Brief Report

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Design, synthesis and biological evaluation of naphthalimide-based fluorescent probes for α_1-adrenergic receptors

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Summary **α_1-Adrenergic receptors (α_1-ARs), as one of the most important members of G protein-coupled receptors (GPCRs), can mediate lots of physiological responses of the sympathetic nervous system. Until now, α_1-ARs have been divided into at least three subtypes, α_{1A}, α_{1B}, and α_{1D}, which distribute in various tissues and organs. In this research, we designed and synthesized several napthalimide-based small-molecule fluorescent probes for α_1-ARs, which mainly contained two parts: the pharmacophore (quinazoline and phenylpiperazine) that binds to α_1-ARs and the fluorophore (naphthalimide) that labels the receptors with fluorescent properties. Moreover, some of these compounds demonstrated potent affinity to α_1-ARs and cell imaging potential.**

Keywords: α1-Adrenergic receptors, fluorescent probes, high affinity, cell imaging

1. Introduction

The α_1-adrenergic receptors (α_1-ARs) are one of the most important members of G protein-coupled receptors (GPCRs), distribute in varieties of organs, tissues and cells, which mediate many important physiological effects in the human body. Until now α_1-ARs have been divided into at least three subtypes, α_{1A}, α_{1B} and α_{1D}, according to the differences on their gene structure, tissue distribution and pharmacological action (*1-3*).

Studies have confirmed that α_1-ARs are closely related to various diseases, such as hypertension, benign prostatic hyperplasia, prostate cancer, and so on (*4-6*). Therefore, it is extremely useful to develop drugs for diseases' examination and clinical diagnosis. So far, researchers have designed and synthesized several α_1-ARs antagonists, including quinazoline and phenylpiperazine-based derivatives, to prevent and treat diseases related to α_1-ARs abnormally expressed (*7*). However, in the absence of their three-dimensional crystal structures, it is difficult to study the biological and pharmacological characteristics of α_1-ARs with conventional research approaches.

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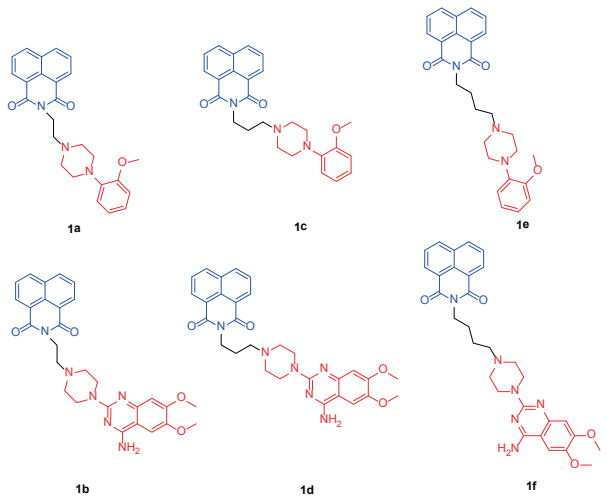
Nowadays, fluorescence analysis technology has developed rapidly in various areas and small-molecule fluorescent probes have demonstrated high sensitivity and selectivity in the detection of biomacromolecules, such as proteins, enzymes, *etc*. (*8-10*). Small-molecule fluorescent probes usually consist of two parts: the pharmacophore that could be bind to the targets, and the fluorophore that labels the targets with fluorescent properties.

Based on our previous work (*11-14*), we chose quinazoline and phenylpiperazine moiety as the pharmacophore with high affinity to α_1-ARs, and naphthalimide as the fluorophore to provide fluorescent properties. Biological evaluation confirmed that our probes demonstrated high affinity to α_1-ARs and reasonable cell fluorescence imaging potential. These interesting results indicated that these fluorescent probes could be employed as fluorescent competitive substrates in α_1-ARs ligand activity screening.

