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

Hui-Yan Zha[a,#], **Bing Shen**[b,c,#], **Kwok-Hei Yau**[a], **Shing-To Li**[a], **Xiao-Qiang Yao**[b,*], and **Dan Yang**[a,*]

[a]Morningside Laboratory for Chemical Biology, Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong, P. R. China.

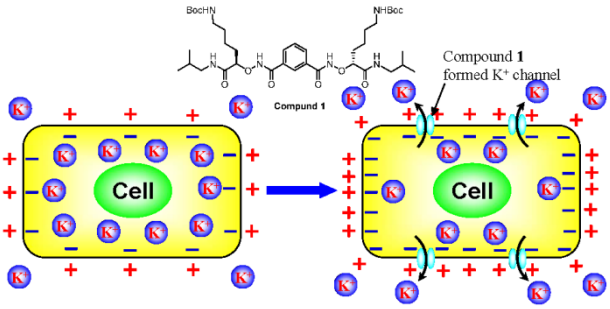
[b]School of Biomedical Sciences,, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China.

[c]Department of Physiology, Anhui Medical University, Hefei, China.

[#]These authors contributed equally to this work.

Correspondence to: Dan Yang, yangdan@hku.hk; Xiao-Qiang Yao, yao2068@cuhk.edu.hk

Abstract



In living cell membranes, K^+ permeability is higher than other ions such as Na^+ and Cl^- owing to abundantly expressed K^+ channels. Polarized membrane potential is mainly established by K^+ outward flowing because K^+ concentration in intracellular side is much higher than that in extracellular side. We have found that small synthetic molecule **1** is capable of self-assembling into selective K^+ channels, enhancing K^+ permeability and hyperpolarizing liposome membrane potential. Interestingly, molecule **1** also functions as K^+ channel hyperpolarizing living cell membrane potential and relaxing agonist-induced blood vessel contraction. Therefore, it may have potential to become a lead compound for the treatment of human diseases associated with K^+ channel dysfunction.

Key words: ion channel, synthetic ion channel, potassium channel, membrane potential

Introduction

Ion channels, membrane proteins that mediate ion flow between plasma membranes, participate in various pivotal biological processes. Specifically, potassium (K^+) channel is expressed in all kinds of cells involving in numerous physiological and pathological processes, such as the excitation of excitable cells, secretion of gland cells, cytosolic H^+/OH^- balance, apoptosis and arrhythmia.[1-4] The basic structure of natural K^+ channel is a tetrameric molecule, which is composed of four identical subunits. The subunits form a four-fold symmetrical protein complex, which is arranged around a central pore through which ions are conducted.[5-16] In literature, most of studies focus on finding new molecules, such as nicoranil, pinacidil, flupirtine, to regulate natural K^+ channels. How to create synthetic K^+ channel and mimic natural K^+ channel function still remains challenging. Although several synthetic cation channels have been created, small synthetic K^+ channel candidates possessing good K^+ channel behavior not only in artificial lipid membrane but also in living system have not been reported yet.[17-20] Previously, we discovered that a small C_2-symmetric molecule with an isophthalamide scaffold linking two *R*-(α)-aminoxy leucine units (**C11**: Scheme 1) was capable of self-assembling into selective Cl^- channels and modulating the membrane potential of living cells.[21] Herein we report compound **1** (Scheme 1), which has the isophthalamide scaffold linking two Boc-protected (α)-aminoxy lysine residues, can self-assemble into K^+ channels in liposomes and living cell membranes.

Results and Discussion

We prepared compound **1** in four steps from readily available (R)-6-(tert-butoxycarbonylamino)-2-(1,3-dioxoisoindolin-2-yloxy) hexanoic acid in an overall yield of 30% (see ESI †).

We first used the pH-sensitive fluorescent dye 8-hydroxy-1,3,6-pyrene-trisulfonate (HPTS, pyranine) to test ion transport ability and selectivity of compound **1**. The addition of a KOH solution created a gradient of approximately one pH unit. The resulting pH gradient caused H^+ efflux (outward flow) or OH^- influx (inward flow) and built up an electrostatic potential, which was expected to be compensated by the exchange of intra- and extravesicular electrolytes across the lipid bilayers of the liposomes, mediated by compound **1**.[22] In KCl-filled liposomes suspended in an isotonic KCl solution, the application of increasing concentrations of compound **1** did induce a rapid exchange of intra- and extravesicular electrolytes as indicated by the increase in relative fluorescence that accompanied the rise in intravesicular pH. This indicated that compound **1** mediated ion transport in a concentration dependent fashion (Fig. 1a/b). To clarify the species of ions mediated by compound **1**, potassium-binding benzofuran isophthalate (PBFI, see ESI †), a K^+ sensitive fluorescence dye, was employed.[23] Liposomes encapsulating 100 mM NMDG-Cl and 500 μM PBFI were prepared and then suspended in a 100 mM KCl solution. The result showed that 25 μM compound **1** markedly enhanced PBFI fluorescence intensity compared to DMSO control (Fig. 1c/d), which suggests K^+ ions moving into

liposomes and binding with PBFI. Therefore, the PBFI assay directly demonstrates that compound **1** indeed transports K^+ ions. In addition, because both K^+ and Na^+ are very important monovalent cations in biological systems, the HPTS assay was also used to demonstrate the difference in transport activity between Na^+ and K^+ for compound **1**. 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^+ (blue line), K^+ (red line)). At t = 100 s, 20 mL 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 this point, the exterior pH reached 7.8 while the interior pH remained at 6.8, which results in the electrostatic potential across the membrane. Since compound **1** could function as a cation transporter, the resulting electrostatic potential caused by H^+ efflux or OH^- influx from the liposomes can be compensated by K^+ or Na^+ influx. Since the relative fluorescence intensity increased faster in extravesicular KCl solution than in extravesicular NaCl solution (Fig. 1e/f), we concluded that compound **1** could transport K^+ faster than Na^+.

