Supporting Information

A step forward in the sigma enigma: a role for chirality in the sigma1 receptor-ligand interaction?

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1. Chemistry

1.1 General

All reagents and solvents were purchased from commercial suppliers and used without any further purification. All reactions involving air-sensitive reagents were performed under nitrogen atmosphere. Anhydrous solvents were obtained according to standard procedures. All solvents were evaporated under reduced pressure using a Heidolph Laborota 4000. Melting points were determined in open capillaries on SMP3 Stuart Scientific apparatus and are uncorrected. Proton nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 400 spectrometer operating at 400.13 MHz. Proton chemical shifts (δ) are reported in ppm with the solvent reference relative to tetramethylsilane (TMS) employed as the internal standard (CDCl3, δ = 7.26 ppm; CD2Cl2, δ = 5.32 ppm; [D6]acetone, δ = 2.05 ppm). The following abbreviations are used to describe spin multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad signal, dd = doublet-doublet, td = triplet-doublet. Reaction courses were checked by thin layer chromatography (TLC) on silica gel (Fluka Kieselgel 60 F254, Merck) pre-coated glass-backed plates purchased from Fluka and the chromatograms were detected by UV radiations, potassium permanganate and acidic ammonium molybdate (IV). Intermediates and final compounds were purified by flash chromatography using Silica Gel 60 (particle size 230–400 mesh) purchased from Nova Chimica (Cinisello Balsamo, Italy).

Optical rotation values were measured on the Jasco photoelectric polarimeter DIP 1000 with a 1 dm cell at the sodium D line (λ = 589 nm); sample concentration values c are given in g 10-2 mL-1. Circular dichroism spectra were recorded on a Jasco J-710 dichrograph.

1.2 Synthesis of (R,S)-1

Synthesis of 4-Piperidin-1-yl-butan-2-one (6).

A solution of piperidine (0.1 mL, 1 mmol) and but-3-en-2-one (0.12 mL, 1.5 mmol) in PEG 400 (2.5 g) was stirred at rt for 35 min. Subsequently, 10% HCl was added to the mixture until pH 2 was reached and an acid-basic work-up was performed. Initially, the aqueous phase was washed with dichloromethane (CH_2Cl_2), then made alkaline with 1 N NaOH solution (pH 10) and extracted with CH_2Cl_2. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure affording the desired product as yellow oil (96 mg, 62%).

Synthesis of 2-([1,1'-biphenyl]-4-yl)-4-(piperidin-1-yl)butan-2-ol [(R,S)-1].

To a solution of 4-bromo-1,1'-biphenyl (12.5 mmol) in anhydrous diethyl ether (Et_2O, 50mL), cooled to −40°C, *tert*-BuLi (25 mmol, 1.7 M in pentane) was added with stirring under nitrogen atmosphere (N_2), keeping the temperature for 20 min. The reaction mixture was then slowly

allowed to warm to room temperature. After 1 h stirring, a solution of 4-(piperidin-1-yl)butan-2-one (10 mmol) in dry Et_2O (15 mL) was added dropwise at -78°C. The reaction mixture was slowly allowed to warm to 0°C and stirring was continued for 3 h; then the reaction mixture was treated with water (30 mL). The aqueous phase was extracted with Et_2O and the combined organic phases were extracted with 5% DL-tartaric acid aqueous solution until pH 4. The acid aqueous layer was made alkaline with 1 N NaHCO to pH 8, extracted with CH_2Cl_2 and concentrated in vacuum, yielding a white solid. The crude product was further purified by crystallization from methanol/water (8/2, v/v) and transformed into the salts **(*R,S*)-1·DL-tartrate** (molar ratio 1/1).

