SUPPORTING INFORMATION

Discovery of Diphenylacetamides as CXCR7 Inhibitors with Novel β-arrestin Antagonist Activity

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In Vitro Biology

CXCR7 radiolabeled ligand binding assay

The binding affinity of test compounds for the human CXCR7 chemokine receptor was determined by their ability to displace ^{125}I-CXCL12 (Perkin Elmer) from membranes obtained from CHO-K1 cells overexpressing the human CXCR7 receptor (DiscoveRx). K_i values are reported as the geometric mean of at least three independent experiments each of which was run in duplicate. The K_d for CXCL12 in our system was determined with saturation binding experiments to be 35 pM and used for calculating the K_i values. Our experimentally determined K_d value was lower than the value of 400 pM reported in the literature (Balbanian et al. *J Biol Chem* **2005**, 280, 35760-35766).

Test compounds were serialized in 100% DMSO and spotted into 96 well plates (NBS). Total binding wells were spotted with diluent. Non-specific wells were defined by the addition of potent CXCR7 agonist. 10 μL of ^{125}I-CXCL12 with a final concentration of 100 pM was added to each well of the plate, followed by the addition of 90 μL of membranes. Both the membranes and ^{125}I-CXCL12 were diluted to the appropriate concentration in assay buffer (HBSS containing 10 mM HEPES and 0.2% BSA). After the addition, membranes plates were incubated at room temperature for 2 h (shaking). Reactions were terminated by rapid filtration through poly(ethyleneimine) (0.3%) treated 96 well GF/C Unifilter plates (Perkin Elmer). Unbound ligand was removed by washing the filters with ice cold wash buffer. Filters were allowed to dry thoroughly prior to the addition of Ready Safe scintillation fluid (Perkin Elmer). The amount of bound ^{125}I-CXCL12 was quantitated by reading plates on the Trilux (Perkin Elmer).

CXCR7 β-arrestin functional assay

The agonist activity of test compounds was determined by their ability to induce β-arrestin recruitment in CHO-K1 cells overexpressing the human CXCR7 receptor (DiscoveRx). EC_{50} values are reported as the geometric mean of at least three independent experiments each of which was run in duplicate. The antagonist activity of test compounds was determined by their ability to inhibit β-arrestin recruitment in CHO-K1 cells overexpressing the human CXCR7 receptor (DiscoveRx). In order to run the assay in antagonist mode, CXCL12 at a concentration of 28 nM (EC_{80}) was added as challenge to compete with the test compounds. IC_{50} values are reported as the geometric mean of at least three independent experiments each of which was run in duplicate.

The receptor is fused with the small enzyme fragment ProLink™ and co-expressed in the cells stably expressing a fusion protein of β-arrestin and the larger, N-terminal deletion mutant of β-gal (called enzyme acceptor or EA). Agonist activation of the receptor by test compounds causes binding of β-arrestin (EA) to the ProLink-tagged CXCR7

resulting in the formation of active β-gal enzyme. Enzyme activity is measured using chemiluminescent PathHunter® detection reagents (DiscoveRx).

Prior to the assay, cells are removed from culture flasks and plated at a density of 6K viable cells per well in white solid bottom 384 well plates. After the addition of cells, plates were placed in humidified 37 ℃ 5% CO_2 incubator for 18-24 h. Test compounds were serialized in 100% DMSO and diluted in assay buffer (HBSS containing 10 mM HEPES and 0.1% BSA) prior to their addition to the cell plates. Media was removed from the cell plates and 20 µL of compound or diluent was added to the appropriate wells of the plate. Following compound addition, cell plates were incubated at 37 ℃ in 5% CO_2 for 30 min. After incubation 20 µL of PathHunter® detection reagents (DiscoveRx) were added to each well of the plate. Plates were incubated at 23 ℃ in the dark for 1 h prior to reading on the Envision (Perkin Elmer).

CXCR4 binding assay

The binding affinities of the test compounds for the CXCR4 receptor were determined by their ability to displace ^{125}I-CXCL12 (PerkinElmer) from Jurkat cells, an immortalized line of human T-lymphocyte cells that endogenously express the CXCR4 receptor.

