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Supporting Information for

Identification of a β-arrestin-biased negative allosteric modulator for the β_2-adrenergic receptor

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Supporting Information Text

Supplemental Methods

Analysis of G_s activation by GTPγS binding. 18 μM of purified G_s heterotrimer in 2% 3:1 dimyristoyl phosphatidylcholine (DOPC):3-([3-cholamidopropyl]dimethyl-ammonio)-2-hydroxy-1-propanesulfonate (CHAPSO) bicelles with 1.13 mM cholesterol hemisuccinate (CHS), 20 mM HEPES, pH 7.5, and 100 mM NaCl was incubated in the presence of 1.5 μM β_2AR for 2 hr on ice to allow protein incorporation into the lipid bicelles. 2 μl of reconstituted β_2AR-G_s was diluted 200-fold in 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$ and 38.5 nM [^{35}S]GTPγS with or without 10 μM DFPQ and incubated for 30 min. 20 μl reactions were initiated by the addition of 1 μM ISO and incubated for 15 min at room temperature while negative control samples had no ISO. Bound [^{35}S]GTPγS was collected by rapid filtration on GF/B filters, washed 4 times with 4 ml of cold GTPγS wash buffer (20 mM Tris-HCl, pH 8, 25 mM $MgCl_2$, 100 mM NaCl) and analyzed by liquid scintillation counting.

Human airway smooth muscle cell scratch assay. HASM cells were seeded into 24-well plates, and a line was scratched in the center of the cell monolayer using a sterile 200 μl pipette tip and then washed three times with PBS to remove the cell debris. Migration of HASM cells into the cleared area was determined at 0 and 24 hours in the presence of PDGF-BB (20 ng/ml) stimulation. All images were captured by an EVOS FL Auto Cell Imaging System inverted microscope (Life Technologies, Carlsbad, CA). Cell-free area was quantitated using ImageJ.

Mutagenesis. β_2AR ECL3 mutants were synthesized by Integrated DNA Technologies and cloned into the pcDNA3-β_2AR-RlucII BRET construct with restriction enzyme digestion. β_2AR point mutants were created with the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) according to the manufacturers' protocol.

Pharmacological screening of β_2AR mutants. HEK 293 cells were transiently transfected with β_1AR-Rluc, β_2AR-Rluc or mutant β_2AR-Rluc and β-arrestin 2-GFP in a 96-well plate using Metafectene Pro (Biontex, München, Germany) following the manufacturer's protocol. Forty-eight hours after transfection, media was removed, and cells were incubated with increasing concentrations (30 nM to 100 μM) of NAM for 30 min, followed by addition of 1 μM ISO in the presence of 5 μM DBC for 20 min. Signals at 395 nm and 530 nm were recorded in an Infinite F500 plate reader. Results from concentration/activity curves are shown as mean ± SEM from six independent experiments. For normalization, we first subtracted the basal signal (wells stimulated with PBS in the absence of ligand) from each stimulated well, and the values of all replicates were then divided by the mean of ISO-alone induced responses and multiplied by 100 for any given read-out.

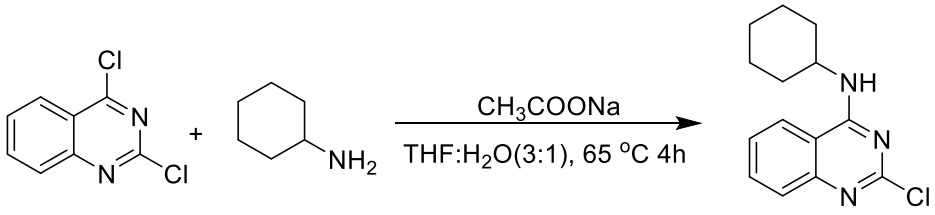
Measurement of cAMP production using BRET. To measure the impact of higher concentrations of DFPQ on ISO-mediated cAMP response, HEK293 cells with endogenous

β_2ARs were transfected with the BRET-based intramolecular cAMP sensor CAMYEL (both the donor and acceptor are fused to the cAMP binding domain of Epac) that upon cAMP binding undergoes a conformational change resulting in a change in the BRET signal. Forty-eight hours after transfection, media was removed, and cells were incubated with increasing concentrations (1.5 μM to 100 μM) of DFPQ for 30 min, followed by the addition of 1 μM ISO in the presence of 5 μM Coelenterazine H for 20 min. The impact of DFPQ on basal cAMP production was assessed by incubating the cells with or without 10 μM DFPQ for 30 min, followed by the addition of 5 μM Coelenterazine H for 20 min. Results from concentration/activity curves are shown as mean ± SEM from three independent experiments. For normalization, we first subtracted the basal signal (wells stimulated with PBS in the absence of ligand) from each stimulated well, and the values of all replicates were then divided by the mean of ISO-alone induced responses and multiplied by 100 for any given read-out.

Synthetic Procedures

General Methods for Chemistry. All commercially obtained solvents and reagents were used as received. Flash column chromatography was performed using silica gel 60 (230-400 mesh). Analytical thin layer chromatography (TLC) was carried out on Merck silica gel plates with QF-254 indicator and visualized by UV, PMA, or $KMnO_4$. ^1H and ^{13}C NMR spectra were recorded on a Bruker Advance 400. Chemical shifts are reported in parts per million (ppm, δ) using the residual solvent line as a reference. Splitting patterns are designated using the following abbreviations: s, singlet; d, doublet; t, triplet; dd, doublet of doublet; m, multiplet; br, broad. Coupling constants (J) are reported in hertz (Hz). Tetramethylsilane was used as an internal standard for proton nuclear magnetic resonance for samples run in $CDCl_3$ or DMSO-d_6. LC–MS data were acquired on a Waters Acquity UPLC/MS system equipped with a UPLC binary pump, an SQD 3100 mass spectrometer with an electrospray ionization (ESI) source and a PDA detector (210–400 nm). High-resolution mass spectra were obtained using the Q Exactive HF-X mass spectrometer which provided high-resolution, accurate mass, and total ion and extracted ion chromatograms. All compounds tested were present within a 5 ppm mass error. The purity of all final compounds was determined by HPLC, and the compounds are at least ≥95% pure.

2-Chloro-N-cyclohexylquinazoline-4-amine (AP-06-082):



AP-06-082

To a stirred solution of 2,4-dichloroquinazoline (1.0 g, 5.025 mmol) and cyclohexanamine (522 mg, 5.28 mmol) in 20 ml of THF/H_2O (3:1) at room temperature, sodium acetate (453 mg, 5.53 mmol) was added and heated to 65 ^oC for 4h. Completion of the reaction was confirmed by TLC, the solution was diluted with ethyl acetate, the layers were separated,

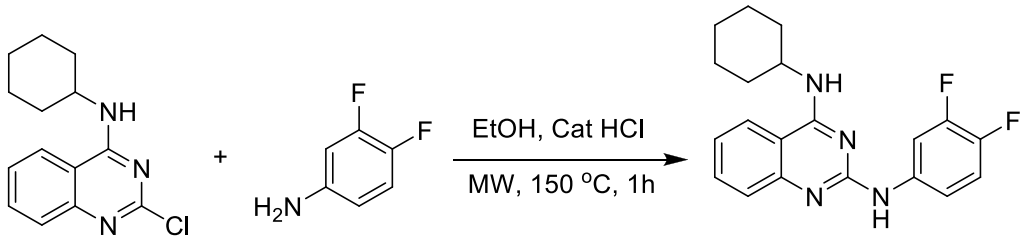
the organic phase was washed with water and brine solution dried over anhydrous Na_2SO_4, and solvent was evaporated under reduced pressure. The crude product purified by flash column chromatography afforded title products as white solid (1.1 g, 4.2 mmol, 83%). The product was confirmed by ^1H NMR, ^{13}C NMR and MS.

^1H NMR (400 MHz, $CDCl_3$) δ 7.78 – 7.68 (m, 2H), 7.64 (d, *J* = 8.0 Hz, 1H), 7.44 (ddd, *J* = 8.2, 6.6, 1.6 Hz, 1H), 5.70 (d, *J* = 7.4 Hz, 1H), 4.29 (tdt, *J* = 11.8, 7.9, 4.0 Hz, 1H), 2.19 – 2.07 (m, 2H), 1.87 – 1.75 (m, 2H), 1.75 – 1.66 (m, 1H), 1.57 – 1.41 (m, 2H), 1.38 – 1.17 (m, 3H).

^{13}C NMR (101 MHz, DMSO-d6) δ 160.63 (s), 157.44 (s), 150.63 (s), 133.99 (s), 126.84 (s), 126.28 (s), 123.85 (s), 113.93 (s), 50.31 (s), 32.22 (s), 25.68 (s), 25.38 (s).

Mass m/z: calculated for $C_{14}H_{16}ClN_3$ = 261.10. Found $[M + H]^+$ = 262.04.

N4-Cyclohexyl-N2-(3,4-difluorophenyl) quinazoline-2,4-diamine (AP-06-202): DFPQ



AP-06-082 **AP-06-202**

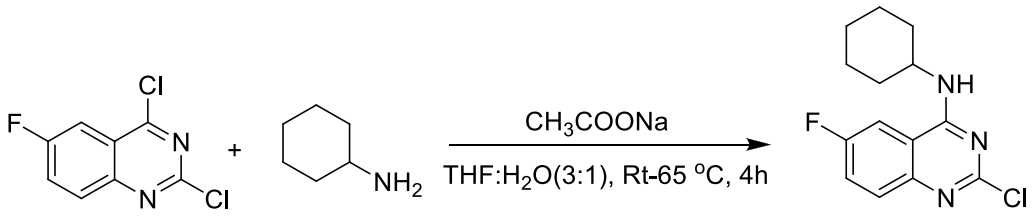
To a stirred solution of 2-chloro-N-cyclohexylquinazoline-4-amine (100 mg, 0.38 mmol) and 3,4-difluoroaniline (985 mg, 0.76 mmol) in 3 ml of ethanol at room temperature, 0.05 mL of 1N HCl was added and heated to 150 °C for 1h using a Microwave reactor. Completion of the reaction was confirmed by TLC, volatiles were evaporated under reduced pressure. Crude product was diluted with water and neutralized with *aqueous* saturated $NaHCO_3$, the product was extracted with ethyl acetate, the layers were separated, the organic phase was washed with water and brine solution dried over anhydrous Na_2SO_4, and solvent was evaporated under reduced pressure. Crude product purified by flash column chromatography afforded title products as white solid (101 mg, 0.28 mmol, 74%). The product was confirmed by ^1H NMR, ^{13}C NMR and MS.

^1H NMR (400 MHz, DMSO) δ 9.27 (s, 1H), 8.27 (ddd, *J* = 14.5, 7.6, 2.4 Hz, 1H), 8.17 (d, *J* = 7.7 Hz, 1H), 7.80 (d, *J* = 7.8 Hz, 1H), 7.64 – 7.54 (m, 1H), 7.49 (d, *J* = 8.9 Hz, 1H), 7.40 (d, *J* = 7.9 Hz, 1H), 7.29 (dd, *J* = 19.8, 9.3 Hz, 2H), 7.22 – 7.13 (m, 1H), 4.30 – 4.10 (m, 1H), 2.00 (d, *J* = 10.5 Hz, 2H), 1.82 (d, *J* = 11.8 Hz, 2H), 1.69 (d, *J* = 12.6 Hz, 1H), 1.54 – 1.30 (m, 3H), 1.30 – 1.12 (m, 2H).

^{13}C NMR (100 MHz, DMSO-d6) δ 159.79 (s), 157.07 (s), 151.54 (s), 150.56 (d, J = 12.9 Hz), 148.17 (d, J = 12.9 Hz), 145.12 (d, J = 13.0 Hz), 142.76 (d, J = 12.9 Hz), 139.38 (d, J = 9.6 Hz), 133.02 (s), 125.70 (s), 123.56 (s), 121.92 (s), 117.22 (d, J = 17.4 Hz), 114.67 (dd, J = 5.1, 2.8 Hz), 112.20 (s), 107.27 (d, J = 22.4 Hz), 50.08 (s), 32.61 (s), 25.85 (s), 25.65 (s).

Mass m/z: calculated for $C_{20}H_{20}F_2N_4$ = 354.17; found [M + H] $^+$ = 354.75.
HRMS (ESI): calculated for $C_{20}H_{20}F_2N_4$ = 354.1656, found $[M+H]^+$ = 355.15832.

2-Chloro-N-cyclohexyl-6-fluoroquinazoline-4-amine (AP-06-260):



AP-06-260

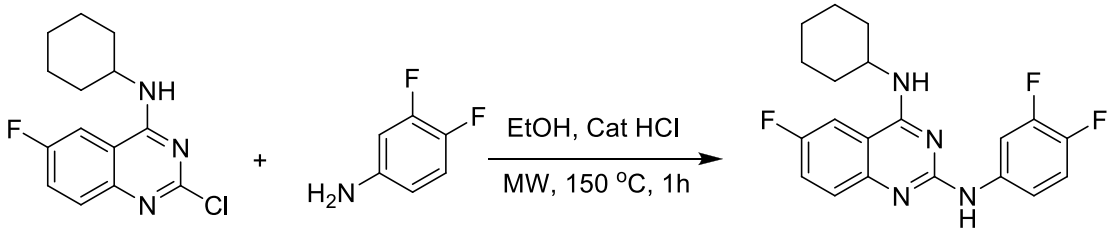
To a stirred solution of 2,4-dichloro-6-fluoroquinazoline (500 mg, 2.30 mmol) and cyclohexanamine (353 mg, 2.53 mmol) in 10 ml of THF / H_2O (3:1) at room temperature, sodium acetate (453 mg, 2.7 mmol) was added and heated to 65 ^{o}C for 4h. Completion of the reaction was confirmed by TLC, the solution was diluted with ethyl acetate, the layers were separated, the organic phase was washed with water and brine solution dried over anhydrous Na_2SO_4, and solvent was evaporated under reduced pressure. Crude product purified by flash column chromatography afforded title products as white solid (471 mg, 1.68 mmol, 73%). The product was confirmed by ^{1}H NMR and ^{13}C NMR and MS.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (dd, *J* = 9.2, 5.2 Hz, 1H), 7.48 (ddd, *J* = 9.1, 8.2, 2.7 Hz, 1H), 7.33 – 7.24 (m, 1H), 5.55 (d, *J* = 7.2 Hz, 1H), 4.35 – 4.18 (m, 1H), 2.22 – 2.07 (m, 2H), 1.89 – 1.75 (m, 2H), 1.70 (ddd, *J* = 16.3, 10.1, 6.2 Hz, 1H), 1.58 – 1.40 (m, 2H), 1.39 – 1.18 (m, 3H).

^{13}C NMR (100 MHz, DMSO-d6) δ 160.87 (s), 160.27 (d, J = 3.9 Hz), 158.44 (s), 157.11 (d, J = 2.1 Hz), 147.72 (s), 129.63 (d, J = 8.6 Hz), 123.03 (d, J = 24.7 Hz), 114.57 (d, J = 9.0 Hz), 108.48 (d, J = 24.2 Hz), 50.42 (s), 32.18 (s), 25.67 (s), 25.32 (s).