[(*R,S*)-1] 2-([1,1'-biphenyl]-4-yl)-4-(piperidin-1-yl)butan-2-ol: white solid (105 mg, 68%). Mp: 110 – 111°C. Rf: 0,46 (TLC: AcOEt/NH_3, 100/0,1, v/v); ^{1}H-NMR δ_H(400 MHz; DMSO) 1,40 (2H, bs, N(CH_2CH_2)CH_3), 1,44 (3H, s,CCH_3), 1,51 (4H, m, N(CH_2CH_2)2CH_2), 1,98 (2H, m, CH_2CH_2N), 2,25 (4H, m, N(CH_2CH_2)$_2CH_2$), 2,41 (2H, m, ArCH_2CH_2), 6,23 (1H, bs, OH), 7,38 (1H, m, aromatic), 7,54 (4H, m, aromatics), 7,69 (4H, m, aromatics).

2. Chiral Resolution of (*R,S*)-1

Analytical chiral resolution of **1** was performed via chiral high performance liquid chromatography (HPLC) using a Jasco (Cremella, LC, Italy) system equipped with a Jasco AS-2055 plus autosampler, a PU-2089 plus quaternary gradient pump, and a MD-2010 plus multiwavelength detector, and using the following columns: Chiralcel OJ-H (4.6 mm diameter x 150 mm length, 5µm), Chiralpak AS-H (4.6 mm diameter x 250 mm length, 5µm) and Chiralpak IC (4.6 mm diameter x 250 mm length, 5µm). Experimental data were acquired and processed by Jasco Borwin PDA and Borwin Chromatograph Software. Solvents used for enantioselective chromatography were HPLC grade and supplied by Carlo Erba (Milan, Italy). All HPLC analyses were performed at room temperature (rt). Sample solutions were prepared dissolving analyte in MeOH (c: 0.5 mg/mL) and filtering the solution through 0.45 µm PTFE membranes before analysis. The injection volume was 10 µL, the flow rate of 0.5 mL/min and the analysis were carried out by UV detector at 254 nm. The retention factor (k) was calculated using the equation $k = (t_R - t_0)/ t_{0,}$ where t_R is the retention time and t_0 the dead time (t_0 was considered to be equal to the peak of the solvent front and was taken from each particular run). The enantioselectivity (α) and the resolution factor (R_s) were calculated as follows: $\alpha = k_2 / k_1$ and $R_s = 2\ (t_{R2} - t_{R1}) / (w_1 + w_2)$ where t_{R2} and t_{R1} are the retention times of the second and the first eluted enantiomers, and w_1 and w_2 are the corresponding base peak widths.

Pure enantiomers of **1** were obtained by a semi-preparative process using a Chiralcel OJ-H column (10 mm diameter × 250 mm length, 5 µm), eluting with MeOH/diethylamine 100/0.1 (v/v) at rt with a flow rate of 3 mL/min (UV detector: 254 nm). Sample solutions were prepared dissolving analyte in MeOH (c: 3 mg/mL), filtered through 0.45 µm PTFE membranes before analysis, and the injection volume was 1 mL. The fractions were collected as reported in Figure S1. Analytical control of collected fractions was performed on Chiralcel OJ-H eluting with MeOH/diethylamine 100/0.1 (v/v), at a flow rate 0.5 mL/min, UV detector at 254 nm. The fractions obtained containing the enantiomers were evaporated at reduced pressure.

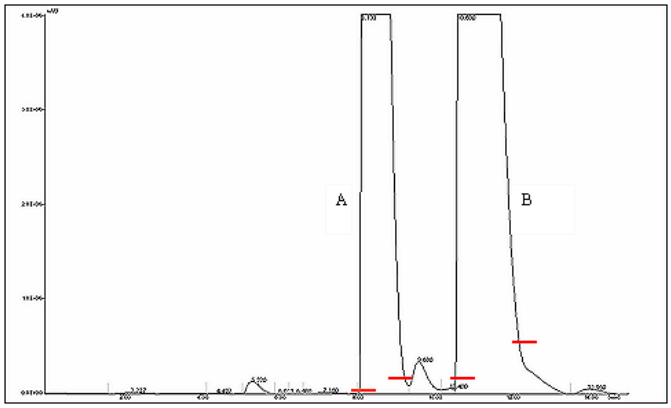

Fig. S1. Semi-preparative enantiomer separation of (*R,S*)-**1**. Chromatographic conditions: Chiralcel OJ-H (10 mm × 250 mm, 5 µm), MeOH/diethylamine 100/0.1 (v/v), flow rate: 3 mL/min, UV detector at 254 nm, injected amount 3 mg, cut points given by dashes (—).