The test compounds were serialized in 100% DMSO and spotted into 96-well plates (NBS). Total-binding wells were spotted with diluent. Nonspecific wells were defined by the addition of cold CXCL12. To each well of the plate, 10 µL of ^{125}I-CXCL12 at a final concentration of 100 pM was added, followed by 90 µL of the Jurkat cell suspension. The Jurkat cells and ^{125}I-CXCL12 were diluted as needed in assay buffer (HBSS containing 10 mM HEPES and 0.2% BSA). After the addition of the Jurkat cells, the plates were incubated at rt for 2 h with shaking. The reactions were terminated by rapid filtration through poly(ethylenimine) (0.3%)-treated 96-well GF/C Unifilter plates (PerkinElmer). Unbound ligand was removed when the filters were washed with ice-cold wash buffer. The filters were allowed to dry thoroughly prior to the addition of the Ready Safe scintillation fluid (PerkinElmer). The amount of bound ^{125}I-CXCL12 was quantitated by reading the plates on a Trilux luminometer (PerkinElmer).

Animal Studies

Animal Care

All the procedures performed on animals in this study were in accordance with established guidelines and regulations and were reviewed and approved by the Pfizer Institutional Animal Care and Use Committee. The Pfizer animal care facilities that supported this work are fully accredited by AAALAC International.

Pharmacokinetics

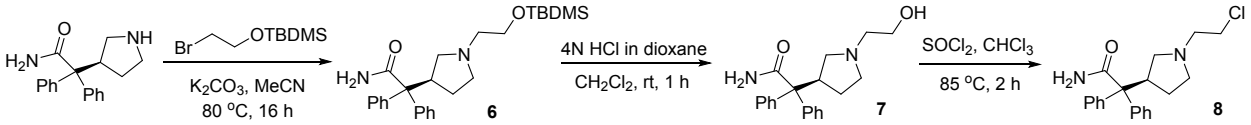
Compound **10** was administered to fasted (food was returned 4 h post dose) male CD-1 mice (n=3) by subcutaneous route. The 30 mg/kg bolus was formulated as solution in a vehicle of DMSO/PEG-400/water (10/50/40, v/v) and administered in a dose volume of 5 mL/kg. Serial blood samples were taken at 0.083, 0.25, 0.5, 2, 5, and 24 h post dose. Blood was centrifuged to produce plasma which was stored frozen until bioanalysis. Bioanalysis of plasma samples was completed using specific HPLC-MS/MS methods with lower limits of detection for 25 equal to or below 1 ng/mL. Pharmacokinetic parameters were calculated using standard non-compartmental analysis.

Experimental Part Chemistry

General Methods:

All chemicals, reagents and solvents were purchased from commercial sources and used without further purification. Advanced starting materials (S)-2,2-diphenyl-2-(pyrrolidin-3-yl)acetamide, 4-(thiazol-2-yl)phenol (**9a**), 4-(1,3,4-oxadiazol-2-yl)phenol (**9b**), 4-(4,5-dihydrooxazol-2-yl)phenol (**9c**), and 4-(3-methyl-1,2,4-oxadiazol-5-yl)phenol (**9d**) were purchased from commercial sources and were used without further purification. All reactions were performed under an atmosphere of nitrogen unless otherwise noted. Nuclear magnetic resonance spectra (^{1}HNMR) were recorded with a 500 MHz Bruker spectrometer. Chemical shifts are expressed in parts per million downfield from tetramethylsilane. Silica gel chromatography was performed using a medium pressure Biotage or ISCO system using columns pre-packaged by various commercial vendors including Biotage and ISCO. Whatman pre-coated silica gel plates (250 μm) were used for analytical TLC. Library compounds **10** – **13** were > 95% purity as judged by LCMS (UV 254 nm or ELSD detection).