Mass m/z: calculated for $C_{14}H_{15}ClFN_3$ = 279.09. Found $[M + H]^+$ = 280.02.

N4-Cyclohexyl-N2-(3,4-difluorophenyl)-6-fluoroquinazoline-2,4-diamine (AP-06-263): DFPQ-6-F



AP-06-260 **AP-06-263**

To a stirred solution of 2-chloro-N-cyclohexyl-6-fluoroquinazoline-4-amine (60 mg, 0.20 mmol) and 3,4-difluoroaniline (34 mg, 0.27 mmol) in 3 ml of ethanol at room temperature, 0.05 mL of 1N HCl was added and heated to 150 ^{o}C for 1h using a Microwave reactor.

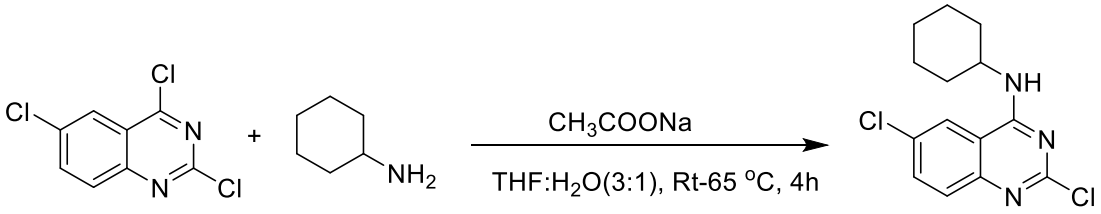
Completion of the reaction was confirmed by TLC, and volatiles were evaporated under reduced pressure. Crude product was diluted with water and neutralized with *aqueous* saturated $NaHCO_3$, product extracted with ethyl acetate, the layers were separated, the organic phase was washed with water and brine solution dried over anhydrous Na_2SO_4, and solvent was evaporated under reduced pressure. Crude product purified by flash column chromatography afforded title products as white solid (65 mg, 0.17 mmol, 85%). The product was confirmed by ^1H NMR, ^{13}C NMR and MS.

^1H NMR (400 MHz, $CDCl_3$) δ 8.13 – 8.00 (m, 1H), 7.55 (dd, *J* = 9.1, 5.2 Hz, 1H), 7.41 – 7.32 (m, 1H), 7.18 (dd, *J* = 8.9, 2.7 Hz, 1H), 7.12 – 7.01 (m, 2H), 6.95 (s, 1H), 5.31 (d, *J* = 7.2 Hz, 1H), 4.15 (dtd, *J* = 10.8, 7.1, 3.8 Hz, 1H), 2.16 (dd, *J* = 9.2, 3.0 Hz, 2H), 1.94 – 1.78 (m, 2H), 1.78 – 1.58 (m, 2H), 1.58 – 1.41 (m, 2H), 1.41 – 1.19 (m, 3H).

^{13}C NMR (100 MHz, DMSO-d6) δ 159.43 (d, J = 3.6 Hz), 158.62 (s), 156.96 (s), 156.25 (s), 150.55 (d, J = 12.8 Hz), 148.48 (s), 148.16 (d, J = 13.0 Hz), 145.15 (d, J = 13.0 Hz), 142.78 (d, J = 12.9 Hz), 139.30 (dd, J = 9.6, 2.1 Hz), 127.93 (d, J = 8.0 Hz), 121.99 (d, J = 24.3 Hz), 117.22 (d, J = 17.6 Hz), 114.68 (dd, J = 5.1, 2.8 Hz), 112.08 (d, J = 8.4 Hz), 108.08 (d, J = 23.4 Hz), 107.27 (d, J = 22.5 Hz), 50.18 (s), 32.57 (s), 25.82 (s), 25.60 (s).

Mass m/z: calculated for $C_{20}H_{19}F_3N_4$ = 372.16. Found $[M + H]^+$ = 372.75.
HRMS (ESI): calculated for $C_{20}H_{19}F_3N_4$ = 372.15618, found $[M+H]^+$ = 373.16346.

2,6-Dichloro-N-cyclohexylquinazoline-4-amine (AP-06-259):



AP-06-259

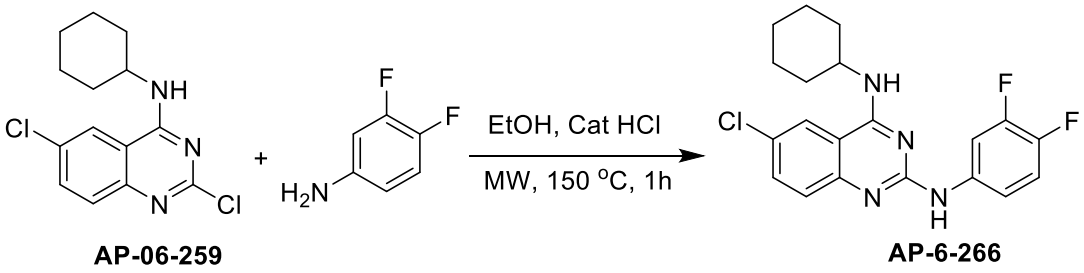
To a stirred solution of 2,4-dichloro-6-fluoroquinazoline (500 mg, 2.14 mmol) and cyclohexanamine (328 mg, 2.36 mmol) in 10 ml of THF/H_2O (3:1) at room temperature, sodium acetate (210 mg, 2.57 mmol) was added and heated to 65 °C for 4h. Completion of the reaction was confirmed by TLC, the solution was diluted with ethyl acetate, the layers were separated, the organic phase was washed with water and brine solution dried over anhydrous Na_2SO_4, and solvent was evaporated under reduced pressure. Crude product purified by flash column chromatography afforded title products as white solid (493 mg, 1.65 mmol, 77%). The product was confirmed by ^1H NMR, ^{13}C NMR and MS.

^1H NMR (400 MHz, DMSO) δ 8.54 (d, *J* = 2.2 Hz, 1H), 8.46 (d, *J* = 7.7 Hz, 1H), 7.81 (dd, *J* = 8.9, 2.2 Hz, 1H), 7.62 (d, *J* = 8.9 Hz, 1H), 4.23 – 3.96 (m, 1H), 1.92 (t, *J* = 12.2 Hz, 2H), 1.76 (t, *J* = 17.8 Hz, 2H), 1.66 (d, *J* = 12.4 Hz, 1H), 1.44 – 1.29 (m, 3H), 1.21 (ddd, *J* = 16.3, 15.0, 6.1 Hz, 2H).

^{13}C NMR (100 MHz, DMSO-d6) δ 159.78 (s), 157.92 (s), 149.52 (s), 134.15 (s), 130.41 (s), 129.08 (s), 123.13 (s), 114.91 (s), 32.15 (s), 25.68 (s), 25.32 (s).

Mass m/z: calculated for $C_{14}H_{15}Cl_2N_3$ = 295.06. Found $[M + H]^+$ = 295.77.

6-Chloro-N4-cyclohexyl-N2-(3,4-difluorophenyl)quinazoline-2,4-diamine (AP-06-266): DFPQ-6-Cl



AP-06-259 AP-6-266

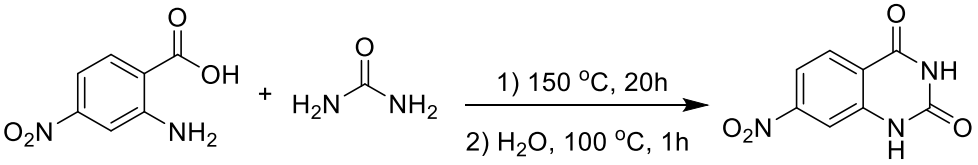
To a stirred solution of 2,6-dichloro-N-cyclohexylquinazoline-4-amine (60 mg, 0.20 mmol) and 3,4-difluoroaniline (34 mg, 0.26 mmol) in 3 ml of ethanol at room temperature, 0.05 mL of 1N HCl was added and heated to 150 °C for 1h using a Microwave reactor. Completion of the reaction was confirmed by TLC, and volatiles were evaporated under reduced pressure. Crude product was diluted with water and neutralized with *aqueous* saturated $NaHCO_3$, product extracted with ethyl acetate, the layers were separated, the organic phase was washed with water and brine solution dried over anhydrous Na_2SO_4, and solvent was evaporated under reduced pressure. Crude product purified by flash column chromatography afforded title products as white solid (68 mg, 0.18 mmol, 86%). The product was confirmed by ^{1}H NMR, ^{13}C NMR and MS.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 – 7.97 (m, 1H), 7.59 – 7.45 (m, 3H), 7.14 – 6.94 (m, 3H), 5.41 (d, *J* = 7.3 Hz, 1H), 4.26 – 4.03 (m, 1H), 2.23 – 2.06 (m, 2H), 1.83 (dt, *J* = 21.3, 9.1 Hz, 2H), 1.73 (dd, *J* = 9.2, 3.6 Hz, 1H), 1.59 – 1.39 (m, 2H), 1.39 – 1.17 (m, 3H).

^{13}C NMR (100 MHz, DMSO-d6) δ 159.00 (s), 157.40 (s), 150.54 (d, J = 12.9 Hz), 150.32 (s), 148.15 (d, J = 12.9 Hz), 145.29 (d, J = 12.9 Hz), 142.92 (d, J = 12.9 Hz), 139.08 (dd, J = 9.6, 2.3 Hz), 133.22 (s), 127.68 (s), 125.84 (s), 122.84 (s), 117.25 (d, J = 17.4 Hz), 114.88 (dd, J = 5.3, 2.9 Hz), 112.94 (s), 107.49 (d, J = 22.4 Hz), 50.26 (s), 32.51 (s), 25.82 (s), 25.59 (s).

Mass m/z: calculated for $C_{20}H_{19}ClF_2N_4$ = 388.13. Found $[M + H]^+$ = 388.95.
HRMS (ESI): calculated for $C_{20}H_{19}ClF_2N_4$ = 388.12663. Found $[M+H]^+$ = 389.13391.

7-Nitroquinazoline-2,4(1H,3H)-dione (AP-06-258):



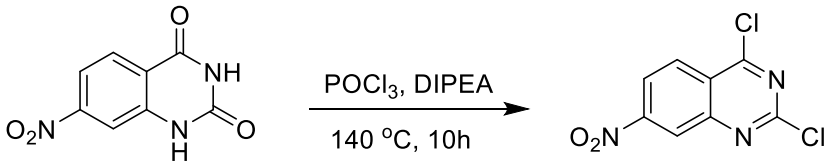
AP-06-258

Anthranilic acids (**2 g**, 10.98 mmol) and urea (6.5 g, 109.80 mmol) were poured into a 100 mL round bottom flask and the reaction mixture was heated at 150 °C for 20 h. Completion of the reaction was confirmed by TLC and cooled to room temperature. 50 mL water was added and the reaction mixture was heated at 100 °C for 1 h. The reaction mixture was cooled in and ice bath and the white solid was precipitated, filtered and washed with water and hexane. The residue was dried *in vacuo* afforded as a white solid (**1.74 g**, 8.34 mmol, 76%) and used in next step without further purification. The product was confirmed by ^{1}H NMR and MS.

^{1}H NMR (400 MHz, DMSO) δ 11.58 (s, 2H), 8.12 (d, *J* = 8.5 Hz, 1H), 7.92 (dd, *J* = 11.1, 2.5 Hz, 2H).

Mass m/z: calculated for $C_8H_4N_3O_4$ = 207.03. Found $[M - H]^-$ = 206.04.

2,4-Dichloro-7-nitroquinazoline (AP-06-275):

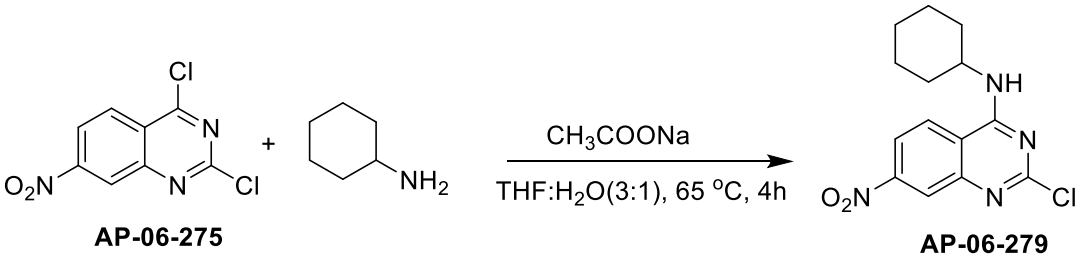


AP-06-258 **AP-06-275**

The compound 7-nitroquinazoline-2,4(1H,3H)-dione (1.73 g, 8.36 mmol) was dissolved in N-ethyl-N-isopropylpropan-2-amine (2.15 g, 16.71 mmol) and $POCl_3$ (12.8 g, 83.6 mmol) was slowly added to the reaction mixture. The reaction mixture was heated at 115 °C for 20 h and completion of the reaction was confirmed by TLC and the reaction solvents were evaporated with toluene. The residue was diluted with water and extracted with ethyl acetate several times. The organic layer was washed with brine solution dried over anhydrous Na_2SO_4 and the solvent was evaporated under in a reduced pressure evaporator. Crude product purified by flash column chromatography afforded title products as white solid (1.62 g, 6.69 mmol, 80%). The product was confirmed by ^{1}H NMR.

^{1}H NMR (400 MHz, DMSO) δ 11.65 (s, 1H), 11.52 (s, 1H), 8.10 (t, *J* = 7.7 Hz, 1H), 8.03 – 7.83 (m, 2H).

2-Chloro-N-cyclohexyl-7-nitroquinazoline-4-amine (AP-06-279):

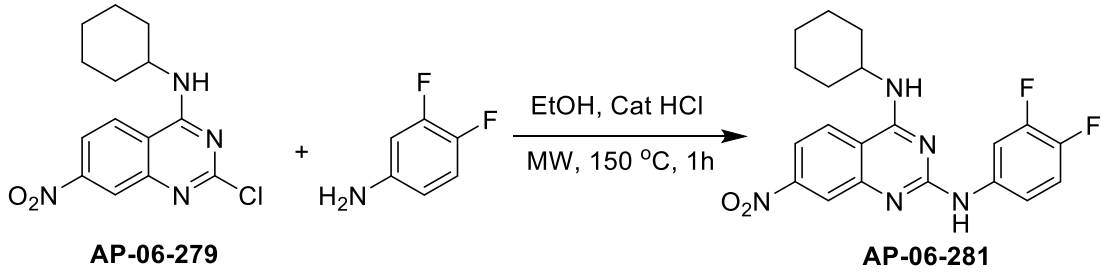


To a stirred solution of 2,4-dichloro-7-nitroquinazoline (300 mg, 1.24 mmol) and cyclohexanamine (129 mg, 1.30 mmol) in 15 ml of THF/ H_2O (3:1) at room temperature Sodium acetate (111 mg, 1.36 mmol) was added and heated to 65 °C for 4h. Completion of the reaction confirmed by TLC, the solution was diluted with ethyl acetate, the layers were separated, and the organic phase was washed with water, and brine solution dried over anhydrous Na_2SO_4, Solvent was evaporated under reduced pressure. Crude product purified flash column chromatography afforded title products as white solid (250 mg, 0.89 mmol, 66%). The product was confirmed by ^{1}H NMR, ^{13}C NMR and MS.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.59 (d, *J* = 2.2 Hz, 1H), 8.19 (dd, *J* = 9.0, 2.2 Hz, 1H), 7.82 (d, *J* = 9.0 Hz, 1H), 5.88 (d, *J* = 7.3 Hz, 1H), 4.47 – 4.15 (m, 1H), 2.16 (dd, *J* = 12.2, 3.1 Hz, 2H), 1.96 – 1.78 (m, 2H), 1.73 (dd, *J* = 9.3, 3.7 Hz, 1H), 1.60 – 1.43 (m, 2H), 1.31 (dtd, *J* = 24.9, 12.2, 3.5 Hz, 3H).

