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Discovery of Quinazoline-Based Fluorescent Probes to α_1-Adrenergic Receptors

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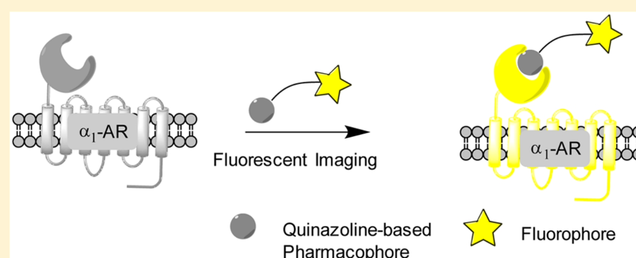
Supporting Information

ABSTRACT: α_1-Adrenergic receptors (α_1-ARs), as the essential members of G protein-coupled receptors (GPCRs), can mediate numerous physiological responses in the sympathetic nervous system. In the current research, a series of quinazoline-based small-molecule fluorescent probes to α_1-ARs (**1a**−**1e**), including two parts, a pharmacophore for α_1-AR recognition and a fluorophore for visualization, were well designed and synthesized. The biological evaluation results displayed that these probes held reasonable fluorescent properties, high affinity, accepted cell toxicity, and excellent subcellular localization imaging potential for α_1-ARs.



KEYWORDS: *α_1-Adrenergic receptors, fluorescent probes, quinazoline, cell imaging, subcellular localization*

α_1-Adrenergic receptors (α_1-ARs), as the important members of G protein coupled receptors (GPCRs), distribute in a variety of organs, tissues, and cells, which mediate many crucial physiological effects in the human body. These receptors are classified into at least three subtypes (α_{1A}, α_{1B}, and α_{1D}) based on their differences on the biological structure, tissue distributions, pharmacological properties, and signaling pathways.[1] Various studies confirmed that α_1-ARs are closely involved in benign prostatic hyperplasia (BPH), hypertension, prostate cancer, and other diseases.[2−4] Functional experiments manifested that α_{1B}-AR is mainly in charge of the vasomotion of small resistance vessels, while α_{1A}- and α_{1D}-ARs take responsibility for the contraction of the main arteries in animal species.[5]

So far, there are many challenges to fully understand the biological and pharmacological characteristics of each α_1-AR subtype. This dilemma is mainly caused by the difficulties either to determine their distribution in various organs and tissues or to define the functional response mediated by each one in the different species by using classical approaches without their three-dimensional crystal structure and tissue-selective α_1-AR antagonists.[6] Fortunately, with the rapid development of fluorescence analysis technology in various areas, small-molecule fluorescent probes with many advantages, including high sensitivity, selectivity, and visualization, have been widely applied to track the biological macromolecules, especially GPCRs.[7−10] Small-molecule fluorescent probes for GPCRs generally consist of two essential components: the pharmacophore moiety that is used to bind with the target through the receptor−ligand interaction and the fluorophore group that can trace the target by the fluorescent properties.[11] Moreover, good optical characteristics and high affinity of probe can ensure its successful labeling of the target. It needs to be noted that in the case of α_1-ARs, only one small-molecule fluorescent probe, BODIPY-FL-prazosin, is available. However, the application of this probe is restricted to its complicated preparation. Recently, some QD-based fluorescent probes have been reported as well.[12,13] Although rational results about α_{1B}-AR study were given by these QD probes, the studies of probes on purity, structure, cytotoxicity, and receptor affinity were inadequate.[10] Therefore, more small-molecule fluorescent probes with diversity of structure and fluorescence property are demanded for the current molecular pharmacology study and drug discovery of α_1-AR.

