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Supporting Information

Visualization of hERG channels in living cells via fluorescent probe regulated by the synergy between solvatochromism and molecular rotation based on a simple targeting group 4-benzylaniline

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Table of Contents	
Experimental Procedures	S2
Materials and apparatus	S2
Synthesis of CBH, ZBH and BBH	S3
Fluorescent and UV spectroscopy measurement	S3
Cell culture	S3
Cytotoxicity test	S4
Cell membrane preparation	S4
Fluorescent assay of CBH	S4
Electrophysiology	S5
Western blotting	S6
Confocal imaging of hERG cells	S6
Immunofluorescence	S6
Supplemental Schemes	S7
Supplemental Figures	S8
NMR Data	S19

Experimental Procedures

Materials and apparatus

Reagents for synthesis were purchased from Macklin and reagents of electrophysiology were purchased from Sigma-Aldrich (Mainland China). The primary antibodies were purchased from alomone labs and abcam (Cambridge, UK). The secondary antibody was purchased from Jackson Immuno Research (Philadelphia, USA). Twice-distilled water was applied throughout all sections of experiments. ¹H NMR (500 MHz) and ¹³C NMR (125 MHz) spectra were acquired on a Bruker Avance-500 spectrometer (Germany) with CDCl₃ and d6-DMSO used to dissolve samples. High-resolution mass spectrometry (HRMS) was obtained through Q-TOF6510 spectrograph (Agilent). The absorbance of MTT was measured by microplate reader (Tecan Auatria GmbH A-5082). UV spectra were performed on UV-2600 Spectrophotometer and fluorescent measurements were operated on F-4600 FL Spectrophotometer. Fluorescence polarization was performed on Flexstation 3 (Molecular Device). The confocal images of hERG and hela cells were taken by Nikon A1R MP. The pH value was detected with a pH meter (PHS-3C). Electrophysiological data was recorded in whole-cell patches using a HEKA EPC10 amplifier with PatchMaster software. The images of Western Blot were captured by BIO-RAD molecular imager (ChemiDocTM XRS⁺ imaging system).

Synthesis of CBH, ZBH and BBH

The synthetic routes of CBH, ZBH and BBH were shown in **Scheme S1**. 4benzylaniline (183 mg) was firstly dissolved into dry CH_2Cl_2 (5 mL) and triethylamine (0.5 mL). Then, 7-(diethylamino)-2-oxo-2H-chromene-3-carbonyl chloride (334 mg) solid was added slowly into the solution at room temperature, and stirred for 2 h. The organic solvents were evaporated under reduced pressure, and the crude product was purified by column chromatography to yield yellow solid CBH (265.8 mg, 62.4%). ¹H NMR (500 MHz, CDCl₃) δ 10.82 (s, 1H), 8.78 (s, 1H), 7.65 (d, *J* = 7.5 Hz, 2H), 7.46 (d, *J* = 10 Hz, 1H), 7.30-7.26 (m, 2H), 7.20-7.16 (m, 4H), 7.00 (d, *J* = 10 Hz, 1H), 6.70-6.66 (m, 2H), 6.52 (d, *J* = 5 Hz, 1H), 3.48 (dd, *J* = 5, 10 Hz, 4H), 1.25 (t, *J* = 5 Hz 6H). ¹³C NMR (125 MHz, CDCl₃) δ 163.06, 160.97, 157.72, 148.40, 141.22, 136.89, 136.37, 131.26, 129.39, 128.77, 128.42, 128.35, 126.00, 125.86, 120.52, 45.12, 41.39, 41.06, 12.42. MS: m/z [M+H]⁺ calcd for C₂₇H₂₇N₂O₃: 427.1977, found: 427.2019.