As patch clamp is a powerful tool for characterizing ion channel, single channel recording was applied to identify whether compound **1** can form ion channels in giant liposomes.[24-25] In POPC giant liposomes (POPC:PS = 4:1) with symmetric extracellular and intracellular solutions (200 mM KCl), typical single channel currents were observed in ramp recording from −100 mV to +100 mV when 5 μM compound **1**

was applied into the extracellular solution compared to DMSO control (Fig. 2a/c/d). The results demonstrate that compound **1** is capable of self-assembling into ion channels to transport ions. Furthermore, if the solutions in both sides were changed into 200 mM NMDG-Cl, the single channel currents disappeared (Fig. 2b). Because $NMDG^+$ (*N*-methyl-D-glucamine) is a huge organic cation, normally it is unable to pass through ion channels. Therefore, the results suggested that the ion channel formed by **1** is a K^+ channel, not a Cl^- channel.

To explore the selectivity between K^+ and Na^+ of the ion channel formed by **1**, patch clamp experiments with inside-out configuration were performed. With symmetrical 200 mM KCl or NaCl solution on both sides, single channel currents were observed at both positive and negative holding potentials (Fig. 3a/b), indicating that besides K^+, Na^+ can also be permeated through this synthetic ion channel formed by **1**. Next, the relative permeability of the channel formed by **1** for K^+ and Na^+ ions was examined. The concentration gradients of K^+ and Na^+ ions were created across the membrane by using 180 mM NaCl and 20 mM KCl as the pipette solution and 180 mM KCl and 20 mM NaCl as the bath solution. The I–V relationship showed that the reversal potential was shifted to -15 ± 3 mV (n = 5, Fig. 3c). Calculated from the Goldman equation, the relative permeability of K^+ to Na^+ ion was 2.2, implying that K^+ is more permeable than Na^+ in this ion channel formed by **1**. Therefore, the data of ion permeability from patch clamp corroborate with the results from the HPTS assay.

Membrane potential fluorescence assay with POPC liposomes was utilized to further characterize the ion transport property of **1**.[26] The liposomes were filled with 100 mM KCl and placed into 100 mM NaCl solution containing 60 nM safranin O. Safranin O is a membrane potential sensitive fluorescence dye and its fluorescence intensity increase represents hyperpolarization. Our results revealed that **1** induced membrane potential hyperpolarization in a dose-dependent fashion (Fig. 4a/b). Because the concentration of Cl^- is symmetric at both sides of the lipid membrane, only K^+ efflux will cause membrane potential hyperpolarization. In addition, valinomycin, which is a well-known K^+-specific carrier, was also used in our experiment (Fig. 4c/d). When 0.6 μM valinomycin was added into liposome suspension (inside vesicles: 100 mM KCl, pH 6.8; outside vesicles: 100 mM NaCl, 60 nM safranin O, pH 6.8), K^+ ions inside liposomes were transported out and the membrane potential was hyperpolarized after several minutes. Then the addition of compound **1** further enhanced the hyperpolarization of the membrane potential (Fig. 4d), indicating that **1** facilitates K^+ ion movement through lipid membrane and induces membrane potential hyperpolarization. Moreover, the data also suggest that the permeability of **1** for K^+ is much higher than that for Na^+. Since the outside solution of liposomes was NaCl and the inside solution was KCl, the asymmetric positive charge distribution and polarized potential can only be established when the permeability of **1** for K^+ is much higher than that for Na^+.

Above results are all based on cell-free studies. The big challenge is whether **1** can

self-assemble into functional K^+ channels in living cell membranes. To address this issue, we used current clamp, one type of patch clamp technique, to measure the resting membrane potential and investigate the effect of self-assembled K^+ channels on living cell.[27] HEK293 cell line, commonly used in ion channel research, was used in this study. Interestingly, the result showed that 10 μM compound **1** significantly hyperpolarized the resting membrane potential (–13 ± 1 mV, n = 7) of HEK293 cells (Fig. 4e). It is well known that intracellular solution is composed of high concentration of K^+ but of low concentrations of Na^+ and Cl^-. Reversely, extracellular solution has high concentration of Na^+ and Cl^- but low concentration of K^+. Normally, the resting membrane potential is mainly established by intracellular K^+ efflux via K^+ channels according to electrochemical driving force, because the permeability of K^+ is much higher than that of Na^+ or Cl^- in resting cells. When new K^+ channels self-assembled from **1** are present in cell membrane, K^+ permeability should be enhanced. Then more K^+ ions would move out of cells, leading to hyperpolarization of cell membrane potential. From these data, we conclude that small molecule **1** is capably of self-assembling into functional K^+ ion channels in living cell membrane and regulating resting membrane potential in living cells.

We further explored the biological application of compound **1** by testing its effects on vascular contraction evoked by phenylephrine (PE), an α_1-adrenergic agonist. As shown in Fig. 5, compound **1** relaxed PE-induced contraction in isolated mice aorta in a concentration-dependent manner and maximal relaxation was achieved (68 ± 4 %)

when its concentration reached 30 μM. The relaxant effect of compound **1** on PE-induced contraction was not affected by endothelium denudation (maximal relaxation: 72 ± 10 %), suggesting a direct action on the vascular smooth muscle cells. This is in contrast to the behavior of Cl^- channel-forming compound C11, which relaxes muscle contraction induced by high K^+ concentration but not PE.[27]

The proposed K^+ channel function of compound **1** also explains the ability of **1** to relax the PE-induced contraction (Fig. 5). It is known that one pathway of PE-induced vascular contraction is to depolarize the membrane potential of vascular smooth muscle cells[28-30], leading to Ca^{2+} entry through L-type Ca^{2+} channels.[10] In most living cells, as the equilibrium membrane potential of K^+ is more negative than the resting membrane potential, activation of K^+ channels would result in K^+ efflux and hence membrane hyperpolarization. Conceivably, synthetic K^+ channel-induced hyperpolarization would deactivate L-type Ca^{2+} channels and inhibit depolarization-mediated Ca^{2+} entry in PE-induced contraction.

