Supporting Information

Discovery of 3-piperidinyl-1-cyclopentanecarboxamide as a Novel Scaffold for Highly Potent CCR2 Receptor Antagonists

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EXPERIMENTALS: BIOLOGICAL ASSAYS

a) Preparation of Human Monocytes: Plasma enriched for human leukocytes is obtained by leukophoresis of human blood (Biological Specialty Corporation.) The leukocyte-rich plasma is diluted 1:2 with saline and underlayed with 10 ml of Lymphocyte Separation Media (LSM) (ICN, Aurora, Ohio), and centrifuged at 700 X g for 30 minutes at room temperature. The top layer is aspirated, the mononuclear layer is aliquoted in 4 50ml conical tubes and the cells washed 2X with PBS. Following centrifugation (200 X g, 5 minutes), 5 ml RPMI is added along with sheep red blood cells (4 ml) and the tubes are incubated for 15 minutes at 370C (mix every 5 minutes). Centrifuge the cells (225 X g, 5 minutes), aspirate the supernatant, and incubate the cell pellets at –200C for 6 minutes and then 40C for 15 minutes. Gently resuspend each pellet in 25 ml PBS and underlay with 10 ml LSM. Centrifuge at 600 X g for 15 minutes. Aspirate the top layer to waste and collect the mononuclear cell layer interface. Wash with PBS and centrifuge. Resuspend the pellet in 5 ml ACK lysis buffer for 5 minutes. Centrifuge (225 X g, 5 minutes) and resuspend cells in 40 ml RPMI/10% FCS. Count the cells and store at 40C.

b) human monocyte binding assay: Human monocytes (2 X 105) expressing human CCR2 receptor, CHO cells (5 X 104) expressing CCR2B I40L receptor, or L1.2 cells expressing CCR2A I40L (5 X 104) or CCR2B I40L/V64I (5 X 104) were incubated with 125I-hMCP-1 (20-25 pM, 2200 Ci/mmol; Dupont/New England Nuclear) at room temperature for 45 minutes in buffer containing HEPES (50 mM), MgCl2 (5 mM) and CaCl2 (1 mM), pH 7.4, 0.5% BSA and protease inhibitor cocktail. For experiments in the presence of serum, 4 X 105 human monocytes were used and human serum was added to a final concentration of 15% in the reaction mixture. The reactions were terminated and the separation of bound from free ligand accomplished by filtration over GF/B filters that had been presoaked in 0.10% polyethylenimine using a Packard Cell Harvester. In all cases non-specific binding was determined by the addition of excess (100nM) of unlabeled MCP-1. The filters were washed with 25 mM HEPES, pH 7.5 containing 500 mM NaCl and the plates dried. The plates were counted for 125I radioactivity using Microscint 0 (Packard) and a Topcount NXT (Packard).

c) human monocyte chemotaxis functional assay: Chemotaxis Assay: Assays were performed in 96 well disposable chemotaxis plates (ChemoTx, NeuroProbe, Inc.) with a 5 mm pore size (5.7 mm diameter). Monocytes (1x107cells/ml) were incubated with 2 mM Calcein-AM (Molecular Probes) in Hanks Balanced Salt Solution containing 0.01% BSA at 37°C for 30 minutes. The dye-loaded cells were washed and resuspended at 6 x 106 cells/ml in RPMI 1640 (lacking phenol red) containing 0.01% BSA. Assay was performed with 1.5 x 105cells/well. Known CCR2 antagonist 1-(3,4-dichlorobenzyl)-5-hydroxy-1H-indole-2-carboxylic acid was calculated to have an IC50 of 95 nM in this assay, in good agreement with the reported value of 60 nM (Faull, A. W.; Kettle, J. G. WO 2000046196, 2000).

