Discovery of a potent and orally bioavailable dual antagonist of CC Chemokine Receptors 2 and 5.

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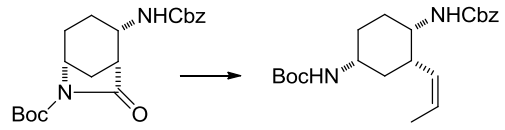
Supporting Information

GENERAL

The synthesis of the compounds described in the manuscript has been previously reported in patent applications, as cited in the manuscript. Given the central nature of compound **13d** to the conclusions of the manuscript, this supporting information file describes the synthesis and full characterization of key compound **13d** (see also Carter, P. H.; Cherney, R. J.; Batt, D. G.; Duncia, John V.; Gardner, D. S.; Ko, S. S.; Srivastava, A. S.; Yang, M. G. PCT Int. Appl. WO 2005021500), including an NMR study to document proof of stereochemistry. In addition, the protocols for assays mentioned in Tables 1 – 3 are described.

SYNTHESIS AND CHARACTERIZATION OF KEY COMPOUND 13D

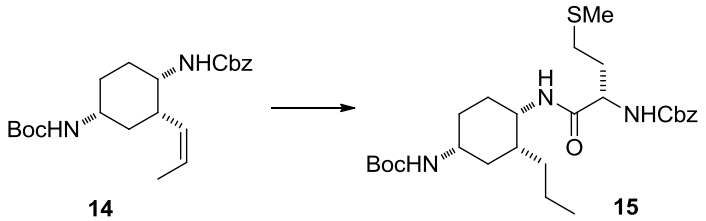


5 **14**

Synthesis of intermediate 14, [(1R, 3R, 4S)-(4-benzyloxycarbonyl-amino-3-propenyl-cyclohexyl)-carbamic acid tert-butyl ester

To a cooled (0 °C) solution of (1R, 2S, 5R)-2-benzyloxycarbonylamino-7-oxo-6-aza-bicyclo[3.2.1]octane-6-carboxylic acid tert-butyl ester (4.6 g, 12.3 mmol) in CH_2Cl_2 (100 mL) was added DIBAL-H (37 mL of a 1.0 M solution in THF). The mixture was stirred for 105 min at 0 °C. The reaction was quenched with 1N HCl and extracted with EtOAc (2 x). The organic extracts were combined, washed with brine, dried (Na_2SO_4), filtered, and concentrated in vacuo to afford tert-butyl (1R, 2S, 5R, 7R/S)-2-(benzyloxycarbonylamino)-7-hydroxy-6-aza-bicyclo[3.2.1]octane-6-carboxylate as a mixture of diastereomers. MS found: $(M\text{-}H_2O+H)^+ = 359.2$.

This material was dissolved in THF (20 mL) and added by cannula (6 mL THF rinse) to a pre-mixed (15 min), pre-cooled (0 °C) solution of ethyltriphenylphosphonium iodide (6.4 g, 14.8 mmol) and KHMDS (31 mL of a 0.5 M solution in toluene). The reaction was stirred for 25 min at 0 °C before being quenched with the addition of sat. NH_4Cl. The biphasic mixture was extracted with EtOAc (2 x). The organic extracts were combined, washed with brine, dried (Na_2SO_4), filtered, and concentrated in vacuo. Purification of the residue via flash chromatography afforded the title compound (**14**) as a colorless oil (3.44 g, 72% yield). MS found: $(M + H)^+ = 389.3$.

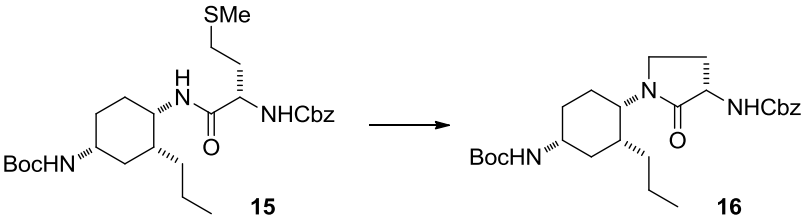


14 **15**

Synthesis of intermediate 15, (1R, 3R, 4S)-[4-((2S)-2-benzyloxycarbonylamino-4-methylsulfanyl-butyrylamino)-3-propyl-cyclohexyl]-carbamic acid tert-butyl ester

A solution of [(1*R*, 3*R*, 4*S*)-(4-benzyloxycarbonyl-amino-3-propenyl-cyclohexyl)-carbamic acid tert-butyl ester (3.44 g) in MeOH (50 mL) was charged with 5% Pd/C, Degussa (1 g). The reaction flask was evacuated and then back-filled with hydrogen; this was repeated three more times. The reaction was stirred under 1 atm of hydrogen for 4 h and then filtered and concentrated in vacuo to afford (1*R*, 3*R*, 4*S*)-(4-amino-3-propyl-cyclohexyl)-carbamic acid tert-butyl ester (quantitative). MS found: $(M + H)^+ = 257.3$.

