Published in final edited form as:

Analyst. 2015 November 23; 140(24): 8101–8108. doi:10.1039/c5an01974e.

Novel Intramolecular Photoinduced Electron Transfer-Based Probe for the Human Ether-a-go-go-Related Gene (hERG) Potassium Channel

Zhenzhen Liu^a, Yubin Zhou^b, Lupei Du^a, and Minyong Li^a

Lupei Du: dulupei@sdu.edu.cn; Minyong Li: mli@sdu.edu.cn

^aDepartment of Medicinal Chemistry, Key Laboratory of Chemical Biology of Natural Products (MOE), School of Pharmacy, Shandong University, Jinan, Shandong 250012, China

^bInstitute of Biosciences & Technology, Texas A&M University Health Science Center, Houston, TX 77030, USA.

Abstract

Drug induced long QT syndrome is a high risk event in clinic, which mainly result from their high affinity with Human Ether-a-go-go-Related Gene (hERG) potassium channel. Therefore, evaluation of the drug's inhibitory activity against hERG potassium channel is a required step in drug discovery and development. In this study, we developed a series of novel conformation-mediated intramolecular photoinduced electron transfer fluorogenic probe for hERG potassium channel. After careful evaluation, probe N4 and N6 showed good activity and may have a promising application in cell-based hERG potassium channel inhibitory activity assay, as well as potential hERG-associated cardiotoxicity. Compared with other assay methods, such as patch clamp, radio-ligand competitive binding assay, fluorescent polarization and potential-sensitive fluorescent probes, this method is convenient and can also selectively measure the inhibitory activity in the native state of hERG potassium channel. Meanwhile, these probes can also be used for hERG potassium channel imaging without complex washing steps.

Graphical abstract

Correspondence to: Lupei Du, dulupei@sdu.edu.cn; Minyong Li, mli@sdu.edu.cn.

Electronic Supplementary Information (ESI) available: [NMR spectra, HRMS and experimental details]. See DOI: 10.1039/x0xx00000x

Our cell imaging work was performed at the Microscopy Characterization Facility, Shandong University.

Introduction

Human Ether-a-go-go-Related Gene (hERG) potassium channel conducts the rapidly activating component of delayed rectified potassium current (I_{Kr}) and plays a critical role in the repolarization phase of the cardiac action potential.¹ The therapeutic potential of targeting hERG channel is best attested by the successful development of antiarrhythmic drugs such as amiodarone, dofetilide and sotalol.² Notably, in recent years, more and more non-antiarrhythmic drugs, such as terfenadine, cisapride, grepafloxacin and terodiline, were withdrawn from market because of their implications in acquired long QT syndrome, a disorder that is characterized by a delay of repolarization and increases the risk of ventricular fibrillation and sudden death.³ This unexpected arrhythmogenic side effect has been mainly attributed to their tight interaction with the hERG channel.⁴ As a result, FDA currently requires careful evaluation of inhibitory activity against hERG for all drugs before clinical trial, which helps to reduce the risk of cardiotoxicity.

To expedite the drug discovery and development pipeline and to save tremendous time and resources, it is most desirable for the pharmaceutical industry to evaluate the hERG inhibitory activity of candidate drugs as early as possible. Hence, there is a critical need for the development of a convenient evaluation system to assess the hERG-associated cardiotoxicity.⁵ To address this issue, various assay methods have been developed, including patch clamp, radio-ligand competitive binding assay, and fluorescence-based assay.⁶⁻⁹ Among these methods, patch clamp assay is considered as the gold standard because of its high accuracy. However, this approach has many limitations, such as low throughput, labor intensive, costly and solely dependence on experienced electrophysiologists. Although automated patch clamp platforms could dramatically upscale the screening throughput, the unaffordability of such special equipment makes them less accessible to the majority of research laboratories. Radio-ligand assay is suitable to screen a large scale of compounds. Nonetheless, it must be conducted in a laboratory with restrictive radiation license. In comparison, the fluorescence-based assay can be conveniently carried out in general labs. Currently, several fluorescent probes were developed for hERG inhibitory activity screening, including potential-sensitive dye (DiSBAC4(3), DiSBAC2(3), CC2-DMPE/DiSBAC2(3), CC2-DMPE/DiSBAC4(3), FMP dye), probes for Tl^+ , a K^+ analogue.^{7, 10, 11} Although these probes have been applied in high-throughput screening, their selectivity for hERG is rather low, which often leads to false positive results. Very recently, Cy3B derived from Dofetilide was reported as a selective fluorescent probe for hERG channel and a high-throughput screening assay based on fluorescence polarization (FP) was established to screen hERG channel inhibitors.^{12, 13} However, the FP-based assay requires the disruption and lysis of assayed cells, and it may not necessarily reflect the native state of the hERG channel. For instance, during cell membrane preparation and the incubation process between ligand and extracted proteins, the structure of hERG channel may be destroyed and/or denatured. Therefore, developing a selective cell-based fluorescent probes for hERG potassium channel inhibitory activity assay is very meaningful, which is minimally perturb the structural integrity of hERG channels.

