Supporting Information

Environment-Sensitive Fluorescent Probe for the Human Ether-a-go-go-Related Gene Potassium Channel

Zhenzhen Liu, Tianyu Jiang, Beilei Wang, Bowen Ke, Yubin Zhou, Lupei Du, and Minyong Li

a Department of Medicinal Chemistry, Key Laboratory of Chemical Biology (MOE), School of Pharmacy, Shandong University, Jinan, Shandong 250012, China
b Laboratory of Anaesthesiology &Critical Care Medicine, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China
c Institute of Biosciences & Technology, Texas A&M University Health Science Center, Houston, TX 77030, USA

Table of Contents

1. Materials and instruments ................................................................. S-2
2. Synthesis of intermediates ............................................................... S-2
3. Fluorescent excitation and emission spectra of free probe S2 ................ S-5
4. hERG potassium channel inhibition assay ........................................ S-5
5. Cell membrane preparation ............................................................. S-6
6. The quantum yields measurement .................................................... S-7
7. Fluorescent properties of SBD fluorophore after binding to hERG transfected membrane ........................................ S-7
8. Fluorescent properties of probe S2 under different pH conditions ......... S-7
9. Fluorescent polarization spectra of probe S2 with hERG transfected HEK293 membranes in buffer .... S-8
10. Cell culture and imaging ................................................................. S-8
11. Patch clamp assay ........................................................................... S-9
12. References ....................................................................................... S-9
13. Characterization of probes ................................................................ S-10
1. Materials and instruments

All reagents and solvents available from commercial sources were used as received unless otherwise noted. Water used for the fluorescence studies was doubly distilled and further purified with a Mill-Q filtration system. Melting points were determined on an electrothermal melting point apparatus and were uncorrected. $^1$H NMR and $^{13}$C NMR were recorded on a Bruker 300 MHz NMR and 600 MHz NMR spectrometer. Mass spectra were performed by the analytical and the mass spectrometry facilities at Shandong University. Absorption spectra and fluorescence spectra were obtained with a Thermo Varioskan microplate reader. The fluorescence polarization assay was conducted on a POLARstar Omega microplate reader. Fluorescence imaging was performed using Zeiss Axio Observer A1 fluorescence microscopy and Zeiss LSM780 confocal fluorescence microscopy.

2. Synthesis of intermediates

Scheme S1. Synthesis route of the intermediates

2-chloro-1-(4-fluorobenzyl)-1H-benzo[d]imidazole (2)

A mixture of 2-Chlorobenzimidazole (1.00 g, 6.55 mmol) and sodium hydroxide (0.52 g, 13.1 mmol) in acetonitrile (30 mL) were refluxed for 30 min. The solution was cooled to room temperature and then 4-fluorobenzyl bromide (1.86 g, 9.83 mmol) was added. The mixture was refluxed for another 3 h. Water was added and the mixture was extracted with ethyl acetate. The combined organic layers were dried over MgSO$_4$, filtered, and concentrated in vacuo. The crude product was recrystallized in petroleum ether to afford 1 as a white solid in 88.5% yield. M.p.: 74.0~78.0°C. $^1$H-NMR (300MHz, CDCl$_3$): $\delta$ 7.74-7.69 (m, 1H), 7.33-7.14 (m, 5H), 7.05-7.00 (m, 2H), 5.52 (s, 2H); ESI-MS: ([M+H]$^+$): 261.0.

Ethyl 4-((1-(4-fluorobenzyl)-1H-benzo[d]imidazol-2-yl)amino)piperidine-1-carboxylate (3)

A mixture of compound 2 (1 g, 3.83 mmol) and ethyl 4-aminopiperidine-1-carboxylate (1.32 g, 7.67 mmol) was subjected to microwave irradiation (180 °C, 2.5 h). After completion of the reaction, the solid was dissolved in dichloromethane and concentrated in vacuo. The crude product was purified by column chromatography to afford 3 as a white solid in 72.0% yield. M.p.: 178.0~180.0°C. $^1$H-NMR (300MHz, DMSO-d$_6$): $\delta$ 7.54 (d, $J=7.8$Hz, 1H), 7.18-1.00 (m, 7H), 7.05-7.00 (m, 2H), 5.52 (s, 2H); 4.16-4.00 (m, 6H), 3.02 (t, $J=1.2$Hz, 2H), 2.10 (m, 2H), 1.33-1.21 (m, 5H); ESI-MS: ([M+H]$^+$): 397.5.
1-(4-fluorobenzyl)-N-(piperidin-4-yl)-1H-benzo[d]imidazol-2-amine hydrobromide (4)