A sample of (1R, 3R, 4S)-(4-amino-3-propyl-cyclohexyl)-carbamic acid tert-butyl ester (1.9 mmol) was dissolved in 1:1 CH_2Cl_2/DMF (40 mL), and the resultant solution was charged with N-Cbz methionine (591 mg, 2.1 mmol), N, N-diethylisopropylamine (1 mL, 5.7 mmol), and BOP (1.0 g, 2.3 mmol). The reaction was stirred for 12 h at RT and then partitioned between EtOAc and sat. $NaHCO_3$; the aqueous phase was back extracted with EtOAc (1 x). The organic phases were combined, washed with brine, dried (Na_2SO_4), filtered, and concentrated in vacuo. The residue was purified by flash chromatography to afford the title compound (375 mg). MS found: $(M + H)^+ = 522.3$.

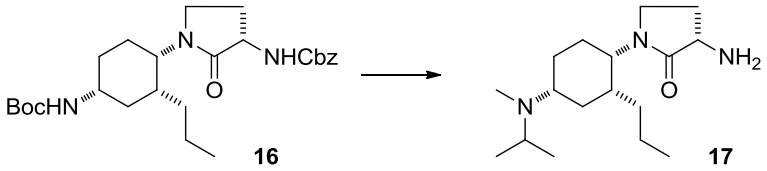


15 **16**

Synthesis of intermediate 16, {(3S)-1-[(1S, 2R, 4R)-4-tert-butoxycarbonylamino-2-propyl-cyclohexyl]-2-oxo-pyrrolidin-3-yl}-carbamic acid benzyl ester

The compound (1R, 3R, 4S)-[4-((2S)-2-benzyloxycarbonylamino-4-methylsulfanyl-butyrylamino)-3-propyl-cyclohexyl]-carbamic acid tert-butyl ester (375 mg) was "wetted" with EtOAc, and then the majority of EtOAc was removed under nitrogen stream. The residue was dissolved in iodomethane (6 mL), and the resulting solution was stirred at RT for 48 h before being concentrated in vacuo. The residue was dissolved in methylene chloride, and the resulting solution was concentrated; this was repeated to afford the salt. MS found: $(M + H)^+ = 536.3$.

This material was dissolved in DMF (12 mL) and the solution was charged with Cs_2CO_3 (470 mg, 1.4 mmol) and stirred for 12 h at RT before being partitioned between EtOAc and brine. The organic phase was dried (Na_2SO_4), filtered, and concentrated in vacuo. The residue was purified by flash chromatography to afford the title compound (185 mg). MS found: $(M + H)^+ = 474.3$.

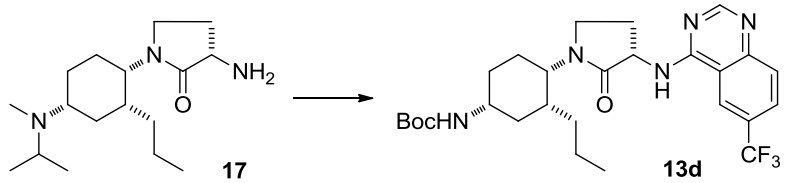


Synthesis of intermediate 17, (S)-3-amino-1-[(1S,2R,4R)-4-(isopropyl(methyl)amino)-2-propylcyclohexyl]-pyrrolidin-2-one

To a solution of {(3*S*)-1-[(1*S*, 2*R*, 4*R*)-4-tert-butoxycarbonylamino-2-propyl-cyclohexyl]-2-oxo-pyrrolidin-3-yl}-carbamic acid benzyl ester (3.88 g, 8.2 mmol) in CH_2Cl_2 (90 mL) was added TFA (45 mL) at RT. The reaction was stirred for 5 h and concentrated in vacuo. The residue was partitioned between 1N NaOH (100 mL) and EtOAc (150 mL). The aqueous layer was extracted with EtOAc (2 x 50 mL) and the organic phases were combined, washed with brine (25 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo to give benzyl (*S*)-1-[(1*S*,2*R*,4*R*)-4-amino-2-propylcyclohexyl]-2-oxopyrrolidin-3-ylcarbamate. MS found: $(M + H)^+ = 374.3$.

The entirety of benzyl (*S*)-1-[(1*S*,2*R*,4*R*)-4-amino-2-propylcyclohexyl]-2-oxopyrrolidin-3-ylcarbamate (assumed 8.2 mmol) was dissolved in methanol (40 mL). The resultant solution was charged with acetone (6 mL, 82 mmol) and stirred at RT for 10 min before sodium cyanoborohydride (2.6 g, 41 mmol) was added in one portion. The reaction was stirred at RT for 10 h and then charged successively with formaldehyde (3.0 mL of 37 wt% aq soln, 41 mmol) and sodium cyanoborohydride (0.52 g, 8.2 mmol). The reaction was stirred for another 9 h at RT and then quenched with sat. $NaHCO_3$ (150 mL). The aqueous mixture was extracted with EtOAc (200 mL, then 2 x 75 mL). The organic extracts were combined, washed with brine (30 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo. After the resulting oil stood, some paraformaldehyde-related products solidified; these were removed by dissolving the mixture in a minimal volume of EtOAc and filtering. Subsequent concentration provided benzyl (*S*)-1-[(1*S*,2*R*,4*R*)-4-(isopropyl(methyl)amino)-2-propylcyclohexyl]-2-oxopyrrolidin-3-ylcarbamate. MS found: $(M + H)^+ = 430.5$.