3. Electronic Circular Dichroism

The solutions of (+)-**1** (c: 2.02x10^{-5}M in n-hexane, optical pathway 1 cm) and (-)-(*S*)-**2** (c: 2.5x10^{-5}M in n-hexane, optical pathway 1 cm) were analyzed in nitrogen atmosphere. ECD spectra were scanned at 50 nm/min with a spectral band width of 2 nm and data resolution of 0.5 nm (Fig.S2).

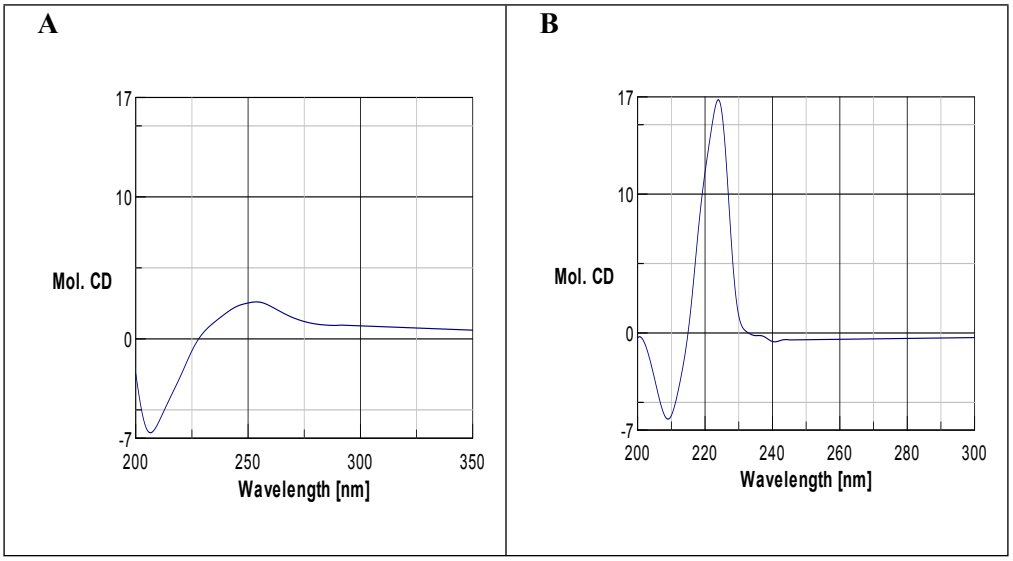

Fig. S2 CD curves of (+)-**1** (c: 2.02x10^{-5}M in n-hexane) (A), and (-)-(*S*)-**2** (c: 2.5x10^{-5}M in n-hexane) (B).

4. Molecular Modeling

The model structures of compounds **1-5** were sketched and geometrically optimized using Discovery Studio (DS, version 2.5, Accelrys, San Diego, CA). A conformational search was then carried out using a well-validated, ad hoc developed combined molecular mechanics/molecular dynamics simulated annealing (MDSA) protocol[S1a-b, S2, S3a-b] using Amber 12[S4] and the ff03 force field.[S5] The optimized compound structures were then docked into the σ_1 putative binding pocket by applying a consolidated procedure performed with Autodock 4.3/Autodock Tools 1.4.6[S6] on a win64 platform. For each compound only the molecular conformation satisfying the combined criteria of having the lowest (i.e., more favorable) Autodock energy and belonging to a highly populated cluster was selected to carry for further modeling.