2. Materials and Methods

2.1. *Chemicals*

In summary, a series of naphthalimide derivatives were well designed and synthesized as fluorescent probes for α_1-ARs (Scheme 1). In this case, quinazolines and phenylpiperazines acted as pharmacophores, and naphthalimide as fluorophores.



Scheme 1. The fluorescent probes for α1-ARs

2.2 *Optical property*

The ideal optical property is of importance for a reasonable fluorescent probe. We firstly measured the optical properties of the synthesized fluorescent probes. The optical properties were performed on a Thermo-Fisher Varioskan microplate reader by dissolving the probes in 50 mM PBS, pH 7.4.

2.3 *Affinity to α_1-ARs*

Besides the optical properties, the affinity to the targets is also a key characteristic for fluorescent probes. Therefore, the affinity activities to three different adrenergic receptor subtypes (α_{1A}-, α_{1B}- and α_{1D}-AR) of these probes were evaluated by radio-ligand binding test, in which the phentolamine was taken as a positive control.

2.4 *Fluorescence imaging*

HEK293A cells were transfected with α_{1A}-AR (HEK293A-α_{1A}-AR cells). The cell lines were grown in DMEM medium supplemented with 10% (v/v) fetal bovine serum (Gibco) in an atmosphere of 5% CO_2 at 37°C.

The cell lines were cultured in 35 mm glass bottom culture dishes (Mat Tek) at 37 °C for 24 h. Then cells were washed with DMEM medium (without fetal bovine serum) and incubated in DMEM medium (without fetal bovine serum) containing the probes for 10 min at 37 °C. Fluorescence imaging was performed on a Zeiss Axio Observer A1.

3. Results and Discussion

The optical results demonstrated that most of the probes had reasonable optical properties (Table 1). After being excited, the probes had a large stokes shift. And as we can see in Table 2, all probes had high affinity to α_1-ARs at the nM level or even at pM level. Further more,

Table 1. The optical properties of the fluorescent probes

Compound	UV λ_{max} (nm)	λ_{ex} (nm)	λ_{em} (nm)
1a	332	333	380
1b	333	333	380
1c	332	333	380
1d	333	333	385
1e	333	333	380
1f	333	333	380

Table 2. The affinity of probes to α_1-ARs

	Ki (nM)			IC_{50} (nM)		
Compound	α_{1A}	α_{1B}	α_{1D}	α_{1A}	α_{1B}	α_{1D}
Phentolamine	0.8	7.7	11.1	1.4	19.2	18.0
1a	0.07	0.2	0.1	0.1	0.5	0.2
1b	19.4	2.3	5.1	41.5	13.5	11.6
1c	0.3	0.5	0.8	0.6	1.1	1.2
1d	5.0	5.6	6.8	10.6	33.6	15.2
1e	0.2	0.5	0.02	0.3	1.1	0.03
1f	35.8	12.3	18.7	76.6	73.9	42.1

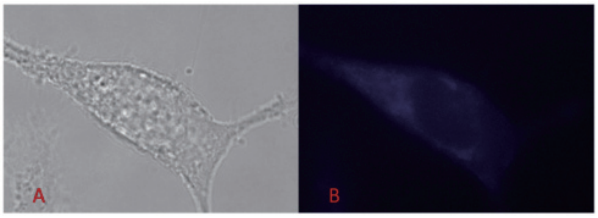


Figure 1. The fluorescence imaging results of 1d. The probe 1d (20 nM) was incubated at 37°C for 10 min. **A:** the bright field of HEK293A-α_{1A}-AR cells; **B:** the fluorescence image of 1d in HEK293A-$\alpha 1_A$-AR cells.

the phenylpiperazine-based probes had about 20-fold higher affinity to α_1-ARs than phentolamine (1a, 1c, 1e), which were more sensitive and can be a direction for developing other probes or antagonists for α_1-ARs. The fluorescent image of 1d (Figure 1B) showed that the probes could target the α_1-ARs expressed in the live cells with fluorescence properties at the nanomolar level (20 nM), which would be a direction for developing longer wavelength fluorescent probes for α_1-ARs. Moreover, these probes could be employed as competitive fluorescent substrates in α_1-ARs ligand activity screening.