The entirety of benzyl (*S*)-1-[(1*S*,2*R*,4*R*)-4-(isopropyl(methyl)amino)-2-propylcyclohexyl]-2-oxopyrrolidin-3-ylcarbamate (assumed 8.2 mmol) was wet with 3 mL of EtOAc and then charged with 30% HBr/AcOH (30 mL). The reaction vessel warms and a vigorous gas evolution occurs. The mixture was stirred for 25 min at RT and then the flask was placed in a cool water bath before the addition of 150 mL of 1:1 Et_2O/H_2O. This mixture was mixed and separated, and the aqueous phase was extracted once with Et_2O. The aqueous phase was basified to pH 14 through the addition of solid NaOH (the temperature of this exothermic process was controlled through the intermittent use of an external ice bath) and the resulting mixture was extracted with EtOAc (75 mL, then 2 x 35 mL). The organic extracts were combined, washed with brine (30 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo to give an orange oil, contaminated with some powdery white solid (presumed to be formaldehyde-related). The mixture was dissolved in a minimal volume of EtOAc, filtered, and concentrated to provide (S)-3-amino-1-[(1S,2R,4R)-4-(isopropyl(methyl)amino)-2-propylcyclohexyl]pyrrolidin-2-one (2.31 g; 1H-NMR shows ~30% EtOAc, indicating an estimated 7.0 mmol of product from Steps 1 -3). MS found: $(M + H)^+ = 296.6$.



Synthesis of key compound 13d, (*S*)-1-[(1*S*,2*R*,4*R*)-4-(isopropyl(methyl)amino)-2-propylcyclohexyl]-3-(6-(trifluoromethyl)quinazolin-4-ylamino)pyrrolidin-2-one (13d)

To a solution of (*S*)-3-amino-1-[(1*S*,2*R*,4*R*)-4-(isopropyl(methyl)amino)-2-propylcyclohexyl]pyrrolidin-2-one (7.0 mmol) in EtOH (23 mL) was added triethylamine (2.5 mL, 17.5 mmol) and 4-chloro-6-(trifluoromethyl)quinazoline (2.03 g, 8.75 mmol). The mixture was heated at 75 °C for 14 h and then concentrated in vacuo. [Note: on smaller reaction scales, this residue could be diluted in water/acetonitrile, filtered, and purified directly by RP-HPLC.] The residue was dissolved in 60 mL of 2:1 H_2O/AcOH and extracted with Et_2O twice. The aqueous phase was basified to pH 14 with solid NaOH (the temperature of this exothermic process was controlled through the intermittent use of an external ice bath) and then extracted with EtOAc thrice. The organic extracts were combined, washed with brine, dried (Na_2SO_4), filtered, and concentrated in vacuo to give a solid. The material was recrystallized from EtOAc twice to provide the title compound, (*S*)-1-[(1*S*,2*R*,4*R*)-4-(isopropyl(methyl)amino)-2-propylcyclohexyl]-3-(6-(trifluoromethyl)quinazolin-4-ylamino)pyrrolidin-2-

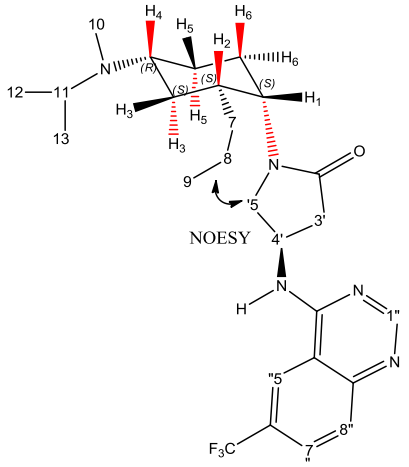
one, as a white microcrystalline solid (1.83 g, 52% yield). MS found: $(M + H)^+$ = 492.4. Elemental Analysis: theoretical for $C_{26}H_{36}N_5OF_3$, C 63.52, H 7.38, N 14.24, F 11.59; observed C 63.24, H 7.64, N 14.18, F 11.29.

[Note: Purification of the the mother liquors using RP-HPLC provided more of the title compound as its bis-TFA salt.]

