Bioorganic & Medicinal Chemistry 18 (2010) 4975–4982

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Evaluation of N-substitution in 6,7-benzomorphan compounds

Lorella Pasquinucci [a,*], Orazio Prezzavento [a], Agostino Marrazzo [a], Emanuele Amata [a], Simone Ronsisvalle [a], Zafiroula Georgoussi [b], Danai-Dionysia Fourla [b], Giovanna M. Scoto [c], Carmela Parenti [c], Giuseppina Aricò [c], Giuseppe Ronsisvalle [a]

[a] Department of Pharmaceutical Sciences, Medicinal Chemistry Section, University of Catania, Viale A. Doria 6, 95125 Catania, Italy
[b] Laboratory of Cellular Signalling and Molecular Pharmacology, Institute of Biology, National Center for Scientific Research "Demokritos" Ag. Paraskevi 15310, Athens, Greece
[c] Department of Pharmaceutical Sciences, Pharmacology Section, University of Catania, Viale A. Doria 6, 95125 Catania, Italy

ARTICLE INFO

Article history:
Received 24 February 2010
Revised 27 May 2010
Accepted 4 June 2010
Available online 09 June 2010

Keywords:
6,7-Benzomorphan derivatives
μ/δ opioid agonist
cAMP accumulation assay
Tail-flick test

ABSTRACT

6,7-Benzomorphan derivatives, exhibiting different μ, δ, and κ receptor selectivity profiles depending on the N-substituent, represent a useful skeleton for the synthesis of new and better analgesic agents. In this work, an aromatic ring and/or alkyl residues have been used with an *N*-propanamide or *N*-acetamide spacer for the synthesis of a new series of 5,9-dimethyl-2′-hydroxy-6,7-benzomorphan derivatives (**12**–**22**). Data obtained by competition binding assays showed that the μ opioid receptor seems to prefer an interaction with the 6,7-benzomorphan ligands having an N-substituent with a propanamide spacer and less hindered amide. Highly stringent features are required for δ receptor interaction, while an *N*-acetamide spacer and/or bulkier amide could preferentially lead to κ receptor selectivity. In the propanamide series, compound **12** (named **LP1**) displayed high μ affinity (K_i = 0.83 nM), good δ affinity (K_i = 29 nM) and low affinity for the κ receptor (K_i = 110 nM), with a selectivity ratio δ/μ and κ/μ of 35.1 and 132.5, respectively. Further, in the adenylyl cyclase assay, **LP1** displayed a μ/δ agonist profile, with IC_{50} values of 4.8 and 12 nM at the μ and δ receptors, respectively. The antinociceptive potency of **LP1** in the tail-flick test after sc administration in rat was comparable with the potency of morphine (ED_{50} = 2.03 and 2.7 mg/kg, respectively), and was totally reversed by naloxone. **LP1**, possessing a μ/δ agonist profile, could represent a lead in further developing benzomorphan-based ligands with potent in vivo analgesic activity and a reduced tendency to induce side effects.

1. Introduction

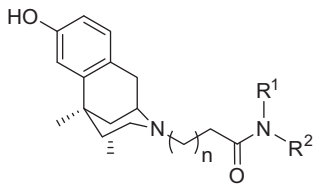
The opioid receptor system is regarded as one of the most important structures in nociception and antinociception. The distribution pattern of the opioid receptor system is closely related to the circuit that selectively controls nociceptive transmission.[1] Opioid agonists modulate neurons of this circuit by supporting a pain-inhibiting state; therefore, the treatment of moderate to severe pain is mainly based on opioid analgesics.[2,3] The opioid system comprises three classical receptor subtypes: μ, δ, and κ receptors, all members of the large G-protein-coupled-receptor family, whose genes are characterized by a high degree of sequence homology.[4]

Opioid receptors are the target of numerous ligands, including endogenous or synthetic peptides, opiates and semi-synthetic opioids, and synthetic derivatives with different structural scaffolds.[5] In the 6,7-benzomorphan class of ligands, the nature of the N-substituent is critical for the modulation of affinity and selectivity versus the μ, δ, and κ receptors as well as agonist and antagonist functional activity.[6] Variable length *N*-alkyl chains and the presence of polar groups or unsaturations in the N-substituent differentially affected the pharmacological profile of 5,9-dimethyl-2′-hydroxy-6,7-benzomorphans.[7–10] Moreover, a higher potency was displayed when an *N*-phenethyl substituent was used instead of *N*-methyl, while an *N*-benzyl substituent produced an inactive compound.[11] In spite of reported data, in the N-substituent the molecular interactions responsible for the in vitro and in vivo characteristics are not evident, and only a small amount of information is known about the N-substituent features required to obtain selectivity for a specific opioid receptor subtype.

