A Small Synthetic Molecule Forms Selective Potassium Channels to Regulate Cell Membrane Potential and Blood Vessel Tone

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Synthesis of compound 1

Compound 1 was prepared according to scheme above. 1a was synthesized according to the procedures described in Yoon et al., J. Org. Chem. 2000, 65, 7667-7675. Freshly distilled CH2Cl2 (50 mL) was added to a flask containing 1a (1.18 g, 3 mmol) under nitrogen atmosphere, followed by the addition of HOAt (0.53 g, 3.9 mmol),
isobutylamine (0.3 mL, 3 mmol), and finally EDC·HCl (863 mg, 4.5 mmol). After being stirred overnight, the reaction mixture was diluted with CH₂Cl₂. The organic layer was washed with 5% aqueous NaHCO₃ and brine and then dried over with anhydrous MgSO₄ and concentrated to afford 1b as an oil (1.2 g, 93%). \([\alpha]_{D}^{20} +42.6^\circ\) (c 0.74, MeOH); \(^1\)H NMR (300 MHz, CDCl₃) \(\delta\) 7.86–7.72 (m, 5H), 4.79 (s, 1H), 4.71 (dd, \(J = 7.2, 4.0\) Hz, 1H), 3.28–3.16 (m, 3H), 3.04–3.0 (m, 1H), 2.07–1.86 (m, 3H), 1.73–1.60 (m, 4H), 1.44 (s, 9H), 0.97 (d, \(J = 6.5\) Hz, 3H), 0.95 (d, \(J = 6.5\) Hz, 3H); \(^1^3\)C NMR (75 MHz, CDCl₃) \(\delta\) 169.7, 163.9, 166.0, 134.9, 133.9, 128.6, 123.8, 123.0, 88.0, 78.8, 46.6, 40.2, 31.9, 29.5, 28.4, 28.3, 22.1, 20.0; IR (CH₂Cl₂) 3392, 1734, 1707, 1670 cm⁻¹; LRMS (FAB) \(m/z\) 448 (M⁺+H); HRMS (EI) for C₂₃H₃₃N₃O₆ (M⁺) calculated 447.2364, found 447.2359.

To a solution of 1b (1.2 g, 2.7 mmol) in CH₃OH (40 mL) was added NH₂NH₂·H₂O (0.58 mL, 9.6 mmol). A white precipitate appeared after 1 hour. After stirred at room temperature for 2.5 hours, the reaction mixture was concentrated on a rotary evaporator. The residue was dissolved in CH₂Cl₂ and washed with 5% aqueous NaHCO₃ and brine. The organic layer was dried over with anhydrous Na₂SO₄ and concentrated to provide a mixture of amine and 2, 3-dihydrophthalazine-1,4-dione as solid. This mixture was immediately used in the next step without further purification. Freshly distilled CH₂Cl₂ (30 mL) was added to a flask containing the mixture got in the last step under nitrogen atmosphere, followed by the addition of HOAt (530 mg, 3.9 mmol), isophthalic acid (225 mg, 1.35 mmol), and finally EDC·HCl (860 mg, 4.5 mmol). After stirred overnight, the reaction mixture was diluted with EtOAc. The organic layer was washed with 5% aqueous NaHCO₃ and brine and then dried over with anhydrous MgSO₄ and concentrated. The crude oil was purified by flash column chromatography to afford compound 1 (688 mg, 4 steps: 30%) as a white solid. Compound 1 was characterized by the following data: \([\alpha]_{D}^{20} +15.6^\circ\) (c 0.61, MeOH); \(^1\)H NMR (300 MHz, CDCl₃) \(\delta\) 11.04 (br, 2H), 8.26 (m, 3H), 8.05 (d, \(J = 7.7\) Hz, 2H), 7.61–7.50 (m, 1H), 4.98 (br, 2H), 4.40–4.32 (m, 2H), 3.16–2.96 (m, 4H), 1.94–1.75 (m, 6H), 1.52–1.45 (m, 8H), 1.36 (s, 18H), 0.92–0.85 (m, 12H); \(^1^3\)C NMR (75 MHz, CDCl₃) \(\delta\) 170.70, 166.28, 156.89, 131.56, 131.40, 129.27, 125.63, 86.42, 79.58, 46.64, 39.85, 30.59, 29.69, 28.36, 28.09, 21.80, 20.07. IR (CH₂Cl₂) 3437, 3321, 1674 cm⁻¹; LRMS (FAB) \(m/z\) 765 (M⁺); HRMS (FAB) for C₃₈H₆₄N₆O₁₀ (M⁺+1³³Cs) calculated 897.3733, found 897.3759.