NMR STUDY OF COMPOUND 13D

^1H and ^{13}C NMR were collected on a Bruker Avance III NMR spectrometer operating at 499.51 or 500.13 MHz resonance frequency and fitted with a 5 mm autotune Z-gradient broadband probe. Due to severe chemical shift overlap in the up-field spectral region, the compound **13d** was dissolved in three different NMR solvents (d_4-Methanol, d_8-Toluene, d_{12}-Cyclohexane) in order to resolve specific ranges of interest, both to determine coupling constants and observe the existence or absence of NOESY crosspeaks. In all cases, 600 µL of NMR solvent was placed in a 5 mm NMR tube with 5-10 mgs of compound. Data was collected at 27°C unless otherwise noted, where increased temperature was used to resolve overlapping multiplets. One and two-dimensional ^1H and ^{13}C NMR experiments (^1H, ^{13}C, ^1H-homoDEC, ^1H-MDEC, COSY, TOCSY, NOESY, dept-^1H-^{13}C-HSQC and ^1H-^{13}C-HMBC) were performed. The complete ^1H and ^{13}C chemical shift assignments were determined by detailed analysis of $J_{H\text{-}H}$ coupling and 2D correlated spectra. Chemical shifts are expressed in units of δ (parts per million, ppm) relative to the solvent peaks (CD3-OD at 3.30 ppm, 49.0 ppm, CD3-C6D5 at 2.09 ppm, C6D12 at 1.38 ppm). Peak multiplicities are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; tt, triplet of triplets; q, quartet; br d, broad doublet; br s, broad singlet; m, multiplet.

The relative stereochemistry of the two chiral centers H2 and H1 was confirmed to be R,S respectively by measuring the JH-H coupling constant (5.0 Hz) between the two protons which established the axial-equatorial relationship. Further JH-H coupling analysis of H1 (td, J=5.0, 1.6 Hz) established its equatorial-axial and equatorial-equatorial relationship between H6a/H6b. A weak to medium NOESY cross peak was observed in d4-methanol between a pyrollidine methylene proton (H5'b) and the methylene protons (H8) of the propyl group. In d12-cyclochexane at 70°C a NOESY cross peak was seen between H1 and the multiplet that was attributed to both H2 and H5(eq).



^1H NMR (500MHz, METHANOL-d_4) δ 8.64 (s, 1H), 8.51 (s, 1H), 7.99 (dd, *J*=8.8, 1.9 Hz, 1H), 7.84 (d, *J*=8.7 Hz, 1H), 5.03 (dd, *J*=11.1, 8.8 Hz, 1H), 4.31 (td, *J*=5.0, 1.6 Hz, 1H), 3.90 - 3.80 (m, 1H), 3.75 (td, *J*=9.7, 6.6 Hz, 1H), 3.13 (dt, *J*=13.0, 6.5 Hz, 1H), 2.70 (tt, *J*=11.6, 3.5 Hz, 1H), 2.55 - 2.42 (m, 1H), 2.38 - 2.27 (m, 1H), 2.26 (s, 3H), 2.00 - 1.95 (m, 1H), 1.96 - 1.93 (m, 1H), 1.94 - 1.89 (m, 1H), 1.87 - 1.81 (m, 1H), 1.80 - 1.76 (m, 1H), 1.67 - 1.58 (m, 1H), 1.57 - 1.52 (m, 1H), 1.49 - 1.45 (m, 2H), 1.44 - 1.40 (m, 1H), 1.38 - 1.32 (m, 1H), 1.09 (d, *J*=6.6 Hz, 3H), 1.09 (d, *J*=6.6 Hz, 3H), 0.95 (t, *J*=7.2 Hz, 3H).

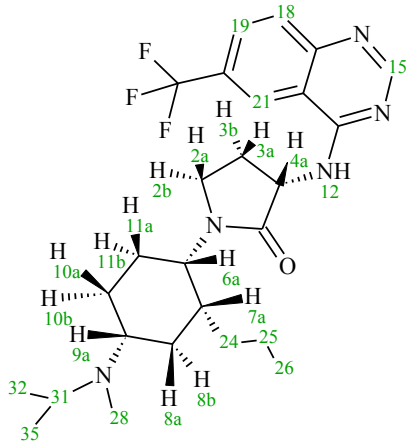
^{13}C NMR (126MHz, METHANOL-d_4) δ 175.3, 161.8, 157.7, 151.8, 129.9 (br q, *J*=2.7 Hz, 1C), 129.2, 128.9 (q, *J*=33.6 Hz, 1C), 125.3 (q, *J*=271.6 Hz, 1C), 122.2 (q, *J*=3.9 Hz, 1C), 115.9, 60.3, 54.0, 51.0, 50.3, 47.3, 40.7, 36.2, 33.2, 32.3, 30.4, 26.8, 26.2, 21.6, 19.4, 19.2, 14.7.