Conclusions

To summarize, we have developed a small molecule **1** that not only mediates selective transport of K^+ ions in lipid membranes, but also obviously functions as K^+ channels in living system. Our data have convincingly proved that compound **1** is capable of self-assembling into K^+ channels and mediating K^+ transport across cell membrane. Therefore, this finding may provide a new avenue in K^+ channel drug development

for the treatment of severe human diseases related to malfunction of natural K^+ channels, such as long QT syndrome, episodic ataxia, Anderson-Tawil syndrome, and familial hyperinsulinemia. Future work will be directed at understanding the self-assembly behavior and exploring other biological applications of synthetic K^+ channels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

†Electronic Supplementary Information (ESI) available: Experimental procedures.

Acknowledgments

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Figure legends

Scheme 1. Chemical structures of compounds **C11** and **1.**

Figure 1. (a and b) HPTS assays with compound **1** at different concentrations from 1-100 μM. (c) Graphical representations of the PBFI fluorescence assay. (d) Representative traces showing that 25 μM compound **1** markedly enhanced PBFI fluorescence ratio. Inside vesicles: 100 mM NMDG-Cl, 500 μM PBFI, pH 7.0; outside vesicles: 100 mM KCl, pH 7.0. Compound **1** (25 μM final concentration) was added at the time indicated at the arrow. (e and f) Comparison of K^+ and Na^+ permeability of compound **1** by HPTS assay.

Figure 2. Single channel currents of compound **1** in POPC liposomes. (a) Single channel ramp recording protocol from -100 mV to +100 mV with a 0 mV holding potential. (b) Representative trace showing that no single channel current could be recorded in symmetric 200 mM NMDG-Cl in the presence of 5 μM compound **1**. (c and d) Representative traces showing that no channel current could be recorded in DMSO control (c) but typical single channel current was found in the presence of 5 μM compound **1** (d) in symmetric 200 mM KCl solution.

Figure 3. (a) Current traces of patch clamp experiment with inside-out configuration in the presence of 5 μM compound **1**. Symmetric 200 mM KCl was used. Channel

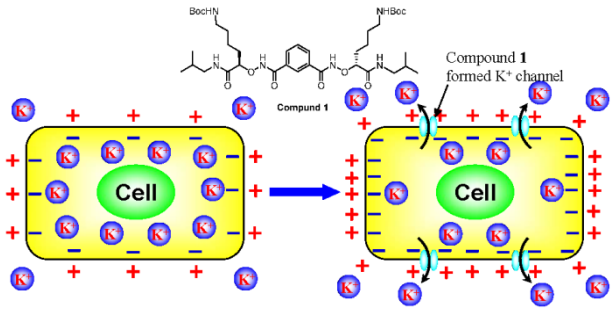
currents were observed at both positive and negative holding potential. K^+ is transported through channel mechanism. (b) Current traces of patch clamp experiment with inside-out configuration with the presence of 5 μM compound **1**. Symmetric 200 mM NaCl was used. Channel currents were observed at both positive and negative holding potential. Na^+ is transported through channel mechanism. (c) I-V relationship of patch clamp experiment with inside-out configuration in the presence of 5 μM compound **1**. 180 mM NaCl and 20 mM KCl were used in the pipette solution. 180 mM KCl and 20 mM NaCl were used in the bath solution. The reversal potential shifts to -15 ± 3 mV (n = 5). The relative permeability of K^+ to Na^+ ion is 2.2.

Figure 4. (a) Graphical representations of the safranin O membrane potential assay. (b) Representative traces showing that the application of 2.5, 5, and 10 μM compound **1** hyperpolarized the membrane potential of liposomes. Inside vesicles: 100 mM KCl, pH 6.8; outside vesicles: 100 mM NaCl, 60 nM safranin O, pH 6.8. Compound **1** was added at the time indicated at the arrow. The concentrations are all final concentrations. In the end of experiments, 10 μM melittin was added to disrupt the lipid membrane of liposomes. (c) Graphical representations of the safranin O membrane potential assay. (d) Representative traces showing that 0.6 μM valinomycin hyperpolarized the membrane potential of liposomes while the later application of 10 μM compound **1** further enhanced the hyperpolarization. Inside vesicles: 100 mM KCl, pH 6.8; outside vesicles: 100 mM NaCl, 60 nM safranin O, pH 6.8. At 100 s, 0.6 μM (final concentration) valinomycin was applied. At 500 s,

compound **1** was applied at 10 μM (final concentration). In the end of experiments, 10 μM melittin was added to disrupt the lipid membrane of liposomes. (e) The representative trace showing the resting membrane potential of HEK293 cell. 10 μM compound **1** application (at the arrow) markedly decreased membrane potential of HEK 293 cell.

Figure 5. Concentration-response curves for the vasorelaxant effect of **1** on the mouse aortic rings preconstricted by 10 μM PE. Data are expressed as mean ± SE.

Table of Contents Entry



A molecule forms a K^+-selective channel in cell membrane to regulate vascular muscle cell membrane potential and blood vessel tone.

Scheme 1

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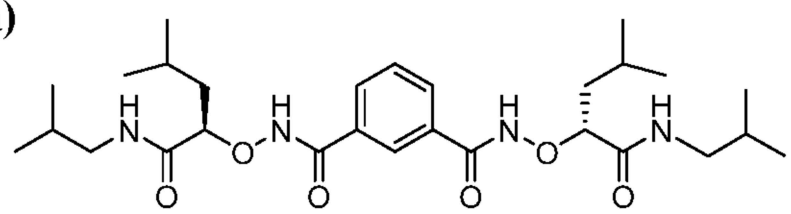

C 11

b)