Each ligand/receptor complex obtained from the docking procedure was further refined in Amber 12 using the quenched molecular dynamics (QMD) method. According to QMD, 1 ns MD simulations at 300 K were employed to sample the conformational space of each ligand/receptor complex in the GB/SA continuum solvation environment.[S7a-b] The integration step was equal to 1 fs. After each picosecond, each system was cooled to 0 K, and the structure was extensively minimized and stored. To prevent global conformational changes of the protein, the backbone atoms of the protein binding site were constrained by a harmonic force constant of 100 kcal/Å, whereas the amino acid side chains and the ligands were allowed to move without any constraint. The best energy configuration of each complex resulting from the previous step was subsequently solvated by a cubic box of TIP3P[S7] water molecules extending at least 10 Å in each direction from the solute. Each system was then neutralized and, furthermore, the solution ionic strength was adjusted to the physiological value of 0.15 M by adding the required amounts of Na^+ and Cl^- ions. Each solvated system was relaxed by 500 steps of steepest descent followed by 500 other conjugate-gradient minimization steps and then gradually heated to a temperature of 300 K in intervals of 50 ps of NVT MD, using a Verlet integration time step of 1.0 fs. The Langevin thermostat was used to control temperature, with a collision frequency of 2.0 ps^{-1}. The SHAKE method[S8] was used to constrain all of the covalently bound hydrogen atoms, while long-range nonbonded van der Waals interactions were truncated by using dual cutoffs 6 and 12 Å. The particle mesh Ewald (PME)[S9] was applied to treat long-range electrostatic interactions. The protein was restrained with a force constant of 2.0 kcal/(mol Å), and all simulations were carried out with periodic boundary conditions.

The density of the system was subsequently equilibrated via MD runs in the isothermal − isobaric (NPT) ensemble (with isotropic position scaling and a pressure relaxation time of 1.0 ps), for 50 ps with a time step of 1 fs. Each system was further equilibrated using NPT MD runs at 300 K, with a pressure relaxation time of 2.0 ps. Five equilibration steps were performed, each 2 ns long and with a time step of 2.0 fs. To check the system stability, the fluctuations of the root-mean-square deviation (rmsd) of the simulated position of the backbone atoms of the σ_1 receptor with respect to those of the initial protein were monitored. All chemico-physical parameters and rmsd values showed very low fluctuations at the end of the equilibration process, indicating that the systems reached a true equilibrium condition.

The equilibration phase was followed by a data production run consisting of 20 ns of MD simulations in the canonical (NVT) ensemble. Only the last 10 ns of each equilibrated MD trajectory were considered for statistical data collections.

The binding free energy, ΔG_{bind}, between all ligands and the σ_1 receptor was estimated by resorting to the MM/PBSA approach. According to this well-validated methodology[S3, S10a-b, S11a-e] the free energy was calculated for each molecular species (complex, receptor, and ligand), and the binding free energy was computed as the difference:

$$\Delta G_{bind} = G_{complex} - (G_{receptor} + G_{ligand}) = \Delta E_{MM} + \Delta G_{sol} - T\Delta S \quad \text{(Eq.1)}$$

The molecular mechanics energy ΔE_{MM} was calculated as the sum of the van der Waals and electrostatic interactions:

$$\Delta E_{MM} = \Delta E_{VDW} + \Delta E_{ELE} \quad \text{(Eq.2)}$$

The solvation free energy term ΔG_{sol} was composed of the polar and nonpolar contributions:

$$\Delta G_{sol} = \Delta G_{PB} + \Delta G_{NP} \quad \text{(Eq.3)}$$

ΔG_{PB} was estimated using DelPhi,[S12] which solves the Poisson−Boltzmann equations numerically and calculates the electrostatic energy according to the electrostatic potential. Dielectric constants of 1 and 80 were used for solute and solvent, respectively. A grid spacing of 0.5 per angstrom, extending 20% beyond the dimensions of the solute, was employed in these calculations.