4. Conclusion

The current paper describes several naphthalimide-based small-molecule fluorescent probes with high affinity for α_1-ARs. These results can provide further direction for structure-based fluorescent probes or ligands for α_1-ARs. Moreover, these fluorescent probes can help us to understand how α_1-ARs bind with their ligands and these probes can be expected to be fluorescent competitive substrates for α_1-ARs activity screening.

Acknowledgements

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Appendix

1. Chemistry: general procedures

All materials were purchased from commercial companies (Aladdin and J&K Scientific) and used without further purification. Twice-distilled water was used throughout all experiments. Mass spectra were performed by the analytical and the mass spectrometry facilities in Drug Analysis Center at Shandong University on Agilent Technologies 1100 infinity HPLC, Applied Biosystems API4000. ^{1}H-NMR and ^{13}C-NMR were recorded on a Bruker 300 MHz NMR spectrometer.

The synthetic routes of these probes were in two different ways in Scheme 2 and Scheme 3. We got the key intermediate **c3** and **d3** through the protection of 3-bromopropan-1-amine and then got the probe **1c** and **1d** (Scheme 2). The key intermediate of probe **1a**, **1b**, **1e**, and **1f** were obtained through the Gabriel reaction (Scheme 3).

1.1. *Benzyl (3-bromopropyl)carbamate (**1**)*

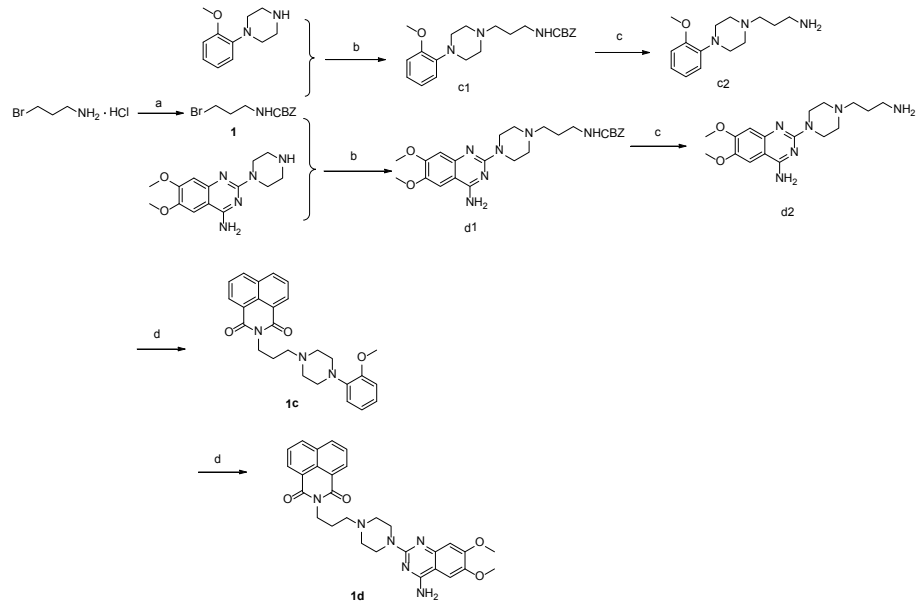
To a solution of 3-bromopropan-1-amine hydrobromide (5 g, 23 mmol) in 3N NaOH (77 mL) and $CHCl_3$ (77 mL) was added benzyl carbonochloridate (Cbz-Cl) at 0°C. Then stirred at room temperature overnight. The $CHCl_3$ layer was separated and washed with H_2O and brine, then dried with $MgSO_4$. The solvent was evaporated in vacuo and separated by column chromatography by using 10% ethyl acetate and 90% petroleum ether to get colorless liquid. ESI-MS calcd for $C_{11}H_{15}BrNO_2$ ($M + H^+$): 272.0; found: 272.3.