A mixture of compound 3 (0.56 g, 1.41 mmol) and 40% HBr aqueous solution (30 mL) was stirred at 100 °C for 18 h. Thereafter, solvent was evaporated in vacuo and the crude product was recrystallized in ethyl acetate to afford 4 as a white solid in 92.6% yield. M.p.: 202.0–205.0°C. 1H-NMR (300MHz, DMSO-d6): δ 7.23–7.11 (m, 5H), 7.05 (d, J=7.5Hz, 1H), 6.94 (td, J=7.5, 1.2Hz, 1H), 6.84 (d, J=7.5, 0.9Hz, 1H), 5.22 (s, 2H), 3.86–3.76 (m, 1H), 2.99 (d, J=12.3Hz, 2H), 2.58 (d, J=12.3Hz, 2H), 1.94 (d, J=9.6Hz, 2H), 1.45-1.32 (m, 2H); ESI-MS: [M+H]+: 325.3.

2-(2-(4-((1-(4-fluorobenzyl)-1H-benzo[d]imidazol-2-yl)amino)piperidin-1-yl)ethyl)isoindoline-1,3-dione (5a)

A mixture of compound 4 (0.24 g, 0.59 mmol), 2-(bromomethyl)isoindoline-1,3-dione (0.25 g, 1.18 mmol), K₂CO₃ (0.18 g, 1.18 mmol) in acetonitrile was refluxed for 5 h. Thereafter, water was added to the suspension, and the mixture was extracted with CH₂Cl₂. The combined organic layers were dried over MgSO₄, filtered, and concentrated in vacuo. The crude product was purified by column chromatography to afford 4 as a white solid in 30.4% yield. M.p.: 198.0–199.0°C. 1H-NMR (300 MHz, DMSO-d6): δ 7.90–7.82 (m, 4H), 7.21–7.10 (m, 5H), 7.05 (d, J=7.5Hz, 2H), 6.93 (td, J=6.6, 1.5Hz, 1H), 6.84 (td, J=7.5, 0.9Hz, 1H), 6.59 (d, J=7.5Hz, 1H), 5.23 (s, 2H), 3.73 (t, J=6.0 Hz, 2H), 2.92 (d, J=10.8 Hz, 2H), 2.57 (t, J=6.0 Hz, 2H), 2.10 (t, J=10.5 Hz, 2H), 1.92 (d, J=11.1Hz, 2H), 1.52-1.43 (m, 2H); ESI-MS: [M+H]+: 499.4.

2-(4-(4-((1-(4-fluorobenzyl)-1H-benzo[d]imidazol-2-yl)amino)piperidin-1-yl)butyl)isoindoline-1,3-dione (5b)

The compound 5b was prepared following the method described for the preparation of compound 5a, employing compound 4 (0.12 g, 0.35 mmol), 2-(4-bromobutyl)isoindoline-1,3-dione (0.15 g, 0.53 mmol) and K₂CO₃ (0.0967 g, 0.70 mmol), and the crude product was purified by silica gel chromatography to afford compound 5b as a white solid in 48.9% yield. M.p.: 93.0–95.0°C. 1H-NMR (300 MHz, CDCl₃): δ 7.86-7.80 (m, 2H), 7.74-7.68 (m, 2H), 7.52 (d, J=7.8Hz, 1H), 7.16-7.09 (m, 3H), 7.07-6.99 (m, 4H), 5.06 (s, 2H), 3.98-3.90 (m, 2H), 3.72 (t, J=6.9 Hz, 2H), 2.87 (d, J=10.2 Hz, 2H), 2.48 (t, J=7.5 Hz, 2H), 2.31 (t, J=10.8 Hz, 2H), 2.12 (d, J=11.4Hz, 2H), 1.75-1.63 (m, 2H), 1.50-1.50 (m, 4H); ESI-MS: [M+H]+: 526.5.