^{13}C NMR (100 MHz, DMSO-d6) δ 160.12 (s), 159.47 (s), 150.93 (s), 150.82 (s), 126.32 (s), 122.08 (s), 119.68 (s), 117.83 (s), 50.81 (s), 31.99 (s), 25.63 (s), 25.29 (s).
Mass m/z: Calcd for $C_{14}H_{15}ClN_4O_2$ = 306.09. Found $[M + H]^+$ = 306.97.

N4-Cyclohexyl-N2-(3,4-difluorophenyl)-7-nitroquinazoline-2,4-diamine (AP-06-281):



To a stirred solution of 2-chloro-N-cyclohexyl-7-nitroquinazoline-4-amine (61 mg, 0.2 mmol) and 3,4-difluoroaniline (51 mg, 4.0 mmol) in 3 ml of ethanol at room temperature, 0.05 mL of 1N HCl was added and heated to 150 °C for 1h using a Microwave reactor. Completion of the reaction was confirmed by TLC and volatiles were evaporated under reduced pressure. Crude product was diluted with water and neutralized with *aqueous* saturated $NaHCO_3$, product extracted with ethyl acetate, the layers were separated, the organic phase was washed with water and brine solution dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure. Crude product purified by flash

column chromatography afforded title products as white solid (71 mg, 0.18 mmol). The product was confirmed by ^{1}H NMR, ^{13}C NMR and MS.

^{1}H NMR (400 MHz, None) δ 9.74 (s, 1H), 8.62 (d, *J* = 9.0 Hz, 1H), 8.48 – 8.32 (m, 2H), 8.28 (s, 1H), 8.09 (dd, *J* = 8.9, 2.1 Hz, 1H), 7.71 (s, 1H), 7.52 (dd, *J* = 19.4, 9.6 Hz, 1H), 4.37 (s, 1H), 2.20 (d, *J* = 13.0 Hz, 2H), 2.01 (d, *J* = 10.9 Hz, 2H), 1.88 (d, *J* = 12.1 Hz, 1H), 1.72 – 1.48 (m, 3H), 1.38 (dd, *J* = 14.4, 7.4 Hz, 2H).

^{13}C NMR (101 MHz, DMSO-d6) δ 159.26 (s), 158.33 (s), 151.92 (s), 150.60 (s), 150.53 (d, J = 14.9 Hz), 148.12 (d, J = 12.9 Hz), 145.57 (d, J = 12.9 Hz), 143.19 (d, J = 12.9 Hz), 138.66 (dd, J = 9.5, 2.3 Hz), 125.93 (s), 120.37 (s), 117.28 (d, J = 17.3 Hz), 116.08 (s), 115.30 (dd, J = 5.2, 2.9 Hz), 114.84 (s), 107.92 (d, J = 22.3 Hz), 50.47 (s), 32.32 (s), 25.78 (s), 25.57 (s).

Mass m/z: Calcd for $C_{20}H_{19}F_2N_5O_2$ = 399.15. Found $[M + H]^+$ = 400.23.

N4-Cyclohexyl-N2-(3,4-difluorophenyl)quinazoline-2,4,7-triamine (AP-06-284): DFPQ-7-NH_2

AP-06-281 AP-6-284

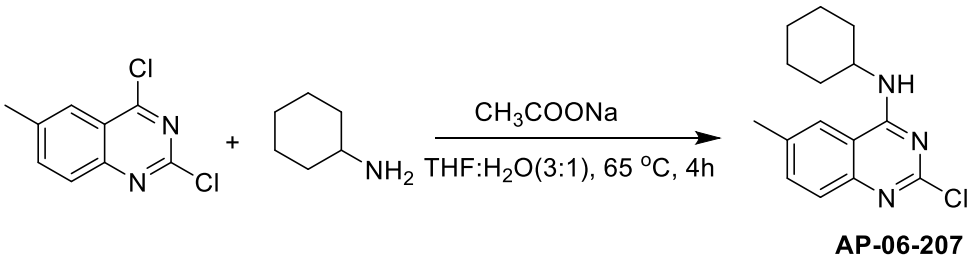
To a stirred solution of N4-cyclohexyl-N2-(3,4-difluorophenyl)-7-nitroquinazoline-2,4-diamine (50 mg, 0.13 mmol) in ethanol (10 mL) at room temperature, Pd/C (10%, 5 mg) was added and stirred under a hydrogen atmosphere at room temperature for 3h. Completion of the reaction was confirmed by LC–MS, the reaction was filtered over Celite, and the filter bed was washed with ethanol (20 mL). The combined organic portions were evaporated to afford the crude product, which was purified by flash column chromatography to afford the title compound (40 mg, 0.11 mmol, 87%). The product was confirmed by ^{1}H NMR, ^{13}C NMR and MS.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 – 8.01 (m, 1H), 7.36 (d, *J* = 8.7 Hz, 1H), 7.05 (dd, *J* = 8.4, 6.3 Hz, 2H), 6.68 (d, *J* = 1.9 Hz, 1H), 6.56 (dd, *J* = 8.7, 2.1 Hz, 1H), 5.40 (s, 1H), 4.10 (s, 3H), 2.16 (d, *J* = 11.0 Hz, 2H), 1.90 – 1.77 (m, 2H), 1.72 (dd, *J* = 9.3, 3.6 Hz, 1H), 1.59 – 1.39 (m, 2H), 1.40 – 1.13 (m, 3H).

^{13}C NMR (100 MHz, DMSO-d6) δ 159.42 (d, J = 3.9 Hz), 157.65 – 156.73 (m), 153.40 (s), 152.96 (s), 151.15 (s), 148.08 (d, J = 21.7 Hz), 139.88 (d, J = 9.9 Hz), 124.38 (d, J = 19.2 Hz), 117.11 (d, J = 16.9 Hz), 114.59 – 113.87 (m), 112.35 (s), 106.92 (dd, J = 21.2, 8.2 Hz), 104.93 (s), 103.05 (s), 49.72 (s), 32.93 (s), 25.90 (s), 25.70 (s).

Mass m/z: Calcd for $C_{20}H_{21}F_2N_5$ = 369.18. Found $[M + H]^+$ = 369.95.
HRMS (ESI): calculated for $C_{20}H_{21}ClF_2N_4$ = 369.1765. Found $[M+H]^+$ = 370.18378.

2-Chloro-N-cyclohexyl-6-methylquinazoline-4-amine (AP-06-207):



AP-06-207

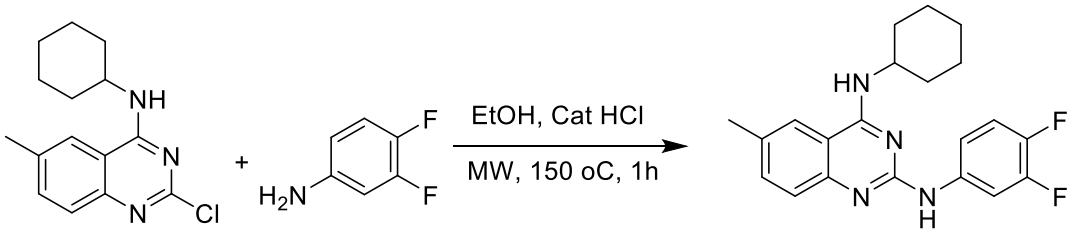
To a stirred solution of 2,4-dichloro-6-methylquinazoline (500 mg, 2.35 mmol) and cyclohexylamine (280 mg, 2.82 mmol) in 20 ml of THF/ H_2O (3:1) at room temperature, sodium acetate (230 mg, 2.82 mmol) was added and heated to 65 ^oC for 4h. Completion of the reaction was confirmed by TLC, the solution was diluted with ethyl acetate, the layers were separated, the organic phase was washed with water and brine solution dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure. Crude product purified by flash column chromatography afforded title products as white solid (518 mg, 1.88 mmol, 80%). The product was confirmed by ^1H NMR, ^{13}C NMR and MS.

^1H NMR (400 MHz, DMSO) δ 8.22 (d, *J* = 7.8 Hz, 1H), 8.15 (s, 1H), 7.61 (dd, *J* = 8.5, 1.4 Hz, 1H), 7.50 (d, *J* = 8.4 Hz, 1H), 4.09 (dd, *J* = 7.3, 3.6 Hz, 1H), 2.45 (s, 3H), 1.93 (d, *J* = 9.6 Hz, 2H), 1.78 (d, *J* = 11.9 Hz, 2H), 1.66 (d, *J* = 12.8 Hz, 1H), 1.50 – 1.27 (m, 4H), 1.18 (dd, *J* = 16.8, 7.8 Hz, 1H).

^{13}C NMR (100 MHz, DMSO-d6) δ 160.25 (s), 156.35 (s), 148.33 (s), 136.12 (s), 135.63 (s), 126.30 (s), 123.00 (s), 113.67 (s), 50.34 (s), 32.24 (s), 25.70 (s), 25.38 (s), 21.47 (s).

Mass m/z: Calcd for $[C_{15}H_{19}ClN_3]^+$ $[M + H]^+$, 276.13; found, 276.03.

N4-Cyclohexyl-N2-(3,4-difluorophenyl)-6-methylquinazoline-2,4-diamine (AP-07-164): DFPQ-6-Me



AP-06-207 **AP-07-164**

To a stirred solution of 2-chloro-N-cyclohexyl-6-methylquinazoline-4-amine (54 mg, 0.19 mmol) and 3,4-difluoroaniline (33 mg, 0.25 mmol) in 3 ml of ethanol at room temperature, 0.05 mL of 1N HCl was added and heated to 150 ^oC for 1h using a Microwave reactor. Completion of the reaction was confirmed by TLC and volatiles were evaporated under

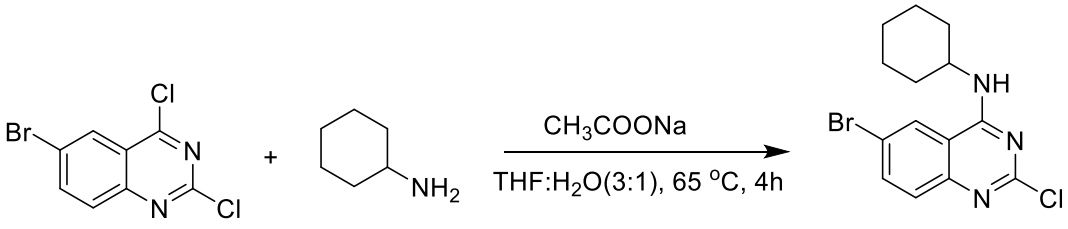
reduced pressure. Crude product was diluted with water and neutralized with *aqueous* saturated $NaHCO_3$, product extracted with ethyl acetate, the layers were separated, the organic phase was washed with water and brine solution dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure Crude product purified by flash column chromatography afforded title products as white solid (59 mg, 0.15 mmol). The product was confirmed by ^1H NMR, ^{13}C NMR and MS.

^1H NMR (400 MHz, DMSO) δ 9.30 (s, 4H), 8.22 (dd, *J* = 14.5, 8.0 Hz, 6H), 8.01 (s, 4H), 7.86 (s, 4H), 7.58 – 7.39 (m, 10H), 7.39 – 7.18 (m, 9H), 4.16 (s, 5H), 2.40 (s, 11H), 1.99 (d, *J* = 9.8 Hz, 8H), 1.82 (d, *J* = 12.1 Hz, 8H), 1.69 (d, *J* = 12.9 Hz, 4H), 1.50 – 1.29 (m, 16H), 1.27 – 1.12 (m, 5H).

^{13}C NMR (100 MHz, CDCl3) δ 164.60 (s), 161.72 (s), 156.06 (d, J = 13.0 Hz), 154.82 (s), 153.66 (d, J = 13.0 Hz), 150.63 (d, J = 13.1 Hz), 148.26 (d, J = 13.1 Hz), 144.33 (dd, J = 9.8, 2.3 Hz), 139.24 (s), 137.56 – 137.33 (m), 136.56 (s), 131.04 (s), 126.51 (s), 121.70 (d, J = 17.8 Hz), 119.19 (dd, J = 5.0, 3.2 Hz), 117.07 (s), 112.36 (d, J = 22.8 Hz), 37.67 (s), 30.83 (s), 30.50 (s), 25.54 (s).

Mass m/z: Calcd for $C_{21}H_{22}F_2N_4^+$ = 368.18. Found $[M + H]^+$ = 369.95.

6-Bromo-2-chloro-N-cyclohexylquinazoline-4-amine (AP-07-161):



AP-07-161

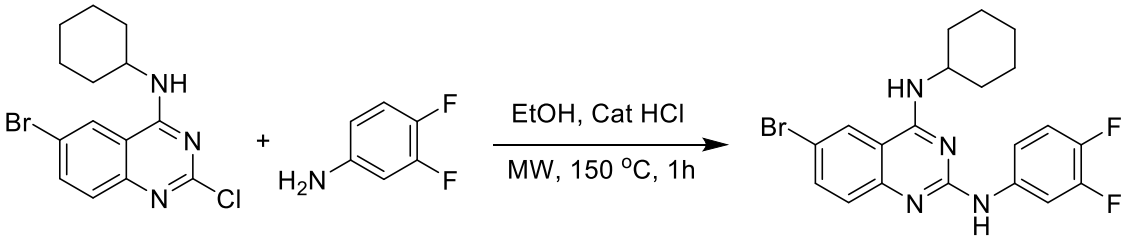
To a stirred solution of 6-bromo-2,4-dichloroquinazoline (500 mg, 1.8 mmol) and cyclohexylamine (187 mg, 1.98 mmol) in 20 ml of THF/ H_2O (3:1) at room temperature, sodium acetate (464 mg, 3.6 mmol) was added and heated to 65 °C for 4h. Completion of the reaction was confirmed by TLC, the solution was diluted with ethyl acetate, the layers were separated, the organic phase was washed with water and brine solution dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure. Crude product purified by flash column chromatography afforded title products as white solid (434 g, 1.28 mmol, 71%). The product was confirmed by ^1H NMR, ^{13}C NMR and MS.

^1H NMR (400 MHz, $CDCl_3$) δ 7.89 – 7.71 (m, 2H), 7.72 – 7.54 (m, 1H), 5.65 (d, *J* = 7.4 Hz, 1H), 4.35 – 4.15 (m, 1H), 2.13 (dd, *J* = 12.3, 3.3 Hz, 2H), 1.93 – 1.77 (m, 2H), 1.77 – 1.64 (m, 1H), 1.62 – 1.40 (m, 2H), 1.38 – 1.13 (m, 3H).

