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RESEARCH ARTICALE

Astemizole-based turn-on fluorescent probes for imaging hERG potassium channel

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Based on the scaffold of astemizole, three novel turn-on fluorescent probes (**N1-N3**) for Human Ether-a-go-go-Related Gene (hERG) potassium channel were developed herein. These probes have reasonable fluorescent properties, acceptable cell toxicity, and potent inhibitory activity, all of which contribute to cell imaging at the nanomolar level. Overall, these probes have the potential for setting up a screening system for hERG channels.

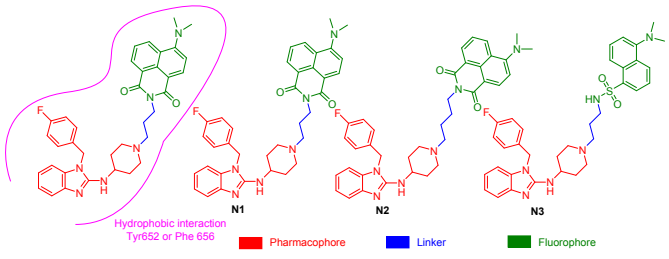
Introduction

The Human Ether-a-go-go-Related Gene encodes hERG potassium channel (Kv11.1), a key effector of cardiac repolarization.[1] hERG channel is a voltage-dependent channel known for its role in repolarizing the cardiac action potential. The alteration of hERG by mutation[2] or pharmacological inhibition[3] produces Long QT syndrome and the lethal cardiac arrhythmia torsade de pointes. In recent years, more and more drugs have serious adverse cardiac reactions due to their blockade on the hERG channel. Most of them were withdrawn from the market, such as Cissapride, Tefenadine, Astemisole and Grepafloxacin.[4] Currently, all drugs should be measured the affinity with hERG channel to evaluate their cardiotoxicity which is required by FDA. Recently, the MacKinnon group[5] discovered that hERG channel is open with the voltage sensors when it is in depolarized conformation and hERG channel's idiosyncratic sensitivity to many drugs may be associated with the very small volume of central cavity, which is embraced by four deep hydrophobic pockets. However, there is still no simple and effective method to high-throughput screening drugs with hERG channel.

Recently, many studies have shown that compared with the corresponding normal cells, the expression of hERG channel is up-regulated in some tumor cell lines[6-8]such as neuroblastoma, breast cancer, and colon cancer cells to facilitate cell proliferation, invasion, and tumor angiogenesis.[9] However, there is no explanation for the up-regulation of hERG channel and the role of hERG channel in these cancers. For the purpose of better analysis and molecular imaging of the hERG channel, there is a stringent demand for a simple, cost-effective, and safe method to label hERG channels. It may help explain the role of hERG channel in these cancers so as to make hERG channel become a biomarker and new target for tumor research.

At the present time, small-molecule fluorescent probes have been diffusely used in pharmacology and biology for the detection of proteins, nucleic acids, and other vital biological molecules, owing to their unique properties, such as high sensitivity, simplicity, and convenient operation. When they label targets, they can provide real-time information and trace the dynamic process on cell even animal level with high spatial-temporal resolution.[10-12]

Now, it has been well studied that when the ligands bind with hERG channel, a hydrophobic interaction between the ligands and the residue Phe 656 or Tyr 652 of hERG channel plays a critical role for high-affinity binding.[1, 13, 14] So we decide to use the molecular mechanism to design environment-sensitive probes. In fact, probes with this off-on mechanism have been utilized to detect structural characterization of the ligand-binding domain, protein–protein interaction, and protein conformation dynamics.[15-17] Generally, three parts are included in a typical fluorescent probe: a pharmacophore, a fluorophore, and a linker. Astemizole, the most potent inhibitor of the hERG channel, was chosen as pharmacophore. In addition, some fluorophores with good fluorescent properties could be used in small-molecule fluorescent probes, such as naphthalimide[18] and dansyl derivative.[19] In order to keep the inhibitory activity of the probe, the major interaction sites of Astemizole's bonding were retained. Herein, the naphthalimide and dansyl fluorophore were conjugated to the recognition moiety using an aliphatic spacer. Soon afterwards, several small-molecule fluorescent probes (**N1–N3**) for the hERG channel were synthesized (**Scheme 1**).