The same method was employed to synthesize ZBH and BBH. ZBH (white solid, 80.3%), ¹H NMR (500 MHz, *d6*-DMSO) δ 10.52 (s, 1H), 8.76 (t, *J* = 5 Hz, 1H), 8.41-8.37 (m, 2H), 7.81 (t, *J* = 5 Hz, 1H), 7.69 (d, *J* = 5 Hz, 2H), 7.27 (t, *J* = 5 Hz, 2H), 7.22 (d, *J* = 5 Hz, 4H), 7.17 (d, *J* = 10 Hz, 1H), 3.91 (s, 2H). ¹³C NMR (125 MHz, *d6*-DMSO) δ 163.57, 148.18, 141.82, 137.53, 137.14, 136.74, 134.56, 130.58, 129.35, 129.07, 128.85, 126.52, 126.37, 122.81, 121.17, 41.03. MS: m/z [M+H]⁺ calcd for C₂₀H₁₇N₂O₃: 333.1194, found: 333.1239.

BBH (white solid, 84.6%), ¹H NMR (500 MHz, *d6*-DMSO) δ 10.56 (s, 1H), 8.77 (d, J = 5 Hz, 1H), 8.42-8.39 (m, 2H), 7.79 (m, 3H), 7.36 (d, J = 5 Hz, 2H), 7.12 (t, J = 10 Hz, 1H), 3.33 (s, 1H). ¹³C NMR (125 MHz, *d6*-DMSO) δ 163.73, 148.18, 139.13, 136.73, 134.59, 129.11, 126.56, 124.56, 122.96, 121.03. MS: m/z [M+H]⁺ calcd for C₁₃H₁₁N₂O₃: 243.0770, found: 243.0763.

Fluorescent and UV spectroscopy measurement

Probe CBH was dissolved in DMSO for a stock solution (1 mM). Test solutions were prepared by diluting 20 μ L of the stock solution into 2 mL with different volume ratio of deionized water and DMSO or Glycerol and DMSO. Test solutions were measured with F-4600 FL and UV-2600 spectrophotometers.

Cell culture

hERG transfected CHO cells were cultured in F12 medium supplemented with 10% fetal bovine serum (FBS) and Hela cells (human cervical cancer cells) were cultivated in MEM (minimum Eagle's medium) with 10% fetal bovine serum in an atmosphere of 5% CO_2 at 37°C. Cells were passaged at 2-day to 3-day intervals and before recording, the cells were plated on 1 cm glass coverslips allowed to adhere for 4h. All the experiments were performed at room temperature.

Cytotoxicity test

hERG transfected CHO cells at a density of 8×10^3 cells per millilitre were seeded in 96-well plate in 100 µL MEM supplemented with 10% fetal bovine serum (FBS) and incubated in humidified atmosphere containing 5% CO₂ at 37.0 °C. Each well was cultivated with different concentrations of CBH for 24 h. Then 10 µL of MTT solution (5 mg/ml in PBS) was added into each well. After treated with MTT for 4 h in a 5% CO₂ condition at 37°C, the previous medium was removed and 100 µL DMSO was added to dissolve the purple crystals for 2h. The optical density (OD) was measured at 490 nm with a Microplate Reader (Tecan Auatria GmbH A-5082). Each well was duplicated for five times.

Cell membrane preparation

The first sixth generations of transfected hERG-CHO cells line were applied to obtain the cell membrane. Before the preparation of cell membrane, transfected hERG-CHO cells were cultured in 10-cm dished. Then the proteins from CHO cells were extracted using RIPA (Thermo,U.S.), and quantified using BCA Protein Assay Reagent Kit (Thermo, U.S.). Then the obtained membrane pellets was resuspended in assay buffer (50mM Tris-HCl, 1 mM MgCl₂, 10 mM KCl) and aliquoted in 1.5 ml tube and stored in -80°C before use.