α_1-Adrenergic receptor antagonists can be structurally categorized into several classes, including quinazolines, 1,4-benzodioxans, dihydropyridines and dihydropyrimidines, fused pyrimidindiones, pyridazinones, imidazolines, *N*-arylindoles, *N*-aryl, and *N*-heteroarylpiperazines, and miscellaneous compounds.[14] Among all chemotypes, quinazoline compounds, including prazosin, terazosin, doxazosin, and alfuzosin, are the most clinically effective α_1-AR antagonists (Scheme 1).[15] In our previous study, a pharmacophore model based on quinazoline derivatives that contribute many effective α_1-AR antagonists was well built, and the proposed interaction model of quinazoline-based antagonists with α_1-ARs was well developed.[16−19] It was found that there is a bulky space around the piperazine group to accommodate the fluorophore moiety, which does not influence the affinity of antagonists to receptors. Therefore, in the current research, we chose the quinazoline

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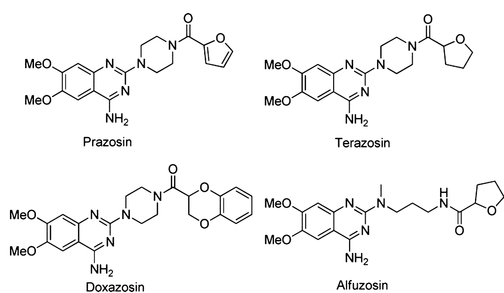
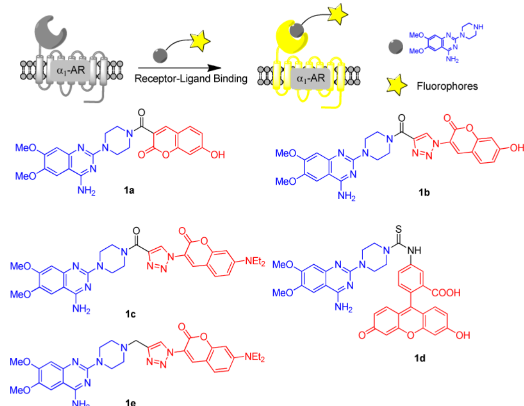
Scheme 1. Structures of Quinazoline Derivatives as α_1-AR Antagonists



moiety as the pharmacophore to generate fluorescent probes for selectively binding with α_1-ARs. In the meanwhile, coumarin and fluorescein groups were selected as fluorophores because of their preferred characteristics, such as high sensitivity, light stability, small molecular weight, water solubility, and reasonable cell permeability. The understanding on how α_1-ARs bind with their ligands[20] and the development of fluorescent labeling analysis method can efficiently promote the emergence of fluorescent probes for α_1-ARs. In view of these evidence, a series of quinazoline derivatives (**1a**−**1e**) were well designed as small-molecule fluorescent probes for α_1-ARs by conjugating pharmacophore (quinazoline) with fluorophores (coumarin and fluorescein) (Scheme 2).

Scheme 2. Designed Quinazoline-Based Fluorescent Probes for α_1-ARs



To establish a preliminary understanding on if these quinazoline-based fluorescent probes can be recognized by α_1-ARs, we first developed docking models of molecules **1a**−**e** with α_{1A}-AR homology model that we developed in 2008.[19] As a result, the docking conformations and orientations of **1a**−**e** around the active site of α_{1A}-AR were highly consistent with of prazosin as depicted in Figures 1 and S1−S6.These computational results clearly propose that compounds **1a**−**e** may be recognized by α_{1A}-AR. More computational details can be found in Supporting Information.

The convenient syntheses of these fluorescent probes were mainly based on CuAAC reaction and amide condensation with mild conditions, high yield, and easy purification (Scheme 3). The fluorescein derivative was obtained through the one-step condensation reaction of fluorescein isocyanate and the quinazoline parent. Synthesis of coumarin derivatives was started from the acetylation or azidation of coumarin. And then, the quinazoline was attached to coumarin by amide or triazole

