Pyrrolone Derivatives as Intracellular Allosteric Modulators for Chemokine Receptors: Selective and Dual-Targeting Inhibitors of CC Chemokine Receptors 1 and 2

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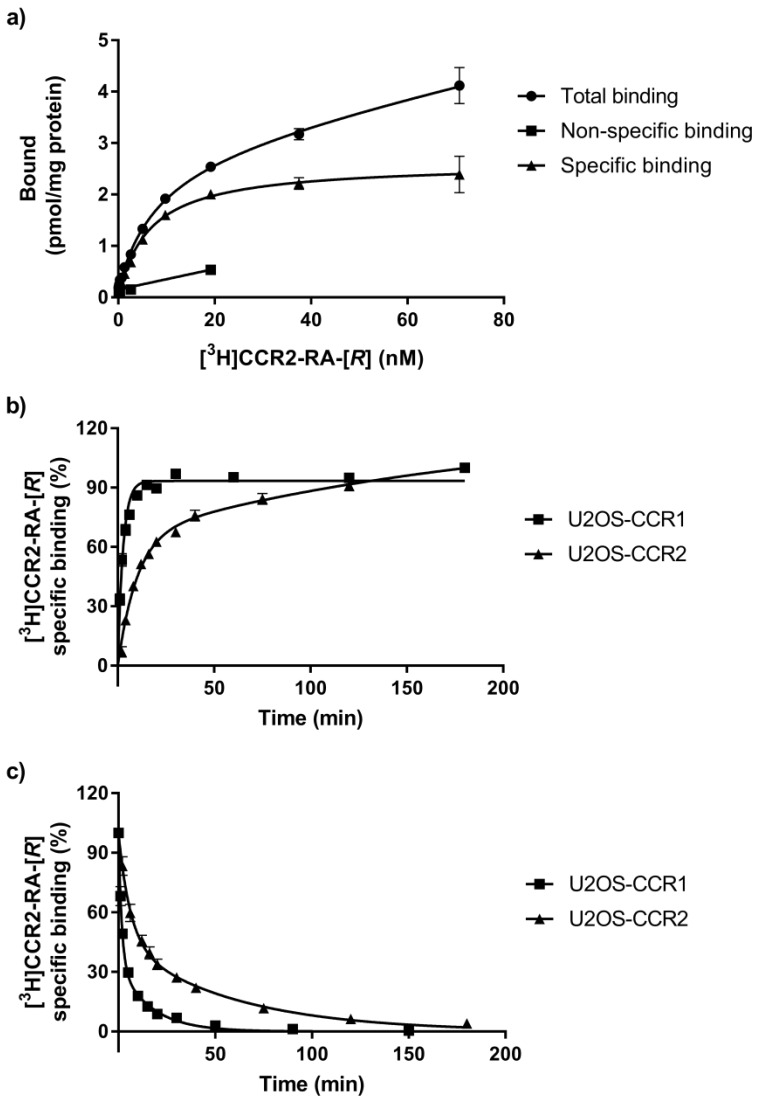

Figure S1. (a) Saturation binding of 0.05 – 70 nM [^{3}H]-CCR2-RA-[*R*] to U2OS-CCR2 at 25°C, in absence (total binding) or presence (non-specific binding) of 10 μM JNJ-27141491. (b) Association kinetics of 6 nM [^{3}H]-CCR2-RA-[*R*] to U2OS-CCR1 (■) and U2OS-CCR2 (▲) at 25°C. In both CCR1 and CCR2, data were best fitted using a two-phase association function. (c) Dissociation kinetics of 6 nM [^{3}H]-CCR2-RA-[*R*] from U2OS-CCR1 (■) and U2OS-CCR2 (▲) at 25°C. In both CCR1 and CCR2, data were best fitted using a two-phase exponential decay function. For all experiments data shown are mean ± SEM of at least three experiments performed in duplicate. See Supplementary Table 1 for pK_D, B_{max}, and rate constants.

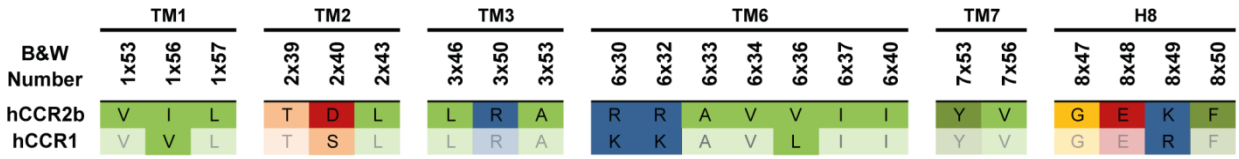

Figure S2. Sequence conservation of the key residues involved in the intracellular binding of CCR2-RA-[*R*] in CCR2,[1] as obtained by sequence alignment of human CCR1 and human CCR2b using the "Structure-based alignment" tool and the "Similarity search" tool of the GPCR database (GPCRdb, http://www.gpcrdb.org).[2] The residues are numbered according to the structure-based Ballesteros-Weinstein system,[2] which corresponds to the system used by the GPCRdb. Different amino acids in both CCR1 and CCR2 are highlighted.