Ethyl 3-methylenecyclopentane carboxylate (**14a**). A solution of 2-[(trimethylsilyl)methyl]-2-propen-1-yl acetate (9.64 mL, 45.36 mmol), ethyl acrylate (5.18g 45.36 mmol), palladium acetate (510 mg, 2.268 mmol) in 50 mL of tetrahydrofuran was thoroughly degassed (vacuum/nitrogen cycle) and triisopropyl phosphite (2.80 mL, 11.34 mmol) was added via syringe. The pale yellow solution was stirred under reflux overnight. The solvent was concentrated in vacuo (80 torr), the residue diluted with water (50 mL) and extracted with diethyl ether (3 x 50 mL). The combined organic extracts were washed with water (2 x 30 mL), brine (1 x 30 mL), dried (anh. sodium sulfate) and the solvent was removed on rotavap (80 torr). The crude product was distilled under reduced pressure to yield 3.96 g (52 %) of pure product. B.P.: 90 - 96 °C (20 torr). ^{1}H NMR (500 MHz, $CDCl_3$): 4.89 (m, 2H), 4.16 (q, 7.0 Hz, 2H), 2.82 (bd, 15.8 Hz, 1H), 2.41 (m, 2H), 2.20 (m, 3H), 1.25 (s, 3H), 1.26 (q, 7 Hz, 3H).

3-Methylenecyclopentane carboxylic acid (**14b**). A solution of ethyl 3-methylenecyclopentane carboxylate (**14a**, 1.689 g, 10 mmol) in THF (6 mL) and water (6 mL) containing 412 mg (20 mmol) of lithium hydroxide monohydrate was homogenized with methanol and stirred at gentle reflux for 30 minutes. The solvent was evaporated to dryness; the residue was dissolved in water, extracted with diethyl ether (3 x 30 mL). The pH was set acidic with 2N HCl, and the desired product was extracted with diethyl ether. The combined organic phases were dried with anhydrous magnesium sulfate, and the solvent was evaporated in vacuo to leave 600 mg (43 %) of the crude acid. Its relatively high volatility made further attempts at purification impractical, and the acid was used in the subsequent reaction step as obtained.

3,5-Bis(trifluoromethyl)benzyl 3-methylene-1-methylcyclopentane-carboxamide (**15**). A solution of 3-methylene-1-methylcyclopentanecarboxylic acid, (**14b**, 600 mg, 4.28 mmol) 3,5-bis(trifluoromethyl)benzylamine hydrochloride (1.196 g, 4.28 mmol), 1-hydroxy-7-azabenzotriazole (583 mg, 4.28 mmol) and diisopropylethylamine (745 µL, 4.28 mmol) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC, 1.230 g, 0.168, 6.42 mmol) in dichloromethane (10 mL) was stirred at room temperature for 1 hr. The reaction mixture was diluted with dichloromethane (40 mL) and washed with water (3 x 30 mL), brine (1 x 30 mL), dried (anhydrous sodium sulfate) and the solvent was evaporated under reduced pressure. The crude product was purified *via* mplc (Lobar Fertigsaule, LiChroprep, 40-63 µm, ethyl acetate/hexanes (1:4)) yielding 777.6 mg (49 %) of pure product. ^{1}H NMR (500 MHz, $CDCl_3$) 7.79 (s, 1H), 7.70 (s, 2H), 6.19 (bs, 1H), 4.98 (bs, 1H), 4.92 (bs, 1H), 4.62 (dd, 15.6 Hz, 6.2 Hz, 1H), 4.54 (dd, 15.8 Hz, 6.0 Hz, 1H), 2.78 (bd, 15.8 Hz, 1H), 2.46 (m, 2H), 2.30 (bd, 15.8 Hz, 1H), 2.18 (m, 1H), 1.70 (m, 1H), 1.31 (s, 3H).