An ideal small molecule fluorescent probe for cell-based screening should be able to produce a significant fluorescence change before and after binding to hERG channel in a

living cell. In fact, unlike enzyme and small active molecules which can utilize their catalytic or reactive activity to design a turn-on switch^{14, 15}, for those biotargets with no catalytic activity, such as GPCRs, ion channels, DNAs and RNAs, how to branch out a fluorescent turn-on probe is still challenging. So far, there are few design strategies to be reported for those biotargets. One approach is environment-sensitive (solvatochromic) probes, which can detect the hydrophobic pocket of the target protein,¹⁶ and another is conformational alteration directed fluorogenic probes^{17–19}, such as intracellular photo-induced electron transfer (PET) effect. Due to not all target can have a hydrophobic pocket, conformational alteration directed PET off-on mechanism may have a wide application. Now, several groups have reported such probes, including 2,7-dichlorofluorescein (DCF) fluorophore derivatives which were used to detect carbonic anhydrase isozymes IX (CA IX), sequence-specific RNA and channel.^{20–22} In this PET system, the probes have four motifs, including fluorophore, recognition group, quencher and linker. In a free state, the probes adopted a folded conformation in which the electron-rich quencher element was brought in proximity to the electron-deficient fluorophore, and the fluorescence is quenched through intracellular photo-induced electron transfer (PET) effect (Scheme 1A). Upon binding to the target, this folded conformation is destroyed, and the distance between the quencher and the fluorophore is expanded, which disables the PET quenching effect, and the fluorescence was recovered simultaneously (Scheme 1A). Inspired by this design strategies and considering naphthalimide ring is also electron-deficient, we speculated that whether a similar conformational directed PET system can be established based on the naphthalimide fluorophore. Therefore, we designed a series of probes with different carbon chain length linker and substituents on the naphthalimide ring, as described in Scheme 1B. In the structure of the probes, we still use the piperazine ring as the linker which can have a fold and unfold conformation changes before and after binding to target (Scheme 1C), and the nitrogen atom in the ring which has a lone pair electrons is the quencher. In a folded state, the nitrogen was in proximity to naphthalimide ring and upon binding to hERG channel, the structure was changed to an unfolded state, and the fluorescence was released (Figure 1C). Different from the reported probes, the quencher is also a part of the recognition, which may increase the fluorescence intensity changes before and after binding to hERG channel, and this is also why we choose Azimilide as the recognition motif.

Results and discussion

Preparation of probes

As depicted in Scheme 2, probes were synthesized by simply substitution reaction between alkyl bromides with secondary amide in the piperazine ring, and the synthesis of the intermediates **18a–e** and **15a–b** were described in Scheme S1. It is found that different substituent in 1,8-Naphthalimide ring and carbon chain linker has a significant effect on the reaction behavior. Therefore, for the probes or intermediates synthesis, we adopted diversity route, the details seen the Scheme S1.

hERG potassium channel inhibition assay

Firstly, the inhibitory activity of the probes against hERG channel was evaluated using the radio-ligand competitive binding assay. The result revealed that the activity of the probes

was greatly improved compared to Azimilide (Table 1 and Figure S2). When the fluorophore and recognition were linked with two carbons alkyl chain, the probe exhibited the most potent activity. Meanwhile, the increase in the length of the linker was accompanied by a decrease in the inhibitory activity. When there is a substituent, acetyl or N,N-dimethyl, the inhibitory activity is also decreased in a degree.

Fluorescent properties of probes combined with hERG transfected HEK293 membranes

In our probe design, it is proposed that the nitrogen atom can form PET effect with the fluorophore. To confirm this, probes (10 μ M) were incubated with different amounts of hERG-transfected HEK293 cell membrane. Since that when there is no substituent on the naphthalimide ring, the excitation and emission wavelength of probes are too short (Figure S1). Therefore, in this assay, we only selected probe N1 and N4-7 for further evaluation. As shown in Figure 1, the fluorescence of all the probes was dramatically enhanced with the increased membrane concentration. When R group on the 4 position of naphthalimide ring is a hydrogen atom or acetyl, the degree of the fluorescence increase is relatively larger. The electron-donating group is not favorable for the formation of PET effect. In addition, it is known that the lone pair electrons of the nitrogen atom were crucial for the PET effect, so factor that can neutralize this electronegativity is able to destroy the PET effect, such as proton. Therefore, we also examined the fluorescence changes in response to varying pH values in Britton-Robinson buffer (Figure S3). The result is similar to membrane assay. However, it is interesting to note that fluorescence intensity of probe N4 was increased approximately 30-fold at acidic solution, which is greater than the results obtained from cell membrane assay, while the fluorescence enhancement of probe N6 and N7 is comparable in both assays (about 10 times). We speculated that when linker consists of two carbons, both of the nitrogen atom in the piperazine ring can form a PET effect with the naphthalimide fluorophore, and when the linker was lengthened to four and six carbons, only the outside nitrogen atom contribute to PET effect. Meanwhile, in probe N4, the PET effect formed by the inside nitrogen may not be easily destroyed by binding to the hERG channel.