**Preparation of HPTS-loaded EYPC Liposomes**

Egg yolk L-α-phosphatidylcholine (EYPC, 91 mg, 120 µmol) was dissolved in a CHCl₃/MeOH mixture. The solution was evaporated under reduced pressure and the resulting thin film was dried under high vacuum for 3 h. The lipid film was hydrated in 1.2 mL of Buffer A, containing 10 mM HEPES, pH = 6.8, 100 mM KCl or NaCl, 0.1 mM HPTS, for 2 h. During hydration, the suspension was submitted to 5 freeze-thaw cycles (liquid nitrogen, water at room temperature). The large multilamellar liposome suspension (1 mL) was submitted to high-pressure extrusion at room temperature (25 extrusions through a 0.1 µm polycarbonate membrane,
affording a suspension of LUVs with an average diameter of 100 nm). The LUV suspension was separated from extravesicular dye by size exclusion chromatography (SEC) (stationary phase: Sephadex G-50, mobile phase: Buffer B containing 10 mM HEPES, pH = 6.8, 100 mM KCl or NaCl, and diluted with the Buffer B to give a stock solution with a lipid concentration of 10 mM (assuming 100% of lipid was incorporated into liposomes).

**Base Pulse Assay**

Typically, 100 µL of HPTS-loaded liposomes (stock solution) was suspended in 1.9 mL of the isotonic corresponding buffer and placed into a fluorometric cell. HPTS emission at 510 nm was monitored with excitation wavelengths at 403 and 460 nm simultaneously. During the experiment, 20 µL of a 1 mM DMSO solution of the compound of interest was added through an injection port, followed by injection of 20 µL of 0.5 M aqueous KOH. Addition of KOH caused a pH increase of approximately 1 pH unit in the extravesicular buffer. Maximal changes in dye emission were obtained at the end of each experiment by lysis of the liposomes with detergent (40 µL of 5% aqueous Triton X-100). The final transport trace was obtained as a ratio of the emission intensities monitored at 460 and 403 nm and normalized to 100% of transport.

In Figure 1a and b, the suspensions of EYPC liposomes containing the pH-sensitive dye HPTS in a HEPES buffer were used. Both the intra- and extravesicular solutions contained 10 mM HEPES, pH 6.8, and 100 mM KCl. At t = 100 s, 20 µL of a DMSO solution of compound 1 at the different final concentrations or 20 µL DMSO (control) was added to extravesicular solution. Then 20 µL of a 0.5 M KOH solution was added. At t = 700 s, 40 µL of 5% Triton X-100 was added to lyse the liposomes.

In figure 1e and f, the suspensions of EYPC liposomes containing the pH-sensitive dye HPTS in a HEPES buffer were used. The intravesicular solution contained 10 mM HEPES, pH 6.8, and 100 mM KCl and the extravesicular solution contained 10 mM HEPES, pH 6.8, and 100 mM MCl (M = Na⁺, K⁺). At t = 100 s, 20 µL of a DMSO solution of compound 1 at the final concentration 10 µM was added to extravesicular solution. Then 20 µL of a 0.5 M KOH solution was added subsequently to create a gradient of approximately one pH unit. At t = 700 s, 40 µL of 5% Triton X-100 was added to lyse the liposomes.