An examination of the structures of endogenous opioid ligands and their synthetic analogs reveals that the Tyr^1 and Phe^3/Phe^4 residues are structural determinants for binding to opioid receptors. Their distances and reciprocal conformations deeply influence the affinity and selectivity for the different opioid receptor subtypes.[12–15] Assuming that the tyramine-like moiety of the benzomorphan nucleus interacts with opioid receptors in a similar mode to Tyr^1 of endogenous ligands, the introduction of a second aromatic ring in the N-substituent, and its localization with respect

* Corresponding author. Tel.: +39 095 7384273; fax: +39 095 222239.
E-mail address: lpasquin@unict.it (L. Pasquinucci).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.06.005



12-18 n = 1
R^1 = Ph, *c*-C_6H_{11}, Bn, CH_2-*c*-C_6H_{11}, *t*-Bu
R^2 = H, Me, Et

19-22 n = 0
R^1 = Ph, *c*-C_6H_{11}
R^2 = H, Me, Et

Figure 1. Structures of N-substituted 6,7-benzomorphan ligands **12**–**22**.

to the tyramine moiety, becomes crucial in achieving a determinate pharmacological profile. Therefore, we prepared compounds **12**–**22** by introducing different functional groups on the N-substituent of (−)-(1*R*,5*R*,9*R*)-5,9-dimethyl-2′-hydroxy-6,7-benzomorphan, such as an aromatic ring and/or alkyl residues linked by an *N*-propanamide or *N*-acetamide spacer (Fig. 1). By having rotatable groups on the N-substituent, these compounds could differentially bind opioid receptor subtypes.

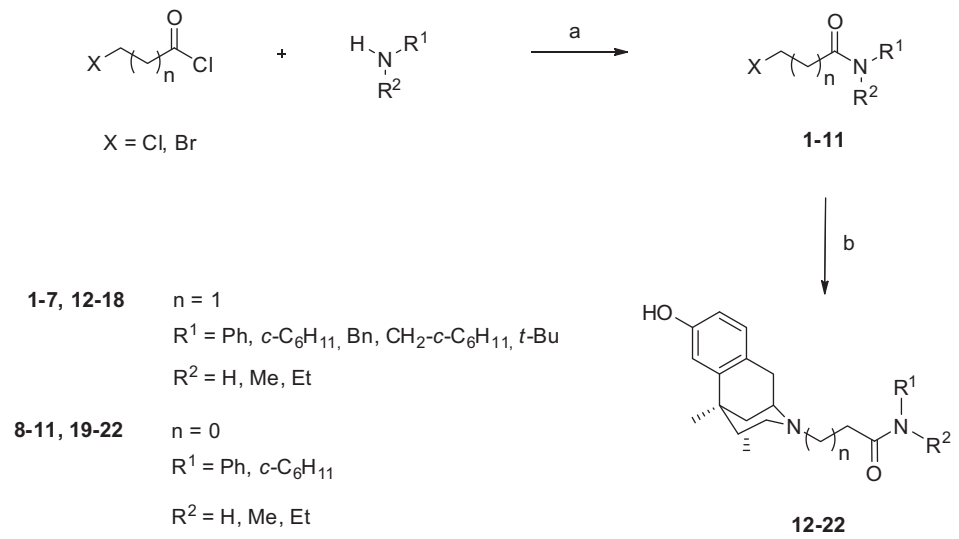
2. Chemistry

The synthesis of the *N*-benzomorphan compounds started by separating *cis*-(−)-(1*R*,5*R*,9*R*)-*N*-normetazocine from a racemic mixture, as previously reported.[16] Amides **1–8** and **9–11** were prepared following reported procedures by amine acylation in anhydrous tetrahydrofuran (THF) at 0 °C with 3-bromopropionyl chloride and 2-chloroacetyl chloride, respectively.[17] The target compounds **12**–**22** were obtained by alkylation of *cis*-(−)-(1*R*,5*R*,9*R*)-*N*-normetazocine with the respective amides **1–11** in *N*,*N*-dimethylformamide (DMF) at 50 °C in the presence of $NaHCO_3$ (Scheme 1).[17]