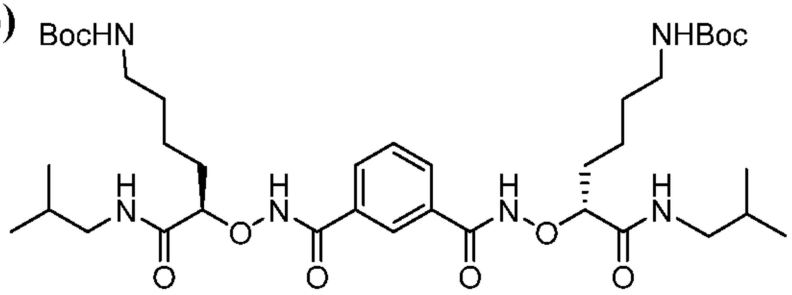

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Figure 1

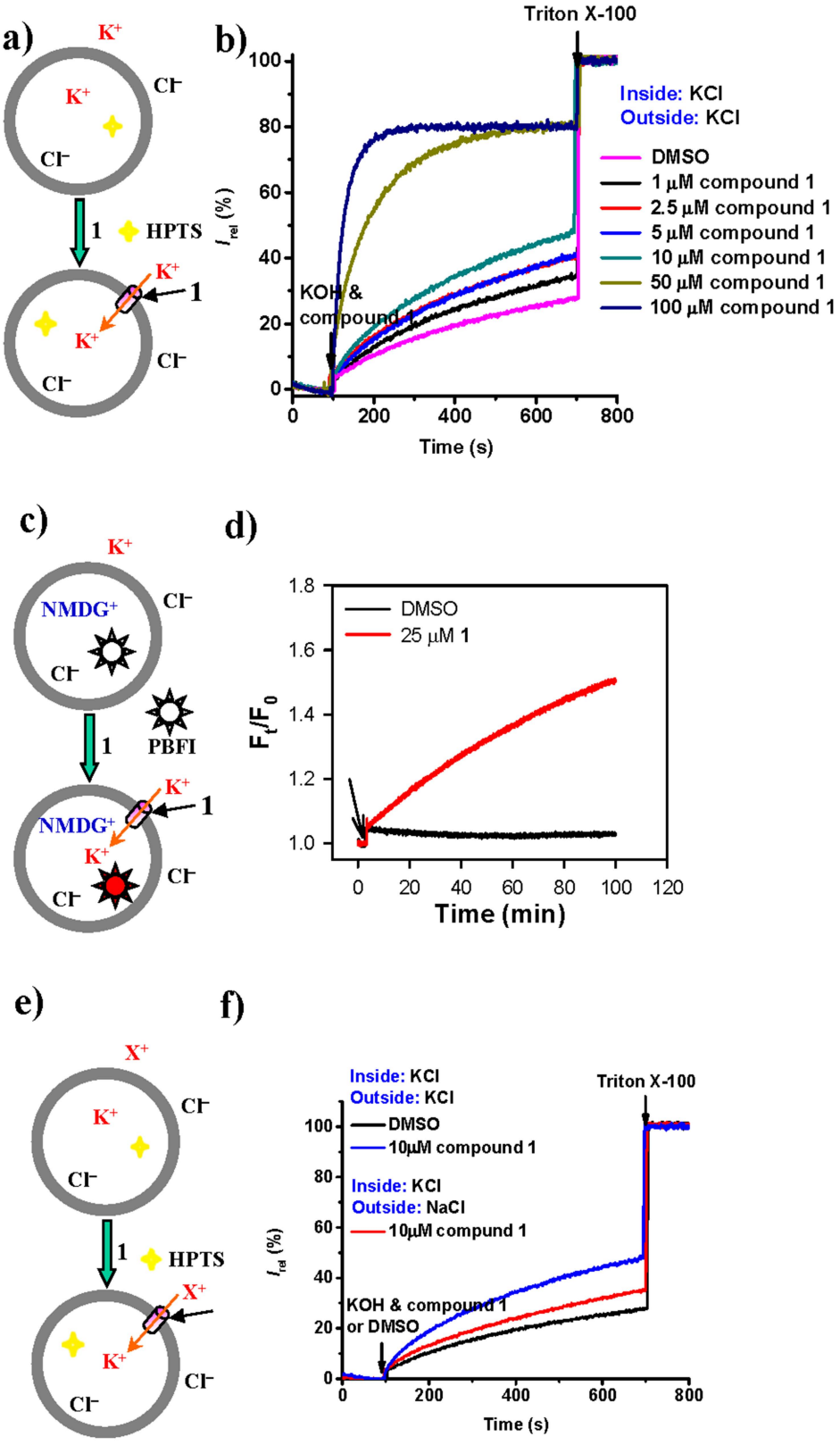
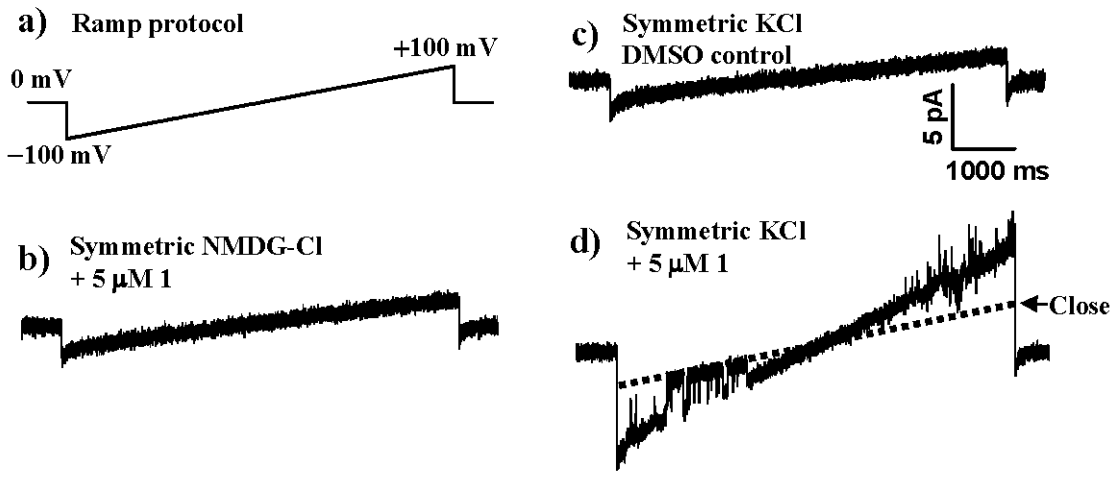


