Supporting Information

Low Doses of Allyphenyline and Cyclomethyline, Effective Against Morphine Dependence, Elicit Antidepressant-like Effect

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Chemistry

General. Melting points were taken in glass capillary tubes on a Büchi SMP-20 apparatus and are uncorrected. IR and NMR spectra were recorded on Perkin-Elmer 297 and Varian Mercury 400 instruments, respectively. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). IR spectral data (not shown because of the lack of unusual features) were obtained for all compounds reported and are consistent with the assigned structures. Mass spectra were obtained using a Hewlett Packard 1100 MSD instrument utilizing electron-spray ionization (ESI). The microanalyses were performed by the Microanalytical Laboratory and the elemental composition of the compounds agreed to within ±0.4% of the calculated value. Optical rotation was measured at a 1 g/100 mL concentration (c = 1) with a Perkin-Elmer 241 polarimeter (accuracy ± 0.002°).All reactions were monitored by thin-layer chromatography (TLC) using silica gel plates (60 F254; Merck), visualizing with ultraviolet light Chromatographic separations were performed on silica gel columns (Kieselgel 40, 0.040-0.063 mm, Merck) by flash chromatography. Compounds were named following IUPAC rules as applied by ChemBioDraw Ultra (version 11.0)

software for systematically naming organic chemicals. The purity of the new compounds was determined by combustion analysis and was ≥ 95%.

Synthetic Procedures

Enantiomeric Separation of Racemic Compounds (±)-2.

The enantiomers of the title compound were separated by chiral HPLC by using a chiral column Chiralcel OD-H (25 cm x 0.46 cm, 5 μm particle size), mobile phase: *n*-hexane/2-propanol 90/10 v/v; flow rate 1 mL/min; detection was monitored at a wavelength of 220 nm: run time 15.0 min. Retention times: 5.9 min and 10.3 min for (+)-**2** and (-)-**2**, respectively; ee >99%. (+)-**2**: $[\alpha]^{20}_D$ = +19.28; (-)-**2**: $[\alpha]^{20}_D$ = -19.59. ^{1}H NMR ($CDCl_3$) δ 0.20 (m, 2, cyclopropyl), 0.53 (m, 2, cyclopropyl), 1.05 (m, 1, cyclopropyl), 1.60 (d, 3, C*H*$_3$CH), 2.56 (m, 2, C*H*$_2$), 3.45-3.76 (m, 4, NC*H*$_2$C*H*$_2$N), 5.05 (q, 1, C*H*CH$_3$), 6.92 (m, 2, Ar*H*), 7.18 (t, 1, Ar*H*), 7.26 (d, 1, Ar*H*), 8.01 (br s, 1, N*H*, exchangeable with D_2O). ^{13}C NMR ($CDCl_3$): δ 5.33, 12.24, 19.11, 37.28, 46.82, 68.41, 113.09, 122.34, 127.91, 130.36, 131.77, 132.85, 154.56, 165.33 ppm. MS (ESI): *m/z* 245.1 $[M+H]^+$. The free bases were transformed into the hydrogen oxalate salts, which were recrystallized from EtOH: mp 176-177 °C. Anal. ($C_{15}H_{20}N_2O.C_2H_2O_4$) C, H, N.

(*S*)-(+)-Methyl 2-(2-(cyclopropylmethyl)phenoxy)propanoate [(*S*)-(+)-3].

A solution of DIAD (3.01 g, 14.9 mmol) in dry THF (10 mL) was added dropwise to a mixture of methyl (*R*)-(+)-lactate (1.36 g, 13.1 mmol), 2-(cyclopropylmethyl)phenol[18] (1.88 g, 12.7 mmol), and triphenylphosphine (3.34 g, 12.7 mmol) in THF (20 mL). The mixture was stirred at room temperature under nitrogen atmosphere overnight. The solvent was evaporated, and diethyl ether/hexane solution was added. The precipitate triphenylphosphine oxide was filtered off, and the removal of the dried solvent gave a residue which was purified by flash chromatography eluting with cyclohexane/AcOEt (95:5): 59% yield; $[\alpha]^{20}_D$ = +8.82 (c 1,$CHCl_3$). ^{1}H NMR ($CDCl_3$) δ 0.20 (m, 2, cyclopropyl), 0.52 (m, 2, cyclopropyl), 1.03 (m, 1, cyclopropyl), 1.64 (d, 3, C*H*$_3$CH), 2.61 (d, 2, C*H*$_2$), 3.78 (s, 3, OC*H*$_3$), 4.79 (q, 1, C*H*CH$_3$), 6.64 (d, 1, Ar*H*), 6.93 (t, 1, Ar*H*), 7.16 (t, 1, Ar*H*), 7.31 (d, 1, Ar*H*). ^{13}C NMR ($CDCl_3$): δ 5.91, 11.36, 19.08, 37.68, 41.70, 75.41, 111.91, 122.68, 128.80, 131.12, 132.99, 133.77, 154.63, 171.27 ppm.