Scheme 1 Design strategy of the fluorescent probes

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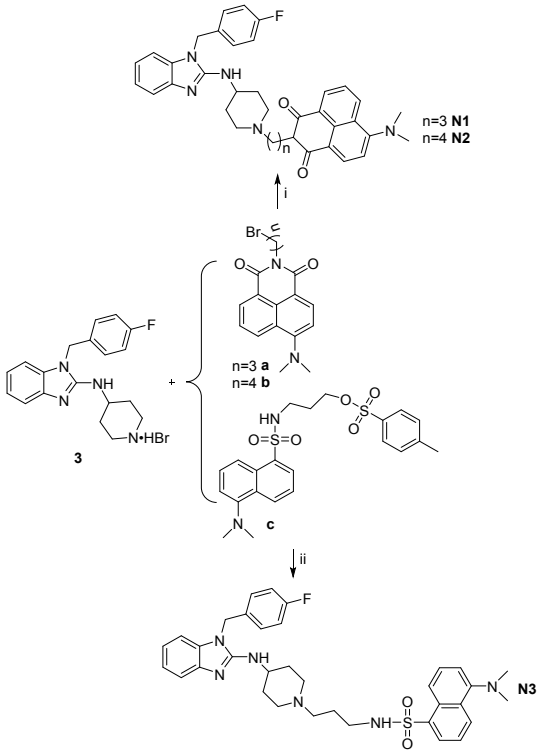
Results and discussion

Chemistry

Subsequently, the fluorescent derivatives of astemizole were synthesized through a convenient reaction as shown in **Scheme 2**. Compound **3** and **a** (**b**, **c**) in the presence of K_2CO_3 in acetonitrile yielded the fluorescent compound **N1** (**N2**, **N3**) through a substitution reaction. Further details about the synthesis of the fluorophores and recognition moiety can be found in the Supporting Information.



Scheme 2 Synthetic routes of the fluorescent probes. (i) K_2CO_3, acetonitrile, 80 °C; (ii) K_2CO_3, acetonitrile, 80 °C.

Spectroscopic properties of the probes

The spectroscopic properties of the probes were measured in 10 μM solution of the corresponding probe (**N1**, **N2**, **N3**) in PBS (pH=7.4). The results indicated that all probes possessed good fluorescent properties. Compared to probe **N3**, the maximum emission of the probes **N1** (**N2)** is red-shifted reaching 535 (545) nm, but the fluorescence quantum yields are relatively low (**Table 1**).

Table 1 Photophysical Properties of Synthesized Probes

compd	λ_{max}	λ_{ex}	λ_{em}	Φ (%)
N1	440	440	535	5
N2	445	430	545	5
N3	330	335	495	15

Binding affinity of probes

Soon afterwards, the inhibitory activities of these probes against the hERG channel were measured by radio-ligand binding assays using hERG transfected HEK293 cells. [20-22]The results showed that probe **N1** displayed best inhibitory effects against the hERG channel, and the calculated IC_{50} and K_i values are 0.053 and 0.030 μM, respectively, which are slightly lower than astemizole (0.011 and 0.006 μM, respectively). Probes **N2** and **N3** also disclosed potent inhibitory activity against the hERG channel, with IC_{50} values of 0.183 and 0.186 μM, respectively, although lower than that of probe **N1**.

Table 2 Inhibitory activity of the synthesized probes against the hERG potassium

compd	IC50 (μM)	K_i[b] (μM)
N1	0.053	0.030
N2	0.183	0.103
N3	0.186	0.104
Astemizole	0.011	0.006

[a] See Supporting Information. [b]The inhibition constant (K_i) was calculated from each IC_{50} value using the Cheng−Prusoff equation.

Cytotoxicity assay

The cytotoxicity of these probes was evaluated by CCK-8 assays using hERG transfected HEK293. The results demonstrated that the IC_{50} of probes **N1–N3** were 3.55±0.28, 2.43±0.12, and 7.03±0.14μM in hERG -HEK293 cells.