The response selectivity of probes to different proteins

As described above, in PET effect assay, probe N1, N4 and N6-7 showed good activity. However, for probe N1, the fluorescent properties were less impressive with short excitation and emission wavelengths and lower fluorescence intensity. Therefore, in the follow-up experiment, we only tested the selectivity of fluorescent intensity enhancement of probe N4 and N6-7. As illustrated in Figure 2, all these three probes displayed excellent selectivity against bovine serum albumin (BSA) and trypsin, which often form a non-specific binding with small molecules. In addition, the fluorescence enhancement of probes incubated with hERG-transfected HEK293 cell membranes can be decreased by an hERG inhibitor, Astemizole. However, this fluorescence enhancement cannot be completely suppressed as the previously-reported probe A1 did. This might be caused by the unavoidable non-specific binding with other proteins in the membrane. Moreover, in comparison with probe N4 and N6, the fluorescence quenching effect of probe N7 by astemizole is less striking, which may be due to its lower activity.

Application of probe N4 and N6 in high-throughput screening for hERG channel inhibitor on live cells

After a series of evaluation described above, probe N4 and N6 exhibited excellent activities with better fluorescent properties and selectivity. Therefore, we further investigated whether probe N4 and N6 can be used in cell-based screening for hERG channel inhibitors using HEK 293 cells stably expressing the hERG channel. In this assay, we initially chose Astemizole as the positive control. In competition binding experiment with increasing concentrations of Astemizole, both probes N4 and N6 displayed the anticipated sigmoidal response curve (Figure 3A and 3C). However, the calculated IC_{50} value is 3.37 ± 1.88 and $3.82 \pm 1.86 \mu M$, respectively (repeated three times and each time, one concentration was triplicate), which was much lower than IC_{50} value (nanomolar level) for Astemizole derived from other cell-based assay, including patch clamp and potential-sensitive dye. Next, we further evaluated the activity of another hERG inhibitor in micromolar range, Sotalolol, using probe N4 and N6. Similarly, the calculated IC_{50} value was decreased from micromolar to millimolar level (Figures 3B and 3D). We speculated that the cause leading to this discrepancy might lie in different test mechanism, competitive binding for our probes and functional screening for patch clamp assay and potential-sensitive dye.

In addition, as mentioned above, our probes were sensitive to pH. Therefore, we also investigated the effect of Astemizole's basicity on the cell-based screening assay. The results demonstrated that the fluorescence had diminutive changes with the increasing concentrations of Astemizole (Figures 4A and B), which indicated that the fluorescence decrease was induced by the binding to hERG channel not the increasing basicity of Astemizole. To evaluate the assay feasibility and quality of probes N4 and N6 for hERG channel inhibitors, Z' factor was also calculated in the presence of $50 \mu M$ Astemizole (Figure 4C and D). The results presented that probes N4 and N6 are suitable for high-throughput screening with a 0.54 and 0.61 Z' factor value, respectively. Therefore, probe N4 and N6 may have a high promising application for high-throughput screening of hERG channel inhibitor.

Cell culture and imaging

To extend the application of probes, we also evaluated whether probes N4 and N6 can be used in hERG channel imaging in living cells. The fluorescent microscopy (performed in Zeiss Axio Observer A1) results revealed that probe N4 and N6 can selectively stained hERG transfected HEK293 cells, while the staining is very low in normal HEK293 cells (Figure 5). Moreover, the fluorescence can be decreased by a potent hERG channel inhibitor, Astemizole. More importantly, obtaining these fluorescent microscopic images did not need a conventional washing procedure, which allows rapid and precise detection of the hERG channel in a real-time manner with low background signal. This appealing result may come from the PET turn-on mechanism introduced in the probe design. Therefore, probes N4 and N6 may be the very promising toolkits for hERG channel detection in living and intact cells.