^{13}C NMR (100 MHz, DMSO-d6) δ 159.64 (s), 157.93 (s), 149.78 (s), 136.83 (s), 129.22 (s), 126.25 (s), 118.60 (s), 115.42 (s), 50.48 (s), 32.15 (s), 25.68 (s), 25.32 (s).

Mass m/z: Calcd for $C_{14}H_{15}BrClN_3$ = 339.01. Found $[M + H]^+$ = 341.92.

6-Bromo-N4-cyclohexyl-N2-(3,4-difluorophenyl)quinazoline-2,4-diamine(AP-07-168): DFPQ-6-Br



AP-07-161 **AP-7-168**

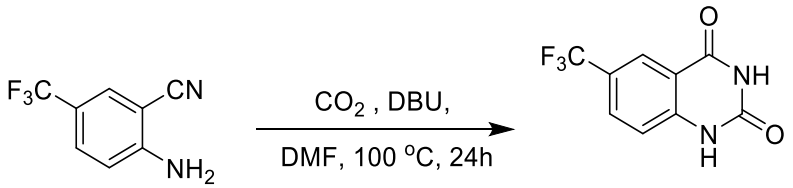
To a stirred solution of 6-bromo-2-chloro-N-cyclohexylquinazoline-4-amine (200 mg, 0.18 mmol) and 3,4-difluoroaniline (98 mg, 0.76 mmol) in 10 ml of ethanol at room temperature, 0.1 mL of 1N HCl was added and heated to 150 °C for 1h using a Microwave reactor. Completion of the reaction was confirmed by TLC and volatiles were evaporated under reduced pressure. Crude product was diluted with water and neutralized with *aqueous* saturated $NaHCO_3$, product extracted with ethyl acetate, the layers were separated, the organic phase was washed with water and brine solution dried over anhydrous Na_2SO_4, and solvent was evaporated under reduced pressure. Crude product purified by flash column chromatography afforded title products as white solid (216 mg, 0.50 mmol, 85%). The product was confirmed by ^1H NMR, ^{13}C NMR and MS.

^1H NMR (400 MHz, $CDCl_3$) δ 8.15 – 7.96 (m, 1H), 7.88 – 7.62 (m, 3H), 7.56 (d, *J* = 8.8 Hz, 1H), 7.14 – 6.90 (m, 2H), 5.66 (d, *J* = 7.4 Hz, 1H), 4.38 – 3.88 (m, 1H), 2.29 – 2.08 (m, 2H), 1.84 (dt, *J* = 27.5, 13.9 Hz, 2H), 1.74 (dd, *J* = 9.6, 3.4 Hz, 1H), 1.59 – 1.39 (m, 2H), 1.38 – 1.13 (m, 3H).

^{13}C NMR (100 MHz, DMSO-d6) δ 158.58 (s), 151.74 (s), 150.61 (d, J = 13.8 Hz), 148.18 (d, J = 13.5 Hz), 138.47 (d, J = 27.0 Hz), 134.46 (d, J = 10.7 Hz), 127.33 (s), 120.22 (s), 118.92 (s), 118.03 (d, J = 17.8 Hz), 117.07 (s), 112.38 (s), 111.76 (d, J = 25.5 Hz), 52.55 (s), 31.70 (s), 25.48 (s), 25.33 (s).

Mass m/z: Calcd for $C_{20}H_{19}BrF_2N_4$ = 432.08. Found $[M + H]^+$ = 433.04.
HRMS (ESI): calculated for $C_{20}H_{19}BrF_2N_4$ = 432.07612. Found $[M+H]^+$ = 433.08340.

6-(Trifluoromethyl)quinazoline-2,4(1H,3H)-dione (AP-07-188):



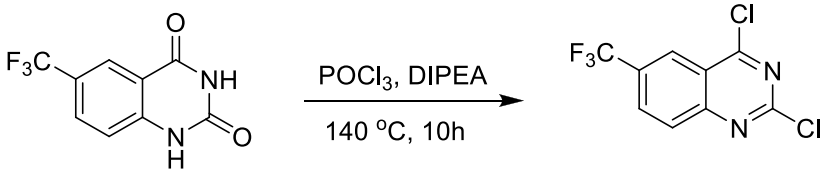
AP-07-188

To a stirred solution of 2-amino-5-(trifluoromethyl)benzonitrile (1.2 g, 6.45 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (2.16 g, 14.19 mmol) in DMF (15 mL), CO_2 gas was passed through the reaction mixture at 100 °C for 30 min, and stired for 24h at 100 °C. Reaction mixture was cooled to room temperature, diluted with water, and the precipitate was filtered, washed with cold water and dried under *high vacuum.* This afforded the title compound as a white solid (1.1 g, 4.77 mmol, 74%). The product was confirmed by ^{1}H NMR and MS.

^{1}H NMR (400 MHz, DMSO) δ 11.55 (s, 2H), 8.11 (d, *J* = 1.4 Hz, 1H), 7.98 (dd, *J* = 8.6, 2.0 Hz, 1H), 7.34 (d, *J* = 8.6 Hz, 1H).

Mass m/z: Calcd for $C_9H_5F_3N_2O_2$ = 230.03. Found $[M - H]^-$ = 229.12.

2,4-Dichloro-6-(trifluoromethyl)quinazoline (AP-07-192):

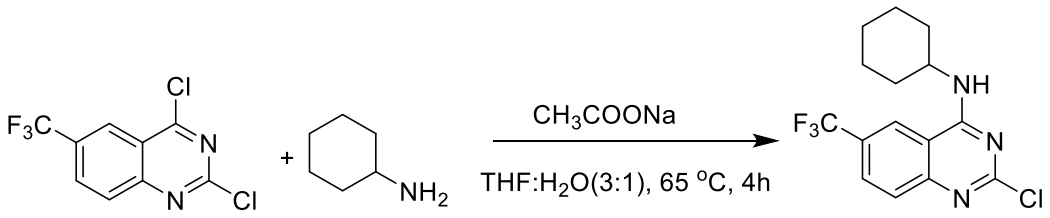


AP-07-188 **AP-7-192**

The compound 6-(trifluoromethyl)quinazoline-2,4(1H,3H)-dione (1.08 g, 4.69 mmol) was dissolved in N-ethyl-N-isopropylpropan-2-amine (1.06 g, 7.04 mmol) and then $POCl_3$ (7.19 g, 46.9 mmol) was slowly added to the reaction mixture and heated at 115 °C for 20 h. Completion of the reaction was confirmed by TLC. The reaction solvents were evaporated with toluene, and the residue was diluted with water, extracted with ethyl acetate several times, washed with brine solution dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure. Crude product purified by flash column chromatography afforded title products as white solid (980 mg, 3.67 mmol, 78%). The product was confirmed by ^{1}H NMR.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57 (s, 1H), 8.24 – 8.07 (m, 2H).

2-Chloro-N-cyclohexyl-6-(trifluoromethyl)quinazoline-4-amine (AP-07-200):



AP-7-192 **AP-7-200**

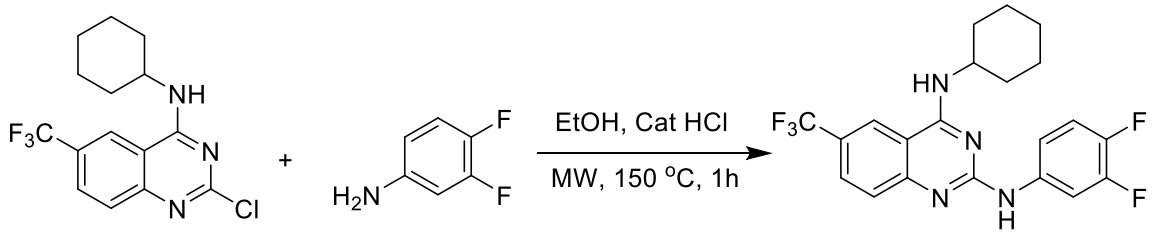
To a stirred solution of 2,4-dichloro-6-(trifluoromethyl)quinazoline (553 mg, 2.07 mmol) and cyclohexylamine (308 mg, 3.10 mmol) in 15 ml of THF/ H_2O (3:1) at room temperature, sodium acetate (187 mg, 2.23 mmol) was added and heated to 65 °C for 4h. Completion of the reaction was confirmed by TLC, the solution was diluted with ethyl acetate, the layers were separated, the organic phase was washed with water and brine solution dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure. Crude product purified by flash column chromatography afforded title products as white solid (500 mg, 1.5 mmol, 73%). The product was confirmed by ^1H NMR, ^{13}C NMR and MS.

^1H NMR (400 MHz, $CDCl_3$) δ 7.91 (d, *J* = 7.7 Hz, 2H), 7.85 (d, *J* = 9.1 Hz, 1H), 5.81 (d, *J* = 7.4 Hz, 1H), 4.40 – 4.20 (m, 1H), 2.16 (dd, *J* = 12.2, 3.1 Hz, 2H), 1.93 – 1.78 (m, 2H), 1.73 (dd, *J* = 9.3, 3.8 Hz, 1H), 1.63 – 1.42 (m, 2H), 1.30 (dddd, *J* = 20.9, 12.5, 10.4, 3.5 Hz, 3H).

^{13}C NMR (100 MHz, DMSO-d6) δ 160.63 (s), 159.77 (s), 152.95 (s), 129.58 (s), 128.39 (s), 127.29 – 125.38 (m), 123.12 (s), 122.46 (d, J = 4.1 Hz), 113.61 (s), 50.65 (s), 32.13 (s), 25.67 (s), 25.33 (s).

Mass m/z: Calcd for $C_{15}H_{15}ClF_3N_3$ = 329.09. Found $[M + H]^+$ = 330.12.

N4-Cyclohexyl-N2-(3,4-difluorophenyl)-6-(trifluoromethyl)quinazoline-2,4-diamine (AP-07-203): DFPQ-6-CF_3:



AP-7-200 **AP-7-203**

To a stirred solution of 2-chloro-N-cyclohexyl-6-(trifluoromethyl)quinazoline-4-amine (30 mg, 0.09 mmol) and 3,4-difluoroaniline (14 mg, 0.11 mmol) in 3 ml of ethanol at room temperature, 0.05 mL of 1N HCl was added and heated to 150 °C for 1h using a Microwave reactor. Completion of the reaction was confirmed by TLC and volatiles were evaporated under reduced pressure. Crude product was diluted with water and neutralized with *aqueous* saturated $NaHCO_3$, product extracted with ethyl acetate, the layers were separated,

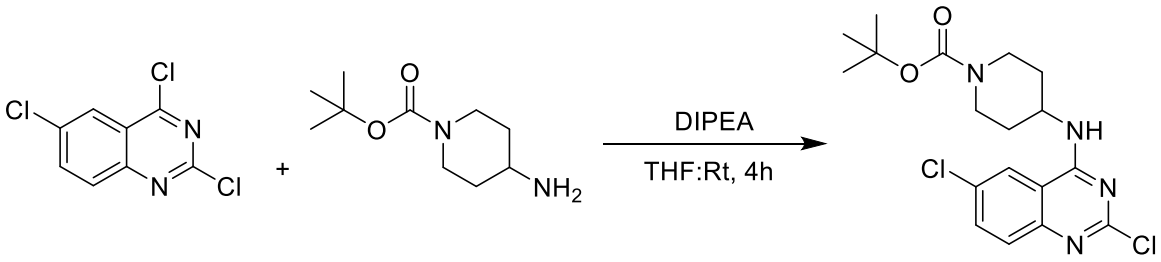
the organic phase was washed with water and brine solution dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure. Crude product purified by flash column chromatography afforded title products as white solid (28 mg, 0.07 mmol, 74%). The product was confirmed by ^1H NMR, ^{13}C NMR and MS.

^1H NMR (400 MHz, $CDCl_3$) δ 8.13 – 7.97 (m, 1H), 7.80 (s, 1H), 7.74 (dd, *J* = 8.8, 1.6 Hz, 1H), 7.58 (d, *J* = 8.8 Hz, 1H), 7.45 (s, 1H), 7.11 – 7.01 (m, 2H), 5.63 (d, *J* = 7.4 Hz, 1H), 4.28 – 3.95 (m, 1H), 2.19 (dd, *J* = 12.2, 2.7 Hz, 2H), 1.95 – 1.79 (m, 2H), 1.75 (dd, *J* = 9.5, 3.4 Hz, 1H), 1.60 – 1.42 (m, 2H), 1.42 – 1.18 (m, 3H).

^{13}C NMR (101 MHz, DMSO-d6) δ 159.85 (s), 158.50 (s), 154.03 (s), 150.53 (d, J = 13.0 Hz), 148.13 (d, J = 12.9 Hz), 145.54 (d, J = 12.8 Hz), 143.17 (d, J = 12.9 Hz), 138.75 (dd, J = 9.5, 2.4 Hz), 128.68 (d, J = 3.1 Hz), 126.64 (s), 124.05 – 120.71 (m), 117.30 (d, J = 17.5 Hz), 115.26 (dd, J = 5.2, 2.9 Hz), 111.44 (s), 107.90 (d, J = 22.4 Hz), 50.44 (s), 32.48 (s), 25.83 (s), 25.62 (s).

Mass m/z: Calcd for $C_{21}H_{19}F_5N_4$ = 422.15. Found $[M + H]^+$ = 422.94.
HRMS (ESI): calculated for $C_{21}H_{19}F_2N_4$ = 422.15299. Found $[M+H]^+$ = 423.16027.

Tert-butyl 4-(2,6-dichloroquinazoline-4-ylamino)piperidine-1-carboxylate (AP-07-148):



AP-07-148

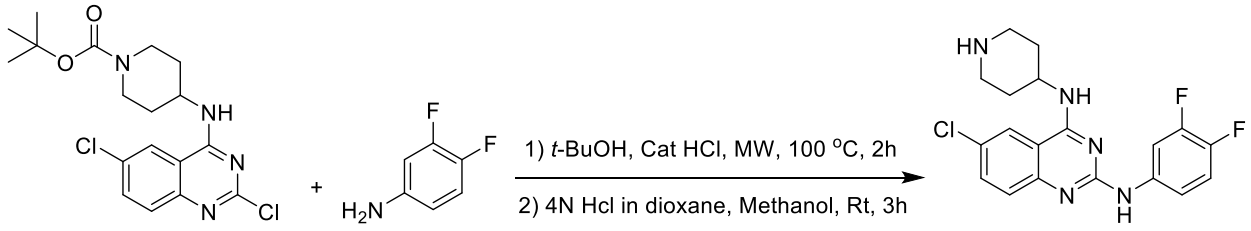
To a stirred solution of 2,4,6-trichloroquinazoline (300 mg, 1.28 mmol) and tert-butyl 4-aminopiperidine-1-carboxylate (386 mg, 1.93 mmol) in 10 mL of THF at room temperature, N-ethyl-N-isopropylpropan-2-amine (332 mg, 2.56 mmol) was added and stirred for 4h. Completion of the reaction was confirmed by TLC, the solution was diluted with ethyl acetate, the layers were separated, the organic phase was washed with water and brine solution dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure. Crude product purified by flash column chromatography afforded title products as white solid (350 mg, 0 .88 mmol, 69%). The product was confirmed by ^1H NMR, ^{13}C NMR and MS.