Table 3 Cytotoxicity results for the synthesized probes

compd	IC_{50} (μM)
N1	3.55±0.28
N2	2.43±0.12
N3	7.03±0.14
Astemizole	17.37±1.07

Fluorescent image assay

As mentioned above, the pharmacophore is environment-sensitive so that the probe was proposed to have a turn-on mechanism for hERG channel. To test this hypothesis, a series of concentrations of hERG transfected HEK293 cell membrane was incubated with the probe **N1** (5 μM). As good as anticipated, with increasing amount of cell membrane, fluorescence intensity was gradually enhanced (**Fig. 1**). When incubated with 0.8 mg/mL cell membrane, the fluorescence intensity was 12-fold higher than the blank group.

Subsequently, the selectivity of fluorescence intensity for hERG potassium channel was also examined. In the assay, taking account of the forming of nonspecific binding with small molecules, trypsin and bovine serum albumin (BSA) were selected as the control group. Probe **N1** (5 μM) was incubated with trypsin, BSA or hERG transfected HEK293 cell membrane at the same concentration (1 mg/mL). As shown in **Fig. 2**, there is a suitable increase for trypsin and BSA, which manifested that probe **N1** may form some

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nonspecific binding with trypsin and BSA. Additionally, when probe **N1** (5 μM) was incubated with cell membrane (1 mg/mL) and Astemizole (a potent hERG channel inhibitor, 25 μM), the fluorescence intensity was decreased compared with the group that was only incubated with cell membrane. However, the decrease degree of fluorescence intensity was not complete, which may be caused by unavoidable nonspecific binding between probe **N1** and other components in the cell membrane, especially the hydrophobic components.

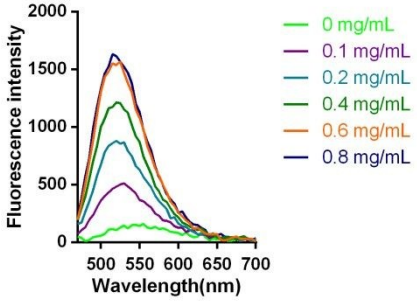

Fig. 1 Fluorescent emission spectra of 5 μM probe **N1** incubated with different concentrations of hERG transfected HEK293 membrane (0.8, 0.6, 0.4, 0.2, 0.1, and 0 mg/mL) for 20−30 min in the assay buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, 10 mM KCl) at room temperature (λ_{ex} = 440 nm).

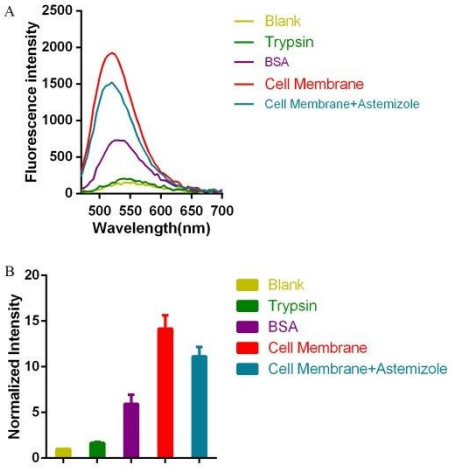

Fig. 2 (A)Fluorescent emission spectra of 5 μM probe **N1** which is respectively incubated with 1 mg/mL trypsin, 1 mg/mL BSA, 1 mg/mL hERG transfected HEK293 membrane, and 1 mg/mL cell membrane combined with hERG channel inhibitor Astemizole (10 μM) for 20−30 min in the assay buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, 10 mM KCl) at room temperature (λ_{ex} = 440 nm). (B) The corresponding fluorescent intensity changes (normalized based on the last point that is seen as 1) at 535 nm (λ_{ex} = 440 nm).