3. Results and discussion

3.1. Opioid receptor affinity

Opioid binding affinities of compounds **12–22** for the μ, δ, and κ receptors were determined by radioligand competition analysis using [^{3}H]DAMGO, [^{3}H]DPDPE, and [^{3}H]U69,593, respectively. These data, in comparison with standard compounds Tyr-D-Ala-Gly-*N*-MePhe-Gly-ol (DAMGO), Tyr-*c*(D-Pen-Gly-Phe-D-Pen (DPDPE), and U50,488 are summarized in Table 1. Compounds **12**–**18**, bearing an *N*-propanamide spacer, had affinity for the μ opioid receptor in the nanomolar range (K_i^{μ} = 0.83–372 nM). Compound **12** (named **LP1**), with a phenyl amide substituent, displayed the highest μ affinity, similar to that of reference compound DAMGO (K_i^{μ} = 0.83 and 0.87 nM, respectively). With respect to **LP1**, all other amide substituents led to reduced μ affinity. Changing the primary amide substituent from phenyl (**LP1**) to cyclohexyl (compound **13**) decreased μ affinity by more than 60-fold (K_i^{μ} = 56 nM). In compounds **14** and **15**, which had a secondary amide with R^1 = Me and Et, respectively, μ affinity decreased as a function of the steric bulk of the alkyl amide substituents (K_i^{μ} = 65 and 136 nM, respectively). On the other hand, attempts to increase the distance between aromatic/alicyclic amide substituents and the benzomorphan core (compounds **16** and **17**) failed to improve the μ affinity (K_i^{μ} = 105 and K_i^{μ} = 107 nM, respectively). Finally, the compound bearing a bulky *tert*-butyl substituent (**18**) displayed the lowest μ affinity (K_i^{μ} = 372 nM). Shortening of the spacer in the *N*-acetamide compounds (**19–22**) produced a significant loss of μ affinity relative to their *N*-propanamide homologs (**12–15**). With respect to **LP1**, compound **19** displayed a reduction in K_i values of almost two orders of magnitude (K_i^{μ} = 0.83 and K_i^{μ} = 722 nM, respectively). **LP1** also exhibited good δ opioid receptor affinity (K_i^{δ} = 29 nM), while all the other compounds had no δ affinity. All compounds displayed a low affinity for the κ receptor, with the partial exception of compound **15** (K_i^{κ} = 70 nM). Moreover, the binding data suggested a preference for the receptor κ of compounds having a shorter spacer (**20–22**) and/or a bulkier amide substituent (**15**, **21–22**), although the affinities were not relevant. In summary, the μ opioid receptor seems to prefer interaction with the 6,7-benzomorphan ligands having an N-substituent with a propanamide spacer and aromatic ring. Highly stringent features are required for δ receptor interaction, while an *N*-acetamide spacer and/or



X = Cl, Br

1-11

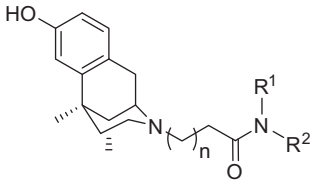
1-7, 12-18 n = 1
R^1 = Ph, *c*-C_6H_{11}, Bn, CH_2-*c*-C_6H_{11}, *t*-Bu
R^2 = H, Me, Et

8-11, 19-22 n = 0
R^1 = Ph, *c*-C_6H_{11}
R^2 = H, Me, Et

12-22

Scheme 1. The synthetic pathway to compounds **12**–**22**. Reagents and conditions: (a) DMAP, THF, 0 °C, 1 h; (b) *cis*-(−)-(1*R*,5*R*,9*R*)-*N*-normetazocine, $NaHCO_3$, KI, DMF, 50 °C, 4 h.