^1H NMR (499MHz, Tol) δ 8.99 (br s, 1H), 8.56 (s, 1H), 8.32 (s, 1H), 7.55 (d, *J*=8.5 Hz, 1H), 7.36 (dd, *J*=8.6, 1.6 Hz, 1H), 4.46 (td, *J*=5.6, 2.1 Hz, 1H), 4.45 (br s, 1H), 3.36 (br t, *J*=9.4 Hz, 1H), 3.14 (dd, *J*=15.4, 9.2 Hz, 1H), 2.98 (dt, *J*=13.0, 6.5 Hz, 1H), 2.41 (tt, *J*=11.2, 3.7 Hz, 1H), 2.17 (s, 3H), 2.07 - 1.94 (m, 2H), 1.89 (br dd, *J*=14.4, 2.2 Hz, 1H), 1.83 (br d, *J*=13.5 Hz, 1H), 1.68 (br d, *J*=11.5 Hz, 1H), 1.64 - 1.59 (m, 1H), 1.58 - 1.54 (m, 1H), 1.52 (d, *J*=5.1 Hz, 1H), 1.50 - 1.43 (m, 2H), 1.43 - 1.33 (m, 1H), 1.29 (br s, 1H), 1.28 - 1.22 (m, 1H), 1.08 (t, *J*=7.3 Hz, 3H), 1.02 (br d, *J*=6.4 Hz, 3H), 1.02 (br d, *J*=6.6 Hz, 3H).

^{1}H NMR at 70 °C (499MHz, Cyclohexane) δ 8.34 (s, 1H), 8.08 (s, 1H), 7.95 (br s, 1H), 7.59 (s, 2H), 4.47 - 4.42 (m, 1H), 4.41 (br s, 1H), 3.75 (t, *J*=8.9 Hz, 1H), 3.62 (td, *J*=9.6, 6.5 Hz, 1H), 3.03 (quin, *J*=6.5 Hz, 1H), 2.69 - 2.62 (m, 1H), 2.60 (tt, *J*=11.3, 4.0 Hz, 1H), 2.23 (s, 3H), 2.06 (t, *J*=9.9 Hz, 1H), 1.98 (br dq, *J*=14.1, 3.0 Hz, 1H), 1.87 (br dt, *J*=13.3, 4.6 Hz, 1H), 1.78 (br d, *J*=4.7 Hz, 2H), 1.72 (tt, *J*=14.1, 4.2 Hz, 1H), 1.63 - 1.58 (m, 1H), 1.57 - 1.52 (m, 1H), 1.48 - 1.43 (m, 1H), 1.51 - 1.42 (m, 2H), 1.32 - 1.28 (m, 1H), 1.02 (d, *J*=6.6 Hz, 6H), 0.98 (t, *J*=7.2 Hz, 3H).

Table of NMR Chemical Shifts (δ, ppm) and Coupling Constants (*J*, Hz) for Compound 13d

ACD Atom No	Chemical Atom	^{1}H (d_4-methanol)	^{1}H (d_8-toluene)	^{1}H (d_{12}-cyclohexane)
2a	5'a	3.75 (td, *J*=9.7, 6.6 Hz, 1H)	3.14 (dd, *J*=15.4, 9.2 Hz, 1H)	3.62 (td, *J*=9.6, 6.5 Hz, 1H)
2b	5'b	3.90 - 3.80 (m, 1H)	3.36 (br t, *J*=9.4 Hz, 1H)	3.75 (t, *J*=8.9 Hz, 1H)
3a	4'a	2.55 - 2.42 (m, 1H)	2.07 - 1.94 (m, 1H)	2.69 - 2.62 (m, 1H)
3b	4'b	2.38 - 2.27 (m, 1H)	2.07 - 1.94 (m, 1H)	2.06 (t, *J*=9.9 Hz, 1H)
4a	3'a	5.03 (dd, *J*=11.1, 8.8 Hz, 1H)	4.45 (br s, 1H)	4.47 - 4.42 (m, 1H)
6a<eq>	1<eq>	4.31 (td, *J*=5.0, 1.6 Hz, 1H)	4.46 (td, *J*=5.6, 2.1 Hz, 1H)	4.41 (br s, 1H)
7a<ax>	2<ax>	1.87 - 1.81 (m, 1H)	1.64 - 1.59 (m, 1H)	1.78 (br d, *J*=4.7 Hz, 1H)
8a<eq>	3a<eq>	1.96 - 1.93 (m, 1H)	1.83 (br d, *J*=13.5 Hz, 1H)	1.87 (br dt, *J*=13.3, 4.6 Hz, 1H)
8b<ax>	3b<ax>	1.44 - 1.40 (m, 1H)	1.29 (br s, 1H)	1.48 - 1.43 (m, 1H)
9a<ax>	4a<ax>	2.70 (tt, *J*=11.6, 3.5 Hz, 1H)	2.41 (tt, *J*=11.2, 3.7 Hz, 1H)	2.60 (tt, *J*=11.3, 4.0 Hz, 1H)
10a<eq>	5a<eq>	1.94 - 1.89 (m, 1H)	1.68 (br d, *J*=11.5 Hz, 1H)	1.78 (br d, *J*=4.7 Hz, 1H)
10b<ax>	5b<ax>	1.67 - 1.58 (m, 1H)	1.43 - 1.33 (m, 1H)	1.63 - 1.58 (m, 1H)
11a<ax>	6a<ax>	1.80 - 1.76 (m, 1H)	1.52 (d, *J*=5.1 Hz, 1H)	1.72 (tt, *J*=14.1, 4.2 Hz, 1H)
11b<eq>	6b<eq>	2.00 - 1.95 (m, 1H)	1.89 (br dd, *J*=14.4, 2.2 Hz, 1H)	1.98 (br dq, *J*=14.1, 3.0 Hz, 1H)
12	NH	Not seen.	δ 8.99 (br s, 1H)	7.95 (br s, 1H)
15	1"	8.51 (s, 1H)	8.56 (s, 1H)	8.34 (s, 1H)
18	8"	7.84 (d, *J*=8.7 Hz, 1H)	7.55 (d, *J*=8.5 Hz, 1H)	7.59 (s, 1H)
19	7"	7.99 (dd, *J*=8.8, 1.9 Hz, 1H)	7.36 (dd, *J*=8.6, 1.6 Hz, 1H)	7.59 (s, 1H)
21	5"	8.64 (s, 1H)	8.32 (s, 1H)	8.08 (s, 1H)
24<'>	7'a	1.38 - 1.32 (m, 1H)	1.28 - 1.22 (m, 1H),	1.32 - 1.28 (m, 1H)
24<">	7'b	1.57 - 1.52 (m, 1H)	1.58 - 1.54 (m, 1H)	1.57 - 1.52 (m, 1H)
25	8'	1.49 - 1.45 (m, 2H)	1.50 - 1.43 (m, 2H)	1.51 - 1.42 (m, 2H)
26	9'	0.95 (t, *J*=7.2 Hz, 3H)	1.08 (t, *J*=7.3 Hz, 3H)	0.98 (t, *J*=7.2 Hz, 3H)
28	10'	2.26 (s, 3H)	2.17 (s, 3H)	2.23 (s, 3H)
31	11'	3.13 (dt, *J*=13.0, 6.5 Hz, 1H)	2.98 (dt, *J*=13.0, 6.5 Hz, 1H)	3.03 (quin, *J*=6.5 Hz, 1H)
32	12	1.09 (d, *J*=6.6 Hz) 3H)	1.02 (br d, *J*=6.4 Hz, 3H)	1.02 (d, *J*=6.6 Hz, 3H)
35	13'	1.09 (d, *J*=6.6 Hz, 3H),	1.02 (br d, *J*=6.6 Hz, 3H)	1.02 (d, *J*=6.6 Hz, 3H)