Conclusion

In summary, we have developed a naphthalimide-based fluorogenic probe N4 and N6 that can be applied in cell-based screening of hERG channel inhibitors. Upon binding to the hERG channel, the probe causes a remarkable 20-fold increase in fluorescence intensity. The wide dynamic range enables us to evaluate confidently the binding affinity of compounds on the hERG channel. Compared with radio-ligand competitive binding assay and FP assay, this cell-based method exerts minimal perturbation to the native hERG channel. Moreover, the turn-on switch we introduced in the probes is an intracellular type so that it does not need a laborious washing procedure as potential-sensitive dye method. Compare to the patch clamp technique, our method excels by its convenience and low demand for instruments. Therefore, the method established herein may provide a promising approach for cardiotoxicity evaluation of drug candidates in early stage development, in particular for those possessing high binding affinities with the hERG channel at nanomolar level. Aside from that, probe N4 and N6 also show great potential for the application of hERG channel imaging. In particular, a laborious washing procedure is not needed when imaging. Taken together, our newly developed hERG probes are not only suitable for real-time imaging of hERG channel in living cells but also compatible with high-throughput screening of small molecule modulators for hERG channel and potential hERG-associated cardiotoxicity.

Experimental section

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. ^1H NMR and ^{13}C NMR were recorded on a Bruker 300M NMR and 600M 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. Fluorescence imaging was performed using Zeiss Axio Observer A1 fluorescence microscope.

Synthesis of fluorescent probes

Synthesis of probe N1—A mixture of **compound 18a** (80 mg, 0.26 mmol), **compound 3** (0.14 g, 0.31 mmol), K_2CO_3 (71.4 mg, 0.52 mmol) in acetonitrile was refluxed for 12 h. The resulting mixture was filtrated and the filtrate was concentrated under reduced pressure. The crude product was purified by silica gel chromatography (eluent: 0%–33.3% CH_2Cl_2 in methanol) to afford compound N1 as a light yellow solid in 52.7% yield. ^1H -NMR (300MHz, CDCl_3): δ 8.60 (dd, $J=7.2$, 0.9Hz, 2H), 8.23 (d, $J=8.4$ Hz, 2H), 7.91 (s, 1H), 7.78 (t, $J=7.8$ Hz, 2H), 7.68 (d, $J=8.4$ Hz, 2H), 7.37 (d, $J=8.7$ Hz, 2H), 6.91 (d, $J=3.6$ Hz, 1H), 6.74 (d, $J=3.6$ Hz, 1H), 4.35 (t, $J=6.9$ Hz, 2H) 4.26 (s, 2H), 3.66-3.61 (m, 2H), 2.88-2.58 (m, 10H), 1.70 (brs, 4H). ^{13}C -NMR (75MHz, CDCl_3): δ 166.79, 164.15, 155.03, 153.27, 148.79, 135.26, 134.12, 133.90, 131.60, 131.19, 129.03, 128.24, 128.20, 126.93, 125.62, 122.70,

115.52, 107.82, 57.83, 55.62, 53.18, 53.08, 48.83, 38.99, 37.50, 25.98, 23.86. HRMS (ESI) m/z calcd. for $C_{36}H_{36}ClN_6O_5$ ($[M + H]^+$) 667.2436; found 667.2430.

Synthesis of probe N2—Probe N2 was prepared following the method described for probe N1, instead employing **compound 18b** (80 mg, 0.25 mmol), **compound 3** (0.13 g, 0.30 mmol) and K_2CO_3 (68.4 mg, 0.49 mmol), and get probe N2 as a light yellow solid in 47.6% yield. 1H -NMR (300MHz, $CDCl_3$): δ 8.60 (d, $J=7.2$ Hz, 2H), 8.23 (d, $J=7.5$ Hz, 2H), 7.93 (s, 1H), 7.78 (t, $J=7.8$ Hz, 2H), 7.69 (d, $J=8.7$ Hz, 2H), 7.38 (d, $J=8.4$ Hz, 2H), 6.92 (d, $J=3.6$ Hz, 1H), 6.74 (d, $J=3.6$ Hz, 1H), 4.27-4.23 (m, 4H), 3.64 (t, $J=6.9$ Hz, 2H), 2.55-2.29 (m, 10H), 1.99-1.90 (m, 2H), 1.72-1.62 (m, 2H), 1.56-1.50 (m, 2H), 1.33-1.24 (m, 2H). ^{13}C -NMR (75MHz, $CDCl_3$): δ 166.79, 164.26, 155.07, 153.28, 148.77, 135.28, 134.16, 133.86, 131.61, 131.16, 129.04, 128.25, 128.20, 126.93, 125.63, 122.80, 115.56, 107.82, 57.78, 56.03, 53.00, 52.81, 48.82, 38.99, 38.91, 25.96, 24.99, 23.79; HRMS (ESI) m/z calcd. for $C_{37}H_{38}ClN_6O_5$ ($[M + H]^+$) 681.2592; found 681.2593.