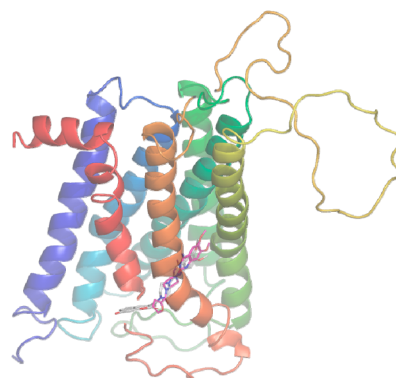
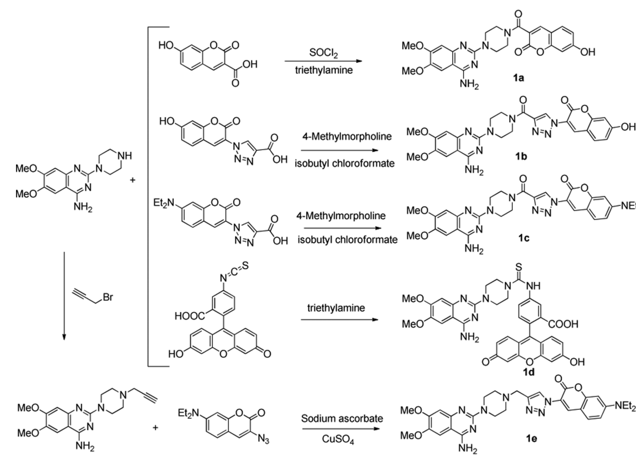


Figure 1. Proposed docking conformation of prazosin (red sticks) and **1a** (white sticks) in the human α_{1A}-AR binding site.

Scheme 3. Synthetic Routes of Designed Fluorescent Probes



moiety. More synthetic details can be found in the Supporting Information.

These probes were first evaluated for in vitro affinities with three human cloned α_1-adrenoceptors subtypes by the radioligand binding assay using [^{3}H]prazosin in membrane from transfected CHO cells.[21,22] All compounds demonstrated up to nanomolar affinities with three α_1-AR subtypes, which are close to the positive control, phentolamine (Table 1). Compound **1e** is approximately 100-fold less potent than compound **1c** because of the lack of carbonyl group. In the absence of the triazole linker, compound **1a** shows the similar affinity with **1b** and **1c** containing the triazole rings. This affinity result also proves that a bulky space exists around the piperazine group to accommodate the fluorophore moiety

Table 1. Comparison of the Probes' Affinity to α_1-ARs

	K_i[a] (nM)			IC_{50} (nM)		
compd	α_{1A}	α_{1B}	α_{1D}	α_{1A}	α_{1B}	α_{1D}
phentolamine	0.6	4.8	7.6	1.1	10.8	12.5
1a	2.1	NA[c]	NA[c]	3.9	<5[b]	<5[b]
1b	1.3	NA[c]	NA[c]	2.5	<5[b]	<5[b]
1c	0.3	0.1	0.4	0.6	1.2	2.2
1d	4.7	7.3	20.7	8.8	16.3	34.0
1e	29.9	29.9	27.5	52.4	52.4	45.2

[a] K_i was calculated from IC_{50} using the Cheng−Prusoff equation. [b] Data was estimated from the unclassical binding-competitive curve. [c] Not available.

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without influencing the affinity to receptors. Certainly, this space is not infinite. When the fluorophore is changed from coumarin (**1a**) to more bulky fluorescein (**1d**), the affinity might decrease slightly.

As far as the spectroscopic properties are concerned (Table 2), all target compounds showed excitation and emission wavelengths comparable to that reported in literature for the parent fluorophores. In addition, the fluorescence quantum yield was checked in a mixed solvent of methanol and PBS buffer. All compounds possessed reasonable fluorescence quantum yields. In particular, compound **1b** showed high quantum yields, which is up to 42%. The solvent effect on fluorescence property was examined as well (Figure S6). The hydroxyl coumarin derivatives, **1a** and **1b**, emitted strong fluorescence in water; however, their fluorescence emissions were quenched in methanol and ethanol. Amino coumarin and fluorescein derivatives behaved in the opposite way.