Synthesis of library template **8**



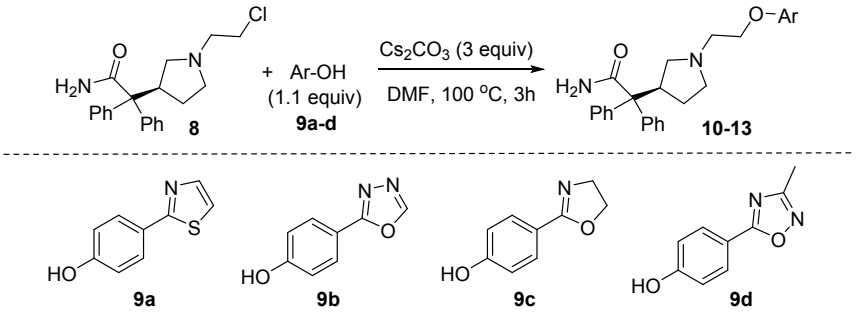
*(S)-2-(1-(2-((tert-butyldimethylsilyl)oxy)ethyl)pyrrolidin-3-yl)-2,2-diphenylacetamide (**6**).* A mixture of (S)-2,2-diphenyl-2-(pyrrolidin-3-yl)acetamide (924 mg, 3.30 mmol) and potassium carbonate (592 mg, 4.28 mmol) in acetonitrile (11.0 mL) was treated with (2-bromoethoxy)(tert-butyl)dimethylsilane (0.780 mL, 3.63 mmol). The heterogeneous mixture was heated to 80 °C for 16 h. The reaction was cooled to room temperature, diluted with ethyl acetate, and was washed with 50% brine solution. The aqueous layer was extracted with additional ethyl acetate. The combined organic extracts were dried over $MgSO_4$, filtered, and evaporated. The crude material was purified by silica gel column chromatography to give **6** as a white solid (1.01 g, 2.30 mmol, 70%). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.36 – 7.19 (m, 10H), .13 (br s, 1H), 7.07 (br s, 1H), 3.67 – 3.56 (m, 1H), 3.53 (t, J = 5.9 Hz, 2H), 2.92 – 2.81 (m, 1H), 2.48 – 2.22 (m, 4H), 1.98 – 1.79 (m, 2H), 159 – 1.47 (m, 1H), 0.81 (s, 9H), -0.02 (s, 3H), -0.03 (s, 3H). LCMS (Waters Acquity HSS T3 C18, 2.1 × 50 mm × 1.7 μM; 5 – 95% acetonitrile (0.1% formic acid)/water (0.1% formic acid)): t_R = 0.74 min. LCMS for $C_{26}H_{38}N_2O_2Si$: 438.3 (calcd), 439.4 $[M + H]^+$ (obsd).

*(S)-2-(1-(2-hydroxyethyl)pyrrolidin-3-yl)-2,2-diphenylacetamide (**7**).* A dichloromethane (3.0 mL) solution of **6** (200 mg, 0.456 mmol) was treated with 4N HCl in dioxane (1.15 mL, 4.56 mmol). The mixture was stirred for 1 h at room temperature at which time consumption of the starting material was observed. The reaction was diluted with additional dichloromethane and was washed with 50% saturated aqueous sodium bicarbonate. The combined organic extracts were dried over $MgSO_4$, filtered, and evaporated. The crude material was purified by silica gel column chromatography to give **7** as a viscous colorless oil (60.0 mg, 0.185 mmol, 41%). ^{1}H NMR (MeOH-d_4, 500 MHz): δ 7.45 – 7.30 (m, 10H), 3.97 (pent, J = 8.2 Hz, 1H), 3.73 (t, J = 5.9 Hz, 2H), 3.70 – 3.63 (m, 1H), 3.52 – 3.39 (m, 1H), 3.29 – 3.05 (m, 4H), 2.52 – 2.40 (m, 1H), 2.09 – 1.97 (m, 1H). LCMS (Waters Acquity HSS T3 C18, 2.1 × 50 mm × 1.7 μM; 5 – 95% acetonitrile (0.1% formic acid)/water (0.1% formic acid)): t_R = 0.54 min. LCMS for $C_{20}H_{24}N_2O_2$: 324.2 (calcd), 325.2 $[M + H]^+$ (obsd).

*(S)-2-(1-(2-chloroethyl)pyrrolidin-3-yl)-2,2-diphenylacetamide hydrochloride (**8**).* A stirred solution of **7** (260 mg, 0.800 mmol) in chloroform (8.0 mL) at 0 °C was treated with thionyl chloride (0.60 mL, 8.00 mmol). The mixture was heated to 85 °C for 2 h at which time consumption of the starting material was observed. All solvent and volatiles were removed *in vacuo* and the residual solid was triturated with diethyl ether. The remaining solid was dried *in vacuo* to give the hydrochloride salt **8** as a tan solid that was used without further purification (280 mg, 0.738 mmol, 92%). ^{1}H NMR (MeOH-d_4, 500 MHz): δ 7.49 – 7.28 (m, 10H), 4.06 – 3.78 (m, 4H), 3.71 – 3.57 (m, 2H), 3.55 – 3.48 (m, 1H), 3.37 – 3.24 (m, 2H), 2.55 – 2.41 (m, 1H), 2.12 – 1.99 (m, 1H). LCMS (Waters Acquity HSS T3 C18, 2.1 × 50 mm × 1.7 μM; 5 – 95% acetonitrile (0.1% formic acid)/water (0.1% formic acid)): t_R = 0.60 min. LCMS for $C_{20}H_{23}ClN_2O$: 342.1 (calcd), 343.2 $[M + H]^+$ (obsd).