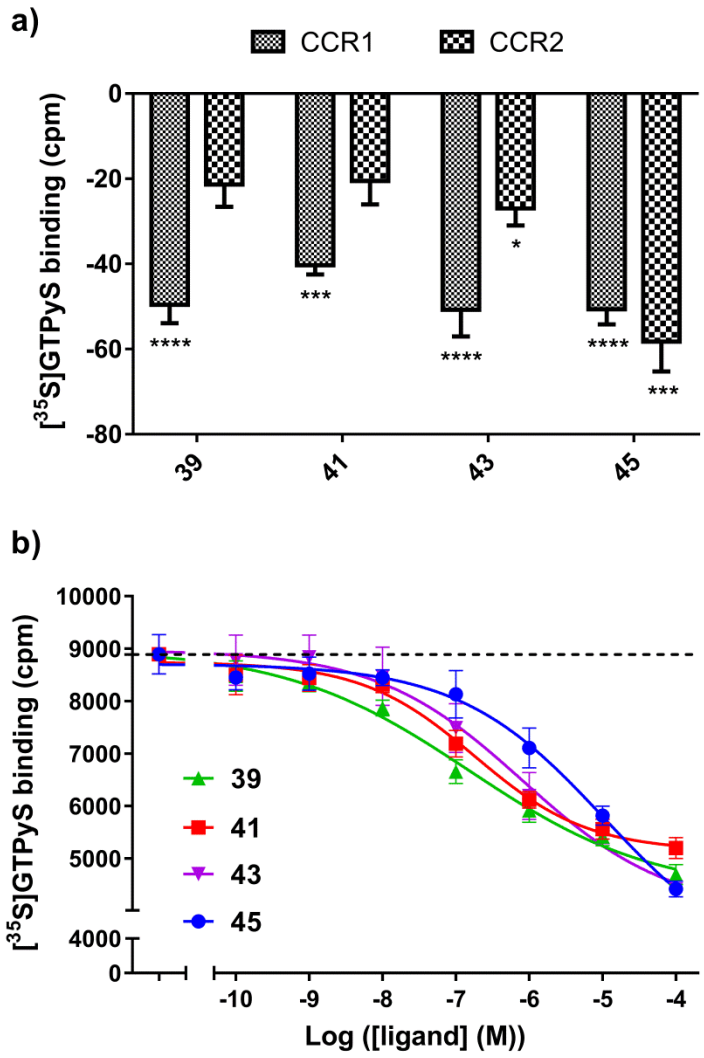

Figure S3. Compounds behave as inverse agonists in CCR1. (a) Maximal inhibition of CCL2 or CCL3-induced [^{35}S]GTPγS binding achieved by the highest concentration tested of compounds **39**, **41**, **43** and **45** in U2OS-CCR1 (100 μM) and U2OS-CCR2 (**45** at 100 μM, **39**, **41**, **43** at 10 μM). One-way ANOVA with Dunnett's post-hoc test was performed to compare the maximal inhibition against basal [^{35}S]GTPγS binding in CCR1 or CCR2. Significant differences are displayed as *, $p < 0.05$; ***, $p < 0.001$; and ****, $p < 0.0001$. (b) Inhibition of basal [^{35}S]GTPγS binding in absence of agonist CCL3 by compounds **39**, **41**, **43** and **45** in U2OS-CCR1. The level of basal activity in U2OS-CCR1 is indicated by a dashed line. Data shown are mean ± SEM of at least three experiments performed in duplicate.

