Supporting Information

A Bivalent Ligand Targeting on the Putative Mu Opioid Receptor–Chemokine Receptor CCR5 Heterodimers: Binding Affinity versus Functional Activities

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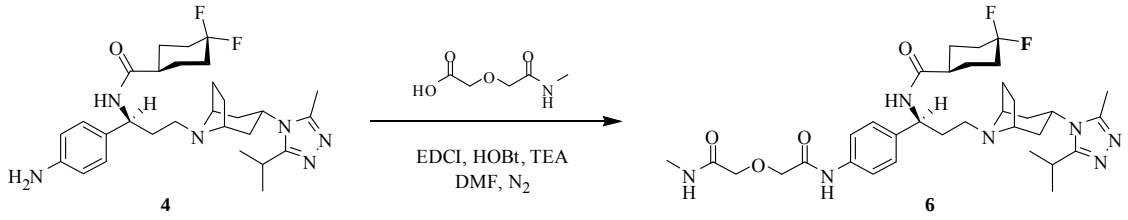
Chemical synthesis and characterization of compound 6

General methods. Chemical reagents were purchased from Sigma-Aldrich. TLC analyses were carried out on Analtech Uniplate F254 plates. Chromatographic purification was accomplished on silica gel columns (230–400 mesh, Merck). Melting points were obtained with a Fisher scientific micro melting point apparatus without correction. IR spectra were recorded on either a Nicolet iS10 IR Instrument. Proton (400 MHz) and Carbon-13 (100 MHz) nuclear magnetic resonance (NMR) spectra were acquired at ambient temperature with tetramethylsilane as the internal standard on a Bruker Ultrashield 400 Plus spectrometer. MS analysis was performed on an Applied Bio Systems 3200 Q trap with a turbo V source for TurbolonSpray. HPLC analysis of the final compounds was done with a Varian ProStar 210 system on Microsorb-MV 100–5 C18 column (250 mm × 4.6 mm) at 254 nm eluting with acetonitrile (0.1% TFA)/water (50/50) at 1 mL/min over 30 min. A satisfying purity of compound **6** (> 95%) was achieved as assessed by HPLC.

Synthetic scheme of 6.



4 6

Synthesis and characterization of 6. On an ice-water bath, a solution of methylcarbamoylmethoxy-acetic acid[1] (22 mg, 0.15 mmol) in DMF (2 mL), was added EDCI (29 mg, 0.15 mmol), HOBt (20 mg, 0.15 mmol), molecular sieves, and TEA (28 μL, 0.2 mmol) with N_2 protection. After 15 min, a solution of **4**[2] (53 mg, 0.1 mmol) in DMF (1 mL) was added dropwise. The resulted mixture was allowed to warm up to

ambient temperature gradually. Upon completion of the reaction as monitored by TLC, the reaction mixture was filtered through celite. The filtrate was concentrated in vacuum to remove DMF and the residue was then purified by column chromatography (DCM/MeOH = 8/1) to afford 41 mg light yellow solid, in 62% yield. The compound was transferred into hydrochloride salt with 1.25 M HCl/MeOH (200 μL) and fully characterized before subjected to biological screening. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.24 (brs, 1H, exchangeable), 10.03 (s, 1H, exchangeable), 8.49 (d, J = 8.36 Hz, 1H, exchangeable), 8.09 (q, J = 4.48 Hz, 1H, exchangeable), 7.61 (d, J = 8.60 Hz, 2H), 7.31 (d, J = 8.56 Hz, 2H), 4.84 (q, J = 9.16 Hz, 1H), 4.47 (m, 1H), 4.23 (m, 1H), 4.16 (m, 1H), 4.14 (s, 2H), 4.03 (s, 2H), 3.82 (m, 1H), 2.93 (m, 2H), 2.75 (s, 3H), 2.72–2.66 (m, 2H), 2.67 (d, J = 4.68 Hz, 3H), 2.42–1.95 (m, 1H), 1.95–1.70 (m, 4H), 1.70–1.50 (m, 2H), 1.29 (d, J = 6.68 Hz, 6H); ^{13}C NMR (100 MHz, CD_3OD): δ 177.88, 172.32, 170.18, 162.35, 154.18, 138.63, 138.36, 128.40 (× 2), 122.12 (× 2), 71.83, 71.56, 63.43, 62.68, 51.67, 50.38, 43.49, 34.81, 34.75, 34.07, 33.82, 33.58, 31.94, 27.19, 27.10, 26.71, 25.94, 25.83, 21.62, 21.58, 15.44, 12.07 (× 2); mp 149–151 °C; IR v (Diamond, cm^{-1}): 3242, 2963, 1659, 1531, 1106, 961, 837; MS (ESI) m/z found 659 $(M + H)^+$; Anal. RP-HPLC (purity: 99.9%).