Figure 2



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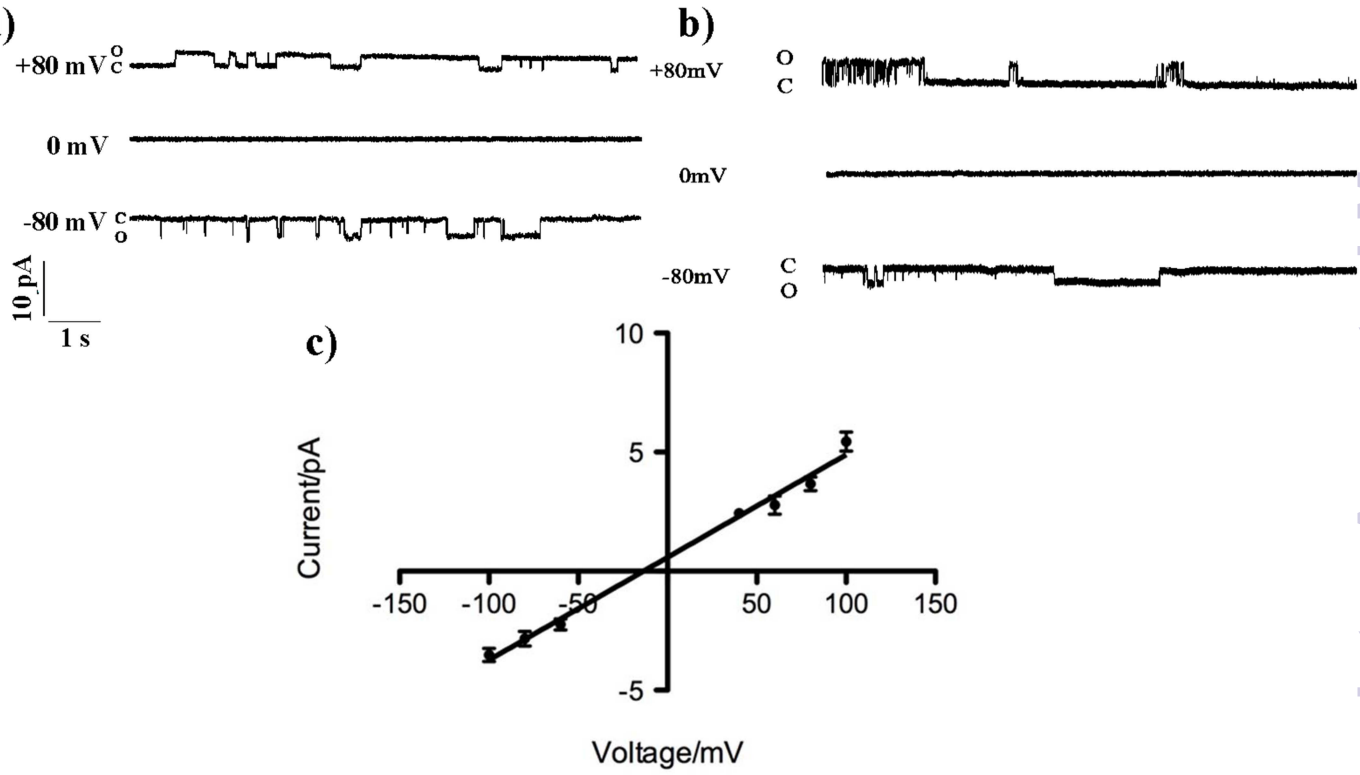


Figure 4

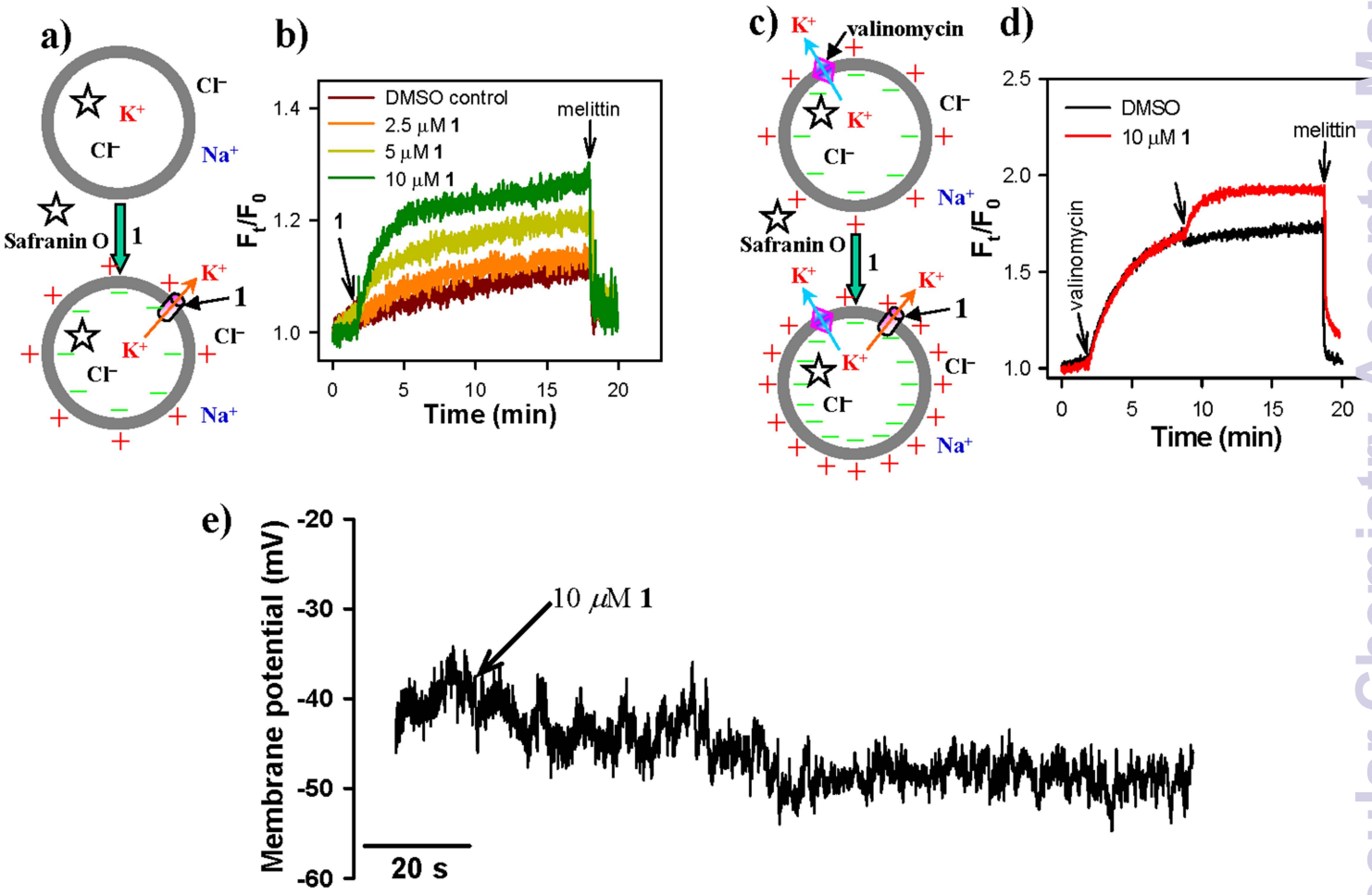
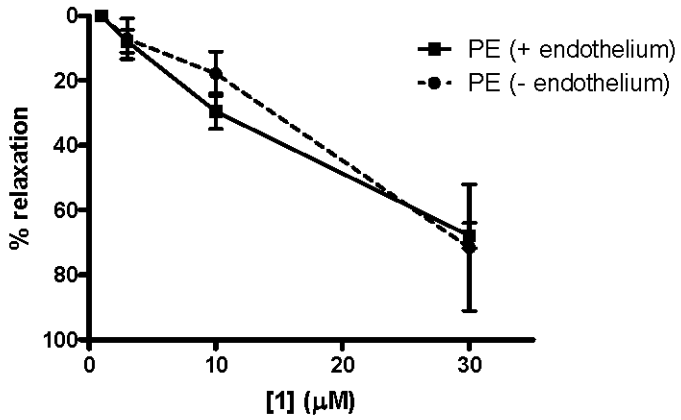