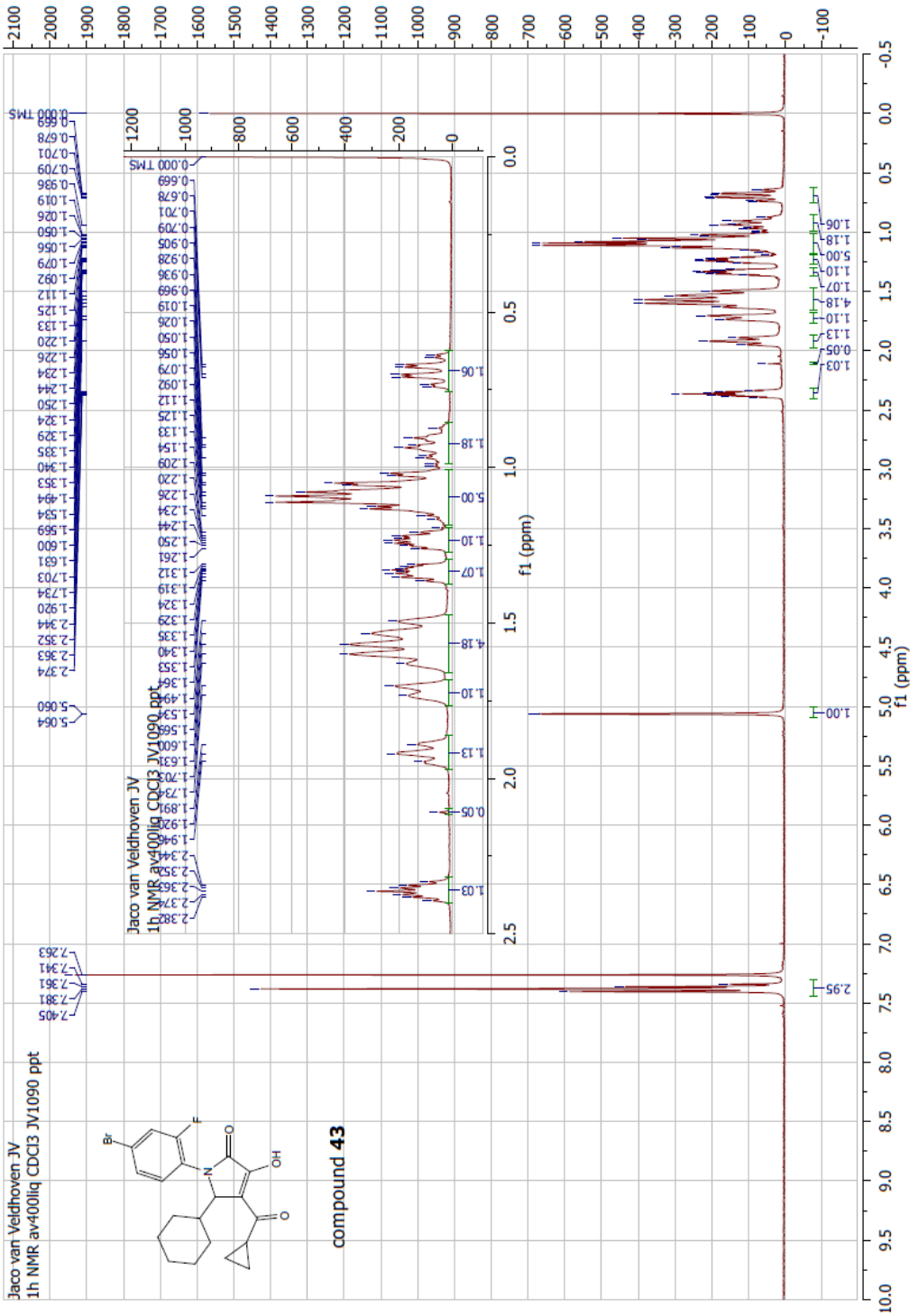

Figure S4. ^{1}H NMR spectrum of compound **43**, as an example of NMR interpretation.

Table S1. Characterization of [^{3}H]CCR2-RA-[*R*] in U2OS-CCR1 and U2OS-CCR2.[a]

	U2OS-CCR1	**U2OS-CCR2**
pK_D (K_D, nM)	7.87 ± 0.03 (13.5)[b]	8.20 ± 0.05 (6.3)[c]
B_{max} (pmol/mg)	6.13 ± 0.24[b]	2.63 ± 0.28[c]
$k_{obs,fast}$ (min^{-1})[d]	0.69 ± 0.08	0.12 ± 0.01*
$k_{obs,slow}$ (min^{-1})[d]	0.11 ± 0.007	0.01 ± 0.001**
%fast	62 ± 2	55 ± 3
$k_{off,fast}$ (min^{-1})[e]	0.62 ± 0.08	0.18 ± 0.02*
$k_{off,slow}$ (min^{-1})[e]	0.06 ± 0.003	0.02 ± 0.002***
%fast	66 ± 2	54 ± 4

[a]Values are means ± SEM of at least three independent experiments performed in duplicate. Unpaired *t*-test analysis with Welch's correction was performed to analyze differences in kinetic rates between receptors, with differences noted as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. [b]Values obtained from homologous displacement of 3, 6 and 12 nM [^{3}H]-CCR2-RA-[*R*] from U2OS-CCR1 at 25°C. [c]Values obtained from saturation binding of 0.05 – 70 nM [^{3}H]-CCR2-RA-[*R*] to U2OS-CCR2 at 25°C. [d]Observed association and [e]dissociation rate constants of [^{3}H]-CCR2-RA-[*R*] in U2OS-CCR1 or -CCR2 at 25°C.

Table S2. Inhibition of basal [^{35}S]GTPγS binding, i.e. in the absence of agonist CCL3, by compounds **37, 39, 41 and 43** in U2OS-CCR1.

Compound	pIC_{50} ± SEM (IC_{50}, μM)[a]	Hill slope[a]
39	6.78 ± 0.04 (0.17)**	-0.4 ± 0.06*
41	6.70 ± 0.08 (0.21)**	-0.6 ± 0.05
43	6.13 ± 0.15 (0.85)	-0.4 ± 0.07*
45	5.17 ± 0.01 (6.73)	-0.5 ± 0.07*

[a]All values are means ± SEM of at least three independent experiments performed in duplicate. Unpaired *t*-test analysis with Welch's correction was performed to analyze differences in their inhibitory potencies and pseudo-Hill slopes as antagonists and inverse agonists in CCR1, with differences noted as *, $p < 0.05$; and **, $p < 0.01$.

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