Fluorescent polarization assay of CBH

50 μ L of 10 μ M CBH in assay buffer (50mM Tris-HCl, 1 mM MgCl₂, 10 mM KCl) was incubated with 50 μ L different concentrations (1, 0.5, 0.25, 0.125, 0.0625, 0.03125 mg/ml) of transfected hERG-CHO cells membranes for 1 h at 37°C. Then the fluorescence of the solutions was detected at 405 nm with Flexstation 3 (Molecular Device). Subsequently, using the same method, different concentrations (300, 100, 30, 10, 3, 1, 0.3, 0.1 μ M) of CBH were incubated with 2.5 mg/ml cell membrane. The normalized polarization was plotted against an increasing concentration of CBH and the equilibrium binding constant (K_d) was calculated by nonlinear curve fit to one site binding saturation equation.

Electrophysiology

Macroscopic currents from hERG transfected CHO cells were recorded in whole-cell patches using a HEKA EPC10 amplifier with PatchMaster software. Pipette solutions were contained with 130 mM KCl, 1 mM MgCl₂, 12.5 mM EGTA, 10 mM HEPES (pH= 7.3, adjusted with KOH). Extracellular solutions were contained with 137 mM NaCl, 4 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂,10 mM HEPES and 10 mM glucose (pH =7.4, adjusted with NaOH). Holding potential was -80 mV for 100 ms, and membrane potential was switched from holding -60 to 60 mV for 2 s following return to -50 mV for 2 s in intervals of 30 to 45 s. Tail currents were measured at -50 mV. For ramp recordings of over-expressing TRPV2 HEK-293 cells, both bath and pipette solutions were contained with 130 mM NaCl, 0.2 mM EDTA, and 3 mM HEPES (PH 7.2-7.4, adjusted with NaOH). Membrane potential was held at 0 mV and current in response to 500 ms voltage ramps from -100 to +100 mV at 1 s intervals. For wholecell recordings of Nav.1.5 channel, the bath solution were contained with 140 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES, and 10 mM glucose (pH 7.3, adjusted with NaOH). The recording pipette was filled with a solution contained 140 mM CsF, 10 mM NaCl, 1 mM EGTA, and 10 mM HEPES (pH 7.3, adjusted with CsOH). Membrane potential was held at -120 mV, and voltage pulses was -10 mV for 100 ms at 1s intervals. For whole-cell recordings of KCNQ1, the bath solution were contained with 160 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES (pH 7.4). The recording pipette was filled with a solution contained 160 mM KCl, 5 mM MgCl₂, and 5 mM HEPES (pH 7.2). For wholecell recordings of Kv1.5 channel, the bath solution were contained with 140 mM NaCl, 4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES-Na and 10 mM glucose (adjusted to pH 7.40 with NaOH). All patch recording experiments were performed at room temperature and all recording data were analyzed with Igor Pro (Wave-metrics) and Origin 8.6 (OriginLab).

Western blotting

Cells were lysed with RIPA lysis buffer containing cocktail phosphatase inhibitor and protease inhibitor (Thermo, US). The protein concentration in the supernatant was

determined with the BCA protein assay kit (Thermo Fisher, US). Protein samples were separated by running on a 10% SDS-PAGE, and then transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% powdered nonfat milk in 1X-TBST (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20) for 1 h at room temperature and then the membranes were incubated with primary antibodies (1:10000; Abcam) and β -actin (1:1000; Boster) at 4°C overnight. After washing in 1X-TBST buffer for 15 min, the membranes were incubated with their corresponding secondary antibody for another 1 h at room temperature, and then washed again for 30 min. Signals were detected using the ECL Western blotting detection system (Bio-Rad).

Confocal imaging of hERG cells

hERG cells were incubated with CBH (10 μ M) for 3 minutes and washed three times with PBS buffer. Then the cells were placed under a confocal fluorescence microscope (Nikon A1R MP) with 40X objective lens for fluorescence imaging with the excitation at 405 nm and collection at 470-520 nm.