(*R*)-(-)-Methyl 2-(2-(cyclopropylmethyl)phenoxy)propanoate [(*R*)-(-)-3].

This was prepared as described for (*S*)-(+)-**3** starting from methyl (*S*)-(-)-lactate (81% yield): $[\alpha]^{20}_D$ = -8.54 (c 1,$CHCl_3$).

(*S*)-(+)-2-(1-(2-(Cyclopropylmethyl)phenoxy)ethyl)-4,5-dihydro-1H-imidazole [(*S*)-(+)-2].

A solution of ethylenediamine (0.47 mL, 6.5 mmol) in dry toluene (9 mL) was added dropwise to a stirred solution of 2M trimethylaluminum (3.5 mL, 6.5 mmol) in dry toluene (5 mL) at 0 °C in nitrogen atmosphere and stirred at room temperature for 1 h. The solution was cooled to 0°C and a solution of (*S*)-(+)-**3** (0.76 g; 3.5 mmol) in dry toluene (8 mL) was added dropwise. The reaction mixture was heated to 65°C for 3 h, cooled to 0°C, and quenched cautiously with MeOH (0.8 mL) followed by H_2O (0.25 mL). After addition of $CHCl_3$ (11.0 mL), the mixture was left for 30 min at room temperature to ensure the precipitation of the aluminum salts. The mixture was filtered and the organic layer was extracted with 2N HCl. The aqueous layer was made basic with 10% NaOH and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4, filtered, and evaporated to give an oil, which was purified by flash chromatography eluting with EtOAc/MeOH/33% NH_4OH (9:1:0.1) to give an oil: (0.42 g, 54% yield). The enantiomeric purity, determined by ^1H NMR on addition of chiral shift reagent (*S*)-(+)-2,2,2-trifluoro-1-(9-anthryl)-ethanol was about 85%. $[\alpha]^{20}_D$ = +13.42 (c 1, MeOH).

(*R*)-(-)-2-(1-(2-(Cyclopropylmethyl)phenoxy)ethyl)-4,5-dihydro-1H-imidazole [(*R*)-(-)-2].

This was prepared as described for (*S*)-(+)-**2** starting from (*R*)-(-)-**3** (49% yield). The enantiomeric purity, similarly determined, was about 85%. $[\alpha]^{20}_D$ = -12.99 (c 1, MeOH).

In vitro ADME profile and hERG activity

Solubility, Metabolic stability and Permeability assays were performed according to the previous reported methods.[9] Activity on human ether-a-go-go-related gene (hERG) K^+ channels was determined by electrophysiological recording of hERG currents in a KCNH2-stably expressing HEK293 cell line. For this purpose, the automated patch clamp platform Nanion PatchLiner, integrated with HEKA amplifier, was employed. After 2 or 3 days in culture, cells were resuspended in external solution and placed in a 4-chamber planar chip where every 10 seconds hERG currents were evoked by a three-steps pulse consisting of a 100 ms step to -40 mV, a conditioning prepulse (500 ms duration, +40 mV) followed by a test pulse (500 ms duration, -40 mV) from a holding potential of -80 mV. Compound effects on the hERG channel was examined following the alteration in the tail current amplitude after a 240 s period of incubation with increasing concentrations of the compound, and normalizing the recorded value against the current

recorded on the same cell at the end of each experiment after incubation with 500 nM of the known blocker E4013.

Biological Assays

Animals

Male CD-1 mice (Harlan SRC, Milan, Italy) weighing 25 g to 35 g were used. The mice were kept in a dedicated room, with a 12:12 h light/dark cycle (lights on at 09:00), a temperature of 20°C to 22°C, and a humidity of 45% to 55%. They were provided with free access to tap water and food pellets (4RF18, Mucedola, Settimo Milanese, Italy). Each mouse was used in only one experimental session. All of the procedures were conducted in adherence to the European Community Directive for the Care and Use of Laboratory Animals. Ethical guidelines for the investigation of experimental pain in conscious animals were followed. The procedures were carried out according to the EEC ethical regulations for animal research (ECC Council 86/609; D.Lgs. 27/01/1992, No. 116), and tests have been approved by the local ethical committee.

Chemicals and drugs

Morphine-hydrochloride was purchased from Salars (S.p.a, Como, Italy), yohimbine-hydrochloride, WAY 100635 and naloxone hydrochloride from Sigma (St. Louis, MO). Drugs were dissolved in distilled water immediately before use, with the exception of morphine-hydrochloride that was dissolved in saline. Compounds **1** and **2** and their enantiomers, yohimbine-hydrochloride (1.25 mg/kg) and naloxone (5 mg/kg) were injected intraperitoneally (i.p.), while morphine-hydrochloride (10 mg/kg) and WAY 100635 (0.1 mg/kg) was administered subcutaneously (s.c.).