The nonpolar solvation contribution was determined using the following relationship:[S13]

$$\Delta GNP = \gamma \times SA + \beta \quad \text{(Eq.4)}$$

in which γ = 0.00542 kcal/(mol Å^2), β = 0.92 kcal/mol, and SA is the molecular surface area estimated by means of the MSMS software.[S14]

The conformational entropy (translation, rotation, and vibration) upon ligand binding (−TΔS in Eq. (1)) was estimated using normal-mode analysis[S15] with the Nmode module of Amber 12. Prior to normal-mode calculations, each MD snapshot of each receptor/ligand complex was energy minimized using a distance-dependent

dielectric constant $\varepsilon = 4r_{ij}$ until the root-mean-square of the elements of the gradient vector was less that 10^{-4} kcal/mol Å. To minimize the effects due to different conformations adopted by individual snapshots, and due to the high computational demand of this approach, we averaged the estimation of entropy over MD 100 snapshots for each molecular complex that were evenly extracted from the last 10 ns of each corresponding MD trajectory.

The per residue binding free energy decomposition was performed exploiting the MD trajectory of each given compound/receptor complex, with the aim of identifying the key residues involved in the ligand-receptor interaction. This analysis was carried out using the MM/GBSA approach[S16] and was based on the same snapshots used in the binding free energy calculation.

All simulations were carried out using the Sander and Pmemd modules of Amber 12, running on the EURORA-CPU/GPU calculation cluster of the CINECA supercomputer facility (Bologna, Italy). The entire MD simulation and data analysis procedure was optimized by integrating Amber 12 in modeFRONTIER, a multidisciplinary and multiobjective optimization and design environment.[S17]