**Preparation of NMDG-Cl-filled and KCl-filled liposomes**

POPC (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine) (Avanti Polar Lipids) (80 mg, 105 µM) and PS (L-α-phosphatidylserine) (20 mg, 25 µM) were dissolved in a CHCl₃/MeOH mixture. The solution was evaporated under reduced pressure and then the thin film was dried under high vacuum for 3 h. The lipid film was hydrated in 1.3 mL of intravesicular solution (500 µM PBFI, 10 mM HEPES, pH 7.0, 100 mM NMDG-Cl) for NMDG-Cl-filled liposomes or intravesicular solution (10 mM HEPES,
pH 6.8, 100 mM KCl) for KCl-filled liposomes for 2 h. During hydration, the suspension was subjected to 5 freeze-thaw cycles from liquid nitrogen to water at room temperature. The large multilamellar liposome suspension (1 mL) was submitted to high-pressure extrusion at room temperature (25 extrusions through a 100 nm polycarbonate membrane, affording a suspension of LUVs with an average diameter of 100 nm). The LUV suspension was diluted with intravesicular solution to give a stock solution with a lipid concentration of 10 mM (assuming that 100 % of lipid was incorporated into liposomes).

Note: potassium-binding benzofuran isophthalate, PBFI, tetraammonium salt *cell impermeant* (P1265MP, Invitrogen)
CAS Name/Number: 1,3-Benzenedicarboxylic acid, 4,4'-[1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7,16-diylibis(5-methoxy-6,2-benzofurandiyl)]/ 124549-11-7

**Single-channel recording on giant liposomes**

Giant liposomes were prepared according to a modified procedure.[1] Briefly, 20 mg POPC and PS with an 80:20 (w/w) ratio were dissolved in 2 mL of distilled water. The mixture was intermittently stirred with Vortex for 20 min, and then sonicated for 10 min under nitrogen protection. The suspension was centrifuged at 160,000 g for 1 h, and then the pellet was resuspended with 200 µL of 10 mM MOPS buffer (pH 7.2) containing 5% (w/v) ethylene glycol. The resuspended sample was deposited on a clean glass slide in 15 µL aliquot and submitted to partial dehydration (3–6 h) at 4°C. Before use, the sample was rehydrated for 10 h at 4°C by using 15 µL of 200 mM KCl, NaCl or NMDG-Cl. For patch-clamp measurements of giant liposomes, 1–3 µL of hydrated liposome suspension was dropped on a Petri dish and diluted with bath solutions. Single-channel currents through giant lipidosome membranes in the presence of compound 1 were measured with cell-attached patch configuration of the patch-clamp technique. Patch pipettes (resistance, 7–10 MΩ) were filled with internal pipette solution containing (in mM): 200 KCl (or NaCl, NMDG-Cl), 10 HEPES, pH 7.4. The bath solution was composed of (in mM) 200 KCl (or NaCl, NMDG-Cl), 10 HEPES, pH 7.4. When the seal resistance reached up to 10 GΩ, single channel currents were recorded with EPC 9 patch clamp amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) in voltage-clamp mode. Pipette and membrane capacitance were electronically compensated. Ramp protocol was applied with Pulse (HEKA) software. Single channel currents were digitized at 0.15 ms sampling interval, filtered at 0.5 kHz.

**Single channel recording for potassium ions**

Symmetrical 200 mM KCl solution was used for bath and pipette solutions. When the seal resistance reached up to 10 GΩ, single channel currents were recorded with EPC 10 patch clamp amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) in voltage-clamp mode with inside-out configuration. The holding potential was kept at either +80 mV or −80 mV. Single channel current was obtained and filtered at 0.1 kHz.
Single channel recording for sodium ions
Symmetrical 200 mM NaCl solution was used for bath and pipette solutions. When the seal resistance reached up to 10 GΩ, single channel currents were recorded with EPC 10 patch clamp amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) in voltage-clamp mode with inside-out configuration. The holding potential was kept at either +80 mV or −80 mV. Single channel current was obtained and filtered at 0.1 kHz.

Permeability of 1 towards potassium ions and chloride ions
Pipette solution was composed of 200 mM KCl while bath solution was composed of 400 mM manitol. The holding potential was kept at either +80 mV or −80 mV. Single channel current was obtained and filtered at 0.1 kHz.

Relative permeability of 1 towards sodium ions and potassium ions
Unsymmetrical solutions were used. The pipette solution was composed of 180 mM NaCl and 20 mM KCl. The bath solution was composed of 180 mM KCl and 20 mM NaCl. Voltage steps from −100 mV to +100 mV were applied. Single channel current were obtained and plotted into an I-V graph. The relative permeability was calculated according to the Goldman-Hodgkin-Katz Equation.

