20 µl buffer, 50 µl Optiphiase (for MicroLux)
5. Completely remove remaining assay buffer from cell plate
6. Solubilize cells with 1% SDS; than add MicroScint or Optiphase
    - 96-well Plate: 30 µl SDS, 170 µl MicroScint 20 (for TopCount)
    - 384-well plate: 20 µl SDS, 50 µl Optiphiase (for MicroLux)
7. Seal both cell and supernatant plates and count

Data Calculation- Radioactivity associated with the assay plate is normalized to the total radioactivity (assay + cell plates) to provide % efflux, under each condition. % efflux in the presence of 10 mM $BaCl_2$ is subtracted from each experimental point to provide the ROMK-sensitive component of $^{86}Rb^+$ efflux. In the absence of test compound, this number corresponds to 100% control efflux. IC_{50} values represent the concentration of compound that inhibits 50% of ROMK efflux. Normally, a control compound is included to support that the assay is giving consistent results compared to previous measurements, although the control is not required to obtain the results for the test compounds.