Supporting Information for

Might Adrenergic α_{2C}-Agonists/α_{2A}-Antagonists Become Novel Therapeutic Tools for Pain Treatment with Morphine? [1]

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[a] Abbreviations: IBS, imidazoline binding sites; α_2-ARs, α_2-adrenoreceptors; MR, molar refraction; CHO, chinese hamster ovary; DME, 1,2-dimethoxyethane; MPE, maximum possible effect.

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Chemical Methodology

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 EM-390 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 spectra data, not shown because of the lack of unusual features, were obtained for all compounds reported and are consistent with the assigned structures. The microanalyses were performed by the Microanalytical Laboratory of the Department of Chemical Sciences. The elemental composition of the compounds agreed to within ± 0.4% of the calculated value. Chromatographic separations were performed on silica gel columns (Kieselgel 40, 0.040-0.063 mm, Merck) by flash chromatography. The eluting solvent used for the imidazoline purification was a mixture of cyclohexane/EtOAc//MeOH/33%NH_4OH (7:3:1:0.1). The term "dried" refers to the use of anhydrous sodium sulphate. Compounds were named following the IUPAC rules proposed by Beilstein-Institut AutoNom (version 2.1), a software for systematic names in organic chemistry.

Biological Experiments

Culture of CHO clones expressing α_2-AR subtypes. The plasmids used to stably express the human α_{2A}-, α_{2B}- or α_{2C}-AR in CHO cells have been previously described.[19] Briefly, each vector contains an expression cassette comprising the CMV promoter/enhancer, the coding region of α_2C10-, α_2C2- or α_2C4-gene, the IRES derived from the encephalomyocarditis virus, the neomycin phosphotransferase gene, and a fragment from the rabbit β-globin gene containing an intron and a polyadenylation signal. Cells were transfected using the calcium-phosphate method and clones were selected in the presence of G418-sulfate (1 mg/mL). CHO-α_{2A}, CHO-α_{2B} and CHO-α_{2C} were routinely subcultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum. The clones used in this study respectively express 1.45 ± 0.3, 4.2 ± 0.8, and 2.7 ± 0.5 pmol of receptor /mg of membrane protein.

Binding experiments. Binding studies were performed on crude cell membrane preparations using the selective α_2-antagonist, [^{3}H]RX 821002, as radioligand. Briefly, membranes were incubated in a final volume of 400 μL of Tris-Mg buffer (50 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 7.5) containing the radioligand at a concentration corresponding to twice its *K*d value for the considered subtype (3 nM for CHO-α_{2A}, 10 nM for CHO-α_{2B} and 6 nM for CHO-α_{2C}) and increasing concentrations (10^{-9} to 10^{-4} M) of the competitor to be tested. After 30 minutes of incubation at room temperature, membrane bound radioactivity was collected by filtration through a fiberglass filter (Whatman GFC) using a Skatron Cell Harvester. Filters were rapidly washed, and transferred to scintillation vials, and counted for radioactivity. Inhibition data were analyzed using the GraphPad Prism computer program (GraphPad Software Inc., San Diego, CA, USA), allowing non-linear regression analysis according to a one- or a two-site inhibition model.

Binding to cloned human α_1-AR subtypes was performed in membranes from CHO cells transfected by electroporation with DNA expressing the gene encoding each α_1-AR subtype.

Cloning and stable expression of the human α_1-AR gene were performed as described in Ref. 20. CHO cell membranes (30 μg proteins) were incubated in 50 mM Tris-HCl, pH 7.4, with 0.1-0.4 nM [^{3}H]prazosin, in a final volume of 1.02 mL for 30 min at 25 °C, in absence or presence of competing drugs (1 pM-10 μM). Nonspecific binding was determined in the presence of 10 μM phentolamine. The incubation was stopped by addition of ice-cold Tris-HCl buffer and rapid filtration through 0.2% polyethyleneimine pretreated Whatman GF/B or Schleicher & Schuell GF52 filters.