^1H NMR (400 MHz, DMSO) δ 8.49 (d, *J* = 2.2 Hz, 1H), 8.46 (d, *J* = 7.6 Hz, 1H), 7.82 (dd, *J* = 8.9, 2.2 Hz, 1H), 7.64 (d, *J* = 8.9 Hz, 1H), 4.42 – 4.17 (m, 1H), 3.99 (d, *J* = 11.9 Hz, 2H), 2.90 (s, 2H), 2.02 – 1.84 (m, 2H), 1.56 – 1.45 (m, 2H), 1.41 (d, *J* = 12.0 Hz, 9H).

^{13}C NMR (100 MHz, DMSO-d6) δ 159.91 (s), 157.78 (s), 154.38 (s), 149.52 (s), 134.34 (s), 130.56 (s), 129.18 (s), 123.09 (s), 114.87 (s), 79.17 (s), 48.51 (s), 28.56 (s).

Mass m/z: Calcd for $C_{18}H_{22}Cl_2N_4O_2$ = 396.11. Found $[M + H]^+$ = 397.15.

6-Chloro-N2-(3,4-difluorophenyl)-N4-(piperidin-4-yl)quinazoline-2,4-diamine hydrochloride (AP-07-213): DFPQ-6-Cl-piperidine:



AP-07-148 **AP-07-213**

To a stirred solution of 2-chloro-N-cyclohexyl-6-fluoroquinazoline-4-amine (100 mg, 0.25 mmol) and 3,4-difluoroaniline (52 mg, 0.33 mmol) in 10 ml of *t*-butanol at room temperature, 0.05 mL of 1N HCl was added and heated to 100 °C for 2h using a Microwave reactor. Completion of the reaction was confirmed by TLC and volatiles were evaporated under reduced pressure. The crude product was treated with 4N HCl in dioxane (1 mL) and methanol (2 mL) at room temperature for 3h, completion of the reaction was confirmed by TLC. Volatiles were evaporated under reduced pressure, crude product was diluted with water and neutralized with *aqueous* saturated $NaHCO_3$, extracted with ethyl acetate, the layers were separated, and the organic phase was washed with water and brine solution dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure. Crude product purified by flash column chromatography afforded title products as white solid (60 mg, 0.15 mmol, 61% over two steps). The product was confirmed by ^{1}H NMR, ^{13}C NMR and MS.

^{1}H NMR (400 MHz, DMSO) δ 10.79 (s, 1H), 9.75 (s, 1H), 9.28 (d, *J* = 9.3 Hz, 1H), 9.06 (s, 1H), 8.77 (s, 1H), 7.89 (d, *J* = 8.1 Hz, 1H), 7.83 – 7.71 (m, 1H), 7.65 (d, *J* = 8.9 Hz, 1H), 7.59 – 7.42 (m, 1H), 7.35 (s, 1H), 4.25 (s, 1H), 3.41 (d, *J* = 11.8 Hz, 2H), 2.94 (d, *J* = 10.4 Hz, 2H), 2.06 (dd, *J* = 32.6, 11.1 Hz, 4H).

^{13}C NMR (100 MHz, DMSO-d6) δ 159.34 (d, J = 3.9 Hz), 152.02 – 151.67 (m), 150.67 (d, J = 19.1 Hz), 148.37 – 147.97 (m), 138.47 (d, J = 14.1 Hz), 136.96 – 135.38 (m), 134.26 (s), 129.29 (s), 124.80 (s), 120.18 (s), 119.21 (s), 118.08 (d, J = 18.1 Hz), 112.00 (s), 48.40 (s), 42.56 (s), 27.56 (s).

Mass m/z: Calcd for $C_{19}H_{18}ClF_2N_5$ = 389.12. Found $[M + H]^+$ = 390.15.
HRMS (ESI): calculated for $C_{19}H_{18}ClF_2N_5$ = 389.12188. Found $[M+H]^+$ = 390.12916.

Supplemental Figure 1: Identifying small-molecule allosteric modulators of β-arrestin recruitment to the β_2AR

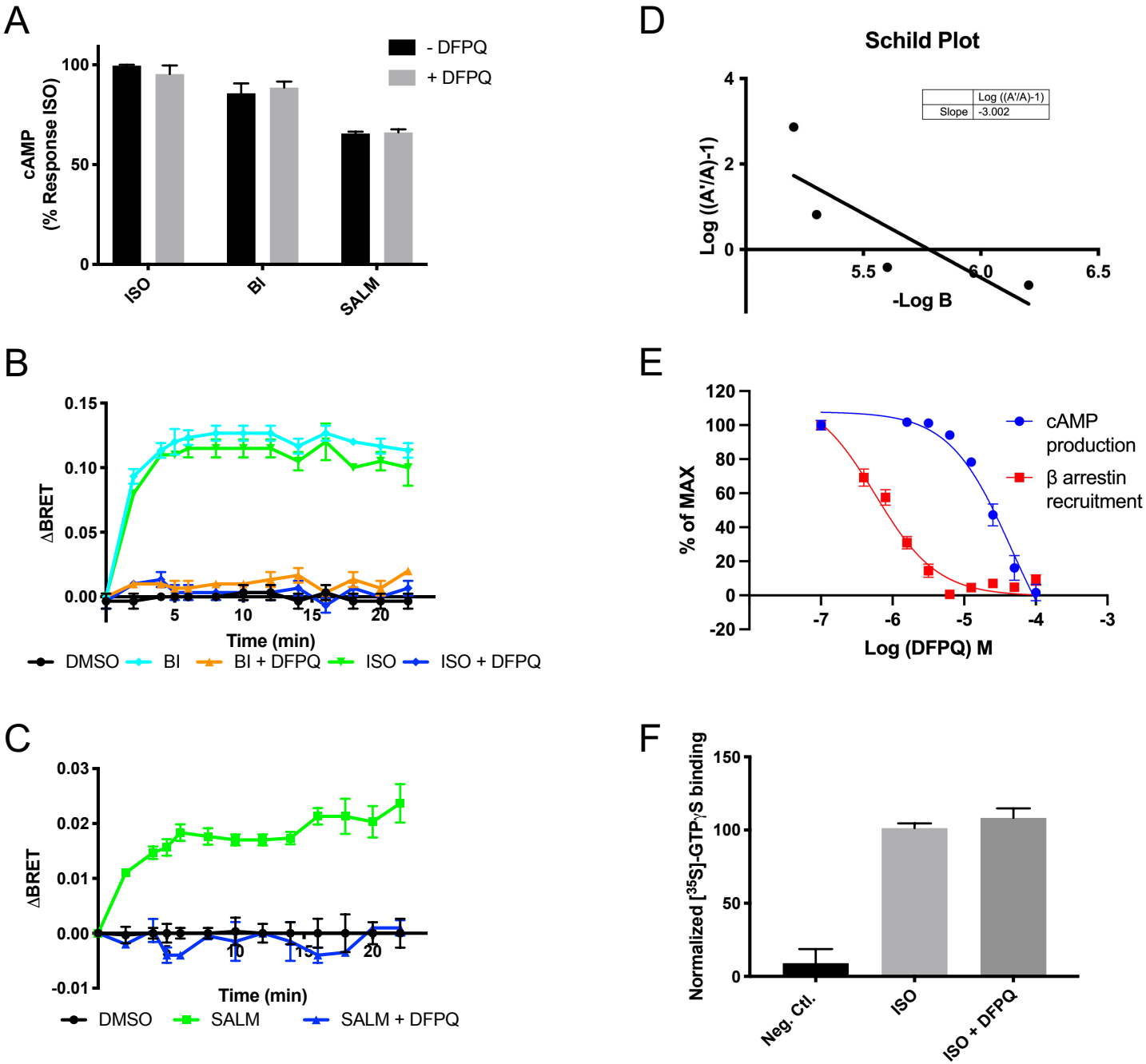


Figure S1. Identifying small-molecule allosteric modulators of β-arrestin recruitment to the β_2AR. **(A)** β_2AR expressing HEK 293 cells were preincubated with 0.1% DMSO or 10 μM DFPQ for 30 min. Cells were then stimulated with 1 μM ISO, BI or SALM for 10 min and cAMP production was measured by ELISA. HEK 293 cells co-transfected with β-arrestin2-GFP10 and β_2AR-RLucII were preincubated with 0.1% DMSO or 10 μM DFPQ for 30 min. Cells were then incubated with Coelenterazine 400a for 2 min and then stimulated with **(B)** 1 μM BI, 1 μM ISO or **(C)** 1 μM SALM**.** BRET signal for β-arrestin recruitment was recorded every 2 min post agonist addition. **(D)** Log((A'/A)-1) and -LogB were extrapolated from the concentration activity curves shown in Fig. 1E and a simple linear regression was performed using the built-in equation in GraphPad Prism. **(E)** Effect of high concentrations of DFPQ on ISO-mediated cAMP production and β-arrestin recruitment. Cells were incubated with increasing concentration ($10^{-6.4}$ to 10^{-4} M) of DFPQ for 30 min, followed by the addition of 1 μM ISO. Concentration/activity curves were generated and plotted as mean ± SEM, n=3. The impact of DFPQ on basal cAMP production and β-arrestin recruitment was assessed by incubating the cells with or without 10 μM DFPQ for 30 min**.** DFPQ had no effect on basal cAMP production (PBS: 0.65 ± 0.01 *vs* DFPQ: 0.64 ± 0.01) or basal β-arrestin recruitment (PBS: 0.12 ± 0.01 *vs* DFPQ: 0.13 ± 0.01) **(F)** Lipid bicelles containing reconstituted β_2AR and G_s heterotrimer were preincubated with 0.1% DMSO or 10 μM DFPQ and then stimulated with 1 μM ISO. Negative control samples did not contain ISO. Bound [^{35}S]-GTPγS was collected by rapid filtration on GF/B filters, washed 4 times with 4 ml of cold GTPγS wash buffer and analyzed by liquid scintillation.

Supplemental Figure 2: β-agonist regulation of primary human airway smooth muscle cell migration

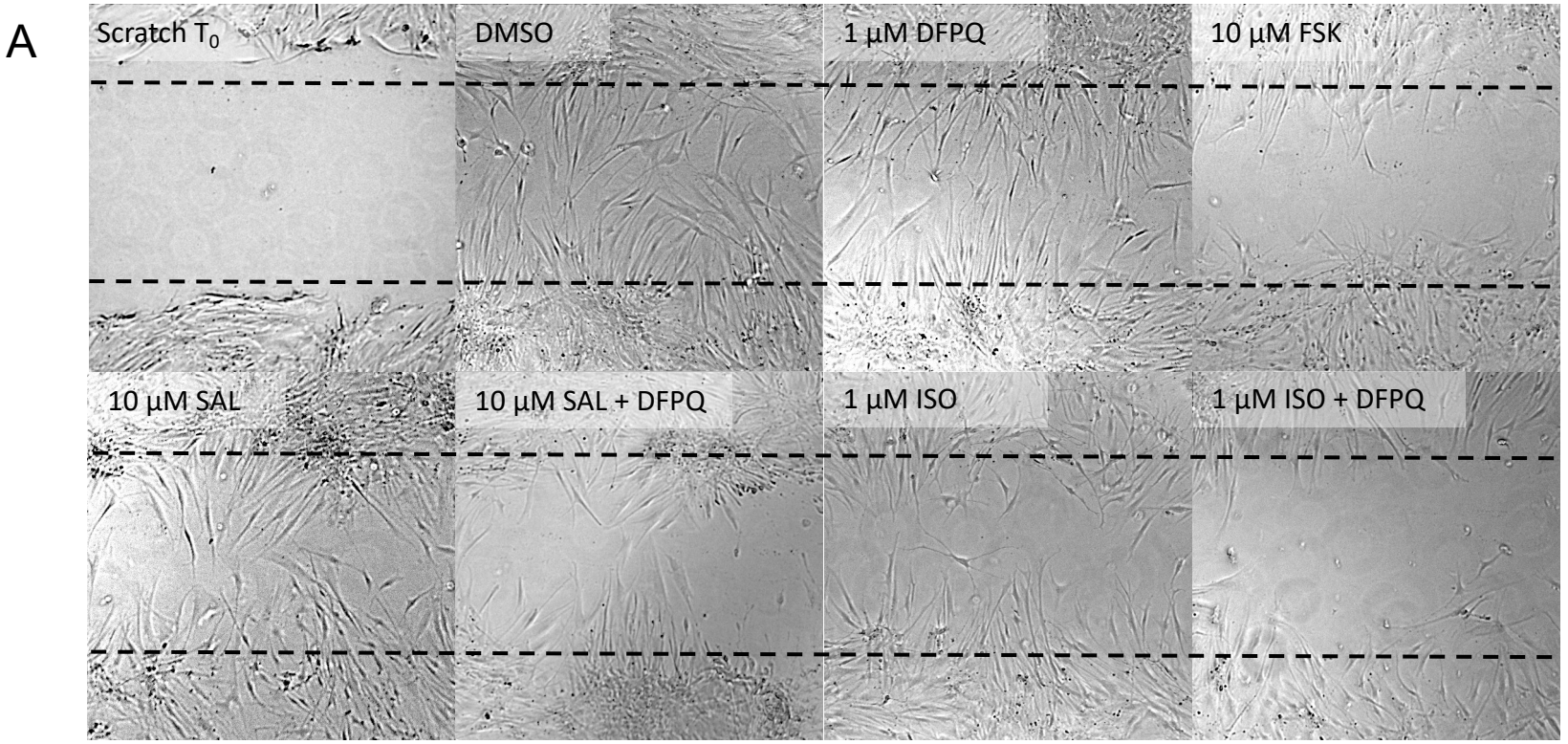


Figure S2. β-agonist regulation of primary human airway smooth muscle cell migration. **(A)** HASM cells were scratched and stimulated with the different conditions shown. **(B)** Cell-free area in the scratch line was quantified with ImageJ and statistical comparison was assessed by t test with Welch's correction. P values were considered significant when <0.05.