Figure 5



Electronic Supplementary Information

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

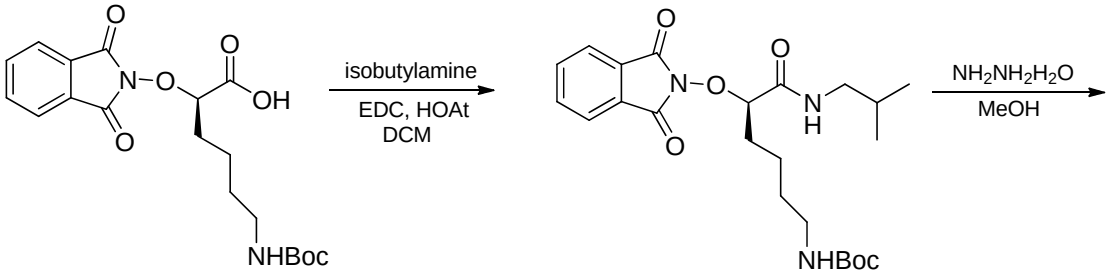
Hui-Yan Zha[a,#], Bing Shen[b,c,#], Kwok-Hei Yau[a], Shing-To Li[a], Xiao-Qiang Yao[b,*], and Dan Yang[a,*]

[a]Morningside Laboratory for Chemical Biology, Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong, P. R. China.

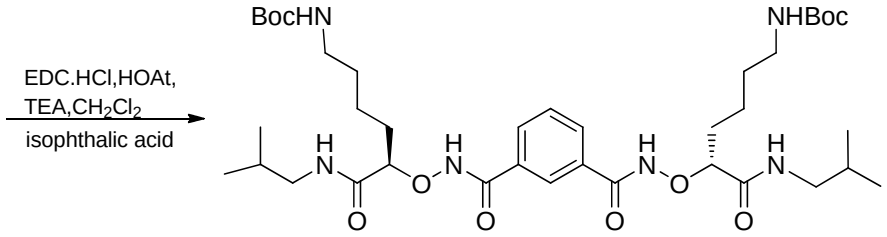
[b]School of Biomedical Sciences,, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China.

[c]Department of Physiology, Anhui Medical University, Hefei, China.

Synthesis of compound 1



1a **1b**



compund 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 CH_2Cl_2 (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 CH_2Cl_2. The organic layer was washed with 5% aqueous $NaHCO_3$ and brine and then dried over with anhydrous $MgSO_4$ and concentrated to afford **1b** as an oil (1.2 g, 93%). $[\alpha]^{20}_D$ +42.6° (*c* 0.74, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$) δ 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); ^{13}C NMR (75 MHz, $CDCl_3$) δ 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 (CH_2Cl_2) 3392, 1734, 1707, 1670 cm^{-1}; LRMS (FAB) *m/z* 448 (M^++H); HRMS (EI) for $C_{23}H_{33}N_3O_6$ (M^+) calculated 447.2364, found 447.2359.

To a solution of **1b** (1.2 g, 2.7 mmol) in CH_3OH (40 mL) was added $NH_2NH_2{\cdot}H_2O$ (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 CH_2Cl_2 and washed with 5% aqueous $NaHCO_3$ and brine. The organic layer was dried over with anhydrous Na_2SO_4 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 CH_2Cl_2 (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 $NaHCO_3$ and brine and then dried over with anhydrous $MgSO_4$ 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: $[\alpha]^{20}_D$ +15.6° (*c* 0.61, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$) δ 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); ^{13}C NMR (75 MHz, $CHCl_3$) δ 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 (CH_2Cl_2) 3437, 3321, 1674 cm^{-1}; LRMS (FAB) *m/z* 765 (M^+); HRMS (FAB) for $C_{38}H_{64}N_6O_{10}$ (M^++^{133}Cs) 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 $CHCl_3$/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 $CHCl_3$/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-diylbis(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 liposome 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 % CO_2 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 $CaCl_2$, 1 $MgCl_2$, 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).