Functional Assays. Cytosensor Microphysiometry. Extracellular acidification was measured using an eight-channel Cytosensor microphysiometry instrument (Molecular Devices, Menlo Park, CA). CHO cells expressing individually human α_2-AR subtypes or α_{1A}-AR were seeded into 12 mm capsule cups at a density of 3×10^5 cells/cup and incubated at 37 °C under 5% CO_2 atmosphere for 24 h. The capsule cups were loaded into the sensor chambers of the instrument and perfused with a running medium (bicarbonate-free DMEM containing 0.584 g/L glutamine and 2.59 g/L NaCl) at a flow rate of 100 µl/min. Agonists were diluted into running medium and injected through a second fluid path. Valves directed the flow from either fluid path to the sensor chamber. For each 90 s pump cycle, the pump was on for 60 s and was then switched off for the remaining 30 s, the pH value was recorded for 20 s (from second 68 to 88). Cells were exposed to agonists for 4 min and consecutive agonist exposures were separated by a 30-min washing period. This stimulation protocol was validated in preliminary experiments with four known agonists, (-)-noradrenaline, clonidine, UK 14304, and BHT 920 for α_2-AR CHO cells and (-)-noradrenaline and cirazoline for α_{1A}-AR CHO cells. The rate of acidification of the chamber was calculated by the Cytosoft program (Molecular Devices). Changes in the rate of acidification were calculated as the difference between the maximum effect after agonist addition and the average of three measurements taken prior to agonist addition. For antagonist studies, a control concentration-response curve was first obtained with clonidine and the cells were then exposed to antagonist for at least 30 min prior to construction of another clonidine concentration-effect curve in the presence of the antagonist. Therefore, each chamber acted as its own control. Data were analysed as the ability of the antagonist to shift the agonist concentration-effect curve and defined as K_b.

Statistical Analysis. The values of K_i and EC_{50} and the extent of maximal response (E_{max}) were calculated from the computer analysis of binding inhibition data and dose-response curves using the program GraphPad Prism (GraphPad Software, San Diego, CA). The K_b values were calculated as M/concentration ratio-1, where concentration ratio is the EC_{50} obtained in the presence of the antagonist divided by that obtained in the absence of the antagonist.[21] Data were expressed as pK_b [-log10(K_b)] and reported values are means ± SEM of three to six separate experiments.

Measurement of MAPK activation. CHO cells were seeded at a density of 8 x 10^5 cells/cm^2 in 60-mm culture dishes. One day post-seeding, cells were placed for 24 h in FCS-free DMEM. Cell layers were then exposed for 10 min to the compound to be tested, rapidly rinsed with ice-cold PBS and harvested in 0.3 mL of RIPA buffer (10 mM Tris-HCl pH 7.5, 1% NP-40, 1% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM Na-orthovanadate, 1 mM PMSF, and 0.5 mM aprotinin). Proteins were separated by SDS-PAGE and blotted onto a nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Bucks, UK). The active forms of MAPK were revealed by chemiluminescence (ECL Western Blotting Detection Reagents, Amersham Biosciences) using anti-phosphorylated Erk antibody (SantaCruz Biotechnologies, Santa Cruz, CA, USA). Equal protein loading was assessed by reprobing the blots with anti-Erk2 antibody (SantaCruz Biotechnologies) that do not distinguish phosphorylated and unphosphorylated forms of MAPKs.

In vivo Experiments

Animals. Male CD-1 mice (Harlan SRC, Milan, Italy), weighing 25–35 g were used. Animals were kept in a room with a 12:12 h light/dark cycle (lights on at 9:00 a.m.), a temperature of 20–22 °C and a humidity of 45–55%. They were offered free access to tap water and food pellets (4RF18, Mucedola, Settimo Milanese, Italy). The animals were used only in one experimental session. Animal testing was carried out according to the European Community Council Directive of 24

November 1986 (86/609/EEC).