Induction of morphine dependence

To induce morphine dependence, naïve mice were treated with morphine (10mg/kg; s.c.) twice daily at 12-h intervals for 6 days. Two hours after the last dose of morphine, the withdrawal syndrome (abstinence), as an index of morphine dependence, was precipitated by an i.p. injection of naloxone (5mg/kg).[28,29] The combination of morphine with high dose of naloxone on day 6 has been demonstrated to induce more severe symptoms, including autonomic signs, since naloxone precipitates dose-dependent withdrawal symptoms in animals acutely or chronically dependent upon morphine.[29] Ten minutes before naloxone treatment, the mice were placed in a transparent acrylic cylinder (20 cm diameter, 35 cm high) to habituate them to the new environment. Immediately after the naloxone challenge, each mouse was again placed gently into the cylinder, and then monitored for 15 min for the occurrence of withdrawal symptoms (jumping, rearing,

forepaw tremor, teeth chatter). To examine the effects of (±)-**2** and its enantiomers on morphine dependence, (±)-**2** (0.05 mg/kg), (*R*)-(-)-**2** and (*S*)-(+)-**2** (0.025 mg/kg) were given i.p., to one group of mice chronically treated 15 min prior to each morphine injection (acquisition; n = 40), or to another group of mice acutely treated 15 min before naloxone (expression; n = 32) as described above. In addition, the effects of **2** and its enantiomers alone on naloxone-induced withdrawal symptoms were examined in non-dependent mice (n = 48), where they received single or repeated administration of these compound or vehicle. The assessment of naloxone-precipitated withdrawal symptoms after the administration of (±)-**2**, (*R*)-(-)-**2** and (*S*)-(+)-**2** has been described above.

Antidepressant-like activity.

To evaluate the antidepressant-like activity, the forced-swimming test was used, the best recognized pharmacological model for assessing antidepressant-like activity in rodents.[30-32] The development of learned helplessness syndrome, when mice are placed in a cylinder filled with water that they cannot escape from, reflects the cessation of persistent escape-directed behavior, as seen by increased periods of immobility.[33] The reduction in immobility is considered as a behavioral profile that is consistent with an antidepressant-like action.[34]

Briefly, the animals were randomly divided into different groups, each of which received i.p. administration of vehicle, (±)-**1** (0.012, 0.025, 0.05, 0.2 and 0.5 mg/kg), (±)-**2** (0.025, 0.05, 0.2 mg/kg) or the reference drug fluoxetine (20 mg/kg). Moreover, related enantiomers (*R*)-(-)-**1**, (*S*)-(+)-**1**, (*R*)-(-)-**2** and (*S*)-(+)-**2** were tested at the dose of 0.025 mg/kg. Fifteen min later, the mice were placed individually in a glass cylinder (20 cm in height, 14 cm in diameter) filled to a 10 cm depth with water (23 ± 1 °C). At this water depth, the mice could touch the bottom of the jar with their tail, but they could not support themselves with their hind limbs. Each mouse was given a 6 min swimming test, and the duration of immobility was noted during the final 4 min interval of the test, since the first 2 min were used to allow the animals to familiarize themselves with the surroundings. All the swim-test sessions were recorded by a video camera positioned directly above the cylinder. Two experienced observers, who were blind to the treatment conditions, scored the videotapes. An immobility period was regarded as the time spent by the mouse floating in the water without struggling and while making only the very slight movements that are necessary to keep its head above the water. Following these swimming sessions, the mice were towel dried and returned to their housing. Each animal was tested only once.

Finally, to verify the involvement of the 5-HT_{1A}-R and α_{2C}-AR activation on the antidepressant-like effect of the enantiomers, WAY 100635 and yohimbine, 5-HT_{1A}-R and α_2-AR antagonists

respectively, were used. They were administered at the dose of 0.1 mg/kg, s.c. and 1.250 mg/kg, i.p. respectively 30 or 15 min before enantiomers injection.

Statistical analysis

All the results obtained from the different tests are presented as mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA), following a *post hoc* comparison Newman-Keuls test to determine differences between groups. Statistical significance was set at $p<0.05$.