PROTOCOLS FOR ASSAYS LISTED IN TABLES 1 – 3

Protocols are excerpted here from our previously published patent applications.

Antagonism of MCP-1 Binding to Human Periperheal Blood Mononuclear Cells (PBMC)

Millipore filter plates (#MABVN1250) are treated with 100 µl of binding buffer (0.5% bovine serum albumin, 20 mM HEPES buffer and 5 mM magnesium chloride in RPMI 1640 media) for thirty minutes at room temperature. To measure binding, 50 µl of binding buffer, with or without a known concentration compound, is combined with 50 µl of 125-I labeled human MCP-1 (to give a final concentration of 150 pM radioligand) and 50 µl of binding buffer containing 5x105 cells. Cells used for such binding assays can include human peripheral blood mononuclear cells isolated by Ficoll-Hypaque gradient centrifugation, human monocytes (Weiner et al., J. Immunol. Methods. 1980, 36, 89), or the THP-1 cell line which expresses the endogenous receptor. The mixture of compound, cells and radioligand are incubated at room temperature for thirty minutes. Plates are placed onto a vacuum manifold, vacuum applied, and the plates washed three times with binding buffer containing 0.5M NaCl. The plastic skirt is removed from the plate, the plate allowed to air dry, the wells punched out and counted. The percent inhibition of binding is calculated using the total counts obtained in the absence of any competing compound and the background binding determined by addition of 100 nM MCP-1 in place of the test compound.

Antagonism of MCP-1-induced Human PBMC Chemotaxis

Neuroprobe MBA96-96-well chemotaxis chamber, Polyfiltronics MPC 96 well plate, and Neuroprobe polyvinylpyrrolidone-free polycarbonate PFD5 8-micron filters are warmed in a 37 °C incubator. Human Peripheral Blood Mononuclear Cells (PBMCs) (Boyum et al., Scand. J. Clin. Lab Invest. Suppl. 1968, 97, 31), freshly isolated via the standard ficoll density separation method, are suspended in DMEM at 1 x 10 7 c/ml and warmed at 37°C. A 60nM solution of human MCP-1 is also warmed at 37°C. Dilutions of test compounds are made up at 2x the concentration needed in DMEM. The PBMC suspension and the 60nm MCP-1 solution are mixed 1:1 in polypropylene tubes with prewarmed DMEM with or without a dilution of the test compounds. These mixtures are warmed in a 370C tube warmer. To start the assay, add the MCP-1/compound mixture into the wells of the Polyfiltronics MPC 96 well plate that has been placed into the bottom part of the Neuroprobe chemotaxis chamber. The approximate volume is 400µl to each well and there should be a positive meniscus after dispensing. The 8 micron filter is placed gently on top of the 96 well plate, a rubber gasket is attached to the bottom of the upper chamber, and the chamber is assembled. A 200µl volume of the cell suspension/compound mixture is added to the appropriate wells of the upper chamber. The upper chamber is covered with a plate sealer, and the assembled unit is placed in a 370C incubator for 45 minutes. After incubation, the plate sealer is removed and all the remaining cell suspension is aspirated off. The chamber is disassembled and the filter gently removed. While holding the filter at a 90 degree angle, unmigrated cells are washed away using a gentle stream of phosphate buffered saline and the top of the filter wiped with the tip of a rubber squeegee. Repeat this wash twice more. The filter is air dried and then immersed completely in Wright Geimsa stain for 45 seconds. The filter is then washed by soaking in distilled water for 7 minutes, and then a 15 second additional wash in fresh distilled water. The filter is again air dried. Migrated cells on the filter are quantified by visual microscopy.