2-(6-(4-(4-(1-(4-fluorobenzyl)-1H-benzo[d]imidazol-2-yl)amino)piperidin-1-yl)hexyl)isoindoline-1,3-dione (5c)

The compound 5c was prepared following the method described for the preparation of compound 5a, employing compound 4 (0.12 g, 0.35 mmol) and 2-(6-bromohexyl)isoindoline-1,3-dione (0.15 g, 0.53 mmol) and K₂CO₃ (0.10g, 0.74 mmol), and the crude product was purified by silica gel chromatography to afford compound 5b as a white solid in 48.9% yield. M.p.: 177.0–180.0°C. 1H-NMR (300 MHz, DMSO-d6): δ 7.89-7.81 (m, 4H), 7.23-7.11 (m, 5H), 7.06 (d, J=7.5Hz, 1H), 6.94 (t, J=6.9Hz, 1H), 6.85 (t, J=7.5Hz, 1H), 5.26 (s, 2H), 3.77 (brs, 1H), 3.59 (t, J=7.2Hz, 2H), 2.83 (s, 2H), 2.72 (d, J=2.1Hz, 2H), 1.98-1.90 (m, 2H), 1.59-1.24 (m, 10H); ESI-MS: [M+H]+: 554.4.

N-(1-(2-aminoethyl)piperidin-4-yl)-1-(4-fluorobenzyl)-1H-benzo[d]imidazol-2-amine (6a)

A mixture of compound 4 (0.1 g, 0.20 mmol) and 2 mL 80% NH₃-NH₃·H₂O aqueous solution in ethanol 10 mL was refluxed 9 h. Thereafter, the solution was cooled under refrigeration and then the precipitation was filtered. The filtrate was concentrated under vacuo. The crude product was dissolved in CH₂Cl₂, filtered and the solvent of the filtrate was evaporated in vacuo to afford 6a as a white solid without further purification.

N-(1-(4-aminoethyl)piperidin-4-yl)-1-(4-fluorobenzyl)-1H-benzo[d]imidazol-2-amine (6b)

The compound 6b was prepared following the method described for the preparation of compound 6a,
Supporting Information

employing compound 5b (0.24 g, 0.39 mmol) and 3 mL 80% NH₂-NH₂-H₂O aqueous solution to afford compound 6b as a white solid without further purification.

N-(1-(6-aminohexyl)piperidin-4-yl)-1-(4-fluorobenzyl)-1H-benzo[d]imidazol-2-amine (6c)
The compound 6c was prepared following the method described for the preparation of compound 6a, employing compound 6c (0.35 g, 0.63 mmol) and 3 mL 80% NH₂-NH₂-H₂O aqueous solution to afford compound 6b as a white solid without further purification.

7-chlorobenzo[c][1,2,5]oxadiazole-4-sulfonamide chloride (7)
CISO₄H (5 mL) was dropped slowly into 4-chloro-2,1,3-benzoxadiazole solid (0.52 g, 3.36 mmol) in ice bath. After addition, the mixture was stirred for 30 min at room temperature and then heated at 120°C oil bath for 6 h. Subsequently, the reaction mixture was poured into ice slowly. The precipitation was filtered and washed with water and the crude product was recrystallized in petroleum ether to afford 7 as a white solid in 49.4% yield. M.p.: 87.0-88.5°C. 1H-NMR (300MHz, CDCl₃): δ 8.19 (d, J=7.5Hz, 1H), 7.65 (d, J=7.5Hz, 1H).

4-(N,N-dimethylaminosulfonyl)-7-chloro-2,1,3-benzoxadiazole (8)
A mixture of dimethyleamine hydrochloride (76.8 mg, 0.95 mmol) and triethylamine (0.24 mL) in THF (5 mL) was stirred for 30 min at room temperature and then was added into the solution of compound 7 (0.20 g, 0.79 mmol) in THF (5 mL). The reaction was stirred for 30 min. Water was added and the mixture was extracted with CH₂Cl₂. The combined organic layers were dried with MgSO₄, filtered and concentrated in vacuo. The crude product was purified by silica gel chromatography to afford compound 8 as a white solid in 43.7% yield. M.p.: 142.0-143.0°C. 1H-NMR (300MHz, CDCl₃): δ 7.98 (d, J=7.5Hz, 1H), 7.56 (d, J=7.5Hz, 1H).