Supplemental Figure 3: Mutagenesis studies using β_2AR/β_1AR chimeras with DFPQ

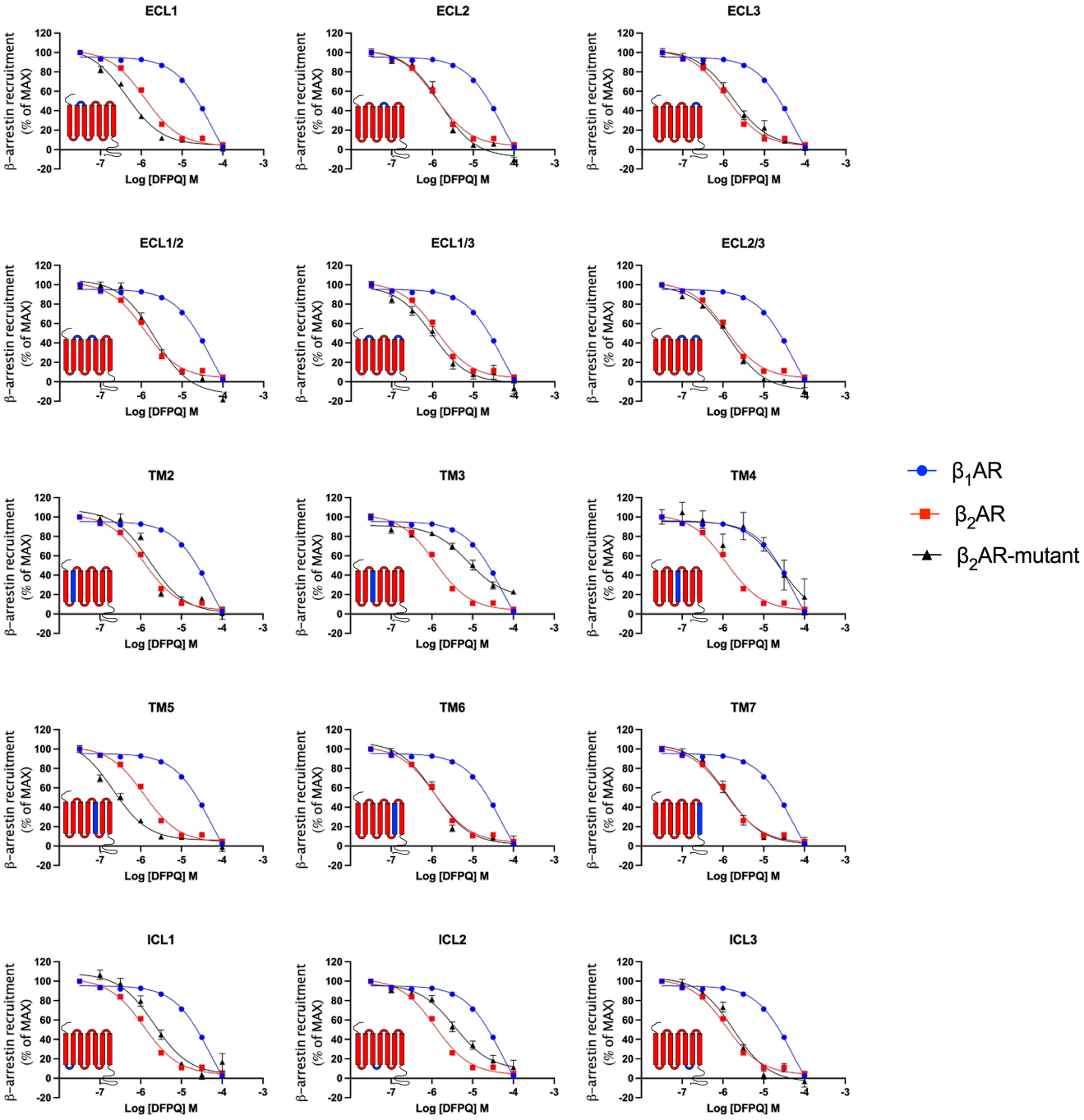


Figure S3. Mutagenesis studies using β_2AR/β_1AR chimeras with DFPQ**.** HEK 293 cells were transfected with β_1AR-Rluc (blue), β_2AR-Rluc (red) or β_2AR-chimeras-Rluc (black) and β-arrestin2-GFP. Cells were incubated with increasing concentrations ($10^{-7.5}$ to 10^{-4} M) of DFPQ for 30 min, followed by addition of 1 μM ISO. Concentration/activity curves were generated and plotted as mean ± SEM, n=6. A diagram of the chimeras is shown with the β_2AR domains in red and the swapped domain from the β_1AR in blue.

Supplemental Figure 4: Mutagenesis studies using β_2AR/β_1AR chimeras with AP-7-168

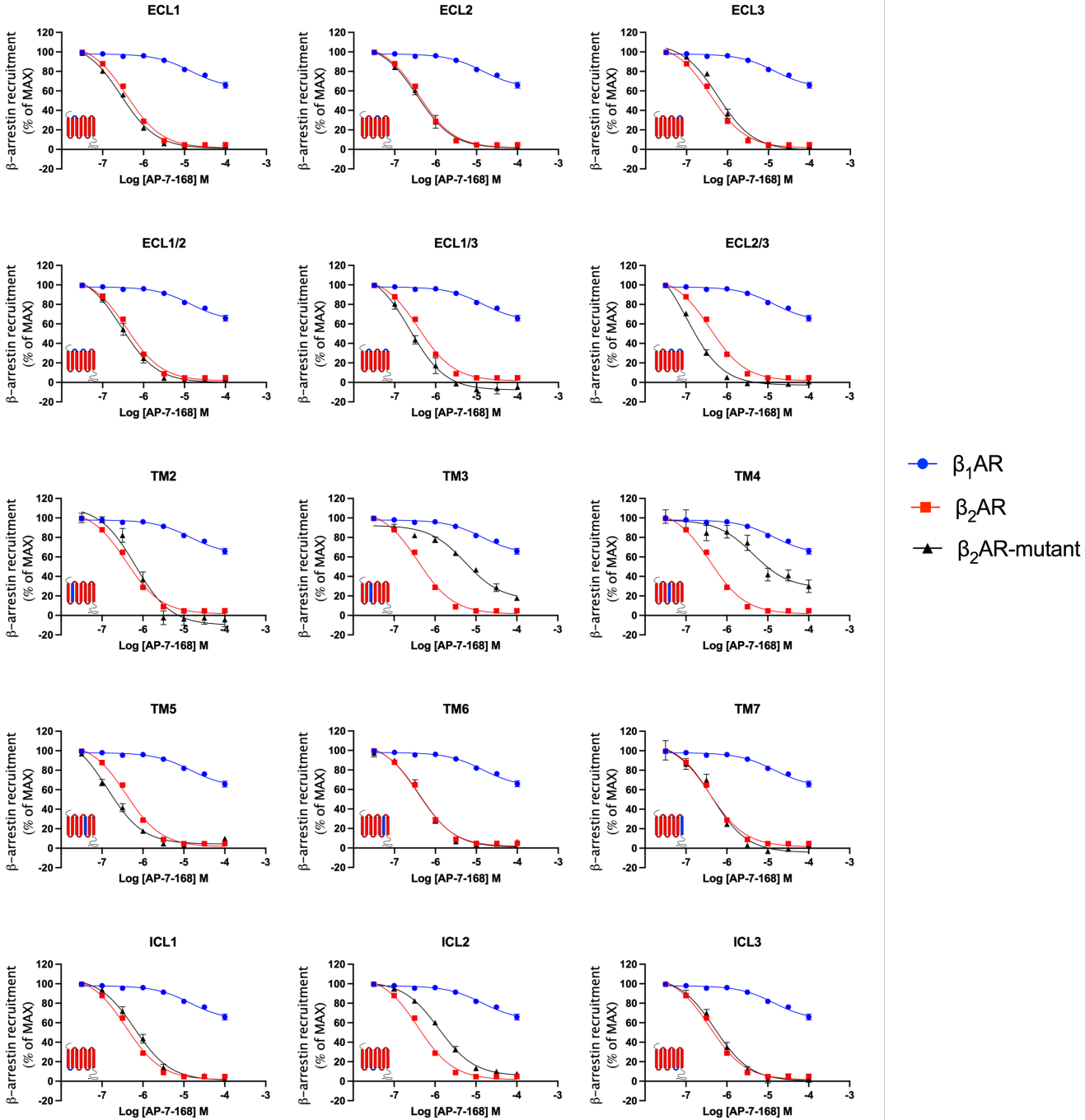


Figure S4. Mutagenesis studies using β_2AR/β_1AR chimeras with AP-7-168**.** HEK 293 cells were transfected with β_1AR-Rluc (blue), β_2AR-Rluc (red) or β_2AR-chimeras-Rluc (black) and β-arrestin2-GFP. Cells were incubated with increasing concentration ($10^{-7.5}$ to 10^{-4} M) of AP-7-168 for 30 min, followed by addition of 1 μM ISO. Concentration/activity curves were generated and plotted as mean ± SEM, n=6. A diagram of the chimeras is shown with the β_2AR domains in red and the swapped domain from the β_1AR in blue.

Supplemental Figure 5: Mutagenesis studies using β_2AR/β_1AR chimeras with AP-7-203

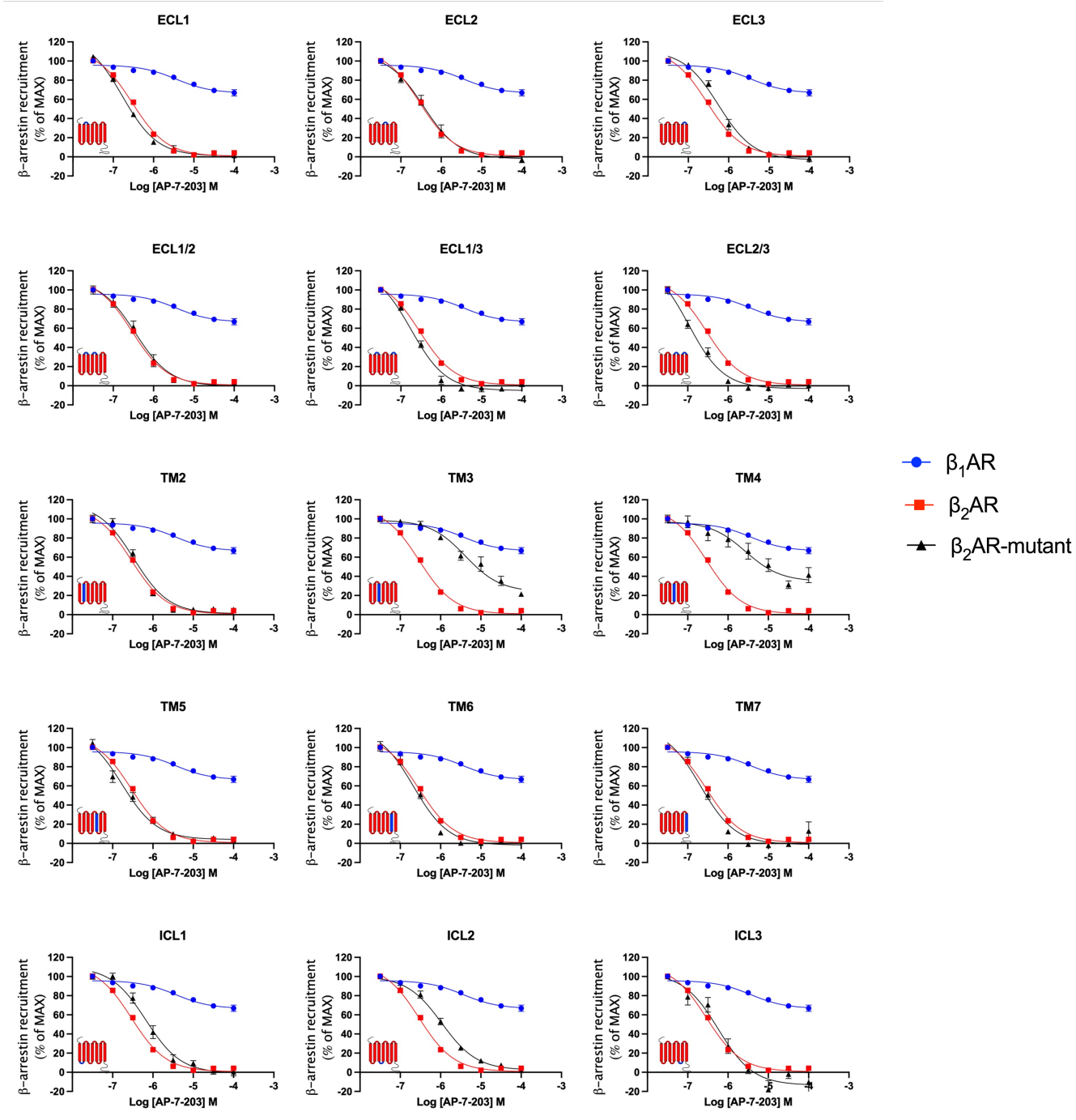


Figure S5. Mutagenesis studies using β_2AR/β_1AR chimeras with AP-7-203**.** HEK 293 cells were transfected with β_1AR-Rluc (blue), β_2AR-Rluc (red) or β_2AR-chimeras-Rluc (black) and β-arrestin2-GFP. Cells were incubated with increasing concentration ($10^{-7.5}$ to 10^{-4} M) of AP-7-203 for 30 min, followed by addition of 1 μM ISO. Concentration/activity curves were generated and plotted as mean ± SEM, n=6. A diagram of the chimeras is shown with the β_2AR domains in red and the swapped domain from the β_1AR in blue.

Supplemental Figure 6: Effect of selected β_2AR/β_1AR chimeras on ISO-promoted β-arrestin recruitment

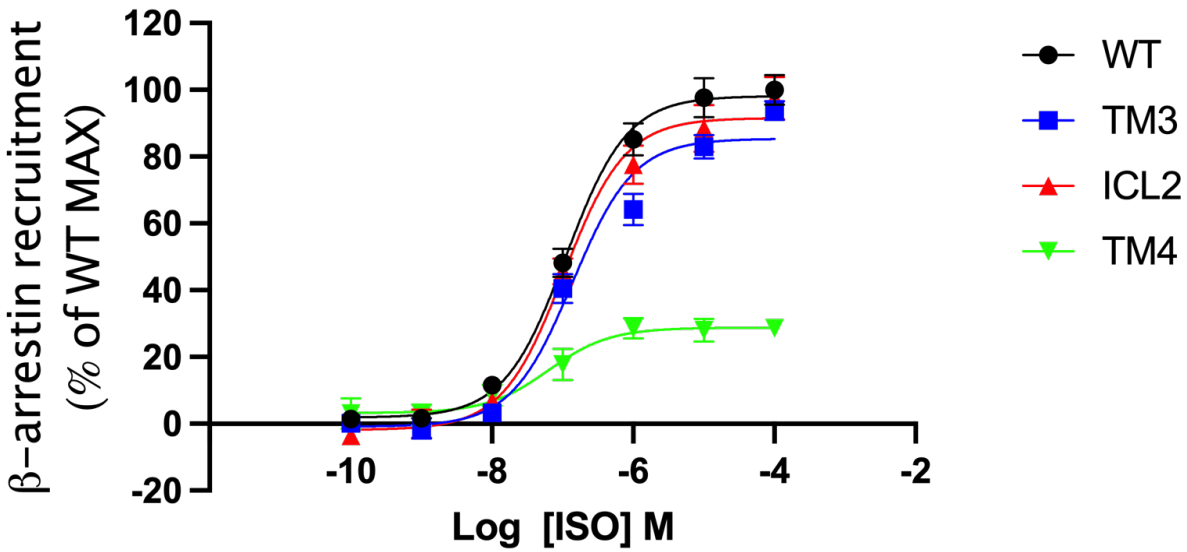


Figure S6. Effect of selected β_2AR/β_1AR chimeras on ISO-promoted β-arrestin recruitment**.** HEK 293 cells were transfected with WT or chimera (TM3, ICL2, TM4) β_2AR-Rluc and β-arrestin2-GFP and stimulated with increasing concentrations ($10^{-7.5}$ to 10^{-4} M) of ISO. Concentration/activity curves were generated and plotted as mean ± SEM, n=6.