CCR5 Binding Assay

Cell derivation and cell culture: A pool of HT1080 cells stably expressing endogenous CC chemokine receptor 5 (CCR5) were developed using the methods outlined by Harrington, Sherf, and Rundlett (see United States patents US 6,361,972 and US 6,410,266). The highest-expressing clones were isolated using repetitive flow cytometry, followed by sub-cloning. These cells were then cultured in 6-well dishes at 3 x 105 cells/well and transfected with a DNA vector containing the chimeric HA-tagged G protein Gqi5 (Molecular Devices; 5 micrograms of linearized vector DNA in 15 microL of Ex-Gen from Fermentes was used for the transfection). Two days after transfection, the wells were combined and plated into P100 plates. Seven days after plating, colonies were picked, expanded, and analyzed for Gqi5 content by Western blot. A clone (designated as 3559.1.6) having high expression of Gqi5 (from transfection) and of CCR5 (endogenous) was selected and used for the experiments described below. The HT1080 cells (clone 3559.1.6) were cultured with alpha-MEM supple-

mented with 10% dialyzed fetal bovine serum, 2% penicillin/streptomycin/glutamine, and 500 microgram/mL hygromycin B (final concentration) at 37°C with 5% CO_2 in a humidified atmosphere.

Membrane Preparation: A cell pellet containing 1 x 108 HT1080 cells (clone 3559.1.6) was resuspended in 5 mL of ice-cold Membrane Prep Buffer (50 mM HEPES, 5 mM $MgCl_2$, 1 mM $CaCl_2$) and homogenized at high-speed on a Polytron homogenizer for 20 sec on ice. The homogenate was diluted with another 25 mL of Membrane Prep Buffer and centrifuged for 12 min (48,000 x g at 4°C). The cell pellet was resuspended in 5 mL of Membrane Prep Buffer before being re-homogenized as described previously. The homogenate was diluted with 5 mL of Membrane Prep Buffer and assayed for CCR_5 protein concentration.

Binding assay: The freshly-prepared homogenate from the Membrane Preparation described above was diluted in Binding buffer (50 mM HEPES, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% BSA; one complete protease inhibitor tablet was added before assay) to achieve a final protein concentration of 10 micrograms/well (solid white 96-well plates from Corning, Inc.). This membrane preparation was mixed with WGA-SPA beads (Amerhsam; pre-soaked in Binding buffer) to give a concentration of 200 micrograms/well. The membrane/SPA bead mix (100 microliters/well) was then added to a plate that had been pre-dotted with 2 microliters DMSO containing various concentrations of test articles (pure DMSO for negative control; various concentrations of examples of this invention for test articles; 500 nM MIP-1 beta as a positive control). The binding assay was initiated through the addition of 50 microliters of [125I]-MIP-1 beta (Perkin Elmer; material was diluted in Binding buffer such that the addition of 50 microliters/well gives a final concentration of 0.1 nM [125I]-MIP-1 beta). The plate was sealed and allowed to stand at room temperature for 4 – 6 h before being counted on a Packard TopCount. The percentage bound for the test article was calculated, using negative and positive controls to define the window for each experiment.

hERG Flux Assay

HEK293 cells stably-expressing hERG channels were grown (37 °C, 5% CO_2) in Dulbecco's Modified Eagle's Media supplemented with 10% Sigma fetal bovine serum, non-essential amino acids, 2mM L-glutamine and 500 µg/ml G418, at incubator. Cell dissociation buffer was used to extract the cells from flasks, which were then plated into 384-well CORNING® poly-D-lysine coated black/clear plates at a density of 2 x 104 cells per well (20 µl) in 10% serum media, and incubated for 15-24 hours at 37 °C in a 5% CO_2 incubator until a confluent monolayer of cells was obtained.

A 2 mM stock of BTC-AM dye (Molecular Probes, Eugene, OR) was prepared in 100% DMSO and then added 1:1 to 10% (w/v) pluronic acid in DMSO on the day of assay. The dye was then diluted in hERG external EP buffer (140 mM NaCl, 4.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM HEPES, pH 7.3 and 10 mM glucose; all buffer components obtained from Sigma Chemical). This BTC dye mixture (30 µl) was added to the cells and produced a final loading concentration of 2.5 µM. Cells are incubated at 21 °C for 45 minutes.