Table 1
Opioid receptor binding affinities of compounds **12–22**



Compound	*n*	R^1	R^2	K_i (nM) ± SEM[a,b]			K_i ratio	
				μ	δ	κ	δ/μ	κ/μ
12 (**LP1**)	1	H	Ph	0.83 ± 0.05	29.1 ± 1	110 ± 6	33.8	132.5
13	1	H	*c*-C_6H_{11}	56 ± 3	>5000	501 ± 25	>89.2	8.9
14	1	Me	Ph	65 ± 3	>5000	261 ± 14	>76.9	4.0
15	1	Et	Ph	136 ± 7	>5000	70 ± 4	>36.7	0.51
16	1	H	Benzyl	105 ± 6	>5000	237 ± 13	>47.6	2.2
17	1	H	CH_2-*c*-C_6H_{11}	107 ± 6	>5000	134 ± 7	>46.7	1.2
18	1	H	*t*-Bu	372 ± 18	>5000	422 ± 20	>13.4	1.1
19	0	H	Ph	722 ± 40	>5000	>5000	>6.9	>6.9
20	0	H	*c*-C_6H_{11}	2930 ± 161	>5000	612 ± 27	>1.7	0.20
21	0	Me	Ph	1370 ± 69	>5000	339 ± 17	>3.6	0.24
22	0	Et	Ph	1120 ± 66	>5000	335 ± 17	>4.4	0.29
DAMGO				0.87 ± 0.6	2670 ± 112	>5000		
DPDPE				2120 ± 112	2.72 ± 0.9	>5000		
U50,488				1750 ± 93	>5000	1.14 ± 0.7		

[a] Values are means ± SEM of three separate experiments, each carried out in duplicate.
[b] K_i values were obtained as [^{3}H]DAMGO displacement for the μ receptor, [^{3}H]DPDPE displacement for the δ receptor, and [^{3}H]U69,593 displacement for the κ receptor.

bulkier amide substituents could preferentially lead to κ receptor interactions.

The most promising compound, **LP1**, was further evaluated for its ability to inhibit adenylyl cyclase in HEK293 cells stably expressing either the μ or δ opioid receptor, and by the tail-flick test in male Sprague–Dawley rats in order to characterize its in vivo antinociceptive effects.

3.2. Adenylyl cyclase-mediated effects

Opioid receptors typically signal through coupling to G_i/G_o proteins to inhibit adenylyl cyclase.[18] To examine the functional role of **LP1** in opioid receptor signaling, the ability of **LP1** to affect forskolin-stimulated adenylyl cyclase activity in HEK293 cells stably expressing the μ or the δ opioid receptor was tested and the results are reported in Table 2. Treatment of HEK293 cells stably expressing the μ opioid receptor with **LP1** from 10 pM to 10 μM revealed a dose-dependent inhibition of cAMP accumulation, with an IC_{50} of 4.8 nM and an efficacy of 73%. The efficacy of **LP1** at inhibiting adenylyl cyclase was similar to that displayed by the μ selective agonist DAMGO (IC_{50} = 3.18 nM; I_{max} = 73%) (Fig. 2). To elucidate the effect of **LP1** at the δ opioid receptor, similar measurements of cAMP accumulation were performed in HEK293 cells stably expressing the δ opioid receptor. **LP1** from 100 pM to 10 μM revealed a dose-dependent inhibition of cAMP accumulation, with an IC_{50} of 12 nM and an efficacy of 83%. δ-Selective agonist DPDPE displayed a lower IC_{50} value of 0.14 nM, with a maximal inhibition of 69% (Fig. 3). **LP1** was approximately a 100-fold less potent than DPDPE at inhibiting cAMP accumulation. This data was unexpected compared to the binding values at the δ receptor, with **LP1** having only a 10-fold lower affinity with respect to DPDPE. Naloxone at 10 μM completely reversed **LP1**-mediated cAMP inhibition in HEK293 cells stably expressing both μ or δ opioid receptors (data not shown). These results suggest that **LP1** acts in vitro as a potent μ opioid agonist with a lower δ agonist activity.