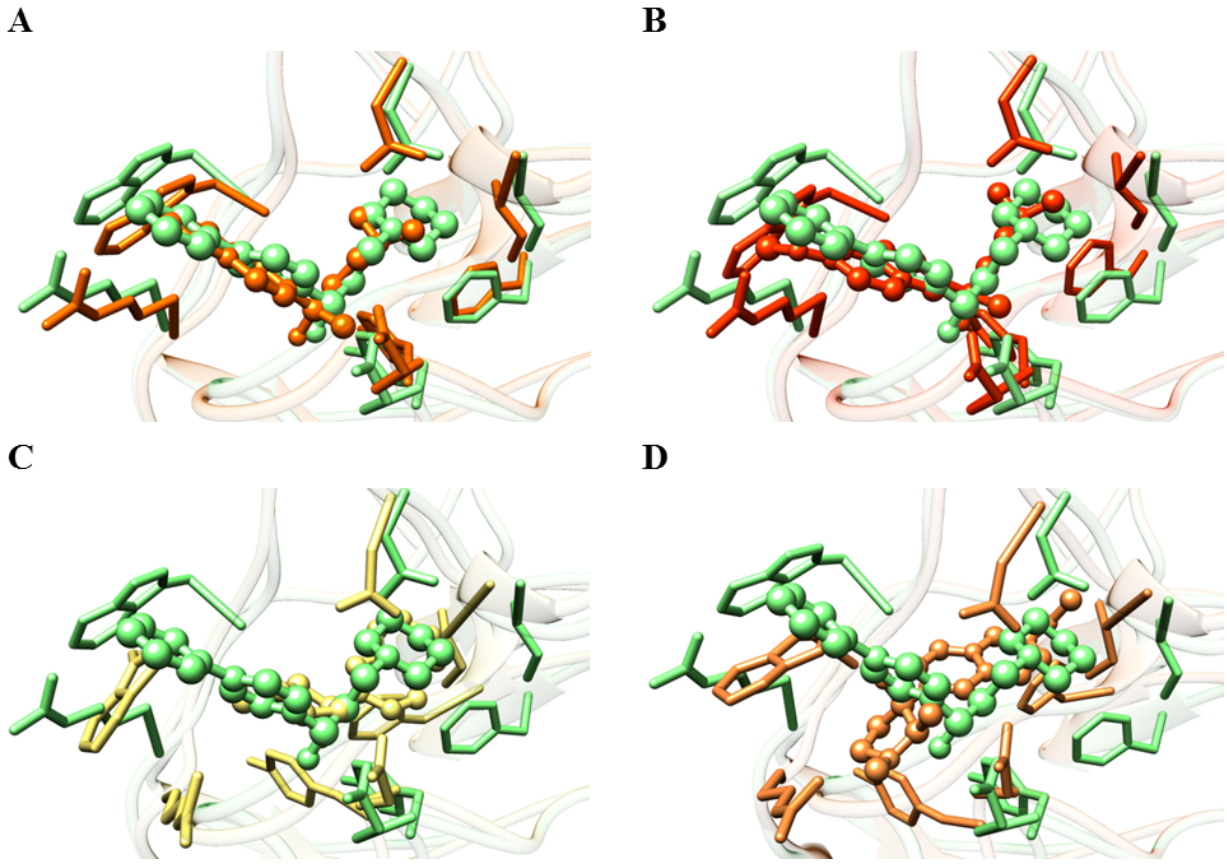

Fig. S3. Superposition of equilibrated MD snapshots of the s_1 receptor in complex with (S)-**1** (green) in comparison with (S)-**2** (**A**, orange), (S)-**3** (**B**, orange red), (2S,3R)-**4** (**C**, khaki) and (2S,3R)-**5** (**D**, sandy brown). Hydrogen atoms, water molecules, ions and counterions are omitted for clarity.

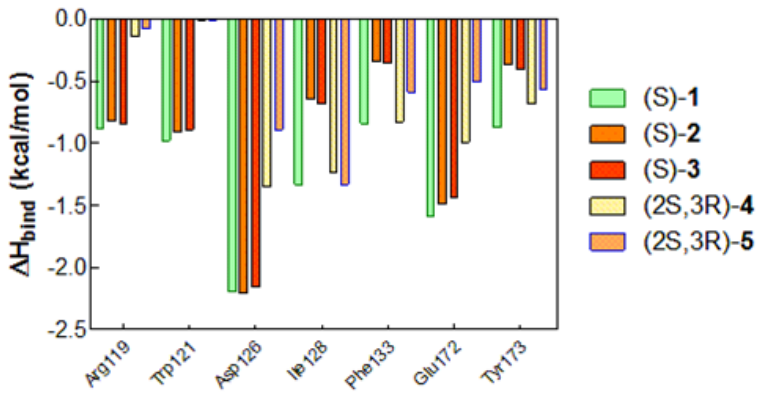

Fig. S4. Per residue energy decomposition for s_1 receptor in complex with (S)-**1** (green), (S)-**2** (orange), (S)-**3** (orange red), (2S,3R)-**4** (khaki) and (2S,3R)-**5** (sandy brown) showing those residues involved in key binding interactions.

5. Biological investigation

5.1 Binding Assays

Materials: Guinea pig brains for the σ_1 receptor binding assays were commercially available (Harlan–Winkelmann, Borchen, Germany). Homogenizer: Elvehjem Potter (B. Braun Biotech International, Melsungen, Germany) and Soniprep 150, MSE, London, UK). Centrifuges: Cooling centrifuge model Rotina 35R (Hettich, Tuttlingen, Germany) and High-speed cooling centrifuge model Sorvall RC-5C plus (Thermo Fisher Scientific, Langenselbold, Germany). Multiplates: standard 96-well multiplates (Diagonal, Muenster, Germany). Shaker: self-made device with adjustable temperature and tumbling speed (scientific workshop of the institute). Vortexer: Vortex Genie 2 (Thermo Fisher Scientific, Langenselbold, Germany). Harvester: MicroBeta FilterMate-96 Harvester. Filter: Printed Filtermat Type A and B. Scintillator: Meltilex (Type A or B) solid-state scintillator. Scintillation analyzer: MicroBeta Trilux (all PerkinElmer LAS, Rodgau-Jügesheim, Germany). Chemicals and reagents were purchased from various commercial sources and were of analytical grade.