7-(2-(4-((1-(4-fluorobenzyl)-1H-benzo[d]imidazol-2-yl)aminopiperidin-1-yl)ethyl)amino)-N,N-dimethylbenzo[c][1,2,5]oxadiazole-4-sulfonamide (S1)
A mixture of compound 8 (40.2 mg, 0.15 mmol), 6a (23.1 mg, 68.8 µmol) and N-methyl morpholine (0.2 mL) in acetonitrile (6 mL) was stirred for 24 h. Water was added and the mixture was extracted with CH₂Cl₂. The combined organic layers were dried over MgSO₄, filtered and concentrated in vacuo. The crude product was purified by silica gel chromatography to afford compound A1 as a yellow solid in 21.1% yield. M.p.: 106.0-110.0°C. 1H-NMR (300MHz, CDCl₃): δ 7.89 (d, J=8.1Hz, 1H), 7.53 (d, J=7.8Hz, 1H), 7.26-7.12 (m, 3H), 7.06-6.99 (m, 4H), 6.53 (brs, 1H), 6.10 (d, J=8.1Hz, 1H), 5.12 (s, 2H), 4.26-4.06 (m, 1H), 3.64 (s, 1H), 3.44 (d, J=4.2Hz, 2H), 2.89-2.80 (m, 10H), 2.38 (brs, 2H), 2.17 (d, J=11.1Hz, 2H), 1.57-1.51 (m, 2H); 1H-NMR (75MHz, DMSO-d₆) δ: 162.93, 153.50, 121.65, 121.51, 118.10, 118.07, 115.64, 115.59, 110.99, 103.50, 99.91, 98.49, 94.74, 91.99, 88.96, 82.74, 43.79, 32.37; HRMS (ESI) m/z calcd. for C₂₉H₂₄F₅N₂O₃S ([M + H]+): 593.2459; found 593.2463.

7-(4-(4-((1-(4-fluorobenzyl)-1H-benzo[d]imidazol-2-yl)aminopiperidin-1-yl)butyl)amino)-N,N-dimethylbenzo[c][1,2,5]oxadiazole-4-sulfonamide (S2)
The compound A2 was prepared following the method described for the preparation of compound S1, employing compound 8 (50 mg, 0.19 mmol), compound 6b (30 mg, 75.8 µmol) and N-methyl morpholine (0.2 mL) to afford compound A2 as a yellow solid in 34.6% yield. M.p.: 113.0-116.0°C. 1H-NMR (400MHz, DMSO-d₆): δ 8.45 (t, J=5.2Hz, 1H), 7.83 (d, J=8.4Hz, 1H), 7.22-7.12 (m, 5H), 7.06 (d, J=8.0Hz, 1H), 6.94 (t, J=7.6Hz, 1H), 6.84 (t, J=7.6Hz, 1H), 6.59 (brs, 1H), 6.33 (d, J=8.0Hz, 1H), 5.26 (s, 2H), 3.74 (brs, 1H), 3.40-3.37 (m, 2H), 2.86 (brs, 2H), 2.68 (s, 6H), 2.33 (brs, 2H), 1.98-1.90 (m, 2H), 1.68-1.67 (m, 2H), 1.55 (brs, 4H); 1H-NMR (75MHz, DMSO-d₆): δ 162.93, 153.90, 146.48, 144.29, 142.79, 141.37, 140.40, 134.24, 133.32, 128.96, 128.86, 120.44, 118.33, 115.41, 115.13, 115.00, 107.77, 104.93, 98.61, 52.22, 43.65, 42.86,
37.41, 31.77, 25.53; HRMS (ESI) m/z calcd. for C₃₁H₇₈FN₉O₃S ([M + H]⁺) 621.2772; found 621.2773.

7-((6-(4-(1-(4-fluorobenzyl)-1H-benzo[d]imidazol-2-yl)amino)piperidin-1-yl)hexyl)amino)-N,N-dimethylbenzo[c][1,2,5]oxadiazole-4-sulfonamide (S3)