Table 2
Measurements of adenylyl cyclase

Compound	IC_{50} (nM) ± SD[a,b]		I_{max} ± SD[c]	
	μ	δ	μ	δ
LP1	4.8 ± 0.5	12 ± 1.2	73 ± 3.8	83 ± 1.2
DAMGO	3.18 ± 0.3	ND[d]	73 ± 0.3	ND
DPDPE	ND	0.14 ± 0.03	ND	69 ± 0.7

[a] Agonist properties of compounds in the inhibition of forskolin-stimulated cAMP accumulation by μ and δ opioid receptors. The inhibition of cAMP accumulation was measured as described in Section 5.
[b] IC_{50} value is the concentration of the compound needed to produce half maximal inhibition, with all values presented as the average ± SD of triplicate determinations from four independent experiments.
[c] IC_{max} value is the maximal percent inhibition obtained with the compound.
[d] ND, not determined.

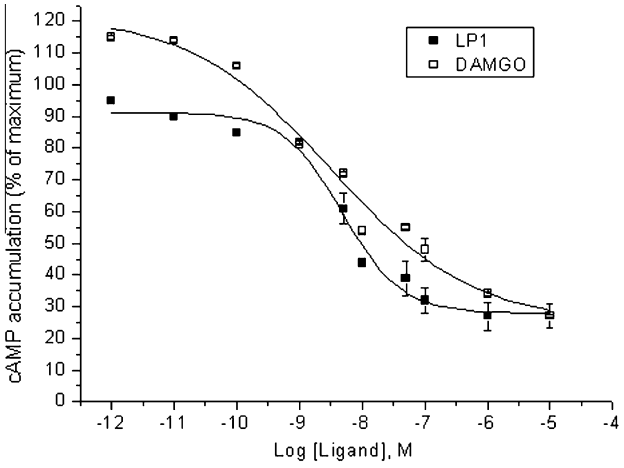


Figure 2. The effect of **LP1** on cAMP accumulation by the μ opioid receptor (■). HEK293 cells stably expressing the μ opioid receptor were labeled with [^{3}H]adenine (1.5 μCi/mL), and generation of [^{3}H]cAMP was measured in response to treatment with 50 μM forskolin. Forskolin-stimulated cAMP accumulation was also measured in the presence of various concentrations of the μ opioid receptor agonist DAMGO (□). The data are presented as cAMP accumulation (percentage of maximum) and represent the average ± SD of triplicate determinations from four independent experiments.

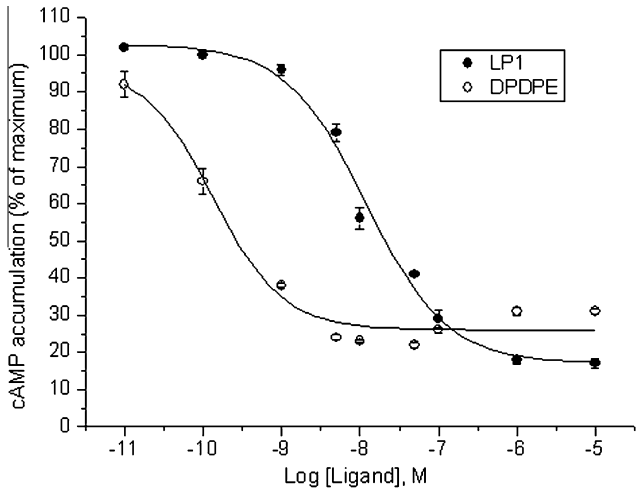


Figure 3. The effect of **LP1** on cAMP accumulation by the δ opioid receptor (●). HEK293 cells stably expressing the δ opioid receptor were labeled as described in Figure 2. Forskolin-stimulated cAMP accumulation was also measured in the presence of various concentrations of the δ opioid receptor agonist DPDPE (○). Data are presented as cAMP accumulation (percentage of maximum) and represent the average ± SD of triplicate determinations from five different experiments.