Results

Effects of (±)-**2** *and its enantiomers on the expression of morphine dependence*

Figure 1 shows the effects of (±)-**2** on naloxone-precipitated withdrawal syndrome behavior. Repeated administration of morphine produced physical dependence, as assessed by a summary of characteristic set of behavioral responses, which included jumping, rearing, forepaw tremor and teeth chattering following the naloxone challenge, as compared to vehicle ($p<0.001$). As shown in Figure 1, acute administration of (±)-**2**, (*R*)-(-)-**2** and (*S*)-(+)-**2** 15 min prior to the naloxone injection significantly decreased both the number of jumping, a sign featuring the morphine withdrawal syndrome [($F(4,36) = 26.425$; $p<0.001$] (Figure 1A), that the frequencies of others withdrawal manifestations, reported as the sum of the frequency of rearing, forepaw tremor, teeth chattering [($F(4,36) = 15.660$; $p<0.001$] (Figure 1B) in morphine-dependent mice. Particularly, reduction of jumping by (±)-**2**, (*R*)-(-)-**2** and (*S*)-(+)-**2** was 60%, 75% and 55% respectively. No differences were seen for the control mice treated with (±)-**2** or its enantiomers (0.05 or 0.025 mg/kg, respectively), as compared with vehicle ($p>0.05$) (data not shown).

Effects of (±)-**2** *and its enantiomers on the acquisition of morphine dependence*

Figure 1 shows also the effects of repeated co-administration of (±)-**2**, (*R*)-(-)-**2** and (*S*)-(+)-**2** with morphine on the naloxone-precipitated withdrawal symptoms. Following the naloxone challenge, the mice that received repeated administrations of morphine showed severe signs of withdrawal as compared with the saline control group ($p<0.01$). Pretreatment of the mice with (±)-**2** and its enantiomers 15 min before each morphine injection significantly attenuated both the development of jumping [$F(7,64) = 42.190$; $p<0.001$] (Figure 1C) and the total signs of withdrawal [$F(7,64) = 16.856$; $p<0.001$] (Figure 1D). Particularly, jumping was reduced by (±)-**2** , (*R*)-(-)-**2** and (*S*)-(+)-**2** to about 55%, 62% and 56% respectively. The mice treated with only (±)-**2** or its enantiomers (0.05

or 0.025 mg/kg, respectively) did not show any significant differences in their withdrawal symptoms, as compared with the control group ($p>0.05$).

Antidepressant-like effect of (±)-**1** *and* (±)-**2** *on FST*

As shown in Figure 2A, in the forced-swimming test, administration of (±)-**1** in the range of 0.025-0.2 mg/kg resulted in significant reduction in the total duration of immobility. Particularly, the effect was greater in the group treated with the central dose of 0.05 mg/kg ($p<0.01$) and it was comparable to that induced by the reference drug fluoxetine (20 mg/kg). The analysis of variance confirmed the significant effects of (±)-**1** treatment [$F(6,52)=6.490$; $p<0.001$].

In the case of (±)-**2** the obtained results (Figure 2B) showed a tendency to decrease immobility time for all tested doses; however, only the reduction induced by the dose of 0.05 mg/kg was statistically significant [$F(4,35) = 2.692$; $p<0.05$]. This reduction, amounting to 50%, is comparable to that observed with the reference drug fluoxetine (20 mg/kg).

Antidepressant-like effect of (*R*)-(-)-**1**, (*S*)-(+)-**1**, (*R*)-(-)-**2** *and* (*S*)-(+)-**2** *on FST*

As shown in Figure 3, administration of (*S*)-(+)-**1**, (*R*)-(-)-**2** and (*S*)-(+)-**2 (**0.025 mg/kg) resulted in marked and statistically significant reductions in the total durations of immobility [$F(4,35)=6.940$; $p<0.001$]. Conversely, (*R*)-(-)-**1** was unable to reduce the immobility time in depressant-mice.

Influence of the 5-HT$_{1A}$R and α_2-AR antagonists on the antidepressant-like effect of (*S*)-(+)-**1**, (*R*)-(-)-**2** and (*S*)-(+)-**2** *on FST*

Results showed that both administration of the 5-HT$_{1A}$R antagonist WAY 100635 (0.1 mg/kg) [$F(7,57)=10.446$; $p<0.001$] and the α_2-AR antagonist yohimbine (1.25 mg/kg) [$F(7,58)=7.316$; $p<0.001$] similarly contrasted the antidepressant effect of (*S*)-(+)-**1**, (*R*)-(-)-**2** and (*S*)-(+)-**2** (0.025 mg/kg), when administered before their injections, while they were ineffective when administered alone (Figure 4A and 4B, respectively).

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Elemental Analysis of (*S*)-(+)-**2** and (*R*)-(-)-**2**

Compd	Formula	Calculated			Found		
		C%	H%	N%	C%	H%	N%
(*S*)-(+)-**2**	$C_{15}H_{20}N_2O.C_2H_2O_4$	61.07	6.63	8.38	61.12	6.58	8.22
(*R*)-(-)-**2**	$C_{15}H_{20}N_2O.C_2H_2O_4$	61.07	6.63	8.38	61.21	6.59	8.17