1.2. *Benzyl (3-(4-(2-methoxyphenyl)piperazin-1-yl) propyl)carbamate (**c1**)*

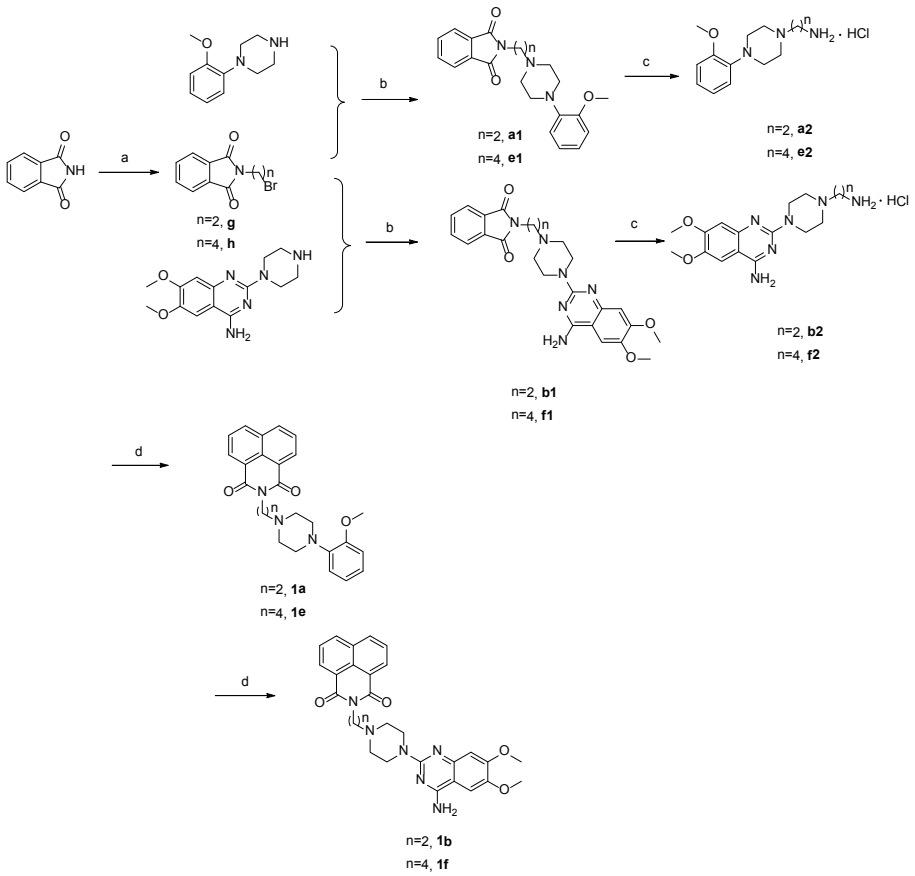
The 1-(2-methoxyphenyl)piperazine (0.83 g, 4.3 mmol), the **1** (1.43 g, 5.3 mmol) (1.2 equiv) and K_2CO_3 (1.25 g, 9 mmol) (2.1 equiv.) were heated to reflux in 25 mL CH_3CN solvent for 5 h under nitrogen atmosphere. Then the mixtures cooled down to room temperature. Then evaporated the solvent in vacuo and the residue dissolved in H_2O and extracted with ethyl acetate. The organic layer was washed by brine and dried by $MgSO_4$. Filtered and solvent evaporated in vacuo, separated by column chromatography (50% petroleum ether and 50% ethyl acetate) to give the product as yellow oil (1.52 g, 92%). ESI-MS calcd for $C_{22}H_{30}N_3O_3$ ($M + H^+$): 384.2; found: 384.4.