Supplemental Figure 7: Mutagenesis studies using β_2AR point mutants with DFPQ

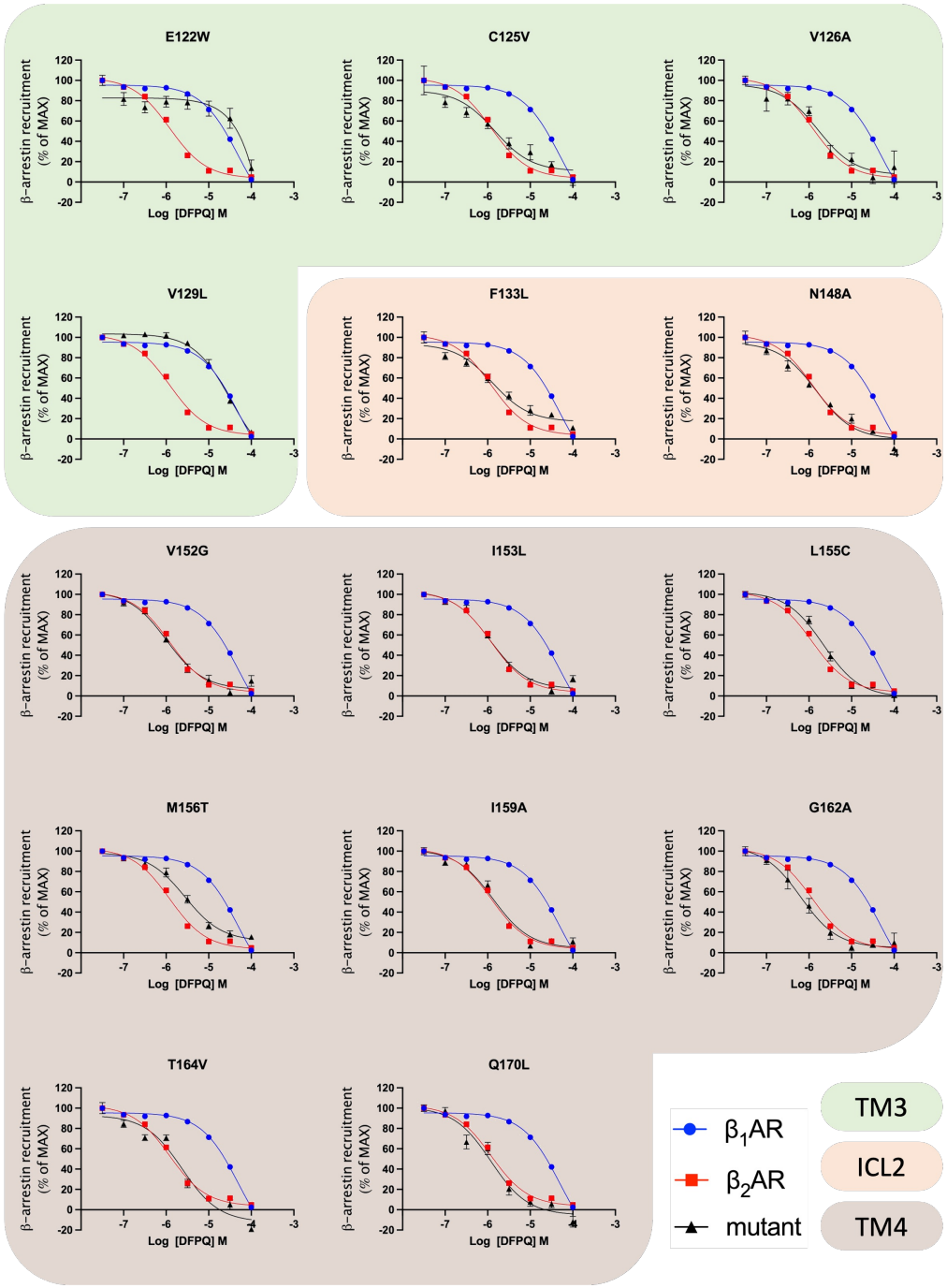


Figure S7. Mutagenesis studies using β_2AR point mutants with DFPQ**.** HEK 293 cells were transfected with β_1AR-Rluc (blue), β_2AR-Rluc (red) or β_2AR-point mutant-Rluc (black) and β-arrestin2-GFP. Cells were incubated with increasing concentration ($10^{-7.5}$ to 10^{-4} M) of DFPQ for 30 min, followed by addition of 1 μM ISO. Concentration/activity curves were generated and plotted as mean ± SEM, n=6. Mutations in TM3 are in green, mutations in ICL2 are in orange and mutations in TM4 are in brown.

Supplemental Figure 8: Mutagenesis studies using β_2AR point mutants with AP-7-168

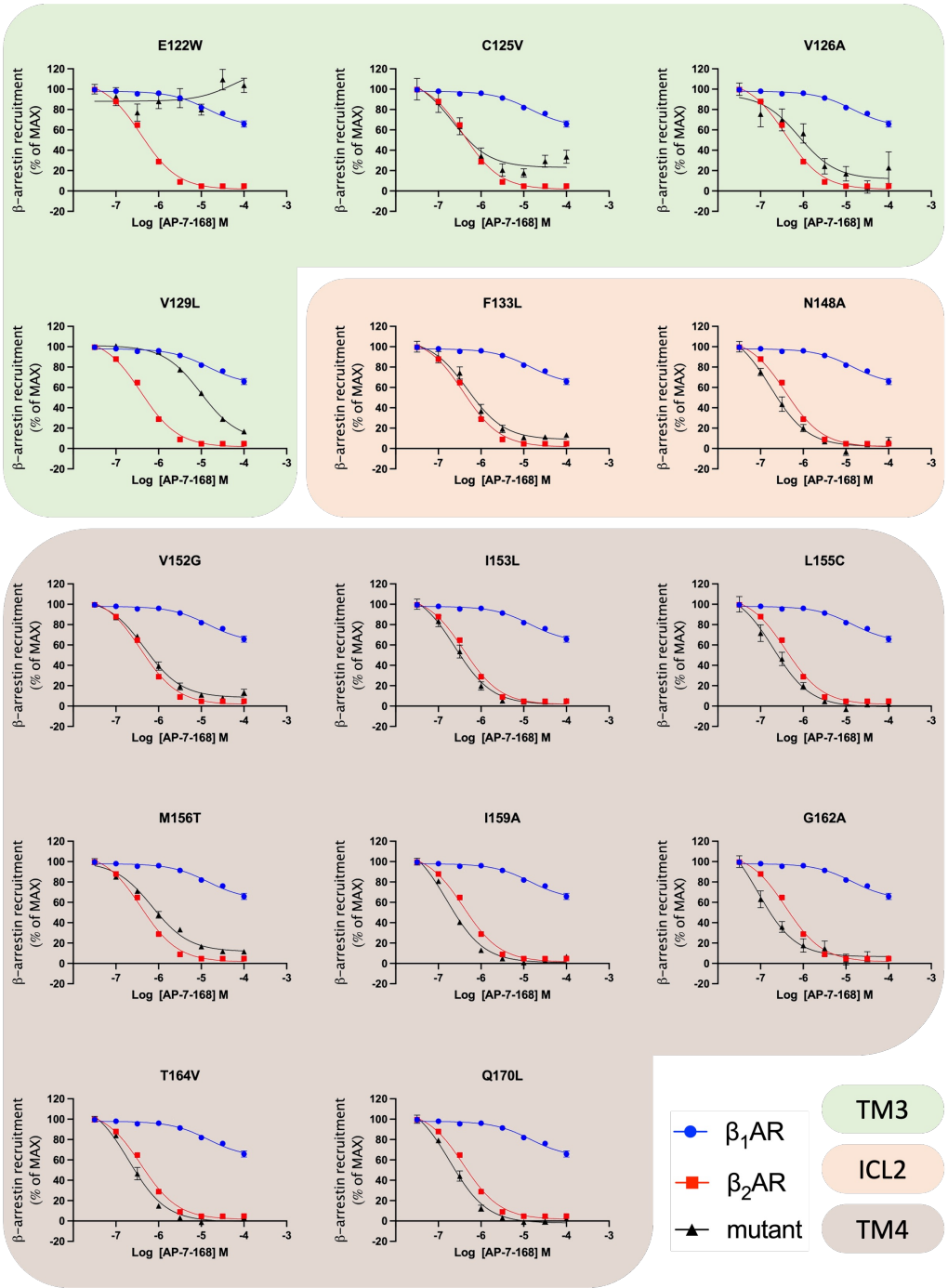


Figure S8. Mutagenesis studies using β_2AR point mutants with AP-7-168**.** HEK 293 cells were transfected with β_1AR-Rluc (blue), β_2AR-Rluc (red) or β_2AR-point mutant-Rluc (black) and β-arrestin2-GFP. Cells were incubated with increasing concentration ($10^{-7.5}$ to 10^{-4} M) of AP-7-167 for 30 min, followed by addition of 1 μM ISO. Concentration/activity curves were generated and plotted as mean ± SEM, n=6. Mutations in TM3 are in green, mutations in ICL2 are in orange and mutations in TM4 are in brown.

Supplemental Figure 9: Mutagenesis studies using chimeras β_2AR point mutants with AP-7-203

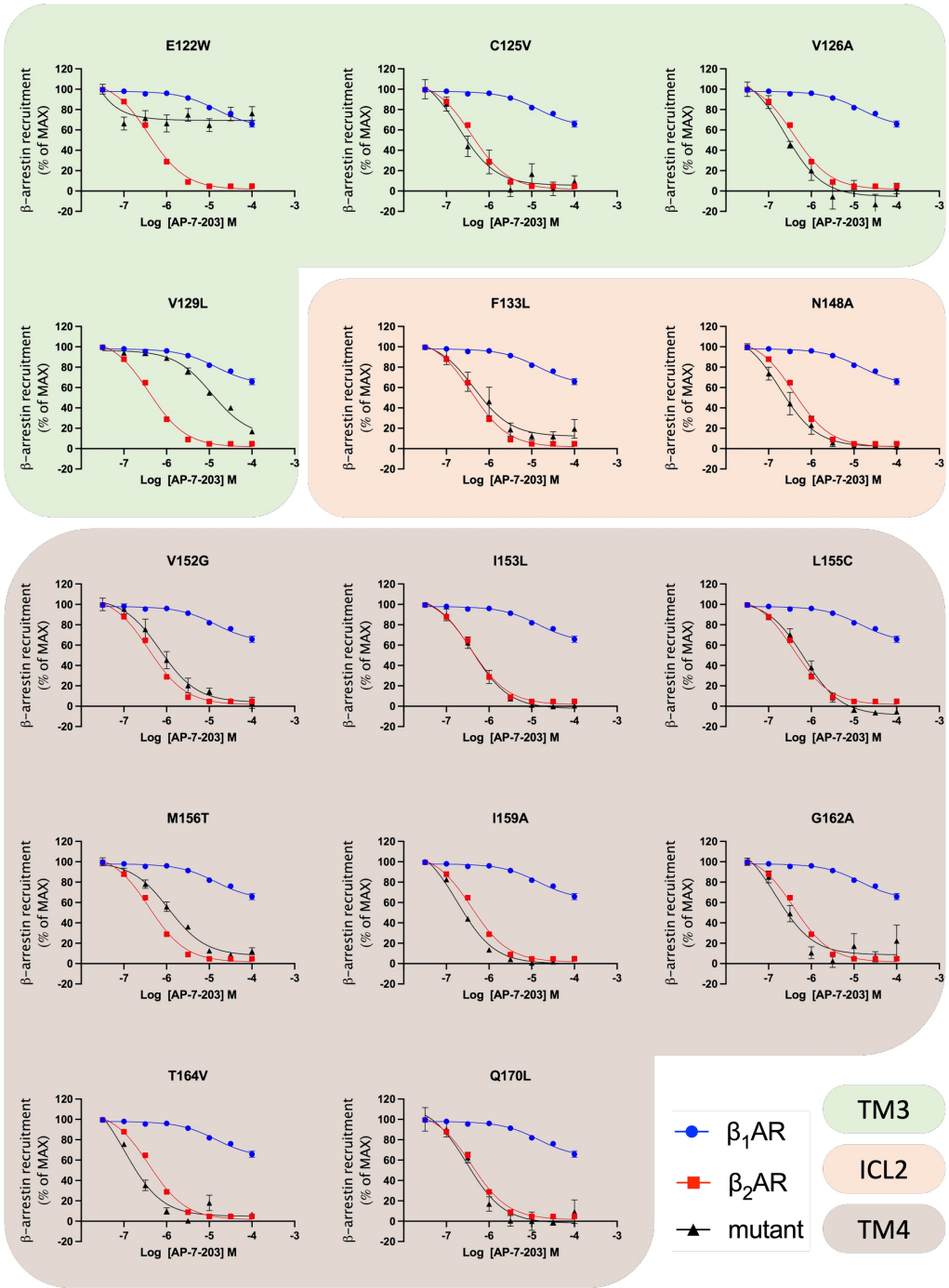


Figure S9. Mutagenesis studies using β_2AR point mutants with AP-7-203**.** HEK 293 cells were transfected with β_1AR-Rluc (blue), β_2AR-Rluc (red) or β_2AR-point mutant-Rluc (black) and β-arrestin2-GFP. Cells were incubated with increasing concentration ($10^{-7.5}$ to 10^{-4} M) of AP-7-203 for 30 min, followed by addition of 1 μM ISO. Concentration/activity curves were generated and plotted as mean ± SEM, n=6. Mutations in TM3 are in green, mutations in ICL2 are in orange and mutations in TM4 are in brown.

Supplemental Figure 10: Effect of selected β_2AR point mutations on ISO-promoted β-arrestin recruitment

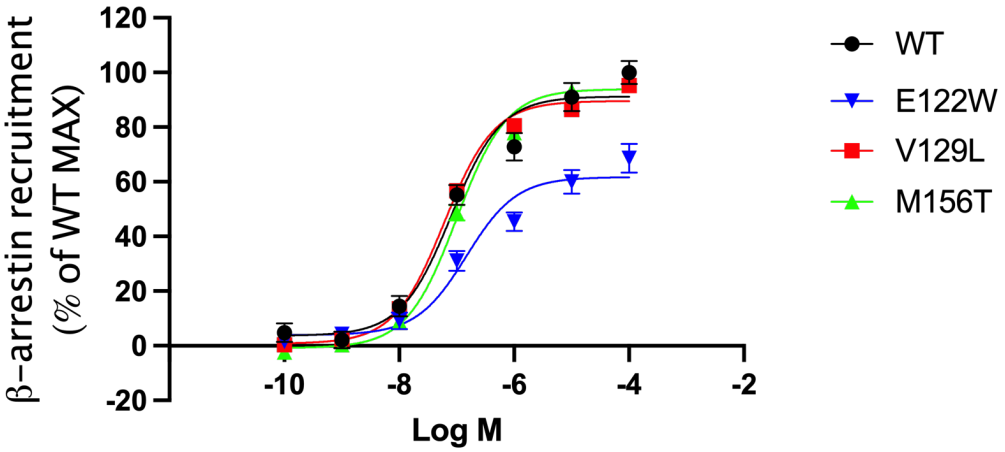


Figure S10. Effect of selected β_2AR point mutations on ISO-promoted β-arrestin recruitment**.** HEK 293 cells were transfected with WT or mutant (E122W, V129L or M156T) β_2AR-Rluc and β-arrestin2-GFP and stimulated with increasing concentrations ($10^{-7.5}$ to 10^{-4} M) of ISO. Concentration/activity curves were generated and plotted as mean ± SEM, n=6.