Drugs. Compounds **8** and **11** were synthesized as reported in the text, while cirazoline was commercially available (Tocris). Morphine-hydrochloride was purchased from Salars (S.p.a, Como, Italy), and yohimbine-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. **8** (0.05, 0.2, 0.5 mg/kg), **11** (0.05, 0.2, 0.5 mg/kg), cirazoline (0.5 mg/kg), clonidine (0.5 mg/kg) and yohimbine-hydrochloride (1.250 mg/kg) were injected intraperitoneally (i.p.), with the exception of morphine-hydrochloride that was administered subcutaneously (s.c.) at the dose of 5.0 mg/kg. The dose of cirazoline and clonidine was chosen based on previous studies in which doses lower than 0.5 mg/kg were ineffective to increase morphine analgesia.

Nociceptive test. Nociception was evaluated by the radiant heat tail-flick test: briefly, it consists of the irradiation of the lower third of the tail with an I.R. source (Ugo Basile, Comerio, Italy). The basal pre-drug latency, ranged between 2-3 s, was calculated as the mean of two trials performed at 30 min interval. Then mice received tested-drugs or related vehicle, 15 min before morphine or saline administration. The antinociceptive activity was evaluated 30, 60, 90, 120, 240 min after morphine injection. A cut-off latency of 12 s was established to minimize tissue damage. In order to demonstrate the involvement of α_{2C}-AR subtype on the enhancement of morphine antinociception induced by **8** and **11**, the α_2-AR antagonist yohimbine was administered 15 min before the tested agonists and its effect evaluated as described above.

Pentobarbital sodium-induced sleep time. In order to rule out a possible sedative effect elicited by **8** or **11**, these compounds were evaluated on pentobarbital sodium-induced sleep time test, a classical test that is routinely used to evaluate general sedative activity of drugs. They were evaluated at the dose that evoked an increase of morphine analgesia comparable to that exhibited by clonidine. Briefly, groups of mice (n = 10) were injected i.p. with sodium pentobarbital (50 mg/kg) 15 min after **8** (0.05 mg/kg), **11** (0.05 mg/kg), clonidine (0.5 mg/kg) or vehicle injection. The time between losing and regaining righting reflex (duration of sleeping) was monitored (in minutes) for each animal.[22,23] Diazepam was used as sedative control drug.

All the experiments were replicated independently in separate rounds of animals and the results were registered by two observers who were blind to the treatment conditions.

Statistical analysis. Antinociceptive effect was expressed as a percent of the Maximum Possible Effect (MPE) according to the following formula: %MPE=(measured latency−basal latency) / (cut-off time−basal latency)×100%. Reported values are means ± S.E.M. and data were analyzed using two- or one-way analysis of variance (ANOVA) according to the test, followed by Student–Newman–Keuls *post-hoc* test for multiple comparisons. Statistical significance was set at $p<0.05$.

Results.

Effect of clonidine and cirazoline on morphine-induced analgesia. The analgesic effect of morphine (5 mg/kg, s.c.) was maximal 30 min after administration and then decreased progressively so that it became ineffective 4 h after injection. Pretreatment with either clonidine or cirazoline significantly increased the morphine-induced antinociception as confirmed by ANOVA [$F(2,22)=2.428$; $p<0.01$]. Post-hoc comparisons showed that this increase starts at 60 min and remains over the entire time course ($p<0.01$) (Figure 2A). Both drugs had no analgesic effect when given alone [$F(2,20)=1.425$; $p>0.05$] (data not shown).

Effect of **8** *on morphine-induced analgesia.* Compound **8** had no analgesic effect when given alone at all doses tested [$F(3,24)=0.930$; $p>0.05$] (Figure S2A), but significantly and dose-dependently increased the analgesic response to morphine [$F(3,25)=4.684$; $p<0.01$]. Post-hoc analysis confirmed that morphine-induced analgesia was significantly increased over the entire time course at all doses tested. Particularly, the lowest dose of 0.05 mg/kg of **8** showed a strong (MPE near to 100%) and long lasting effect (until 240 min) starting from 60 min ($p<0.01$) (Figure 2B).