Radioligand binding assay

The hMOR-CHO competitive radioligand binding assay was performed as described before.[3]

The CCR5 competitive radioligand binding assay was conducted by EMD Millipore, St. Charles, MO. Ligands solutions were prepared in equilibrium binding buffer (water supplemented with 50 mM HEPES, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% BSA at pH 7.4)

and mixed 1:1 with [^{125}I]-MIP-1α (fixed concentration at 0.25 nM, K_d = 3.2 nM). The reaction was initiated by the addition of CCR5, rhesus macaque membranes (Chem-1 as the host cells) prepared in assay buffer at (2X) with a final 1 unit/well. After all sample additions, the assay plate was allowed to incubate at room temperature in a non-binding plate for 120 minutes. Prior to harvesting, the FC filter plate was pre-coated with 0.3% PEI for one hour. Samples were collected and the filter plate was washed three times in wash buffer (50 mM HEPES, pH 7.4, 500 mM NaCl, and 0.1% BSA). The filtration plate was allowed to dry, followed by the addition of scintillation fluid at 50 μL per well. Radiolabeled samples were measured on a Perkin Elmer (Wallac) 1450 Microbeta TriLux liquid scintillation counter to determine assay counts per minute. Binding K_i values were derived from IC_{50} values calculated with the Chang-Prusoff equation using GraphPad Prism 3.0 software (San Diego, CA).

Ca^{2+} flux inhibition assay

The hMOR-CHO, CCR5-MOLT-4 cell culture were conducted as described in the literature.[4, 5]

MOR calcium mobilization assay

hMOR-CHO cells[4] were first transfected with Gqi5 pcDNA1[6] using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommended procedure. Then cells were incubated for 6 hours at 37 °C and 5% CO_2 and then trypsinized and transferred to a clear bottom, black 96-well plate (Greiner Bio-one) at 20,000 cells per well in DMEM/F-12 supplemented with 5% fetal bovine serum, 100 u/mL penicillin, 100 µg/mL streptomycin, and 0.25 mg/mL hygromycin B. Forty eight hours after transfection the growth media was decanted and wells were washed with 100 µL of 50:1 HBSS:HEPES

assay buffer. Cells were then incubated with 55 μL of Fluo4 loading buffer [30 μL 2 μM Fluo4-AM (Invitrogen), 84 μL 2.5 mM probenacid, in 5.5 mL assay buffer] for 30 minutes. Varying concentrations of ligands and controls were added to the wells to bring the total volume up to 80 μL in each well and the plates were subsequently incubated for 15 minutes. Plates were then read on a FlexStation3 microplate reader (Molecular Devices) at 494/516 ex/em for a total of 90 seconds. After 15 seconds of reading, 20 μL of 1.25 μM DAMGO in assay buffer, or assay buffer alone, was added to the wells to bring the total volume up to 100 μL. The changes in Ca^{2+} mobilization were monitored and peak height values were obtained using SoftMaxPro software (Molecular Devices). Non-linear regression curves and IC_{50}s were generated using GraphPad Prism 3.0 (San Diego, CA). All experiments were repeated a total of three times.

CCR5 calcium mobilization assay

CCR5-MOLT-4 cells (Obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH, from Dr. Masanori Baba, Dr. Hiroshi Miyake, Dr. Yuji Iizawa[5]) were transfected with Gqi5 pcDNA1[6] using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommended procedure and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 u/mL penicillin, 100 μg/mL streptomycin, and 1 mg/mL G418 at 37 °C and 5% CO_2. Forty eight hours after transfection, a total of 2,500,000 cells were spun down and brought back up in 8 mL of 50:1 HBSS:HEPES assay buffer. Cells were then plated at 25,000 cells per well into a clear bottom, black 96-well plate (Greiner Bio-one) and 50 μL of Fluo4 loading buffer [40 μL 2 μM Fluo4-AM (Invitrogen), 100 μL 2.5 mM probenacid, in 5 mL assay buffer] was added to bring the volume up to 130 μL. After incubating for 45 minutes, 50 μL of

varying concentrations of ligands and controls were added and the plate was incubated for an additional 15 minutes. Plates were then read on a FlexStation3 microplate reader (Molecular Devices) at 494/516 ex/em for a total of 120 seconds. After 16 seconds of reading, 20 μL of 200 nM RANTES (Biosource) in assay buffer, or assay buffer alone, was added to the wells to bring the total volume up to 200 μL. The changes in Ca^{2+} mobilization were monitored and peak height values were obtained using SoftMaxPro software (Molecular Devices). Non-linear regression curves and IC_{50}s were generated using GraphPad Prism 3.0 (San Diego, CA). All experiments were repeated a total of three times.

HIV-1_{SF162} infection assay in human astrocytes

Primary human astrocytes (HA, ScienCell catalog # 1901) were cultured in 24-well plates and transfected with the plasmid pBlue3'LTR-luc (NIH AIDS Research and Reference Reagent Program) using Lipofectamine 2000 (Invitrogen) followed by treatment with maraviroc (MVC, 10, 50, 100, 500 nM, Sigma-Aldrich, St. Louis, MO), naltrexone (NTX, 1.5 μM, Sigma-Aldrich, St. Louis, MO), the mixture of maraviroc (100 nM) and naltrexone (1.5 μM), and bivalent ligand **1** (10, 50, 100, 500 nM) as indicated 30–60 min prior to R5 HIV-1_{SF162} (originally isolated by Dr. Jay Levy[7] and obtained through the NIH AIDS Research and Reference Reagent Program) infection at a concentration of p24 50 pg /10^6 cells[8]. Eighteen hours later, supernatant was removed. Cells were rinsed twice in 1× PBS then lysed and the relative Tat protein expression was determined by measuring luciferase using the Luciferase Assay System (Promega) according to manufacturer's protocol. Light units were measured using a PHERAstar FS plate reader (BMG Labtech).

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