Table 2. Characterization and Properties of Compounds 1a–1e

compd	λ_{max} (nm)	λ_{ex} (nm)	λ_{em} (nm)	Φ (%)
1a	391	391	456	8.5 ± 1
1b	392	392	454	42 ± 1
1c	420	420	500	7.8 ± 0.2
1d	494	485	517	2.6 ± 0.3
1e	430	430	505	4.7 ± 0.1

It should be pointed out that a reasonable fluorescent probe should be harmless while labeling the target. In view of this reason, the cytotoxicities of these probes were checked by a standard sulforhodamine B (SRB) colorimetric assay.[23] As demonstrated in Table 3, the cytotoxicity of compounds **1c** and **1e** in HEK293A (human embryonic kidney) cells transfected with α_{1A}-AR (HEK293A-α_{1A}-AR) or HEK293A cells transfected with α_{1D}-AR (HEK293A-α_{1D}-AR) was similar to the positive control, doxazosin, while compounds **1a**, **1b**, and **1d** had slight cytotoxicity. Obviously, as presented in Table 3, only compound **1c** and **1e** had moderate cytotoxicities with IC_{50} values at the micromolar level. The results showed that cell viability was not significantly changed upon treatment, indicating the low cytotoxicity and good biocompatibility of these probes in living cell study at the nanomolar concentration without any cell damage.

Table 3. Cytotoxicity Results of the Synthesized Probes

	IC_{50} (μM)	
compd	HEK293A-α_{1A}-AR	HEK293A-α_{1D}-AR
doxazosin	19 ± 2	22 ± 2
1a	>100	>100
1b	>100	85 ± 2
1c	12 ± 0.2	32 ± 2
1d	>100	>100
1e	26 ± 3	17 ± 0.2

The above-mentioned results demonstrated that most of the probes displayed high affinities to α_1-ARs and accepted cytotoxicities that laid a solid foundation for fluorescence imaging in living cells. Although their fluorescence quantum yield left something to be desired, cell imaging potential of high expression of α_1-ARs was extensively evaluated. We incubated HEK293A cells transfected with α_{1A}-AR and α_{1D}-AR with the probes and DiD (a cell membrane dye, red, colocalization) at 37 °C for 5 min, in which normal HEK293A cells (without α_1-AR expression) as a negative control, and HEK293A-α_{1A}-AR and HEK293A-α_{1D}-AR cells incubated with tamsulosin (a potent α_1-AR antagonist) as another negative control. Indeed, the fluorescence of cells was not strong due to the poor quantum yield of compounds.[24] However, as shown in Figure 1, the incubation of cells with compound **1a** allowed visualization of the α_{1A}- and α_{1D}-ARs in stably transfected HEK293 cells using conventional fluorescence microscopy (Figure 2A,B), while a significant reduction in fluorescence labeling was observed in transfected HEK293 cells upon competition with 20-fold excess of tamsulosin (Figure 2D,E). Moreover, very weak fluorescence labeling in negative HEK293 cells showed a spot of nonspecific binding of compound **1a** (Figure 2C). We also proved the application of our probe **1c** in detecting cellular α_1-ARs by flow cytometry. As shown in Figure S20, compound **1c** could identify the HEK293 cells transfected with α_1-ARs successfully. Although compound **1c** has weaker affinity to α_{1d}-ARs than α_{1a}-ARs, stronger staining of α_{1d}-ARs cells than that of α_{1a}-ARs cells was found. This may be caused by the inconsistent expression levels of α_{1a}-AR and α_{1d}-AR in cells, and in this case, the expression level of α_{1d}-AR is higher than α_{1a}-AR.

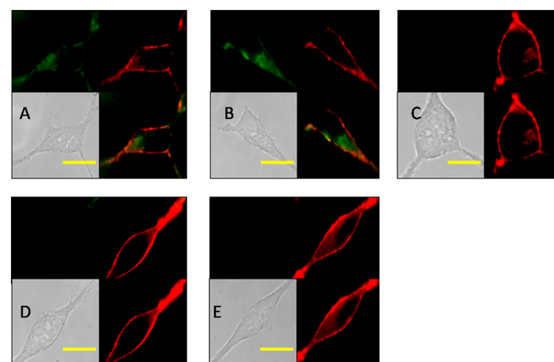

Figure 2. Confocal fluorescence image of HEK293 cell using compound **1a**. All cells are incubated with **1a** at 37 °C for 5 min and washed immediately, and the exposure time is the same. The background was adjusted by ImageJ software. (A) Image of HEK293A-α_{1A}-AR cells incubated with **1a** (15 nM) and DiD; (B) image of HEK293A-α_{1D}-AR cells incubated with **1a** (15 nM) and DiD; (C) image of HEK293A cells incubated with **1a** (15 nM) and DID; (D) image of HEK293A-α_{1A}-AR cells incubated with **1a** (15 nM), DID, and tamsulosin (300 nM); (E) image of HEK293A-α_{1D}-AR cells incubated with **1a** (15 nM), DID, and tamsulosin (300 nM). Scale bar (yellow) = 20 μm.