Preparation of membrane homogenates from rat liver : Two rat livers (Sprague–Dawley rats) were cut into small pieces and homogenized with the potter (500–800 rpm, 10 up-and-down strokes) in six volumes of cold 0.32m sucrose. The suspension was centrifuged at 1200 g for 10 min at 4 °C. The supernatant was separated and centrifuged at 31 000 g for 20 min at 4 °C. The pellet was re-suspended in 5–6 volumes of buffer (50 mm Tris, pH 8.0) and incubated at RT for 30 min. After incubation, the suspension was centrifuged again at 31000 g for 20 min at 4 °C. The final pellet was re-suspended in 5–6 volumes of buffer and stored at - 80 °C in 1.5 mL portions containing ~2 (mg protein)mL^{-1}

Preparation of membrane homogenates from guinea pig brain cortex: Five guinea pig brains were homogenized with the potter (500–800 rpm, 10 up-and-down strokes) in six volumes of cold 0.32m sucrose. The suspension was centrifuged at 1200 g for 10 min at 4°C. The supernatant was separated and centrifuged at 23500 *g* for 20 min at 4°C. The pellet was re-suspended in 5–6 volumes of buffer (50 mm Tris, pH 7.4) and centrifuged again at 23500 *g* (20 min, 4 °C). This procedure was repeated twice. The final pellet was resuspended in 5–6 volumes of buffer and frozen (-80°C) in 1.5 mL portions containing ~1.5 (mg protein)mL^{-1}.

Protein determination: The protein concentration was determined by the method of Bradford[S18] modified by Stoscheck.[S19] The Bradford solution was prepared by dissolving 5 mg of Coomassie Brilliant Blue G 250 in 2.5 mL EtOH (95% v/v). Deionized H_2O (10 mL) and phosphoric acid (85% w/v, 5 mL) were added to this solution, and the mixture was stirred and filled to a total volume of 50 mL with deionized water. Calibration was carried out using bovine serum albumin as a standard in nine concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, and 4.0 mg mL^{-1}). In a 96-well standard multiplate, 10 mL of the calibration solution or 10 mL of the membrane receptor preparation were mixed with 190 mL of the Bradford solution. After 5 min, the UV absorption of the protein–dye complex at l=595 nm was measured with a plate reader (Tecan Genios, Tecan, Crailsheim, Germany).

General protocol for binding assays: The test compound solutions were prepared by dissolving ~10 mmol (usually 2–4 mg) of test compound in DMSO so that a 10 µM stock solution was obtained. To obtain the required test solutions for the assay, the DMSO stock solution was diluted with the respective assay buffer. The filtermats were presoaked in 0.5% aqueous polyethylenimine solution for 2 h at RT before use. All binding experiments were carried out in duplicate in 96-well multiplates. The concentrations given are the final concentrations in the assay. Generally, the assays were performed by addition of 50 µL of the respective assay buffer, 50 µL test compound solution at various concentrations (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}M), 50 µL of corresponding radioligand solution, and 50 µL of the respective receptor preparation into each well of the multiplate (total volume 200 µL). The receptor preparation was always added last. During the incubation, the multiplates were shaken at a speed of 500–600 rpm at the specified temperature. Unless otherwise noted, the assays were terminated after 120 min by rapid filtration using the harvester. During the filtration each well was washed five times with 300 mL of water. Subsequently, the filtermats were dried at 95°C. The solid scintillator was melted on the dried filtermats at 95°C for 5 min. After solidifying of the scintillator at RT, the trapped radioactivity in the filtermats was measured with the scintillation analyzer. Each position on the filtermat corresponding to one well of the multiplate was measured for 5 min with the [^{3}H]-counting

protocol. The overall counting efficiency was 20%. The IC50 values were calculated with GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA) by nonlinear regression analysis. The IC50 values were subsequently transformed into Ki values using the equation of Cheng and Prusoff.[S20] The Ki values are given as mean value ±SEM from three independent experiments.