The test compound was diluted to 10 mM DMSO in 60 µl. The compound was then serially-diluted at a 1:2 ratio in DMSO in columns 1-10 and 11-20 of a 384-well plate. Assay-ready plates were generated by stamping 2.5 µl from the DMSO serially diluted plate, which was prepared on the Velocity 11 BIOCEL®. Aqueous plates were created by adding 48 µl of EP buffer and then were diluted 30 - 45 minutes before the assay was read on the FLIPR®. After dye loading, aqueous-diluted compound was added to the cells of the three replicate plates (10 µl) yielding a ten point concentration range of 80 µM to 0.156 nM. Final DMSO concentration in the assay is 1%. Assay-ready aqueous plates were prepared and diluted on a CyBio liquid handler.

Cells loaded with dye were read on the FLIPR®384 (Molecular Devices, Sunnyvale, CA), which excites the dye using the 488 nm line of an argon laser. Emission was filtered using a 540 ± 30 nm bandpass filter. hERG channels are stimulated to open by the addition of 20 µl/well EP buffer containing 66 mM K_2SO_4 and 1.3 mM Tl_2SO_4 (Sigma/Aldrich). For each plate, data were collected every second for a period of 12 seconds, at which time the Tl+-containing stimulus buffer was added. Data collection proceeded every second for 48 seconds, and then continued every three seconds for an additional 2 minutes.

The dynamic range of the assay was determined from blanks and totals wells. The totals wells (columns 21 and 22) define maximal hERG activation for the plate (no test compound present), and the blanks wells (columns 23 and 24) define 100% hERG inhibition. The blanks wells contain 400 nM of either of the standard hERG inhibitors dofetilide (Ficker et al., 1998) or E-4031. Raw data points in each sample well were first corrected for cell/signal variation, negative control (blanks) background, and normalized to the positive controls (totals) using the online FLIPR® software. Test compound concentration response curves for the hERG Tl+ flux data were then fit using Excel Fit (ID Business Solutions Limited, Surrey, UK) with a single-site logistic equation, Y= A + ((B-A)/1 + ((C/X) ^ D))) where A= maximal inhibition. Data were analyzed by fitting maximum amplitudes of change in fluorescence for Tl+ flux for a given condition of test compound. Potencies (IC_{50} values) of compound were calculated from the average of triplicate wells.

hERG Patch Clamp

Whole-cell patch-clamp was used to directly measure hERG currents in HEK 293 cells stably expressing the cloned hERG potassium channel α subunit. The compound was tested in an aqueous buffer with pH 7.4 at room temperature. Repetitive test pulses (0.05 Hz) were applied from a holding potential of 80 mV to +20 mV for 2 seconds and tail currents were elicited following the test pulses by stepping the voltage to 65 mV. The effects from the compound were calculated by measuring inhibition of peak tail current

Single-Dose Pharmacokinetics in Rats

Male Sprague-Dawley rats (250-300 g) were used for the pharmacokinetic studies. Rats were fasted overnight prior to PO dosing and fed 4 h post dose. Blood samples (~0.3 mL) were collected from the jugular vein into K2EDTA-containing tubes and then centrifuged at 4 °C (1500-2000xg) to obtain plasma. In an oral bioavailability study, 2 groups of animals (N=2-3 per group) received the test compound either as an intravenous (IV) infusion (over 10 min) via the jugular vein or by oral gavage. Serial blood samples were obtained at 0.17 (for IV only), 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, and 24 h post dose. Plasma samples, obtained by centrifugation at 4 °C (1500-2000xg), were stored at 20 °C until analysis by LC/MS/MS.

Single-Dose Pharmacokinetics in Monkeys

The pharmacokinetics of various test compounds were evaluated in male cynomolgus monkeys in a crossover-design. Monkeys were fasted overnight prior to PO dosing and fed 4 h post dose. A group of 1-3 animals (3 to 5 kg) received the compound by IV infusion (over 10 min) via a femoral vein and by oral gavage, with a 1-week washout between treatments. Serial blood samples (~0.3 mL) were collected from a femoral artery at 0.17 (IV only), 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, and 24 h post dose, and centrifuged at 4 °C (1500-2000xg) to obtain plasma. Samples were stored at 20 °C until analysis by LC/MS/MS.

Data Analysis for Pharmacokinetic Assays

The pharmacokinetic parameters were obtained by non-compartmental analysis of plasma concentration vs. time data (Kinetica software, Version 4.2, InnaPhase Corporation, Philadelphia, PA). The peak concentration (Cmax) and time for Cmax were recorded directly from experimental observations. The area under the curve from time zero to the last sampling time (AUC(0-T)) was calculated using a combination of linear and log trapezoidal summations. The total plasma clearance (CLTp), steady-state volume of distribution (Vss), apparent elimination half-life (T1/2) and mean residence time (MRT) were estimated after IV administration. Estimations of T1/2 was made using a minimum of 3 time points with quantifiable concentrations. The absolute oral bioavailability (F) was estimated as the ratio of dose-normalized AUC values following oral and IV doses.