In subcellular localization, the fluorescence of α_{1A}-AR was found on the cell surface, and their location was almost overlapped with the red area stained by DiD. In the case of α_{1D}-AR, its fluorescence mainly distributed in cell plasma, as well as a little bit on the cell surface. These subcellular localization results are highly in accordance with Piascik's conclusion, where α_{1A}-AR fluorescence was detected not only on the cell surface but also intracellularly, and α_{1D}-AR fluorescence was detected mainly intracellularly.[25] Fluorescence imaging of the other four compounds **1b**–**1e** presents similar results (Figures S16–S19). Although the largest emission peak of **1a** or **1b** was near to blue, the blue filter did not give a clear imaging result; therefore, these images were recorded through the green filter.

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Additionally, it is a known fact that α_1-ARs are overexpressed in prostate cancer.[26] After successful staining of α_{1A}- and α_{1D}-AR overexpressing HEK293A cells, we tested **1d** on PC-3 prostate cancer cells because of its most reasonable fluorescence property. As shown in Figure 3, PC-3 cells could be "lighted up" after being incubated with **1d** (1 μM), while HepG2 cells (low α_1-AR expression) showed little fluorescence.[27] Also, **1d** shows high displaceable properties in PC-3 prostate cancer cells with doxazosin. These findings confirm probe **1d** as a labeling tool in α_1-AR overexpressing cells.

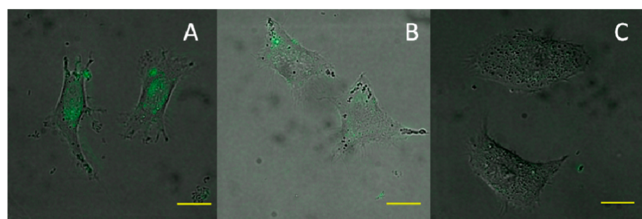

Figure 3. Fluorescence image of cancer cells using compound **1d**. The background was adjusted by ImageJ software. (A) PC-3 cells were with incubated with **1d** (1 μM) at 37 °C for 30 min, then washed immediately; (B) PC-3 cells were with incubated with **1d** (1 μM) and doxazosin (10 μM) at 37 °C for 30 min, then washed immediately; (C) HepG2 cells were incubated with **1d** (1 μM) at 37 °C for 30 min, then washed immediately. Scale bar (yellow) = 20 μm.

In summary, we herein well developed a series of quinazoline-based small-molecule fluorescent probes with high sensitivity, high affinity, and low toxicity for convenient detection of α_1-ARs. The probes have up to nanomolar affinities with three α_1-AR subtypes. These fluorescent probes at the nanomolar level have been successfully used in visualization and subcellular localization of α_1-AR in cell imaging, including both α_1-AR transfected HEK293 cells and prostate cancer cells, thus supplanting the application of radioligand for drug screening and providing extra dimensions of probing receptors that simple competitive radioligands do not present. The study is a preliminary work to establish that the strategy can be useful for cell staining. In addition, these probes have the advantage of easy synthesis from readily available inexpensive starting materials. It is expected that these probes now can be added to the armamentarium of fluorescent ligands that may be utilized as versatile and extremely useful tools for nowadays molecular pharmacology and drug discovery in the area of α_1-ARs.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Full experimental procedures; analytical and spectral characterization data of all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

†These authors contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

α_1-ARs, α_1-adrenergic receptors; $\alpha_{1A/B/D}$-ARs, $\alpha_{1A/B/D}$-adrenergic receptors; CHO, Chinese hamster ovary; CuAAC, Cu-catalyzed azide–alkyne cycloaddition; GPCRs, G protein coupled receptors; HEK, Human embryonic kidney; QD, quantum dot; SRB, sulforhodamine B

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