Immunofluorescence

The glass coverslips with hERG cells were washed with PBS buffer for three times to remove the medium. Then the cells were fixed in 4% paraformaldehyde for 15 minutes and washed with PBS for three times. After that, the cells were covered by 1% blocking solution for 1h and incubated with (0.8mg/ml) anti-Kv11.1 antibody (alomone labs APC-109) with a dilution rate about 1:25 after overnight culture at 4°C. The next day, blocking solution with antibody was removed and the cells were washed with PBS for three times. Then the cells were covered by PBS with a second antibody (Alexa Fluor® 488-conjugated AffiniPure Goat Anti-Rabbit IgG purchased from Jackson ImmunoResearch) with a dilution rate about 1:200 for 1h at 37°C. Finally, the cells were washed with PBS three times and placed under a confocal fluorescence microscope.

.Supplementary Schemes



Scheme S1. Synthesis of CBH, ZBH and BBH

Supplementary Figures



Fig. S1 Particle size distribution of CBH in DMSO/water mixture with a 50% fraction.



Fig. S2 Fluorescent spectrum of CBH in solid state under excitation at 405 nm, inset: fluorescence photographs of CBH in solid state under 365 nm UV irradiation and photographs (insert) of CBH in solid state under sunlight.



Fig. S3 Fluorescent emission spectra of 10 μ M CBH incubated with different concentrations of the membrane (0, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1 mg/ml) for 1h upon excitation at 405 nm. (B) Linearity between the fluorescence intensity of CBH at 465 nm emission wavelength and the concentration of cell membrane from 0 to 1.0 mg/ml. The results are presented as means ± SEM with three replicates (n = 3).



Fig. S4 Curve fitting in red represents dose-dependent inhibition of hERG channel by CBH and the IC_{50} value was calculated by the Origin 86 software.



Fig. S5 Fluorescence polarization analysis of hERG with CBH (0.1, 0.3, 1, 3, 10, 30, 100, 300 μ M) at different concentrations. The normalized polarization was plotted against an increasing concentration of CBH and the equilibrium binding constant (K_d) was calculated by nonlinear curve fit to one site binding saturation equation. K_d value determined for CBH was 13.78 μ M. The results are presented as means ± SEM with three replicates (n = 3).



Fig. S6 No activation or inhibition effects of 10 μ M CBH were found on (A) Kv1.5 channel, (B) KCNQ1 channel, (C) Nav1.5 channel and (D) TRPV2 channel.



Fig. S7 Confocal imaging of fluorescence and bright field images of (left) probe CBH (10 μ M), (mid) coumarin (10 μ M) and (right) co-incubation in expressed hERG cells.



Fig. S8 Confocal imaging of fluorescence and bright field images of probe CBH (10 μ M) in hERG cells, Nav 1.5 transfected HEK 293 cells, Kv 1.5 transfected CHO cells, KCNQ1 transfected HEK 293 cells and TRPV2 transfected HEK 293 cells. Scale bars (white): 50 μ m.



Fig. S9 Fluorescent intensity changes at the maximum emission wavelength (465 nm) of 10 μ M CBH incubated with different concentrations (0, 0.0625, 0.125, 0.25, 0.5, 1 mg/ml) of cell membrane and serum albumin for 1h. The results are presented as means \pm SEM with three replicates (n = 3).



Fig. S10 (A) Confocal imaging of fluorescence and bright field images of probe CBH (10 μ M) in hERG cells (upper panel) and hela cells (lower panel). Scale bars (white): 50 μ m. (B) Western blot detecting the expression of hERG protein in hERG and hela cells with anti-Kv11.1 antibody. (C) The change of fluorescent intensity between hERG cells and hela cells incubated with probe CBH (10 μ M). (D) The normalize values of relative change in the expression of hERG protein between hERG cells and hela cells. The results are presented as means ± SEM with three replicates (n = 3). Significantly different at ***p < 0.001 (Student's t-test).



Fig. S11 Viability of hERG cells after treatment with different concentrations (0.1–30 μ M) of probe CBH for 24 h. Cell viability was measured by MTT assay. The result are presented as means ± SEM with three replicates (n = 3)











0.03