The compound A3 was prepared following the method described for the preparation of compound S1, employing compound 8 (50 mg, 0.19 mmol), compound 6b (25.6 mg, 63.1 µmol) and N-methyl morpholine (0.5 mL) to afford compound A2 as a yellow solid in 24.1% yield. M.p.: 131.0~1433.0°C. 1H-NMR ((300MHz, CDCl₃): δ 7.91 (d, J=8.1 Hz, 1H), 7.52 (d, J=7.8Hz, 1H), 7.19-7.07 (m, 5H), 7.01 (t, J=84Hz, 2H), 6.15 (d, J=7.8Hz, 1H), 5.93 (brs, 1H), 5.26 (s, 2H), 4.35 (brs, 1H),3.49-3.37 (m, 4H), 2.31 (brs, 4H), 1.85-1.74 (m, 4H), 1.47 (brs, 4H); 13C-NMR (75MHz, DMSO-d6): δ 162.93, 153.75, 146.50, 144.28, 142.61, 141.38, 140.46, 134.23, 133.35, 128.96, 128.85, 120.52, 118.49, 115.44, 115.15, 115.06, 107.90, 104.78, 98.58, 43.69, 42.88, 38.47, 37.42, 27.51, 26.16; HRMS (ESI) m/z calcd. for C₃₃H₄₂FN₉O₃S ([M + H]⁺) 649.3085; found 649.3085.

3. Fluorescent excitation and emission spectra of free probe S2

![Fluorescent excitation and emission spectra of free probe S2](image)

**Figure S1.** Fluorescent excitation (left, the emission wavelength was 595 nm) and emission spectra (right, the excitation wavelength was 490 nm) of probe S2 in PBS buffer (pH= 7.4), EtOH and ethyl acetate.

4. hERG potassium channel inhibition assay

![Competitive binding curve of probe S1-3 to hERG potassium channel](image)

**Figure S2.** Competitive binding curve of probe S1-3 to hERG potassium channel was determined by radio-ligand binding assay.
The inhibitory activity on hERG potassium channel was determined by a radio-ligand binding assay. Astemizole (Cat. No.#A2861-10MG, Sigma-Aldrich) and atropin were chosen as positive and negative controls, respectively. The affinity with hERG potassium channel was assessed in the presence of 9 nM [3H] dofetilide. The probe’s binding abilities with the hERG were displayed with displacement curves and compared to the positive and negative controls.

In brief, probe S1-3 was dissolved in DMSO as stock solution (1 mM), which was further diluted with binding buffers (10 folds, 6-8 points) when applied to the binding assays. Cell membranes were prepared following the manufacturer’s instructions described by GenScript USA Inc (Cat. No. # M00355). First, each well of Uni-filter 96 GF/C microplate was incubated with 100 μL 0.5% PEI (Polyethyleneimine, Sigma-Aldrich, dissolved in Milli-Q water) at 4°C for 30-60 min. Then, PEI was discarded by filtration with Millipore vacuum manifold (8-15 mmHg) and plates were washed with 2 mL/well wash buffer (50 mM Tris-HCl, pH 7.4; filtered and stored at 4°C). The reaction mixtures including membrane (10 μg/well), probe A1 and [3H] dofetilide ligand (9 nM) were prepared in 24-well plates in a final volume of 100 μL (binding buffer: 10 mM HEPES, 130 mM NaCl, 60 mM KCl, 0.8 mM MgCl₂, 1 mM NaEGTA, 10 mM glucose, 0.1% BSA, pH 7.4; filtered and stored at 4°C) and incubated at 25 °C for 2 h with a shaking speed of 530 RPM. The reaction system was transferred into the filter plates and filtered with Millipore vacuum manifold. The wells was washed with 2 mL/well cold wash buffer and dried at rt for 120 min. The bottom of the plates was sealed with Bottom SealTM (opaque) (Perkin Elmer) and 50 μL MicroScint 20TM (Perkin Elmer) was added to each well. The plates was sealed with Topseal A (Perkin elmer) and counted on TopCount NXT for 1 min/well. Data were recorded by Topcount NXT and stored on the GenScript computer network for off-line analysis. Data acquisition was performed by Microsoft Excel (version 2003) program; IC50 values were obtained by GraphPad Prism 4 using the Cheng-Prusoff equation. The binding data was converted to % displacement according to the below equation: % displacement=100 × (1-(sample CPM/Total binding CPM)) (in which total binding CPM values were obtained by testing binding of [3H] dofetilide to the targets without competitors).