1.3. *Benzyl (3-(4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)propyl)carbamate (**d1**)*

Using the general synthetic procedure of **c1** above to give the product as a yellow solid. ESI-MS calcd for $C_{25}H_{33}N_6O_4$ ($M + H^+$): 481.3; found: 481.4.



Scheme 2. Reagents and conditions. (a) Cbz-Cl, 3 mol/L NaOH, $CHCl_3$, overnight; **(b)** K_2CO_3, CH_3CN, 80°C, 5 h; **(c)** H_2, Pd/C,30°C, overnight; **(d)** 1,8-naphthalic anhydride, CH_3CH_2OH, 85°C, 3 h.



Scheme 3. Reagents and conditions. (a) K_2CO_3, DMF, 30°C, overnight; **(b)** triethylamine, CH_3CN, 85°C, 6 h; **(c)** (*i*) hydrazine hydrate, EtOH, 85°C, 3 h, (*ii*) HCl/EtOH; **(d)** 1,8-naphathalic anhydride, EtOH, 85°C, 3 h.

1.4. *3-(4-(2-Methoxyphenyl)piperazin-1-yl)propan-1-amine (**c2**)*

The mixture of compound **c1** (0.58 g, 1.5 mmol) and Pd/C (36 mg) in 10 mL CH_3OH stirred overnight under hydrogen atmosphere. Filtered and solvent evaporated *in vacuo* to give the product as a brown oil (0.35 g, 95%). ESI-MS calcd for $C_{14}H_{24}N_3O$ ($M + H^+$): 250.2; found: 250.4.

1.5. *2-(4-(3-Aminopropyl)piperazin-1-yl)-6,7-dimethoxyquinazolin-4-amine (**d2**)*

Using the general synthetic procedure of **c2** above to give the product **d2**. ESI-MS calcd for $C_{17}H_{27}N_6O_2$ (M + H^+): 347.2; found: 347.4.

1.6. *2-(3-(4-(2-Methoxyphenyl)piperazin-1-yl)propyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (**1c**)*

The naphthalimide (150 mg, 0.76 mmol) and **c2** were heated to reflux in 30 mL EtOH for 3 h, and then cooled to room temperature. Solvent evaporated *in vacuo* and separated by column chromatography (40% petroleum ether and 60% ethyl acetate) to give the product as yellow solid (294 mg, 90%). m.p. 151-153°C. ESI-HRMS calcd for $C_{26}H_{28}N_3O_3$ (M + H^+): 430.2125; found: 430.2127. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 8.52 (d, 2H, *J* = 6.9 Hz), 8.46 (d, 2H, *J* = 7.5 Hz), 7.89 (t, 2H, *J* = 7.8Hz), 6.94-6.86 (m, 2H), 6.85 (td, 1H, 7.8 Hz, 2.4 Hz), 6.67 (d, 1H, *J* = 6.9 Hz), 4.17 (t, 2H, *J* = 7.2 Hz), 3.73 (s, 3H), 2.72 (s, 4H), 2.51-2.43 (m, 6H), 1.89-1.8 (m, 2H, *J* = 6.9 Hz). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 163.5(2C), 151.9, 141.1, 134.2(2C), 131.3, 130.6(2C), 127.4, 127.2(2C), 122.3, 122.2(2C), 120.7, 117.7, 111.8, 55.7, 55.2(2C), 52.8(2C), 49.8(2C), 24.1.