Synthesis of probe N3—Probe N3 was prepared following the method described for probe N1, instead employing **compound 18c** (80 mg, 0.23 mmol), **compound 3** (0.12 g, 0.28 mmol) and K_2CO_3 (65.5 mg, 0.47 mmol), and get probe N3 as a light yellow solid in 60.7% yield. 1H -NMR (300MHz, $CDCl_3$): δ 8.61 (dd, $J=7.5, 0.9$ Hz, 2H), 8.23 (d, $J=8.4, 0.9$ Hz, 2H), 7.94 (s, 1H), 7.78 (t, $J=8.1$ Hz, 2H), 7.69 (d, $J=8.7$ Hz, 2H), 7.39 (d, $J=8.7$ Hz, 2H), 6.92 (d, $J=3.6$ Hz, 1H), 6.75 (d, $J=3.6$ Hz, 1H), 4.24-4.18 (m, 4H), 3.66-3.62 (m, 2H), 2.53-2.42 (m, 10H), 1.90-1.49 (m, 10H). ^{13}C -NMR (75MHz, $CDCl_3$): δ 166.80, 164.21, 155.07, 153.28, 148.78, 135.33, 134.16, 133.91, 131.60, 131.22, 129.05, 128.26, 128.18, 126.94, 125.64, 122.72, 115.55, 107.82, 57.17, 57.79, 52.92, 48.86, 40.13, 39.00, 26.13, 25.98, 23.89; HRMS (ESI) m/z calcd. for $C_{38}H_{40}ClN_6O_5$ ($[M + H]^+$) 695.2749; found 695.2749.

Synthesis of probe N4—Probe N4 was prepared following the method described for probe N1, instead employing **compound 18d** (80 mg, 0.22 mmol), **compound 3** (0.14 g, 0.33 mmol) and K_2CO_3 (60.1 mg, 0.44 mmol), and get probe N4 as a light yellow solid in 20.1% yield. 1H -NMR (300MHz, $DMSO-d_6$): δ 10.43 (s, 1H), 8.74 (d, $J=8.4$ Hz, 1H), 8.54 (d, $J=7.2$ Hz, 1H), 8.49 (d, $J=8.1$ Hz, 1H), 8.32 (d, $J=8.1$ Hz, 1H), 7.92 (t, $J=8.4$ Hz, 1H), 7.80-7.77 (m, 3H), 7.54 (d, $J=8.4$ Hz, 2H), 7.19 (d, $J=3.6$ Hz, 1H), 6.99 (d, $J=3.6$ Hz, 1H), 4.18 (brs, 2H), 3.47 (brs, 2H), 2.51 (brs, 2H), 2.28 (s, 4H), 1.56 (brs, 4H). ^{13}C -NMR (75MHz, $DMSO-d_6$): δ 169.57, 167.64, 163.49, 162.91, 153.34, 152.93, 149.32, 140.42, 133.35, 132.61, 131.65, 130.88, 129.39, 129.10, 128.31, 126.34, 125.56, 124.02, 122.18, 119.40, 117.36, 115.64, 109.01, 48.05, 24.04; HRMS (ESI) m/z calcd. for $C_{38}H_{39}ClN_7O_6$ ($[M + H]^+$) 724.2650; found 724.2645.

Synthesis of probe N5—Probe N5 was prepared following the method described for probe N1, instead employing **compound 18e** (60 mg, 0.17 mmol), **compound 3** (0.11 g, 0.25 mmol) and K_2CO_3 (47.0 mg, 0.34 mmol), and get probe N5 as a yellow solid in 26.7% yield. 1H -NMR (300MHz, $CDCl_3$): δ 8.57 (dd, $J=7.5, 0.9$ Hz, 1H), 8.48-8.42 (m, 2H), 7.94 (s, 1H), 7.69-7.60 (m, 3H), 7.39 (d, $J=8.7$ Hz, 2H), 7.13 (d, $J=8.4$ Hz, 1H), 6.92 (d, $J=3.6$ Hz, 1H), 6.74 (d, $J=3.6$ Hz, 1H), 4.34 (t, $J=6.9$ Hz, 2H), 4.26 (s, 2H), 3.66 (t, $J=6.6$ Hz, 2H), 3.10

(s, 6H), 2.75-2.50 (m, 12H), 1.71-1.66 (m, 4H). ^{13}C -NMR (75MHz, CDCl_3): δ 167.80, 163.76, 163.09, 156.74, 153.51, 153.14, 149.54, 133.46, 132.79, 132.45, 131.70, 130.73, 129.78, 129.28, 128.51, 125.75, 125.17, 124.39, 122.46, 115.80, 113.45, 113.16, 109.18, 57.35, 55.38, 53.01, 52.93, 48.20, 44.54, 38.32, 36.98, 25.60, 23.50; HRMS (ESI) m/z calcd. for $\text{C}_{38}\text{H}_{41}\text{ClN}_7\text{O}_5$ ($[\text{M} + \text{H}]^+$) 710.2858; found 710.2850.

Synthesis of probe N6—Probe N6 was prepared following the method described for probe N1, instead employing **compound 15a** (50 mg, 128.45 μmol), **compound 5** (51.84 mg, 116.78 μmol , described in SI) and K_2CO_3 (29.97 mg, 0.23 mmol), and get probe N6 as a yellow solid in 6.94% yield. ^1H -NMR (300MHz, DMSO- d_6): δ 10.41 (s, 1H), 8.73 (d, $J=8.4\text{Hz}$, 1H), 8.54 (d, $J=6.9\text{Hz}$, 1H), 8.49 (d, $J=8.4\text{Hz}$, 1H), 8.32 (d, $J=8.4\text{Hz}$, 1H), 7.91 (t, $J=8.4\text{Hz}$, 1H), 7.81-7.78 (m, 3H), 7.54 (d, $J=8.7\text{Hz}$, 2H), 7.19 (d, $J=3.6\text{Hz}$, 1H), 6.98 (d, $J=3.6\text{Hz}$, 1H), 4.39 (s, 2H), 4.07 (t, $J=6.6\text{Hz}$, 2H), 3.48 (t, $J=6.6\text{Hz}$, 2H), 2.38-2.28 (m, 9H), 1.64-1.45 (m, 8H). ^{13}C -NMR (75MHz, DMSO- d_6): δ 169.55, 167.62, 163.48, 162.92, 153.33, 152.94, 149.34, 140.33, 133.30, 132.61, 131.59, 130.82, 129.26, 129.10, 128.31, 126.33, 125.56, 124.00, 122.27, 119.36, 117.45, 115.62, 109.01, 57.11, 56.92, 52.21, 52.10, 48.02, 38.05, 25.32, 24.04; HRMS (ESI) m/z calcd. for $\text{C}_{40}\text{H}_{43}\text{ClN}_7\text{O}_6$ ($[\text{M} + \text{H}]^+$) 752.2963; found 752.2950.

Synthesis of probe N7—Probe N7 was prepared following the method described for probe N1, instead employing **compound 15b** (60 mg, 0.13 mmol), **compound 5** (67.68 mg, 0.16 mmol) and K_2CO_3 (37.32 mg, 0.27 mmol), and get probe N7 as a yellow solid in 11.7% yield. ^1H -NMR (300MHz, DMSO- d_6): δ 10.40 (s, 1H), 8.73 (d, $J=7.8\text{Hz}$, 1H), 8.54 (d, $J=6.6\text{Hz}$, 1H), 8.49 (d, $J=8.4\text{Hz}$, 1H), 8.32 (d, $J=8.1\text{Hz}$, 1H), 7.91 (t, $J=7.5\text{Hz}$, 1H), 7.81-7.78 (m, 3H), 7.54 (d, $J=8.7\text{Hz}$, 2H), 7.19 (d, $J=3.6\text{Hz}$, 1H), 6.99 (d, $J=3.6\text{Hz}$, 1H), 4.39 (s, 2H), 4.05 (t, $J=6.9\text{Hz}$, 2H), 3.48 (t, $J=6.9\text{Hz}$, 2H), 2.50-2.28 (m, 12H), 1.63-1.33 (m, 12H). ^{13}C -NMR (75MHz, DMSO- d_6): δ 169.55, 167.62, 163.45, 162.89, 153.34, 152.95, 149.34, 140.33, 133.32, 133.01, 132.62, 131.59, 130.81, 130.63, 129.10, 128.93, 128.32, 126.33, 125.56, 124.00, 122.25, 119.35, 117.43, 115.62, 109.01, 48.02, 38.05, 27.37, 26.29, 25.30, 24.04; HRMS (ESI) m/z calcd. for $\text{C}_{42}\text{H}_{47}\text{ClN}_7\text{O}_6$ ($[\text{M} + \text{H}]^+$) 780.3276; found 780.3270.

hERG potassium channel inhibition assay⁸

The inhibitory activity on hERG potassium channel was determined by a radio-ligand binding assay. Astemizole (Cat. No.#A2861-10MG, Sigma-Aldrich) and Atropine 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, probes were dissolved in DMSO as a 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 to the filter plates and filtered with Millipore vacuum manifold. The wells were washed with 2 mL/well cold wash buffer and dried at room temperature for 120 min. The bottom of the plates was sealed with Bottom Seal™ (opaque) (Perkin Elmer) and 50 µL MicroScint 20™ (Perkin Elmer) was added to each well. The plates were 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 = $100 \times (1 - (\text{sample CPM} / \text{Total binding CPM}))$ (in which total binding CPM values were obtained by testing binding of [3H] dofetilide to the targets without competitors).

Application of probe N4 and N6 in high-throughput screening for hERG channel inhibitor base-on live cells

Whether probe N4 and N6 can be used in cell-based screening for hERG channel inhibitors was evaluated using hERG gene stably transfected HEK 293 cells which were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 400 µg/ml G418 (Sigma). In this assay, Astemizole and Sotalol were selected as positive drugs. The detailed procedure was following: Firstly, 96-well plates (#3603) were coated with polylysine (100 mg/mL). An amount of $3-5 \times 10^4$ cells per well were transferred to 96-well plates. After culturing for 18–24 h, the medium was removed and washed with assay buffer (160 mM NaCl, 4.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, pH= 7.4) three times. Then, 90 µL of 1 µM probe N4 or N6 in above assay buffer was added to each well and then incubated for 30 min. Subsequently, 10 µL of different concentrations of positive drugs (Astemizole and Sotalol, prepared in assay buffer) were added to each well and continued to be incubated for 30 min. Plates were read on Biotek Synergy microplate reader at 460 nm, excited at 350 nm. The background fluorescent intensity was determined using wells with only 100 µL assay buffer. The final fluorescent intensity (FI) for each cell induced by the binding of probes to hERG channel was FI (each well) – FI (background signal). Then, the data was analyzed using GraphPad Prism 5 software and IC₅₀ value was calculated (using the equation: $\log(\text{inhibitor})$ vs. $-\text{responseVariable slope}$). IC₅₀ value was repeated three times (each time, one concentration was triplicated).

To assess the quality of high-throughput screening of hERG channel inhibitors, Z' factor was also determined using 96-well plates as the similar procedure described above (Figure S4). In this assay, 20–30 wells were randomly selected as negative groups, and 20–30 wells

were randomly selected as positive groups. For negative groups, 100 μ L of 1 μ M probes were added to each well and for the positive group, the final concentration of Astemizole was 50 μ M. The Z' factor was calculated using the following equation $z' = 1 - (3 * SD(\text{negative}) + 3 * SD(\text{positive})) / |FI(\text{negative}) - FI(\text{positive})|$.

Cell culture and imaging

hERG transfected HEK293 were grown in DMEM medium supplemented with 10% (v/v) fetal bovine serum and 400 μ g/ml G418 (Sigma) in an atmosphere of 5% CO₂, 95% air at 37 °C. Moreover, the control cell lines HEK293 were maintained in the same culture conditions without G418. Cells were plated on the 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 N4 and N6 (10 μ M, prepared in DMEM medium without fetal bovine serum) or co-incubated with N4 or N6 and 50 μ M Astemizole (a potent hERG channel blocker) for 15 min. Fluorescence imaging was performed using Zeiss Axio Observer A1 fluorescence microscope. Then, the contrast of images was adjusted using ImageJ software

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The present project was supported by grants from the National Natural Science Foundation of China (No. 30901836), the Doctoral Fund of Shandong Province (No. BS2012YY008), the Shandong Natural Science Foundation (No. JQ201019), the Scientific Research Foundation for the Returned Overseas Chinese Scholars, the Independent Innovation Foundation of Shandong University, IIFSDU (Nos. 2009TB021 and 2012JC002) and the National Institutes of Health (No. RO1GM112003). We also thank Professor Gui-Rong Li from the University of Hong Kong for his generous gift, the hERG-transfected HEK293 cell.

Notes and references

1. Tristani-Firouzi M, Sanguinetti Michael C. *Cell. Cardiol.* 2003; 35:27–35.
2. He F-Z, McLeod HL, Zhang W. *Trends Mol. Med.* 2013; 19:227–238. [PubMed: 23369369]
3. Brown AM. *Cell Calcium.* 2004; 35:543–547. [PubMed: 15110144]
4. Hancox JC, McPate MJ, El Harchi A, Zhang Yh. *Pharmacol. Ther.* 2008; 119:118–132. [PubMed: 18616963]
5. Hamdam J, Sethu S, Smith T, Alfirevic A, Alhaidari M, Atkinson J, Ayala M, Box H, Cross M, Delaunois A, Dermody A, Govindappa K, Guillon JM, Jenkins R, Kenna G, Lemmer B, Meecham K, Olayanju A, Pestel S, Rothfuss A, Sidaway J, Sison-Young R, Smith E, Stebbings R, Tingle Y, Valentin JP, Williams A, Williams D, Park K, Goldring C. *Toxicol Appl Pharmacol.* 2013; 273:229–241. [PubMed: 23732082]
6. Wolff C, Fuks B, Chatelain P. *J Biomol Screen.* 2003; 8:533–543. [PubMed: 14567780]
7. Zheng W, Spencer RH, Kiss L. *Assay Drug Dev Technol.* 2004; 2:543–552. [PubMed: 15671652]
8. Huang XP, Mangano T, Hufeisen S, Setola V, Roth BL. *Assay Drug Dev Technol.* 2010; 8:727–742. [PubMed: 21158687]
9. Wible BA, Hawryluk P, Ficker E, Kuryshv YA, Kirsch G, Brown AM. *J Pharmacol Toxicol Methods.* 2005; 52:136–145. [PubMed: 15950494]
10. Dorn A, Hermann F, Ebneith A, Bothmann H, Trube G, Christensen K, Apfel C. *J Biomol Screen.* 2005; 10:339–347. [PubMed: 15964935]

11. Maher MP, Wu NT, Ao H. *J Biomol Screen*. 2007; 12:656–667. [PubMed: 17517905]
12. Singleton DH, Boyd H, Steidl-Nichols JV, Deacon M, de Groot MJ, Price D, Nettleton DO, Wallace NK, Troutman MD, Williams C, Boyd JG. *J Med Chem*. 2007; 50:2931–2941. [PubMed: 17536794]
13. Deacon M, Singleton D, Szalkai N, Pasieczny R, Peacock C, Price D, Boyd J, Boyd H, Steidl-Nichols JV, Williams C. *J Pharmacol Toxicol Methods*. 2007; 55:255–264.
14. Chan J, Dodani SC, Chang CJ. *Nat Chem*. 2012; 4:973–984. [PubMed: 23174976]
15. Mizukami S, Hori Y, Kikuchi K. *Acc Chem Res*. 2014; 47:247–256. [PubMed: 23927788]
16. Zhuang Y-D, Chiang P-Y, Wang C-W, Tan K-T. *Angew Chem Int Ed Engl*. 2013; 52:8124–8128. [PubMed: 23780746]
17. Sparano BA, Koide K. *J Am Chem Soc*. 2007; 129:4785–4794. [PubMed: 17385867]
18. Zhang S, Yang C, Lu W, Huang J, Zhu W, Li H, Xu Y, Qian X. *Chem Comm*. 2011; 47:8301–8303. [PubMed: 21681333]
19. Zhang H, Fan J, Wang J, Zhang S, Dou B, Peng X. *J Am Chem Soc*. 2013; 135:11663–11669. [PubMed: 23862760]
20. Zhang S, Yang C, Lu W, Huang J, Zhu W, Li H, Xu Y, Qian X. *Chemical communications*. 2011; 47:8301–8303. [PubMed: 21681333]
21. Sparano BA, Koide K. *Journal of the American Chemical Society*. 2007; 129:4785–4794. [PubMed: 17385867]
22. Liu Z, Wang B, Ma Z, Zhou Y, Du L, Li M. *Anal Chem*. 2015; 87:2550–2554. [PubMed: 25665091]

Figure 1.

A–E: Fluorescent emission spectra of 10 μ M probe N1, N4-7 incubated with different concentrations of membrane (3.2, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0 mg/mL) for 30 min at room temperature (for probe N1, N4, N6-7, excited at 355 nm; for probe N5, excited at 440 nm); **F:** Fluorescent intensity (normalized based on the last point that is seen as 1) at their maximum emission wavelength respectively.

Figure 2. Fluorescent intensity changes of 10 μ M probe N4, N6-7 incubated with 1 mg/mL proteins (blank, trypsin, BSA, hERG-transfected HEK293 cell membrane, hERG-transfected HEK293 cell membrane and Astemizole (20 μ M) for 20–30 min.

Figure 3.
Concentration-response curves of Astemizole and Sotalol determined using 1 μ M probe N4 (A–B) and N6 (C–D) respectively.

Figure 4.

A–B: The effect of basicity of Astemizole to the cell-based screening assay (**A** for probe N4, **B** for probe N6); **C–D:** the calculated Z' factor value of probe N4 (**C**) and N6 (**D**) for screening of hERG channel blockers;

Figure 5.

Fluorescence microscopic imaging of hERG-transfected HEK293 cells and HEK293 cells incubated with 10 μ M probe N4 (Top row: A1, hERG-transfected HEK293 cells in the absence of 50 μ M Astemizole; A2, hERG-transfected HEK293 cells in the presence of 50 μ M Astemizole; A3, HEK293 cells) and N6 (Bottom row: B1, hERG-transfected HEK293 cells in the absence of 50 μ M Astemizole; B2, hERG transfected HEK293 in the presence of 50 μ M Astemizole; B3, HEK293 cells). Objective lens: 63 $\times$.

Scheme 1.

The design strategy of small molecule fluorogenic probes for the hERG channel based on a conformation directed intracellular PET effect.

Scheme 2.
The synthesis route of probes N1–N7

Table 1

Fluorescent properties of probe N1-7 and their inhibitory activity against the hERG channel (determined by radio-ligand competitive binding assay)

	Wavelength (nm)		IC ₅₀ (μ M)	K _i (μ M)
	λ_{ex}	λ_{em}		
Probe N1	348	388	0.054	0.027
Probe N2	340	387	0.16	0.08
Probe N3	338	391	0.25	0.13
Probe N4	355	460	0.24	0.12
Probe N5	440	545	0.246	0.123
Probe N6	354	470	0.653	0.326
Probe N7	353	469	1.39	0.698
Azimilide	-	-	1.91	0.954