5. Cell membrane preparation

HEK 293 cell line stably transfected with hERG gene was obtained as a gift from the Li group at the University of Hong Kong and cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 400 μg/mL G418 (Sigma). The establishment of the hERG-transfected HEK293 cell line was described in their previous paper. Briefly, the vector of hERG/pcDNA3 generously provided by Dr. G. Robertson (University of Wisconsin, Madison, WI, USA) was transfected into HEK 293 cells (ATCC, Manassas, VA, USA) using 10 ml Lipofectamine 2000TM (Invitrogen, Hong Kong) with 4 mg hERG/pcDNA3 plasmid, and selected using 1000 mg/ml G418 (Sigma–Aldrich, St. Louis, MO). Colonies were picked with cloning cylinders and examined for channel expression by whole-cell current recordings as previous reported. Significant tail current of hERG channel was observed.

As we know, although the hERG-HEK293 we use was a stably transfected cell line, with the cells passaged, the transfected gene may be lost. Therefore, to guarantee the quality of the extracted cell membrane, we examined the hERG channel current changes with the increase of the cell generation. The results showed that the hERG channel current was weak after about 6 times of passages. Therefore, to obtain cell membrane pellets for PET assay and FP assay, we only collected membrane from the first sixth generations of cells. Before collecting the membrane, hERG-transfecting HEK 293 cells were cultured in 10-cm dishes. When they reached 90% confluency, cells were collected, and washed with PBS buffer (pH 7.4) three times by centrifugation for 4 min at 800 RMP. The cell pellets were resuspended in assay buffer.
Supporting Information

(50 mM Tris-HCl, 1 mM MgCl₂, 10 mM KCl, pH 7.4, at 4 °C) and stored in -80°C. Until use, the cell suspension was thawed at 4 °C and was lysed by passing 20-30 times through 27G ½ needle on ice bath. The cell lysates were centrifuged at 40,000 g for 20 min (4 °C). The supernatant was discarded and the pellet was suspended in assay buffer and homogenized using 27G ½ needle and centrifuged at 40,000 g for 20 min (4 °C). The supernatant was discarded and the obtained membrane pellets was resuspended in assay buffer and aliquoted in 1.5 mL tube and stored in -80 °C before use. Protein concentration was determined using a Braford kit as manufacturer’s instructions (M2031, Mbchem).

6. The quantum yields measurement

The quantum yields of probe S2 under assay buffer (50 mM Tris-HCl, 1 mM MgCl₂, 10 mM KCl, pH = 7.4) were calculated by comparison with fluorescein in 0.1 M NaOH (ΦST = 0.92) as a reference using the following equation:11

\[ \Phi_X = \Phi_{ST} \left( \frac{A_{ST}}{A_X} \right) \left( \frac{F_X}{F_{ST}} \right) \left( \frac{\eta_X}{\eta_{ST}} \right)^2 \]

Where the subscripts ST and X denote standard and test respectively, Φ is the quantum yield, A is the absorbance value at the maximum absorbance wavelenght, F is the integrated area under the fluorescence spectra using the maximum absorbance wavelength as the excitation wavelength, η is the refractive index of the solvent.

| Table S1 The quantum yields of probe S2 before and after binding to hERG channel |
|-----------------|--------|-----|-----|-----|
|                  | Concentration | A    | F    | η    | Φ    |
| Fluorescein (standard) | 0.25μM | 0.02 | 164896.481 | 1.3352 | 0.92  |
| Probe S2 (free probe)    | 2.5μM  | 0.025 | 987.877 | 1.3356 | 0.0044|
| Probe s2 and 0.0625 mg/mL membrane | 2.5μM  | 0.04  | 5481.057 | 1.336  | 0.0153|

7. Fluorescent properties of SBD fluorophore after incubating with hERG transfected HEK293 membranes

Figure S3. Fluorescent emission spectra changes of SBD fluorophore (7-chloro-N,N-dimethylbenzo[c][1,2,5]oxadiazole-4-sulfonamide, 1 μM) incubated with different concentrations of membrane for 20 min (excitated at 420nm) and B: Fluorescent intensity (normalized based on the last point which is seen as 1) at maximum emission wavelength.

8. Fluorescent properties of probe S2 under different pH conditions
Figure S4. A: Fluorescent emission spectra changes of probe S2 (5 μM) in Britton-Robinson buffer at different pH values (excited at 435 nm); B: Fluorescent intensity (normalized based on the last point which is seen as 1) changes of S2 at their maximum wavelength with pH values.

9. Fluorescent polarization spectra of probe S2 with hERG transfected HEK293 membranes in buffer

Figure S5. Fluorescence polarization (FP) assay used to monitor the interaction between probe S2 (1 μM) and cell membrane.