1.7. *2-(3-(4-(4-Amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl)propyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (**1d**)*

Using the general synthetic procedure of **1c** above to give the product as yellow solid (153mg, 73%). ESI-HRMS calcd for $C_{29}H_{31}N_6O_4$ (M + H^+): 527.2401; found: 527.2400. ^{1}H-NMR (300MHz, DMSO-d6) δ: 8.53 (dd, 2H, *J* = 7.5 Hz, 0.6 Hz), 8.46 (d, 2H, *J* = 8.4 Hz), 7.9 (t, 2H, *J* = 7.5 Hz), 7.38 (s, 1H), 7.06 (s, 2H), 6.69 (s, 1H), 4.18 (t, 2H, *J* = 6.6 Hz), 3.81 (s, 3H), 3.77 (s, 3H), 3.5 (s, 4H), 2.45 (t, 2H, *J* = 6.6 Hz), 2.34 (s, 4H), 1.89 (t, 2H, *J* = 6.9 Hz). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 163.5(2C), 161.0, 158.3, 154.1, 148.8, 144.8, 134.2(2C), 131.3, 130.6(2C), 127.4, 127.2(2C), 122.2(2C), 105.1, 103.6, 102.7, 55.9, 55.8(2C), 55.3, 52.8(2C), 43.3(2C), 24.3.

1.8. *2-(2-Bromoethyl)isoindoline-1,3-dione (**g**)*

Potassium phthalimide (0.93 g, 5 mmol) was added to a solution of 1,2-dibromoethane (1.3 mL, 15 mmol) in DMF (8 mL). The mixture was stirred at room temperature overnight and evaporated the solvent in vacuo, the residue dissolved in H_2O and extracted with ethyl acetate. The organic layer was washed by brine and dried by $MgSO_4$. Filtered and solvent evaporated in vacuo, recrystallized from ethyl acetate to give white solid (566 mg, 45 %).

1.9. *2-(4-Bromobutyl)isoindoline-1,3-dione (**h**)*

Using the general synthetic procedure of **g** above to give the product **h** as a white solid (4.1 g, 85 %).

1.10. *2-(2-(4-(2-Methoxyphenyl)piperazin-1-yl)ethyl) isoindoline-1,3-dione (**a1**)*

The compound **g** (0.36 g, 1.42 mmol), 1-(2-meth oxyphenyl)piperazine (0.35 g, 1.84 mmol) and triethylamine (990 μL, 7.1 mmol) were heated in 20 mL CH_3CN at 85°C for 6 h. The mixture was cooled to room temperature and solvent evaporated, separated by column chromatography (50% petroleum ether and 50% ethyl acetate) to give the product as yellow solid (0.35 g, 67%). ESI-MS calcd for $C_{21}H_{23}N_3O_3$ (M + H^+): 366.2; found: 366.3. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 7.92-7.83 (m, 4H), 6.96-6.91 (m, 2H), 6.87-6.83 (m, 2H), 3.76-3.72 (m, 5H), 2.88 (s, 4H), 2.61-2.57 (m, 6H).

1.11. *2-(4-(4-(2-Methoxyphenyl)piperazin-1-yl)butyl) isoindoline-1,3-dione (**e1**)*

Using the general synthetic procedure of **a1** above to give the product **e1** as yellow solid (1.34 g, 85 %). ESI-MS calcd for $C_{23}H_{28}N_3O_3$ (M + H^+): 394.2; found: 394.4. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 7.89-7.82 (m, 4H), 6.96-6.92 (m, 2H), 6.86-6.84 (m, 2H), 3.76 (s, 3H), 3.62 (t, 2H, *J* = 6.9 Hz), 2.92 (s, 4H), 2.51-2.46 (m, 4H), 2.35 (t, 2H, *J* = 6.9 Hz), 1.68-1.58 (m, 2H), 1.50-1.41 (m, 2H).

1.12. *2-(2-(4-(4-Amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl)ethyl)isoindoline-1,3-dione (**b1**)*

Using the general synthetic procedure of **a1** above to give the product **b1** as yellow solid (553 mg, 60%). ESI-MS calcd for $C_{24}H_{27}N_6O_4$ (M + H^+): 463.2; found: 463.4. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 7.91-7.83 (m, 4H), 7.40 (s, 1H), 7.08 (s, 2H), 6.71 (s, 1H), 3.82 (s, 3H), 3.77 (s, 3H), 3.77 (t, 2H, *J* = 6.3 Hz), 3.62 (s, 4H), 2.59 (t, 2H, *J* = 6.3 Hz), 2.52-2.46 (m, 4H).