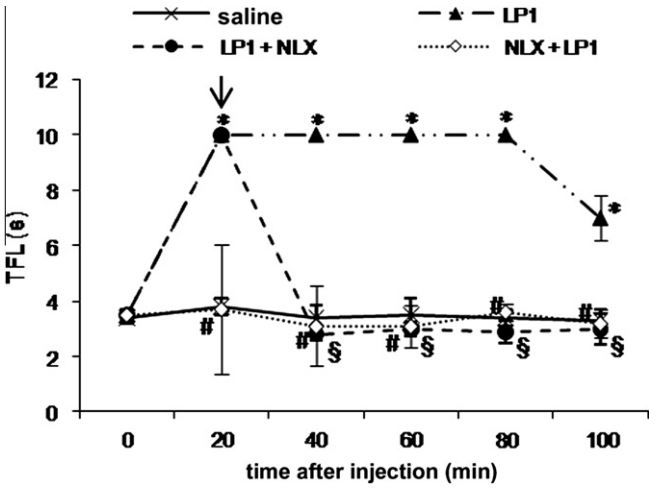


Figure 4. Naloxone (3 mg/kg sc) pre-treatment and post-treatment antagonizes the **LP1** (4 mg/kg sc) analgesic response. The arrow indicates the administration of naloxone at the peak of the **LP1** effect. Data are expressed as means ± SE (n = 8–10). Differences were considered significant when $p < 0.05$.

Table 3
ED_{50} values of **LP1** and morphine in the tail-flick test

Compound	ED_{50} mg/kg sc (C.L.$_{95\%}$)[a]
LP1 (2–5 mg/kg sc)	2.03 (1.53–2.53)
Morphine (1–10 mg/kg sc)	2.7 (2.56–2.94)

[a] ED_{50} value is the dose of the compound needed to produce half maximal effect. Confidence limits 95% are given in brackets.

3.3. Tail-flick test

The antinociceptive effect induced by subcutaneous (sc) administration of **LP1** using the tail-flick test in rats was evaluated. **LP1** produced a dose-dependent analgesic effect in the dose-range of 2–5 mg/kg. Table 3 shows the ED_{50} value of **LP1** with its confidence limits (C.L.$_{95\%}$) compared to the ED_{50} value of morphine. **LP1**, at a dose of 4 mg/kg sc, significantly increased the nociceptive latency, demonstrating a clear analgesic effect compared to the group of rats treated with saline ($^*p < 0.05$ vs saline-treated rats). The cut-off latency of 10 s, established as the maximal analgesic threshold, was reached 20 min after injection, and lasted until 80 min after treatment. Pretreatment with naloxone (3 mg/kg sc, 30 min prior to **LP1**) prevented the analgesic effect induced by **LP1** at a dose of 4 mg/kg sc (Fig. 4). The administration of naloxone at the peak of the **LP1** effect caused a fast decrease of tail-flick latencies (TFLs), as indicated by the arrow in Figure 4. All of these data established that the **LP1** analgesic effect was comparable to morphine and was opioid receptor-mediated.

4. Conclusion

In this study, our attention was focused on the evaluation of N-substitution in 6,7-benzomorphan compounds to extend the body of information about this structural region in the opioid receptor interaction. Collectively, the K_i values obtained from competition binding assays confirmed that the N-substituent nature influences the μ, δ, and κ receptor affinity and selectivity in a very variable way. We observed that an *N*-propanamide chain (compounds **12–18**) allows an improved μ affinity with respect to an *N*-acetamide chain (compounds **19–22**). Among compounds **12–18**, the optimal opioid binding affinities at both μ and δ receptors occurred with **LP1**, which has an *N*-propanamide spacer and a phenyl amide substituent. All the structural modifications evaluated reduced the μ affinity compared to **LP1**, while on the δ receptor, all attempted modulations induced a complete loss of affinity. Higher κ receptor selectivity was present in compounds with an *N*-acetamide spacer and/or bulkier amide substituent.

The in vivo tail-flick test, performed to assess the pharmacological properties of **LP1**, showed that **LP1** is a potent analgesic and that its effect is opioid receptor-mediated. This evidence, together with measurements of cAMP accumulation, suggests that **LP1** possesses a peculiar mixed μ/δ agonist profile. The relevance of simultaneous μ and δ agonist activation to produce analgesia with reduced tolerance is well established, and is important in producing useful drugs for chronic pain treatment.[19] Clinically used opioids exert their pain relief action mainly through the activation of the μ receptor. However, their prolonged administration produces tolerance to the analgesic effects, requiring escalating doses that are associated with side effects such as respiratory depression, constipation, and physical dependence, which limit their therapeutic potential.[20,21] In view of these findings, the mixed μ/δ agonist **LP1** could be useful as an analgesic agent for chronic pain treatment. Further, in vivo experiments are in progress to evaluate its tolerance-inducing capability.