Table S1. Quinazoline structure activity relationship, related to Figure 5

Structure activity data for quinazoline derivatives from library screening and chemical synthesis.

Functional Group Substitution					
R1 Substituent	**R2 Substituent**	**R3 Substituent**	***IC_{50} Arrestin (µM)**	***IC_{50} cAMP (µM)**	****Arrestin fold bias**
		R1 Set			
ethylpropylether	2,4-diaminoquinazoline	cyclohexyl	12 ± 1	34 ± 18	-
cyclohexyl	2,4-diaminoquinazoline	cyclohexyl	4 ± 1	> 50	>12
m-trifluoromethylphenyl	2,4-diaminoquinazoline	cyclohexyl	3 ± 1	9 ± 3	3
m-chlorobenzyl	2,4-diaminoquinazoline	cyclohexyl	21 ± 2	25 ± 4	-
m,p-dimethoxyphenyl	2,4-diaminoquinazoline	cyclohexyl	14 ± 3	> 50	> 4
m-bromophenyl	2,4-diaminoquinazoline	cyclohexyl	2.5 ± 0.5	> 50	> 20
p-chlorophenyl	2,4-diaminoquinazoline	cyclohexyl	0.8 ± 0.2	> 50	> 50
m,p-dichlorophenyl	2,4-diaminoquinazoline	cyclohexyl	3.0 ± 0.7	> 50	> 15
m-chloro,p-fluorophenyl	2,4-diaminoquinazoline	cyclohexyl	3 ± 1	26 ± 11	8
p-fluorophenyl	2,4-diaminoquinazoline	cyclohexyl	0.23 ± 0.07	> 50	> 200
m-fluorophenyl	2,4-diaminoquinazoline	cyclohexyl	0.4 ± 0.1	> 50	> 100
m-difluorophenyl	2,4-diaminoquinazoline	cyclohexyl	0.20 ± 0.05	> 50	> 200
m,p-difluorophenyl	2,4-diaminoquinazoline	cyclohexyl	0.5 ± 0.1	> 50	> 100
m-chlorophenyl	2,4-diaminoquinazoline	cyclohexyl	6.0 ± 0.8	> 50	> 8
phenyl	2,4-diaminoquinazoline	cyclohexyl	3.0 ± 0.7	> 50	> 16
		R2 Set			
phenyl	2N-methyl,4-diaminoquinazoline	cyclohexyl	> 50	> 50	-
m-dichlorophenyl	2,4-diamino-6-chloroquinazoline	cyclohexyl	8 ± 2	> 50	> 6

m,p-difluorophenyl	2,4,7-triaminoquinazoline	cyclohexyl	4.4 ± 1.0	7.6 ± 2.5	-
m,p-difluorophenyl	2,4-diamino-6-fluoroquinazoline	cyclohexyl	0.5 ± 0.15	16 ± 2	32
m,p-difluorophenyl	2,4-diamino-6-chloroquinazoline	cyclohexyl	0.18 ± 0.07	> 50	> 250
m,p-difluorophenyl	2,4-diamino-6-methylquinazoline	cyclohexyl	0.36 ± 0.18	17 ± 7	47
m,p-difluorophenyl	2,4-diamino-6-bromoquinazoline	cyclohexyl	0.031 ± 0.015	28 ± 10	903
m,p-difluorophenyl	2,4-diamino-6-trifluoromethylquinazoline	cyclohexyl	0.07 ± 0.04	> 50	> 500
m,p-difluorophenyl	2,4-diamino-6-chloroquinazoline	piperidine	12 ± 5	50 ± 20	4
		R3 Set			
m-dichlorophenyl	2,4-diaminoquinazoline	H	10 ± 2	4 ± 2	-
m-dichlorophenyl	2,4-diaminoquinazoline	ethyl	13 ± 2	7 ± 2	-
m-dichlorophenyl	2,4-diaminoquinazoline	dimethylpropylamine	26 ± 3	> 50	> 2
m-dichlorophenyl	2,4-diaminoquinazoline	piperidine	22 ± 3	> 50	> 2
m-chloro-p-fluorophenyl	2,4-diaminoquinazoline	piperidine	7 ± 2	> 50	> 7
m-dichlorophenyl	2,4-diaminoquinazoline	p-2-phenylethanol	18 ± 2	15 ± 1	-
m-dichlorophenyl	2,4-diaminoquinazoline	benzyl	30 ± 4	> 50	-
m-dichlorophenyl	2,4-diaminoquinazoline	ethylphenyl	7 ± 2	24 ± 3	3

*IC_{50}s are generated by fitting background subtracted and normalized data from seven triplicate measurements taken over a 1000-fold concentration range (10 nM to 10 μM) to the logarithmic form of the Langmuir binding isotherm with the Hill coefficient fixed at one. The reported uncertainty reflects the 95% confidence interval for the IC_{50} value.

**GloSensor™ IC_{50}/PathHunter™ IC_{50}

Table S1. Quinazoline structure activity relationship table. The structure activity relationship of quinazoline derivatives on β_2AR-promoted activation of cAMP production and β-arrestin2 binding is shown as functional group substitutions effects on PathHunter™ IC_{50} values. The fold bias is denoted as the GloSensor™ IC_{50}:PathHunter™ IC_{50} ratio.

Table S2. Comparison of NAM efficacies and potencies between WT β_2AR and chimeras

A	I_{max} (% of max average) ± SEM (n=9)															
NAM	WT	ECL1	ECL2	ECL3	ECL1/2	ECL1/3	ECL2/3	TM2	TM3	TM4	TM5	TM6	TM7	ICL1	ICL2	ICL3
DFPQ	3.4 ± 1.2	4.5 ± 1.4	**-8.1 ± 2.7****	3.6 ± 2.7	**-13.0 ± 3.1*****	-1.4 ± 3.5	**-8.8 ± 2.1*****	0.8 ± 3.6	**16.9 ± 4.1***	-4.1 ± 27.9	5.7 ± 1.8	1.5 ± 2.4	1.8 ± 2.8	3.7 ± 4.2	8.7 ± 4.0	**-4.2 ± 3.2***
AP-7-168	1.4 ± 0.9	1.0 ± 0.9	1.2 ± 1.8	-0.8 ± 1.6	-0.1 ± 1.8	**-7.8 ± 2.4****	**-2.8 ± 0.9****	**-10.0 ± 4.1***	**16.0 ± 2.9****	**28.0 ± 6.8*****	4.2 ± 1.3	0.7 ± 1.6	-4.4 ± 2.9	0.8 ± 2.0	**5.4 ± 1.6***	0.1 ± 2.2
AP-7-203	0.6 ± 1.0	0.9 ± 1.2	-2.0 ± 2.1	-3.1 ± 1.9	-0.4 ± 2.3	**-5.0 ± 1.9***	-2.9 ± 1.4	1.0 ± 1.8	**23.8 ± 3.8*****	**34.7 ± 5.7*****	4.0 ± 2.0	-1.9 ± 1.9	-1.2 ± 2.5	-1.0 ± 2.8	2.2 ± 2.3	**-13.5 ± 4.5****
B	pIC_{50} (% of max average) ± SEM (n=9)															
NAM	WT	ECL1	ECL2	ECL3	ECL1/2	ECL1/3	ECL2/3	TM2	TM3	TM4	TM5	TM6	TM7	ICL1	ICL2	ICL3
DFPQ	5.9 ± 0.0	**6.3 ± 0.0*****	5.8 ± 0.1	5.8 ± 0.1	**5.7 ± 0.1****	6.0 ± 0.1	5.8 ± 0.0	5.8 ± 0.1	**5.1 ± 0.1*****	**4.6 ± 0.3*****	**6.7 ± 0.1*****	5.9 ± 0.1	5.9 ± 0.1	**5.7 ± 0.1***	**5.4 ± 0.1*****	**5.7 ± 0.1***
AP-7-168	6.4 ± 0.0	**6.5 ± 0.0****	6.4 ± 0.1	6.2 ± 2.6	6.5 ± 0.1	**6.6 ± 0.1***	**7.0 ± 0.0*****	6.2 ± 0.1	**5.3 ± 0.1*****	**5.4 ± 0.2*****	**6.9 ± 0.1*****	6.4 ± 0.0	6.4 ± 0.1	**6.2 ± 0.1****	**5.9 ± 0.0*****	6.3 ± 0.1
AP-7-203	6.5 ± 0.0	**6.8 ± 0.0****	6.4 ± 0.1	6.2 ± 3.3	6.4 ± 5.0	**6.7 ± 0.1***	**7.0 ± 0.1*****	6.5 ± 0.1	**5.4 ± 0.1*****	**5.6 ± 0.2*****	**6.8 ± 0.1***	6.7 ± 0.1	6.7 ± 0.1	**6.2 ± 0.1*****	**6.0 ± 0.0*****	6.2 ± 0.1

Table S3. Comparison of NAM efficacies and potencies between WT β_2AR and point mutants

A	I_{max} (% of max average) ± SEM (n=9)														
NAM	WT	E122W	C125V	V126A	V129L	F133L	N148A	V152G	I153L	L155C	M156T	I159A	G162A	T164V	Q170L
DFPQ	3.4 ± 1.2	nd	10.6 ± 5.1	6.9 ± 6.2	**-26.3 ± 6.9*****	**17.0 ± 2.9****	-0.3 ± 3.1	6.6 ± 2.6	6.8 ± 2.2	-1.3 ± 2.5	**11.3 ± 2.6****	1.6 ± 2.0	4.9 ± 3.9	**-12.2 ± 3.7****	**-5.4 ± 3.6***
AP-7-168	1.4 ± 1.8	nd	**23.1 ± 4.1*****	11.6 ± 6.4	**7.8 ± 2.4***	**8.8 ± 2.7***	2.0 ± 2.1	**8.3 ± 1.7****	1.5 ± 2.1	-0.8 ± 2.6	**11.7 ± 1.6*****	0.8 ± 1.3	6.6 ± 2.9	-0.7 ± 1.6	-2.1 ± 1.6
AP-7-203	1.4 ± 0.9	nd	5.6 ± 4.6	-5.4 ± 4.3	**11.6 ± 3.5***	12.0 ± 4.6	1.9 ± 3.0	3.8 ± 3.9	-2.4 ± 2.2	**-8.8 ± 2.3****	**7.7 ± 2.3***	-0.1 ± 1.1	8.6 ± 4.6	4.9 ± 2.2	-2.0 ± 4.5
B	pIC_{50} (% of max average) ± SEM (n=9)														
NAM	WT	E122W	C125V	V126A	V129L	F133L	N148A	V152G	I153L	L155C	M156T	I159A	G162A	T164V	Q170L
DFPQ	5.9 ± 0.0	nd	5.8 ± 0.2	5.8 ± 0.2	**4.5 ± 0.1*****	5.9 ± 0.1	5.8 ± 0.1	6.0 ± 0.1	5.9 ± 0.1	**5.7 ± 0.0****	**5.5 ± 0.1*****	6.0 ± 0.1	6.2 ± 0.1	**5.6 ± 0.1****	5.9 ± 0.1
AP-7-168	6.4 ± 0.0	nd	6.7 ± 0.2	**6.1 ± 0.2**	**5.0 ± 0.0*****	6.3 ± 0.1	**6.8 ± 0.1****	6.3 ± 0.0	**6.6 ± 0.1***	**6.7 ± 0.1***	**6.1 ± 0.0*****	**6.8 ± 0.0*****	**7.0 ± 0.1****	**6.7 ± 0.0****	**6.7 ± 0.1*****
AP-7-203	6.4 ± 0.0	nd	6.7 ± 0.2	6.6 ± 0.1	**4.9 ± 0.1*****	6.3 ± 0.2	**6.7 ± 0.1***	6.1 ± 0.1	6.4 ± 0.1	**6.2 ± 0.1***	**5.9 ± 0.1*****	**6.7 ± 0.0*****	6.8 ± 0.2	**7.0 ± 0.1*****	6.5 ± 0.1

Table S4. Comparison of ISO efficacies and potencies between WT β_2AR and selected mutants

A	E_{max} (% of max average) ± SEM (n=6)						
Agonist	WT	TM3	ICL2	TM4	E122W	V129L	M156T
ISO	93.6 ± 2.3	**85.4 ± 2.4***	91.6 ± 3.2	**28.8 ± 2.2*****	**61.0 ± 1.9*****	89.6 ± 1.0	93.9 ± 1.5
B	pEC_{50} (% of max average) ± SEM (n=6)						
Agonist	WT	TMD3	ICL2	TMD4	E122W	V129L	M156T
ISO	7.0 ± 0.1	6.9 ± 0.1	7.0 ± 0.3	7.2 ± 0.3	6.9 ± 0.1	**7.2 ± 0.0***	7.0 ± 0.0

Table S2. Comparison of NAM efficacies and potencies between WT β_2AR and chimeras. To obtain values for I_{max} and IC_{50}, data from Figs. S3, 4 and 5 were fitted and plotted by using the function log(inhibitor) vs response (three parameters) of the non-linear curve fitting in GraphPad Prism. **(A)** Values of I_{max} are represented as % of ISO-induced maximal response ± SEM (n=9). **(B)** Potency values are represented as the positive logarithm of the ligand IC_{50} concentration ± SEM (n=9). Statistical significance between WT-induced values for I_{max} and IC_{50} and chimeras-induced values for I_{max} and IC_{50} was assessed by t-test with Welch's correction, *p < 0.05; **p < 0.01; ***p < 0.001; nd = not determined.

Table S3. Comparison of NAM efficacies and potencies between WT β_2AR and point mutants. To obtain values for I_{max} and IC_{50}, data from Figs. S7, 8 and 9 were fitted and plotted by using the function log(inhibitor) vs response (three parameters) of the non-linear curve fitting in GraphPad Prism. **(A)** Values of I_{max} are represented as % of ISO-induced maximal response ± SEM (n=9). **(B)** Potency values are represented as the positive logarithm of the ligand IC_{50} concentration ± SEM (n=9). Statistical significance between WT-induced values for I_{max} and IC_{50} and point mutant-induced values for I_{max} and IC_{50} was assessed by t-test with Welch's correction, *p < 0.05; **p < 0.01; ***p < 0.001; nd = not determined.

Table S4. Comparison of ISO efficacies and potencies between WT β_2AR and selected mutants. To obtain values for E_{max} and EC_{50}, data from Figs. S6 and 10 were fitted and plotted by using the function log(agonist) vs response (three parameters) of the non-linear curve fitting in GraphPad Prism. **(A)** Values of E_{max} are represented as % of ISO-induced WT-mediated maximal response ± SEM (n=6). **(B)** Potency values are represented as the positive logarithm of the ligand EC_{50} concentration ± SEM (n=6). Statistical significance between WT-induced values for E_{max} and EC_{50} and mutant-induced values for E_{max} and EC_{50} was assessed by t-test with Welch's correction, *p < 0.05; **p < 0.01; ***p < 0.001; nd = not determined.