General procedure (Parallel Format)

A suspension of alkyl chloride **8** (30 mg, 0.080 mmol), the corresponding phenol (**9**; 0.087 mmol), and Cs_2CO_3 (77 mg, 0.24 mmol) in DMF (0.8 mL) was heated to 100 °C for 3h. The reaction was cooled to room temperature, and evaporated. The crude material was purified by reverse-phase HPLC to give the desired products (**10**-**13**).



*(S)-2,2-diphenyl-2-(1-(2-(4-(thiazol-2-yl)phenoxy)ethyl)pyrrolidin-3-yl)acetamide **(10)***. Prepared according to the general procedure with 4-(thiazol-2-yl)phenol (**9a**; 15 mg, 0.087 mmol). Purified by preparative HPLC (Sunfire C18 19 × 100 mm × 5 μM; 40–80% acetonitrile (0.05% TFA)/water (0.05% TFA)) to afford **10** (18.4 mg, 40%). t_R = 1.74 min; m/z = 484.3 $[M + H]^+$. ^1H NMR (DMSO-d_6, 500 MHz): δ 7.89 – 7.82 (m, 3H), 7.68 (d, J = 3.5 Hz, 1H), 7.35 – 7.19 (m, 10H), 7.15 (br s, 1H), 7.08 (br s, 1H), 7.02 – 6.97 (m, 2H), 3.99 (t, J = 5.5 Hz, 2H), 3.71 – 3.58 (m, 1H), 2.98 – 2.86 (m, 1H), 2.74 – 2.62 (m, 1H), 2.61 – 2.51 (m, 2H), 2.45 – 2.37 (m, 1H), 1.99 – 1.84 (m, 2H), 1.64 – 1.50 (m, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 175.3, 167.5, 160.4, 144.0, 143.4, 130.5, 130.3, 128.2, 128.0, 127.8, 126.9, 126.8, 126.4, 119.8, 115.6, 67.1, 64.0, 57.2, 54.4, 54.2, 42.1, 28.3. LCMS (Waters Acquity HSS T3 C18, 2.1 × 50 mm × 1.7 μM; 5 – 95% acetonitrile (0.1% formic acid)/water (0.1% formic acid)): t_R = 0.67 min; *m/z* 484.2 $(M + H)^+$. HRMS (*m/z*) $[M + H]^+$ calcd for $C_{29}H_{29}N_3O_2S$, 484.2053; found, 484.2047.

*(S)-2-(1-(2-(4-(1,3,4-oxadiazol-2-yl)phenoxy)ethyl)pyrrolidin-3-yl)-2,2-diphenylacetamide **(11)***. Prepared according to the general procedure with 4-(1,3,4-oxadiazol-2-yl)phenol (**9b**; 14 mg, 0.087 mmol). Purified by preparative HPLC (Sunfire C18 19 × 100 mm × 5 μM; 50–85% acetonitrile (0.05% TFA)/water (0.05% TFA)) to afford **11** (2.6 mg, 7%). t_R = 1.76 min. LCMS for $C_{28}H_{28}N_4O_3$: 468.2 (calcd), 469. 3 $[M + H]^+$ (obsd).

*(S)-2-(1-(2-(4-(4,5-dihydrooxazol-2-yl)phenoxy)ethyl)pyrrolidin-3-yl)-2,2-diphenylacetamide **(12)***. Prepared according to the general procedure with 4-(4,5-dihydrooxazol-2-yl)phenol (**9c**; 14 mg, 0.087 mmol). Purified by preparative HPLC (Sunfire C18 19 × 100 mm × 5 μM; 60–90% acetonitrile (0.05% TFA)/water (0.05% TFA)) to afford **12** (14.5 mg, 39%). t_R = 1.73 min. LCMS for $C_{29}H_{31}N_3O_3$: 469.2 (calcd), 470.3 $[M + H]^+$ (obsd).

*(S)-2-(1-(2-(4-(3-methyl-1,2,4-oxadiazol-5-yl)phenoxy)ethyl)pyrrolidin-3-yl)-2,2-diphenylacetamide **(13)***. Prepared according to the general procedure with 4-(3-methyl-1,2,4-oxadiazol-5-yl)phenol (**9d**; 15 mg, 0.087 mmol). Purified by preparative HPLC (Sunfire C18 19 × 100 mm × 5 μM; 50–80% acetonitrile (0.05% TFA)/water (0.05% TFA)) to afford **13** (7.5 mg, 19%). t_R = 1.94 min. LCMS for $C_{29}H_{30}N_4O_3$: 482.2 (calcd), 483.3 $[M + H]^+$ (obsd).