20 μL of probe S2 (the final concentration is 1 μM) in assay buffer (50 mM Tris-HCl, 1 mM MgCl₂, 10 mM KCl) was incubated with 80 μL different concentration of hERG transfected HEK293 cell membranes for 4 h at room temperature. Then, the fluorescent polarization values were monitored at 550 nm (excited at 450 nm) on POLARstar Omega microplate reader.

10. Cell culture and imaging

hERG transfected HEK293 were grown in DMEM medium supplemented with 10% (v/v) fetal bovine serum and 400 μg/ml G418 (Sigma) in an atmosphere of 5% CO₂, 95% air at 37 °C. Cells were plated on confocal dish and allowed to adhere for 12 h–24 h. After the medium was removed, the cells were carefully washed with DMEM medium without fetal bovine serum, and then incubated at r.t. in the presence of the probe S2 (5 μM, prepared in DMEM medium without fetal bovine serum) or co-incubated with S2 and 50 μM Astemizole (a potent hERG channel blocker) for 15 min. Fluorescence imaging was performed using Zeiss Axio Observer A1 fluorescence microscope. Using the similar procedure, probe S2 (2.5 μM) was co-stained with lysosome tracer which was synthesized by our own lab and the image was obtained on LSM780 confocal fluorescence microscope. To verify that the aggregation of probe S2 in lysosome is induced by its basicity, hERG transfected HEK293 cells were incubated with 5 μM probe S2 in absence or presence of Chloroquine (a reagent that can basified the acidic environment of lysosome). TIRF (Total Internal Reflection Fluorescence)
Supporting Information

internal reflection fluorescence microscopic) imaging is conducted on Carl Zeiss Laser TIRF 3 after cells were incubated with 2.5 μM probe S2 for 30 min.

Figure S6. A1-4: Fluorescence microscopic imaging of hERG transfected HEK293 co-stained with 2.5 μM probe S2 with Lysosome blue tracer (synthesized by our own lab) (A1: bright field, A2: green channel (stained by probe S2), A3: Blue channel (stained by lysosome tracer), A4: merged image; B1-2: TIRF imaging of 2.5 μM probe S2.

11. Patch clamp assay

The inhibition activity of probe S2 was evaluated by whole-cell voltage patch clamp on hERG transfected HEK293 cells. Briefly, hERG transfected HEK293 cells were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum and 400 μg/mL G418 (Sigma) in an atmosphere of 5% CO₂, 95% air at 37 °C. Before recording, the cells were plated on 1 cm coverslip and allowed to adhere for 4 h. Then, the medium was removed and washed with extracellular recording solution (137 mM NaCl, 5.1 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES and 10 mM glucose, pH = 7.4). The coverslip with cells were bathed in extracellular solution. Cell membrane hERG channel current recording was obtained by Axopatch 200B amplifier (Axon Instruments, Inc., Sunnyvale, CA) at room temperature (approximately 25°C). Pipettes (3 to 5 MΩ) were filled with intracellular solution (140 mM KCl, 1.6 mM MgCl₂, 2 mM EGTA, 10 mM HEPES, pH = 7.3). To evaluate the inhibitory activity of probe S2 to hERG channel, the holding potential was 80 mV, and membrane potential was switched from holding -80 to 20 mV for 2 s following return to -50 mV for 5.5 s in intervals of 30 to 45 s. After stable control recording, different concentration of probe S2 was continuously perfused into recording chamber near the cell for 4-10 min. Tail currents were measured at -50 mV. The raw data was analyzed by pClamp 10.0 software and then the results was transferred to GraphPad Prism 5 and the the IC₅₀ value was calculated (using equation: log (inhibitor) vs. response—Variable slope).

12. References

13. Characterization of probes

Figure S7. $^1$H-NMR spectra of probe S1
Supporting Information

Figure S8. $^{13}$C-NMR spectra of probe S1

Figure S9. ESI-HRMS spectra of probe S1
Figure S10. $^1$H-NMR spectra of probe S2

Figure S11. $^{13}$C-NMR spectra of probe S2
Figure S13. $^1$H-NMR spectra of probe S3
Figure S14. $^{13}$C-NMR spectra of probe S3

Figure S15. ESI-HRMS spectra of probe S3