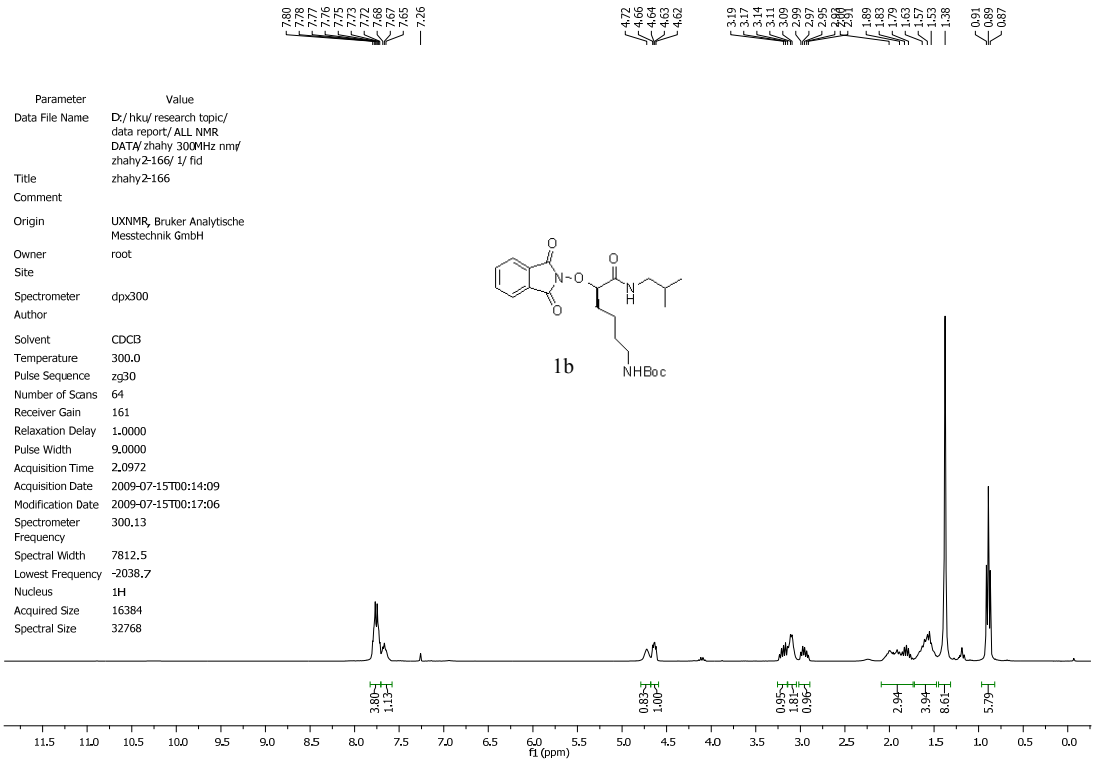
Blood Vessel Preparation and Isometric Tension Measurement

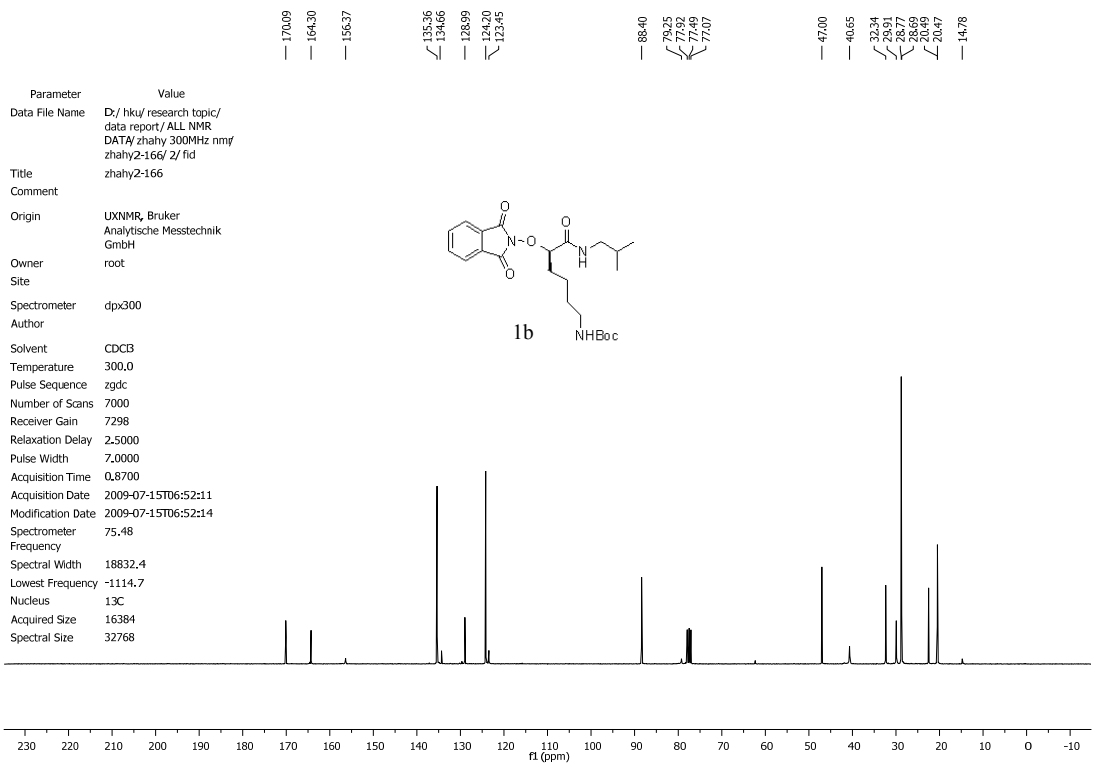
All animal experiments were conducted in accordance with NIH publication no. 8523 and approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong. Experiments were performed on aortae isolated from male C57 mice supplied by the Laboratory Animal Services Centre, the 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 CO_2 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 $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.2 mM $NaHCO_3$, 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% O_2 and 5% CO_2 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 $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.2 mM $NaHCO_3$, 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.