Membrane potential measurement in liposomes
Typically, 100 µL of stock solution of KCl-filled liposomes (10 mM HEPES, pH 6.8, 100 mM KCl) was suspended in 1.9 mL of NaCl-HEPES buffer (10 mM HEPES, pH 6.8, 100 mM NaCl and 60 nM safranin O) and placed into a fluorometric cell. The emission of safranin O at 580 nm was monitored with excitation wavelength of 520 nm. During the experiment, 20 µL of compound 1 in different concentrations was added into the liposome suspension.

Perforated whole-cell membrane potential recording on HEK 293 cells
HEK 293 cell line obtained from the American Type Culture Collection was cultured in DMEM supplemented with 10 % FBS and 100 U/mL penicillin and 0.1 mg/mL streptomycin. Cells were grown at 37°C in a 5 % CO₂ humidified incubator. Perforated whole-cell membrane potential was recorded by using an EPC 9 patch clamp amplifier in current-clamp mode same as previous procedure.[2] Patch pipettes (resistance, 3–5 MΩ) were filled with a solution internal pipette solution containing (in mM): 105 K⁺-gluconate, 30 KCl, 1 MgCl₂, 10 NaCl, 10 HEPES, pH 7.2, with 250 µg/mL amphotericin B. The bath solution contained a normal physiological saline solution (NPSS) that contained in mM: 140 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, 5 HEPES, pH 7.4. After cell-attached model was formed for about 20 min, the series resistance reduced gradually. In the experiment, the cells with <50 mΩ series resistance were used for membrane potential recording. When the perforated whole-cell model was established, the membrane potential would stably maintain at about −40 mV. After the resting membrane potential maintained for 5 min, compound
1 was added into the bath. Changes of membrane potential were detected from the same cells before and after application of compound 1 at the concentration of 10 μM. The data were analyzed by PulsFit software (HEKA). The value of membrane potential after compound 1 was obtained by averaging the continuous membrane potential at the lowest level for 60 s. All experiments were performed at room temperature (22–25°C).

Blood Vessel Preparation and Isometric Tension Measurement
All animal experiments were conducted in accordance with NIH publication no. 8523 and approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong. Experiments were performed on aortae isolated from male C57 mice supplied by the Laboratory Animal Services Centre, the Chinese University of Hong Kong. The animals were housed at constant temperature (21 ±1 °C) under a 12:12-h dark-light cycle and had free access to chow diet and water. The mice were euthanized by CO₂ inhalation. The thoracic aortae were excised and placed in Krebs-Henseleit (K-H) solution containing 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25.2 mM NaHCO₃, and 11.1 mM glucose (pH 7.4 with NaOH). Following the removal of periadventitial fat, each artery was cut into segments of ∼2 mm in length. Each segment was suspended between two tungsten wires in chambers of a Multi Myograph System (610M, Danish Myo Technology A/S, Aarhus N, Denmark) for the measurement of isometric force. In some rings, the endothelium was removed mechanically by rolling the luminal surface with a tungsten wire. Each chamber was filled with 5 mL-K-H solution aerated with 95% O₂ and 5% CO₂ and maintained at 37 °C. The rings were stretched to a previously determined optimal resting tension of 3 mN. After an equilibration period of 1 h, the contractile function of the vessels was tested twice by replacing the K-H solution with a high-K⁺ solution containing 62.7 mM NaCl, 60 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25.2 mM NaHCO₃, and 11.1 mM glucose (pH 7.4 with NaOH) to test the contractility of the vessels, and washed in normal K-H solution, and finally allowed to equilibrate for 30 min. The vessel was contracted once with 10 μM phenylephrine (PE) for 10 min and then relaxed with 10 μM acetylcholine (ACh) for 4 min. The integrity or functional removal of endothelium was verified by the relaxant response to 10 μM ACh. After another washout period, the cumulative concentration-response of compound 1 was tested in aortic rings precontracted with 10 μM PE.

References: