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Letter

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Fluorogenic Probe for the Human Ether-a-Go-Go-Related Gene Potassium Channel Imaging

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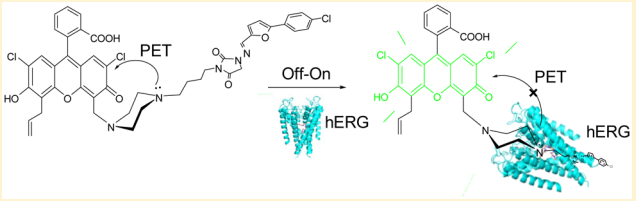
Supporting Information

ABSTRACT: The first small-molecule fluorogenic probe A1 for imaging the human Ether-a-go-go-Related Gene (hERG) potassium channel based on the photoinduced electron transfer (PET) off−on mechanism was described herein. After careful biological evaluation, this probe had the potential of detecting and imaging the hERG channel at the molecular and cellular level. Moreover, the competitive binding mechanism of this probe would presumably minimize the effects on the electrophysiological properties of the hERG channel. Therefore, this probe may serve as a powerful toolkit to the hERG-associated study.



Human Ether-a-go-go-Related Gene (hERG) encodes the main subunit of the rapid rectifier potassium channel that plays a critical role in the repolarization phase of cardiac action potential.[1] Mutation in the hERG gene can result in severe inherit long QT syndrome (LQTS-2).[2] Blocking the hERG channel can prolong the action potential duration, which has been applied to treat ventricular tachycardia. However, presently, more and more nonantiarrythmic drugs were found to be associated with acquired long QT syndrome, such as antibiotics (sufisomezole), antipsychotics (chlorpromazine), antihistamine (terfenadine), gastric motility drugs (cisapride), and so on.[3,4] Subsequent research found that these drugs have high affinity with hERG potassium channel, which contributes to their arrhythmogenic side effect. Currently, FDA requires that all drugs should assay the affinity with hERG channel so as to evaluate their cardiotoxicity. In addition, hERG channel is also expressed in neural tissues, pancreas, smooth muscle,[2] liver, and other tissues.[5−7] In tumors derived from these types of tissues, such as endometrial cancer, colorectal cancer, and neuroblastoma, the expression of hERG channel is up-regulated and is regarded to facilitate cell proliferation, invasion, and tumor angiogenesis.[8] Therefore, the hERG channel may be a potential tumor biomarker for diagnostic purposes.

To aid in better analysis and molecular imaging of the hERG channel, several methods have been developed in recent years, including immunofluorescence and fluorescent protein-based techniques.[9−11] During immunofluorescent manipulation, cells have died and it could record a cell at a certain time without temporal resolution. Fluorescent protein-based technique involves complicated, expensive, and time-consuming procedures by conjugating a fluorescent protein as the reporter. Moreover, it has been reported that fluorophore conjugation to the channel often affects the electrophysiological properties.[12] As a result, there is an urgent need for developing a convenient method to aid both molecular imaging and mechanistic dissection of the hERG channel.

Fluorescence-based techniques, such as small molecule fluorescent probes, fluorescent protein based methods, and quantum dot-based approaches, have been well developed since the first description of fluorescence phenomenon in 1575. Among these techniques, small molecule fluorescent probes, for its unique advantages including high sensitivity, low cost, and convenient operation have been widely used in high-throughput screening for identification of drug candidates, biotarget (protein, DNA, RNA, H_2S, H_2O_2, and others) detection, and imaging.[13,14] In addition, small molecule fluorescent probes can observe the biotarget in a living state and provide dynamic information at spatial and temporal resolution, thereby expanding the repertoire of chemical toolkits that are useful for biological study and medical diagnosis.

Until now, there is only one report that describes the use of small molecule fluorescent probes for hERG channels on the basis of dofetilide.[15] This probe has been applied to establish the high-throughput screening assay for hERG inhibitors based on the fluorescence polarization (FP) assay. Meanwhile, there are several other small molecule potential sensitive probes for indirectly detecting the hERG channel, such as DiSBAC4(3), DiSBAC2(3), CC2-DMPE/DiSBAC2(3), CC2-DMPE/DiSBAC4(3), FMP dye, as well as probes for detecting Tl^+, a K^+ analogue.[16−18] Although these probes have been applied in high-throughput screening, their low sensitivity often hampers

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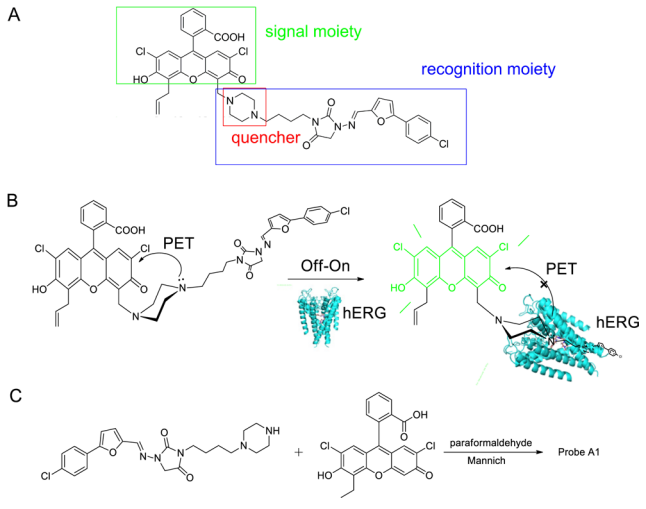
further mechanistic studies. Therefore, in the current study, we undertake an effort on developing a small molecule fluorescent probe that may provide a convenient strategy to image the hERG channel. After careful selection, azimilide, a classical hERG channel inhibitor, was herein selected as the pharmacophore group to develop a small molecule fluorescent probe for hERG channel imaging.

It should be noted that an ideal small molecule fluorescent probe should have an off−on effect, which can reduce the background signal. Until now, a series of fluorescence turn-on mechanisms have been well developed, such as photoinduced electron transfer (PET), intramolecular charge transfer (ICT), and fluorescence resonance energy transfer (FRET).[19,20] In 2004, the Koide group reported a 2,7-dichlorofluorescein (DCF) fluorophore derivation, which can form a intramolecular PET effect on the nitrogen atom in the piperazine ring as the electron donor (or quencher).[21,22] In a free state, the probe adopts a closed conformation in which the nitrogen atom was brought in proximity to the xanthene ring, and the fluorescence is quenched through the PET effect. Upon binding the target molecules, this closed conformation is destroyed and the distance between the nitrogen atom and the xanthene ring is expanded, which disables the PET quenching, and the fluorescence was recovered simultaneously. By using this turn-on mechanism, the Qian group developed a selective fluorogenic probe for detecting carbonic anhydrase IX (CA IX).[23] Inspired by this strategy, we set out to apply this off/on mechanism for designing a fluorescent probe for the hERG channel (Scheme 1A,B). Moreover, this is also the reason why

Scheme 1. Design Strategies of a Small Molecule Fluorescent Probe for hERG Channel Imaging (A and B) and the Synthetic Route of Probe A1



we chose azimilide as the recognition motif, in which there is a piperazine substructure as the quenching group. However, our approach differs from their design since the quencher motif in our probe is part of the recognition structure and involved in the binding with the target, which may facilitate to destroy the PET effect.

Subsequently, probe A1 was synthesized as shown in Scheme 1C. In brief, the azimilide recognition motif and the 2,7-dichlorofluorescein (DCF) fluorophore derivation were prepared as previously described (Scheme S1 in the Supporting Information).[22,24] The recognition moiety was incorporated with fluorophore through a Mannich reaction. After obtaining the probe A1, we evaluated the inhibitory activity against hERG using a radio-ligand competitive binding assay. The results demonstrated that probe A1 displayed good inhibitory effects against the hERG channel, and the calculated IC_{50} and K_i values are 1.60 and 0.801 μM, respectively, which are comparable to azimilide (1.91 and 0.954 μM, see Table 1), while atropine exhibits no activity.

Table 1. Fluorescent Property of Probe A1 and Its Inhibitory Effect on hERG Channel

	wavelength (nm)			
cmpd	λ_{ex}	λ_{em}	IC_{50} (μM)	K_i (μM)
probe A1	515	535	1.60	0.801
azimilide			1.91	0.954
atropine			NA	NA

As described above, the nitrogen atom in our probe structure was proposed to form a PET quenching with the fluorophore. Therefore, with the increased concentration of target protein, this PET quenching effect may be destroyed to release the fluorescence. To confirm this, a series of concentrations of hERG-transfected HEK293 cell membrane was incubated with the same concentration of probe A1 (5 μM). As anticipated, fluorescence intensity was gradually enhanced with increased membrane concentrations (Figure 1A,B). When incubated with 1 mg/mL membrane, the fluorescence intensity presented was approximately 2.5-fold higher than that of the blank group.

Afterward, the selectivity of fluorescent intensity enhancement of probe A1 was examined, in which bovine serum albumin (BSA) and trypsin were selected as negative controls

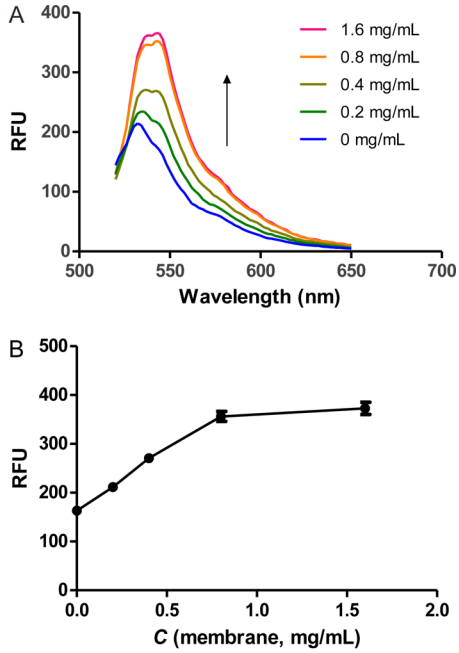


Figure 1. (A) Fluorescent emission spectra of 5 μM probe A1 incubated with different concentrations of membrane (1.6, 0.8, 0.4, 0.2, and 0 mg/mL) for 20−30 min in the assay buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, 10 mM KCl) at room temperature. (B) The corresponding fluorescent intensity changes at 544 nm with increased membrane concentration (λ_{ex} = 490 nm).

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because that they can easily form a nonspecific binding with small molecules. Probe A1 (5 μM) was incubated with these different proteins or hERG-transfected HEK293 cell membrane at the same concentration (1 mg/mL). The experimental results demonstrated that no discernible fluorescence increase for trypsin was detected. However, there is a certain degree of increase for BSA, which indicted that probe A1 may form some nonspecific binding with BSA. When treated with hERG-transfected HEK293 cell membrane, probe A1 exhibited a higher fluorescence response over other proteins. Specifically, the selectivity of this probe for hERG-transfected HEK293 cell membrane is more than 2.5-fold over trypsin and the blank control and 1.5-fold over BSA (Figure 2). In addition, the

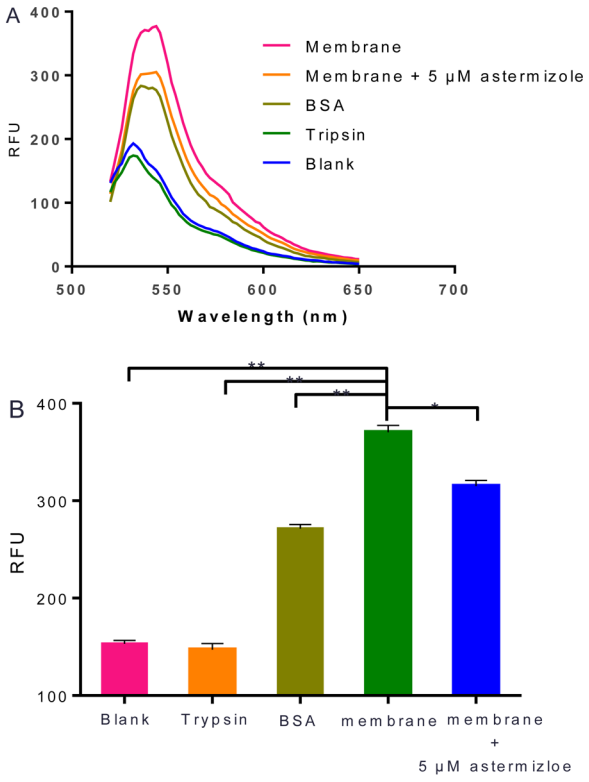

Figure 2. (A) Fluorescent emission spectra of 5 μM probe A1 incubated with 1 mg/mL of different proteins or membrane (trypsin, BSA, and hERG-transfected HEK293 cell membrane in the presence or absence of 5 μM astermizole) for 20−30 min in assay buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, 10 mM KCl) at room temperature (λ_{ex} = 490 nm, λ_{em} = 544 nm). (B) The corresponding fluorescent intensity changes of 5 μM probe A1 at 544 nm incubated with 1 mg/mL trypsin, BSA, and hERG-transfected HEK293 cell membrane in the presence or absence of 5 μM astermizole. ** < 0.001, * < 0.01 (t-test, calculated by GraphPad Prism software).

fluorescence enhancement of probes incubated with hERG-transfected HEK293 cell membranes can be obviously decreased (P < 0.01) by a hERG inhibitor, astemizole. However, this fluorescence enhancement cannot be completely suppressed, which may be caused by the unavoidable nonspecific binding with other proteins in the membrane.

In addition to the radio-ligand competitive binding assay, a fluorescence polarization assay was performed to evaluate the binding affinity of probe A1 with the hERG channel. The results demonstrated that probe A1 exhibited a highly sensitive response to the hERG channel at both the 1 μM and 5 μM concentrations. Meanwhile, when treated with 1 mg/mL cell membrane, the FP value can have a dose-dependent enhancement and the calculated K_d value is 0.14 ± 0.03 μM (Figure 3).

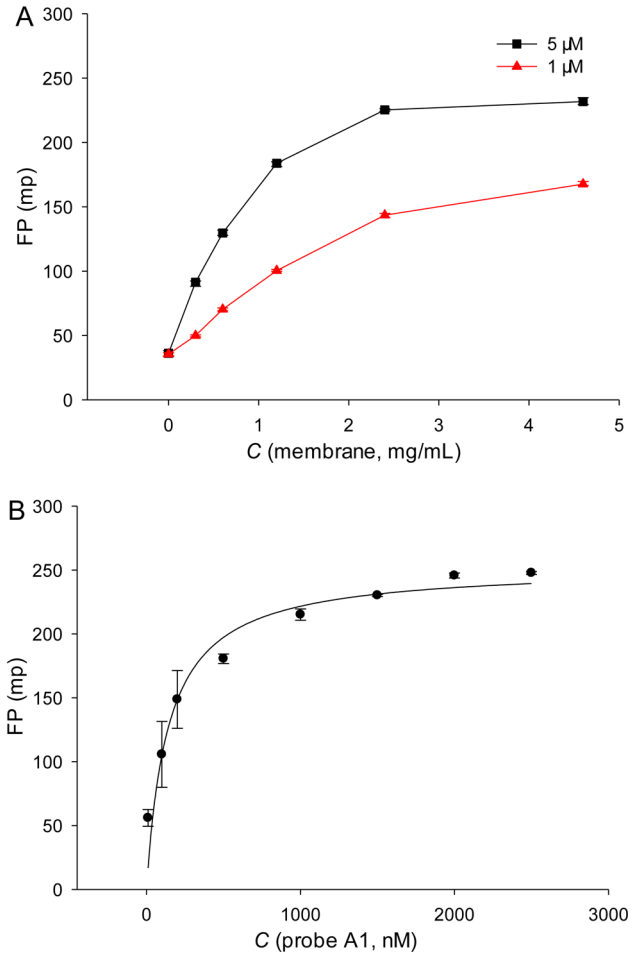

Figure 3. (A) Fluorescence polarization (FP) assay used to monitor the interaction between probe A1 (1 μM and 5 μM) and the cell membrane; (B) fluorescent polarization value changes with the increase of probe A1 concentration at 1.2 mg/mL cell membrane (λ_{em} = 520 nm, λ_{ex} = 485 nm).

In parallel, encouraged by the data described above, we further explored the feasibility of applying this probe to image hERG channels in living cells. The hERG-transfected and wild-type HEK293 cells were selected as positive and negative groups, respectively. The cell imaging (performed in a Zeiss Axio Observer A1 fluorescent microscope) results revealed that probe A1 selectively could label hERG-transfected HEK293 cells (Figure 4A1−B2), while a significant reduction in fluorescence staining was observed in cell imaging upon competition with 20-fold excess of Astermizole, a potent hERG channel inhibitor (Figure S2 in the Supporting Information). In view of the hERG channel as a membrane protein, hERG-transfected HEK293 cells were costained with a commercial membrane dye (DID red dye) (Figure 4C1,C2). The confocal imaging data revealed that probe A1 stained the plasma membrane of HEK293 cells transfected with hERG channels, as indicated by the PM-localized dye DID (red, Figure 4C1,C2). The calculated degree of colocalization, as quantified by the Pearson's correlation coefficient using ImageJ software, is 0.85. In addition to its localization in the cell membrane, a portion of expressed hERG channel protein was

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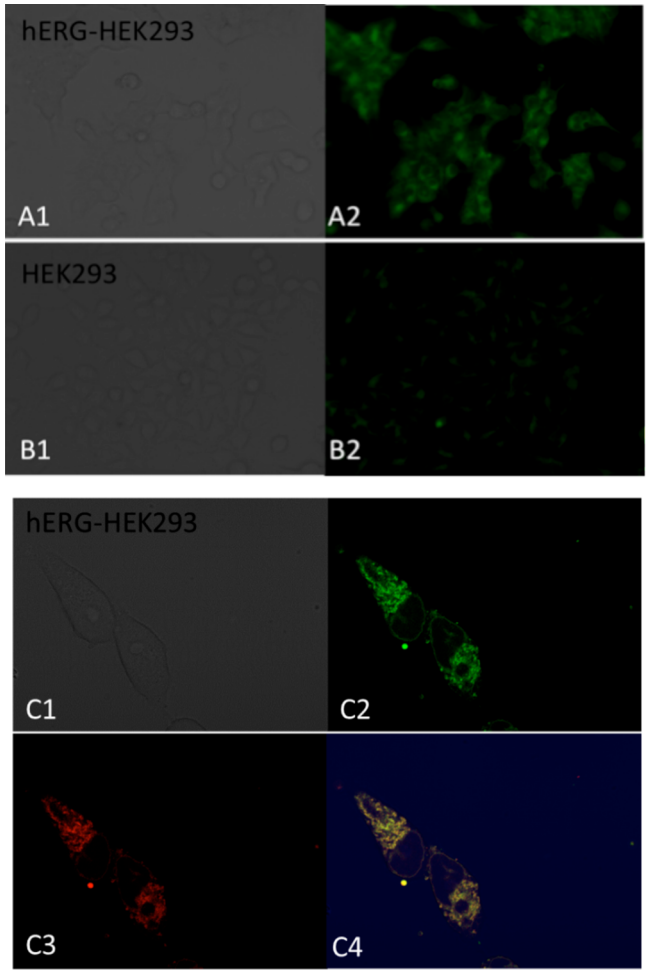

Figure 4. Fluorescence microscopic imaging of hERG transfected HEK293 (A1, bright field; A2, green channel), HEK293 (B1, bright field; B2, green channel) cells incubated with 5 μM probe A1. Objective lens, 40×. The contrast of images was adjusted using ImageJ software. Confocal microscopic imaging of hERG-transfected HEK293 (C1, bright field; C2, green channel; C3, red channel; C4, merged image) incubated with 5 μM probe A. Objective lens, 63×.

retained in the cytoplasm, which corresponds to the reported results of fluorescent protein-based method as well.[11]

Small molecule fluorescent probes have a broad application in the interrogation and mechanistic study of a myriad of biological processes. It is becoming an indispensable tool for biological research. However, there is few highly sensitive and selective small molecule fluorescent probes for ion channel detection.[25,26] In this letter, we designed and synthesized a selective fluorescent probe for imaging the hERG channel at the molecular and cellular level. To the best of our knowledge, this is one of the few examples of an "off−on" selective fluorescent probe for potassium channels. Compared with immunofluorescent and fluorescent protein based approaches, this fluorogenic imaging method is rapid, simple, economic, and more importantly, it can also observe the hERG channel in living and intact cells. In addition, this probe adopted a competitive binding mechanism, which would presumably minimize the effects on the electrophysiological properties of the hERG channel. Therefore, this probe may serve as a powerful tool to the hERG-associated study.

Enzyme and small active molecules can utilize their catalytic or reactive activity to design a turn-on fluorogenic mechanism. However, 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. In the present study, the conformational alteration-directed PET turn-on mechanism may provide a "light-up" example for these types of biotargets without catalytic or reactive activity. On the basis of these interesting results, more efforts will be undertaken toward developing further improved hERG fluorogenic probes in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra, HRMS, and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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