Effect of **11** *on morphine-induced analgesia.* Compound **11** given alone exhibited a weak and dose-dependent analgesic effect [$F(3,24)=2.903$; $p<0.05$]. This effect occurred rapidly and was maximum 30 min after administration of all doses tested; it then decreased so that **11** became ineffective 2 h

after injection (Figure S2B). When administered before morphine, **11** dose-dependently increased opioid antinociception at all doses tested [$F(3,28)=22.008$; $p<0.01$]. Post-hoc analysis showed that this was a long-lasting effect, as it was still present after 120 min even at the lowest dose tested (Figure 2C). Particularly **11** at the highest doses (0.2 and 0.5 mg/kg) increased morphine-induced analgesia starting at 30 min ($p<0.01$) and over the entire time course with a MPE near to 100%. Notably, at the lowest dose of 0.05 mg/kg, **11** was effective to enhance morphine-induced analgesia starting at 60 min ($p<0.01$).

Effect of yohimbine on morphine-induced analgesia increased by **8** *and* **11**. Yohimbine had any analgesic effect when given alone ($p>0.05$).[24] However, previous treatment of mice with yohimbine significantly prevented the increase of morphine-induced analgesic effect by 0.05 mg/kg of **8** and **11**, when tested at 60 min [$F(5,32)=7.116$; $p<0.001$] (Figure S1).

Effect of **8** *and* **11** *on pentobarbital-induced sleeping time.* At tested dose, **8** and **11** were not able to increase the sleeping time induced by pentobarbital as compared to control ($p>0.05$). Conversely, both clonidine and diazepam significantly prolonged the sleeping time ($p<0.01$) (Figure 3).

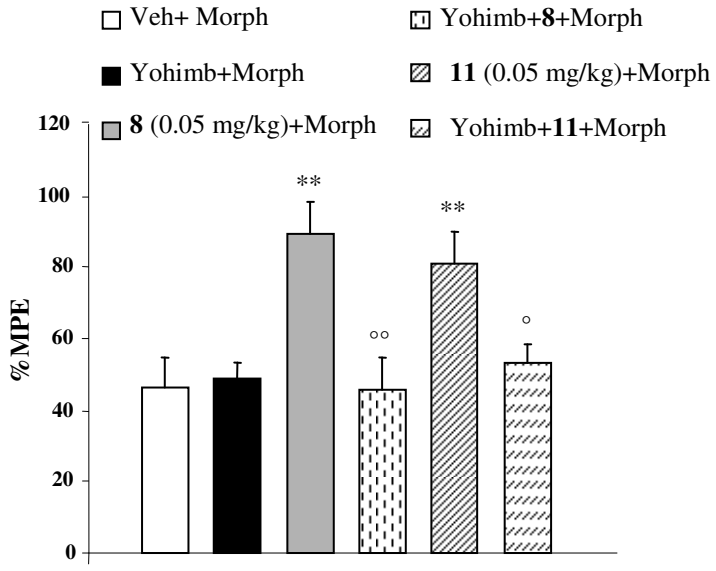

Figure S1. Effect of **8** and **11** on morphine-induced analgesia after yohimbine pretreatment in the tail flick test. The basal pre-drug latency, ranged between 2-3 s, was calculated as the mean of two trials performed at 30 min interval. Then mice received tested-drugs or related vehicle, 15 min before morphine or saline administration. Yohimbine was administered 15 min before α_{2C}-AR agonists. The antinociceptive activity was evaluated 60 min after morphine injection. A cut-off latency of 12 s was established to minimize tissue damage. Each column represents the media±S.E.M. of 6-8 animals. Significant differences: **$p<0.01$ compared to morphine group. °$p<0.05$; °°$p<0.01$ compared to related α_{2C}-AR agonists + morphine treated group.