***N*-[3,5-bis(trifluoromethyl)benzyl]-1-methyl-3-[(1*R*,3'*R*)-3'-methyl-1'*H*-spiro[indene-1,4'-piperidin]-1'-yl]cyclopentanecarboxamide (16).** A solution of the olefin 3,5-bis(trifluoromethyl)benzyl 3-methylene-1-methylcyclopentane-carboxamide (15, 255 mg, 0.698 mmol) in dichloromethane (20 mL) was ozonized at –78 °C. The excess ozone was removed with a stream of nitrogen. Intermediate 1 (165 mg, 0.698 mmol), diisopropyl-ethylamine (121 µL, 0.698 mmol) and 400 mg of molecular sieves (4A, crushed) were added, followed by sodium triacetoxyborohydride (444 mg, 2.094 mmol). The reaction mixture was stirred at room temperature for 48 hrs after which it was diluted with dichloromethane (50 mL). The sieves were filtered off (Celite), the filtrate was washed with a saturated solution of sodium bicarbonate (1 x 50 mL), water (2 x 50 mL) and brine (1 x 50 mL). After drying (anhydrous sodium sulfate), the solvent was evaporated under reduced pressure, and the residue (216 mg) was further purified by preparative thin layer chromatography (Analtech, Silica Gel GF, 1000 µ, 100 % ethyl acetate) to yield 68 mg (18 %) of the higher eluting (1,3-*cis*-cyclopentane) diastereoisomeric pair and 92 mg (24 %) of the lower eluting *trans*-diastereoisomeric pair. The higher eluting diastereoisomeric pair was separated into single enantiomers using Diacel's Chiralcel OD chiral preparative HPLC column, eluent hexane : ethanol (97 : 3) at flowrate of 9 mL/min. The retention times of the individual isomers (analytical 250 x 4.6 mm column, 1.0 mL/min) were 6.93 min (40 %), 7.91 (45 %), 9.63 (9 %) and 12.04 (4 %). ^{1}H NMR (500 MHz, $CDCl_3$) 9.22 (bs, 1H), 7.82 (bs, 2H), 7.78 (bs, 1H), 7.27 (m, 1H), 7.24 (dt, 7.3 Hz, 0.7 Hz, 1H), 7.14 (t, 7.3 Hz, 1H), 6.80 (m, 2H), 6.60 (d, 5.7 Hz, 1H), 4.68 (m, 2H), 3.15 (bd, 11.4 Hz, 1H), 3.02 (bd, 10.5 Hz, 1H), 2.93 (bs, 1H), 2.35 (bd, 14 Hz, 1H), 2.20 (m, 2H), 1.7-2.1 (m, 9 , 1.37 (s, 3H), 1.32 (bdt, 13.7 Hz, 2.5 Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) 177.0, 148.4, 142.8, 142.1, 134.4, 133.9, 127.9, 127.7, 126.4, 121.6, 116.5, 115.3, 67.8, 56.4, 54.2, 51.8, 48.3, 42.7, 39.4, 37.5, 34.8, 32.1, 28.4, 25.8, 12.4. ESI-MS.: for $C_{30}H_{32}F_6N_2O$: calculated 550.24; found: 551.40 (M+H). HiRes MS: for $C_{30}H_{32}F_6N_2O$: calculated 551.24916 $[M+H]^+$, found 551.24774.

Compound purities were determined by two diverse HPLC conditions.

HPLC conditions A: Waters XBridge C18, 150 x 4.6mm, 5 μm Eluents: A = 10 mM ammonium formate in water, B = acetonitrile, flow 1.5 mL/min, oven temperature 40 C, diode-array detector (monitored at 260 nm) and a Shimadzu EV1020 MS detector. Time program: 0.00 min (2%B), 2.00 min (65%B), 10.00 min (95%B). Tr = 5.42 min (100%).

HPLC conditions B: Column: Waters Sunfire C18, 150 x 4.6mm, 5 μm. Eluents: A = 0.1 % formic acid in water, B = 0.1 % formic acid in acetonitrile, flow 1.5 mL/min, oven temperature 40 C, diode-array detector (monitored at 260 nm). Time program: 0.00 min (2%B), 2.00 min (65%B), 10.00 min (95%B).

HPLC and HRMS data:

Compd	HPLC data			MF	HRMS (M+H)	
	Rt (min)	% area	method		Calculated	Found
10a-2	4.25	98	A	$C_{26}H_{28}F_6N_2O$	499.21631	499.21631
	2.52	100	B			
12	4.97	98	A	$C_{29}H_{30}F_6N_2O$	537.23157	537.23175
	4.51	93	B			
16	5.33	99	A	$C_{30}H_{32}F_6N_2O$	551.24916	551.24774
	4.57	95	B			
18	4.76	97	A	$C_{29}H_{30}F_6N_2O$	537.23351	537.23187
	3.04	93	B			