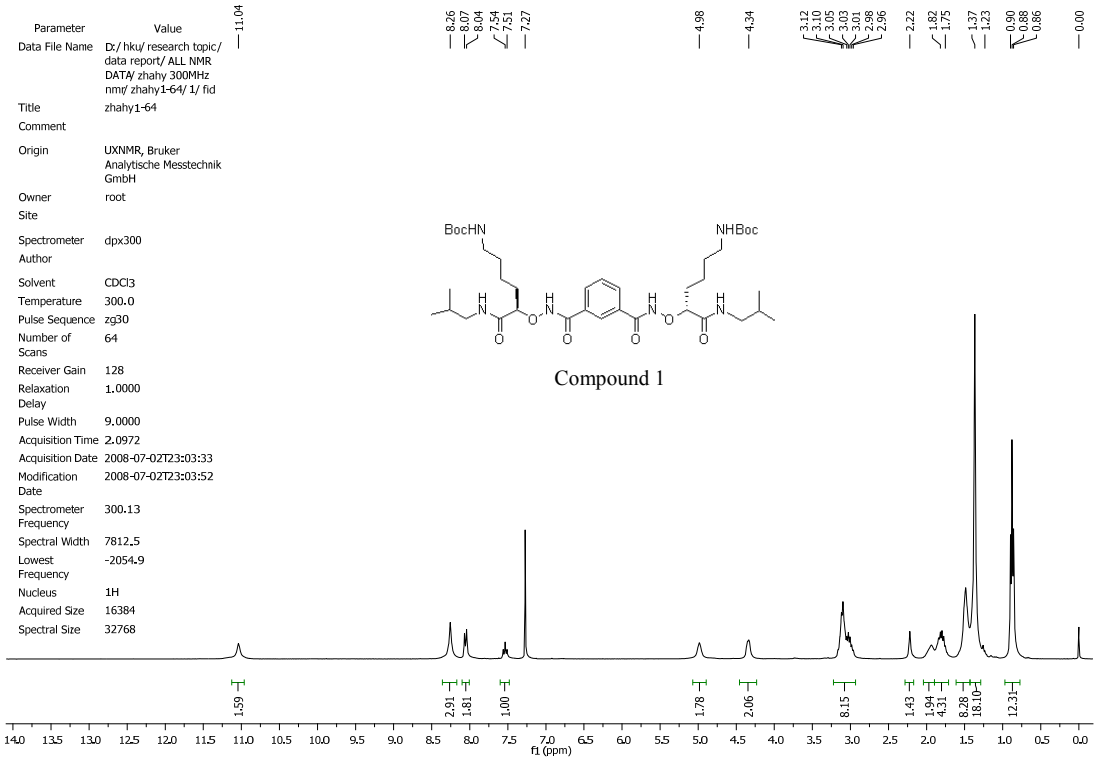
References:

[1] D. M. Engelman and T. A. Steitz, *Cell* 1981, **23**, 411.

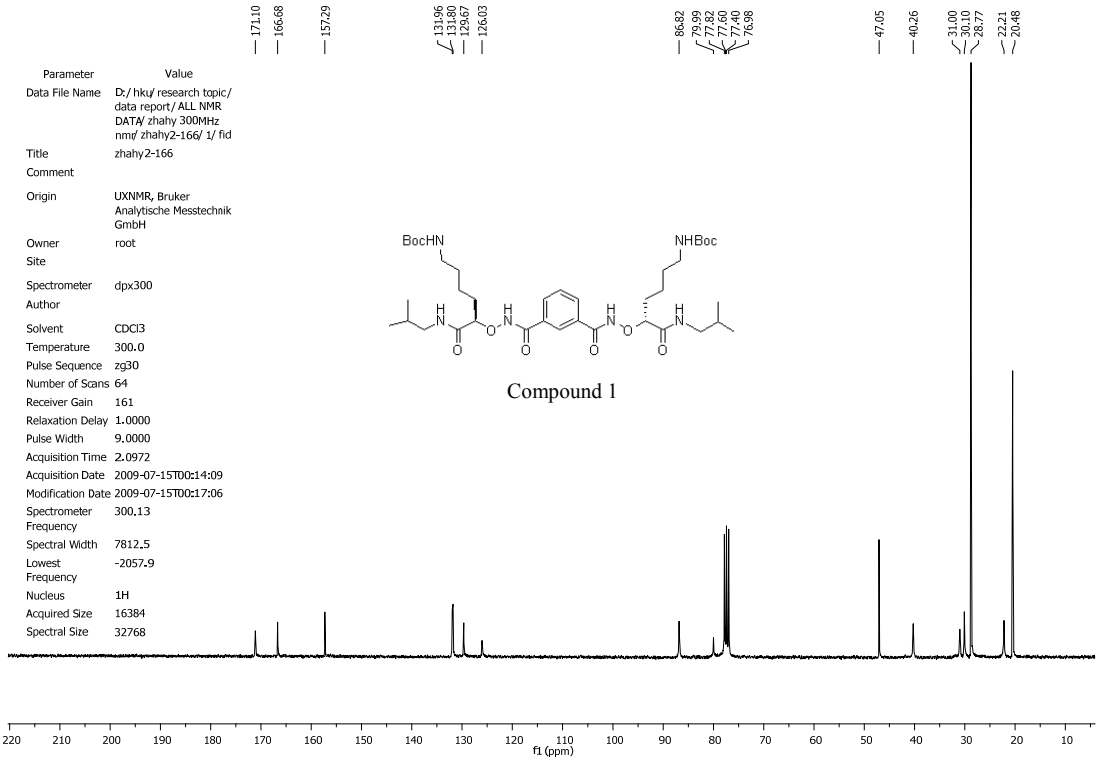
[2] H. Y. Kwan, B. Shen, X. Ma, Y. C. Kwok, Y. Huang, Y. B. Man, S. Yu, and X. Yao, *Circ Res* 2009, **104**, 670.







Compound 1



Compound 1