On account of their good fluorescent properties, acceptable cell toxicity, and potent inhibitory activity, probes **N1-N3** can be used for hERG channel imaging in living cell in order to expand the application of our probes. Thus, microscopic imaging of probes **N1-N3** for hERG channels in living cells was conducted on hERG transfected HEK293 cells. The microscopic imaging results indicated that these probes exhibit rapid responses and strong fluorescence to hERG - HEK293 cells (**Fig. 3**). Astemizole, a potent inhibitor of the hERG channels, was chosen to incubate the cells with each probe. When the cells were coincubated with Astemizole and probe, the microscopic imaging results showed that inhibition of hERG by astemizole resulted in a decrease of fluorescence intensity, which confirmed that the hERG channel can be well selectively labeled by the probe. Particularly, because of their turn-on mechanism, there was not needed a complex washing procedure, which ensured a convenient imaging process.[23, 24] Additionally, we also investigated cell autofluorescence and the effect of astemizole on cell autofluorescence (see Supporting Information). The microscopic imaging results showed that the autofluorescence of the cells is negligible in both the presence and absence of astemizole (**Fig. S9**) so that it would not interfere with the imaging of cells using probes **N1-N3**. In general, the results showed that these probes all possess superior selectivity for hERG channels and could be used in the detection of hERG potassium channel.

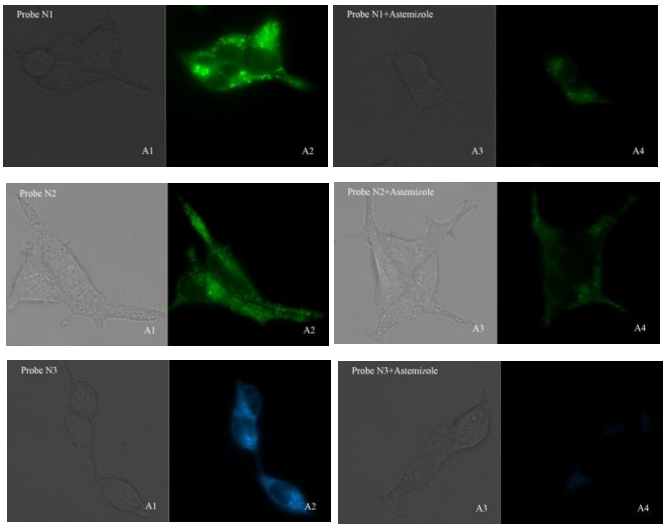

Fig. 3 Fluorescence microscopy imaging of hERG transfected HEK293 cells incubated with 0.3 μM probe **N1** (A1, bright field; A2, GFP channel), 0.3 μM probe **N2** (B1, bright field; B2, GFP channel), and 0.5 μM probe **N3** (C1, bright field; C2, DAPI channel), respectively. The imaging of inhibition of the hERG channels was accomplished by incubating astemizole (3 μM; 3 μM; 5 μM) with probe **N1** (0.3 μM; A3, bright field; A4, GFP channel), **N2** (0.3 μM; B3, bright field; B4, GFP channel), and **N3** (0.5 μM; C3, bright field; C4, DAPI channel). All cells were incubated with each probe at 37 °C for 10 min and washed immediately. The background was adjusted by ImageJ software. Imaging was performed using a Zeiss Axio Observer A1 microscope with a 63× objective lens.

Conclusions

In conclusion, we developed three high-affinity environment-sensitive probe, with commendable fluorescent properties, which can be utilized to the localization and visualization of hERG channel. These probes have been successfully utilized to label the hERG channels in hERG transfected HEK293 (hERG- HEK293) cells at the

nanomolar level. Because of the environment-sensitive switch in the structure, it is not needed a washing procedure, so this method is convenient compared with other imaging techniques, such as fluorescent protein-based approaches and immuno-fluorescence. Meanwhile, the synthesis of these probes is convenient and affordable. When compared with our previously published astemizole-based fluorescent probes, [23, 24] these probes have better turn-on effect and higher fluorescent intensity. They also have good fluorescent properties, acceptable cell toxicity, and potent inhibitory activity, which make the probes favourable for cell imaging. Therefore, these probes are anticipated to be applied in the study of hERG channels. However, there is still a lot of work to be done, such as setting a screening system for hERG channels.

Acknowledgements

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Conflicts of Interest

The authors declare no conflict of interest.

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