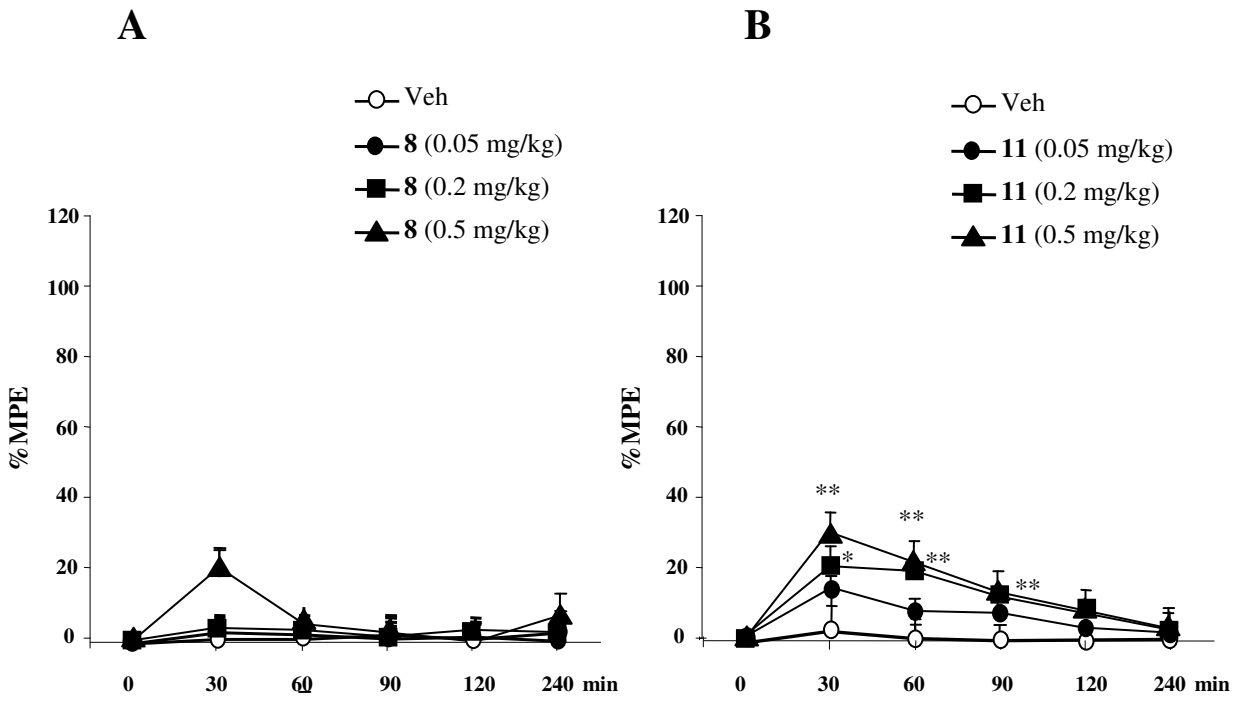

Figure S2. Antinociceptive effects of **8** (A) and **11** (B) in the tail flick test. The reaction latencies were expressed as a percent of the maximum possible effect (%MPE) Each mouse was tested 1 and 0.5 h before vehicle or tested compound to determine baseline latency. Then mice were i.p. administered with compounds **8** (0.05, 0.2, 0.5 mg/kg), **11** (0.05, 0.2, 0.5 mg/kg) or related vehicle. The antinociceptive activity was evaluated 30, 60, 90, 120, 240 min later. Each point represents the media±S.E.M. of 6-8 animals. Significant differences: $*p < 0.05$, $**p < 0.01$ compared to vehicle treated group; where not indicated, the difference was not statistically significant.

Table 2. Elemental Analysis for compounds **10**-**11**

Compd	Formula	Calculated			Found		
		C%	H%	N%	C%	H%	N%
10	$C_{14}H_{20}N_2O \cdot H_2C_2O_4$	59.61	6.88	8.69	59.44	6.70	8.51
11	$C_{14}H_{18}N_2O \cdot H_2C_2O_4$	59.99	6.29	8.74	59.81	6.10	8.83

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