σ_1 receptor binding assay: The assay was performed with the radioligand [^{3}H](+)-pentazocine (22.0 Ci mmol^{-1}; PerkinElmer). The thawed membrane preparation of guinea pig brain cortex (~100 mg protein) was incubated with various concentrations of test compounds, 2 nM [^{3}H](+)-pentazocine, and Tris buffer (50 mM, pH 7.4) at 37°C. The nonspecific binding was determined with 10 mM unlabeled (+)-pentazocine. The Kd value of (+)-pentazocine is 2.9 nM.

σ_1 receptor binding assay: The assays were performed with the radioligand [^{3}H]DTG (specific activity 50 Cimmol^{-1}; ARC, St. Louis, MO, USA). The thawed membrane preparation of rat liver (~100 mg protein) was incubated with various concentrations of the test compound, 3 nM [^{3}H]DTG, and buffer containing (+)-pentazocine (500 nM (+)-pentazocine in 50 mM Tris, pH 8.0) at RT. The non-specific binding was determined with 10 mM unlabeled DTG. The K_d value of [^{3}H]DTG is 17.9 nM.

5.2 NGF-induced neurite outgrowth in PC12 cells.

Cell culture: PC12 cells were cultured at 37°C, under 5% CO_2 in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), 10% heat-inactivated horse serum (HS), 1% Glutamax, 1% Zell (Biochrom). The medium was changed two or three times a week. When NGF with or without the test compounds had to be added, cells were detached from the culture dishes, centrifuged at 150 g for 5 min, re-suspended in RPMI 1640 medium containing 0.5% HS, 1% Glutamax, 1% Zell and plated at 8000 cells mL^{-1} in 24-well tissue culture plates coated with poly-D-lysine; 24 h after plating, the medium was replaced and NGF (2.5 ng mL^{-1}) was added with or without drugs. Stock solutions (10 mM) of compounds (*R,S*)-**1·DL-tartrate**, (*R*)-**1·L-tartrate** and (*S*)-**1·D-tartrate** were dissolved with apyrogenic H_2O to 1 mM solution and added to the cell medium to reach the selected final concentrations (0.25 µM, 2.5 µM, 5 µM). In some experiments, the well-characterized σ_1 receptor antagonist NE-100 was co-administered with (*R,S*)-**1·DL-tartrate**, (*R*)-**1·L-tartrate** or (*S*)-**1·D-tartrate** at a final concentration of 3µM.

Quantification of neurite outgrowth: five days after incubation with NGF (2.5 ng mL^{-1}) with or without drugs, PC12 cells were fixed at RT for 30 min in phosphate-buffered saline (PBS) containing 4% (w/v) paraformaldehyde. Morphometric analysis was performed on digitized images of fixed cells taken under phase-contrast illumination with a microscope (Optika) linked to a digital camera. Images of at least six fields per well were taken at 20 x magnification in order to count an

average of 300 cells. At least three independent experiments were performed for each condition, using different batches of PC12 cells. Neurite outgrowth was scored by measuring the percentage of differentiated cells bearing at least one neurite longer than the cell body diameter. Cell counting and neurite length measurements were performed in a blind manner by two independent observers using NeuronJ plugin[S21] of ImageJ public domain software.

Statistical analysis: Data are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed by two-way analysis of variance (ANOVA) followed by post hoc Bonferroni-Dunnett's test. Values of $p<0.05$ were considered statistically significant.

6. References

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