1.13. *2-(4-(4-(4-Amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl)butyl)isoindoline-1,3-dione (**f1**)*

Using the general synthetic procedure of **a1** above to give the product **f1** as yellow solid (1.3 g, 88 %). ESI-MS calcd for $C_{26}H_{31}N_6O_4$ (M + H^+): 491.2; found: 491.5. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 7.90-7.82 (m, 4H), 7.40 (s, 1H), 7.08 (s, 2H), 6.71 (s, 1H), 3.82 (s, 3H), 3.78 (s, 3H), 3.66 (s, 4H), 3.63 (t, 2H, *J* = 6.9 Hz), 2.35-2.28 (m, 6H), 1.68-1.59 (m, 2H), 1.52-1.45 (m, 2H).

1.14. *2-(4-(2-Methoxyphenyl)piperazin-1-yl)ethanamine hydrochloride (**a2**)*

Hydrazine hydrate (200uL, 3.3 mmol) was added to a solution of **a1** (0.3 g, 0.8 mmol) in EtOH (20 mL). The reaction mixture was stirred at 85°C for 3h. Then cooled to room temperature, filtered the precipitate

and add HCl/EtOH to the filtrate, filtered and got the crude product (161 mg, 72 %), which was used without further purification.

1.15. *4-(4-(2-Methoxyphenyl)piperazin-1-yl)butan-1-amine hydrochloride (**e2**)*

Using the general synthetic procedure of **a2** above to give the product **e2** as gray solid, which was used without further purification.

1.16. *2-(4-(2-Aminoethyl)piperazin-1-yl)-6,7-dimeth oxyquinazolin-4-amine hydrochloride (**b2**)*

Using the general synthetic procedure of **a2** above to give the product **b2** as gray solid, which was used without further purification.

1.17. *2-(4-(4-Aminobutyl)piperazin-1-yl)-6,7-dimeth oxyquinazolin-4-amine hydrochloride (**f2**)*

Using the general synthetic procedure of **a2** above to give the product **f2** as gray solid, which was used without further purification.

1.18. *2-(2-(4-(2-Methoxyphenyl)piperazin-1-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (**1a**)*

Using the general synthetic procedure of **1c** above to give the product **1a** as faint yellow solid (112 mg, 67%). ESI-HRMS calcd for $C_{25}H_{26}N_3O_3$ ($M + H^+$): 416.1969; found: 416.1972. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 8.54-8.47 (m, 4H), 7.92 (t, 2H, *J* = 7.5 Hz), 6.96-6.85 (m, 4H), 4.26 (t, 2H, *J* = 7.2 Hz), 3.77 (s, 3H), 2.93 (s, 3H), 2.64 (s, 6H).

1.19. *2-(4-(4-(2-Methoxyphenyl)piperazin-1-yl)butyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (**1e**)*

Using the general synthetic procedure of **1c** above to give the product **1e** as faint yellow solid (55mg, 16%). ESI-HRMS calcd for $C_{27}H_{30}N_3O_3$ ($M + H^+$): 444.2282; found: 444.2282. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 8.52-8.45 (m, 4H), 7.88 (t, 2H, *J* = 4.5 Hz), 6.96-6.84 (m, 4H), 4.1 (t, 2H, *J* = 6.9 Hz), 3.76 (s, 3H), 2.92 (s, 4H), 2.51-2.5 (m, 4H), 2.37 (s, 2H), 1.73-1.63 (m, 2H), 1.57-1.48 (m, 2H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 163.4(2C), 151.9, 141.2, 134.3(2C), 131.3, 130.7(2C), 127.4, 127.2(2C), 122.3, 122.1(2C), 120.8, 117.8, 111.9, 57.6, 55.3(2C), 53.0(2C), 50.0(2C), 25.6, 23.9.

1.20. *2-(2-(4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (**1b**)*

Using the general synthetic procedure of **1c** above to give the product **1b** as yellow solid (54 mg, 41%). ESI-HRMS calcd for $C_{28}H_{29}N_6O_4$ ($M + H^+$): 513.2245; found: 513.2244. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 8.53-8.46 (m, 4H), 7.91 (t, 2H, *J* = 7.8 Hz), 7.4 (s, 1H), 7.09 (s, 2H), 6.72 (s, 1H), 4.26-4.2 (m, 2H), 3.83 (s, 3H), 3.78 (s, 3H), 3.67 (s, 4H), 3.37 (s, 4H), 2.64 (t, 2H, *J* = 7.2 Hz). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 163.4(2C), 161.0, 158.5, 154.1, 148.8, 144.8, 134.3(2C), 131.3, 130.7(2C), 127.4, 127.2(2C), 122.0(2C), 105.2, 103.6, 102.8, 55.7, 55.3(2C), 55.2, 53.0(2C), 43.7(2C).

1.21. *2-(4-(4-(4-Amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)butyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (**1f**)*

Using the general synthetic procedure of **1c** above to give the product **1f** as yellow solid (230mg, 42%). ESI-HRMS calcd for $C_{30}H_{33}N_6O_4$ ($M + H^+$): 541.2558; found: 541.2558. ^{1}H-NMR (300MHz, DMSO-d_6) δ: 8.51-8.45 (m, 4H), 7.9-7.84 (m, 2H), 7.61 (s, 3H), 4.11 (t, 2H, *J* = 6.6 Hz), 3.85 (s, 3H), 3.82 (s, 3H), 3.36 (s, 6H), 3.07-3.02 (m, 4H), 1.92-1.7 (m, 4H).

2. Affinity test

2.1. *Materials and reagents*

(1) [^{3}H] Prazosin: 1 mCi/mL, 85.3 Ci/mmol, Cat. No.# NET823025UC; Perkin-Elmer;
(2) Phentolamine: Cat. No.# P7547; Sigma-Aldrich;
(3) Human ADRA1A Receptor Membrane Preparation; Cat. No. # M00354; GenScript USA Inc.;
(4) Human ADRA1B Receptor Membrane Preparation; Cat. No. # M00355; GenScript USA Inc.;
(5) Human ADRA1D Receptor Membrane Preparation; Cat. No. # M00405; GenScript USA Inc.;
(6) DMSO: Cat. No.# 0231; Amresco;
(7) UniFilter-96 GF/C filter plates; Cat. No.#6005177; PerkinElmer;
(8) Binding buffer: 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4; filtered and stored at 4°C; Wash buffer: 50 mM Tris-HCl, pH 7.4; filtered and stored at 4°C.

2.2. *Procedures*

(1) Each well of Uni-filter 96 GF/C microplate was incubated with 100 µL 0.5% PEI (Polyethyleneimine, Sigma-Aldrich, Cat. No.# 408727, dissolved in milli-Q water) at 4°C for 30-60 min;
(2) PEI was discarded by filtration with Millipore vacuum manifold (8-15 mmHg) and plates were washed with 2 mL/well wash buffer (4-8°C);
(3) The reaction mixtures including membrane, labeled and unlabeled ligand were prepared in 24-well plates and incubated at 25°C for 2 h with a shaking speed of 500 RPM;
(4) The reaction system was transferred into the filter

plates and filtered with Millipore vacuum manifold (8-15 mmHg);
(5) The wells was washed with 3 mL/well cold wash buffer and dried at RT for 60 min;
(6) The bottom of the plates was sealed with Bottom seal™ (opaque) (Perkin-Elmer);
(7) 50 µL MicroScint 20™ (Perkin-Elmer) was added to each well;
(8) The plates were sealed with Topseal A (Perkin-Elmer) and counted on TopCount NXT for 1 min/well.