5. Experimental

5.1. General

All commercial chemicals were used as received from Aldrich Chemical Co. unless otherwise specified. *cis*-(±)-*N*-Normetazocine was obtained from Fabbrica Italiana Sintetici. Melting points were determined in open capillary tubes with a Büchi 530 apparatus and are uncorrected. Analytical thin-layer chromatography was performed on silica gel 60 F_{254} aluminum sheets (Merck) with fluorescent indicator. Components were visualized by UV light (λ = 254 and 366 nm) and iodine vapor. Flash column chromatography was performed on Merck silica gel 60 (230–400 mesh). Optical rotations were determined in MeOH solution with a Perkin-Elmer 241 polarimeter. Infrared spectra were recorded on a 1600 FT-IR Perkin-Elmer instrument. ^1H and ^{13}C NMR spectra were routinely recorded at a constant temperature of 27 °C on a Varian Inova-200 spectrometer in $CDCl_3$ solution; chemical shifts δ are expressed in

ppm with reference to TMS as an internal standard. The data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constants (Hz) and number of protons. Unless otherwise specified, all final compounds were purified to ⩾95% purity, as determined by elemental analysis using a Carlo Erba analyzer. Receptor radioligands [^{3}H]DAMGO, [^{3}H]DPDPE, and [^{3}H]U69,593 were purchased from Perkin-Elmer Life Sciences. Some of the propanamide and acetamide compounds are known, although we report the experimental procedures and spectral characterization since they were prepared by different synthetic routes.

5.2. Compounds synthesis

5.2.1. General procedure for the preparation of 3-bromo-*N*-substituted- and 3-bromo-*N*,*N*-disubstituted propanamide derivatives (1–7)

To a solution of 3-bromopropionyl chloride (5.14 g, 3.02 mL, 30 mmol) in THF (10 mL) cooled at 0 °C and kept under vigorous stirring, a solution of the appropriate amine (20 mmol) and 4-(dimethylamino)pyridine (DMAP) (1.15 g, 9.4 mmol) in THF (20 mL) was added dropwise. After 1 h, the reaction mixture was quenched with H_2O and extracted with $CHCl_3$. The organic phase was washed with a saturated aqueous solution of $NaHCO_3$, dried over anhydrous Na_2SO_4 and evaporated. The crude product was purified by flash chromatography on silica gel using cyclohexane and ethyl acetate (AcOEt) as solvents.

5.2.1.1. 3-Bromo-*N*-phenylpropanamide (1). White solid (80%); mp 123–124 °C; IR (KBr, cm^{-1}): 1658; ^{1}H NMR ($CDCl_3$): δ 7.91 (s, 1H, exchangeable in D_2O), 7.54–7.50 (m, 2H), 7.37–7.26 (m, 2H), 7.16–7.09 (m, 1H), 3.71 (t, J = 6.6 Hz, 2H), 2.94 (t, J = 6.6 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 168.33, 137.36, 128.95, 124.69, 120.31, 40.38, 27.13. Anal. Calcd for $C_9H_{10}BrNO$: C, 47.39; H, 4.42; N, 6.14. Found: C, 47.55; H, 4.67; N, 6.10.

5.2.1.2. 3-Bromo-*N*-cyclohexylpropanamide (2). White solid (46%); mp 98–100 °C IR (KBr, cm^{-1}): 1640; ^{1}H NMR ($CDCl_3$): δ 5.47 (s, 1H, exchangeable in D_2O), 3.90–3.77 (m, 1H), 3.65 (t, J = 6.6 Hz, 2H), 2.71 (t, J = 6.6 Hz, 2H), 1.96–1.90 (m, 2H), 1.75–1.46 (m, 3H), 1.42–1.12 (m, 5H). ^{13}C NMR ($CDCl_3$): δ 168.57, 48.37, 39.85, 32.97, 27.78, 25.36, 24.71. Anal. Calcd for $C_9H_{16}BrNO$: C, 46.17; H, 6.89; N, 5.98. Found: C, 46.32; H, 6.95; N, 5.92.

5.2.1.3. 3-Bromo-*N*-methyl-*N*-phenylpropanamide (3). Colorless oil (79%); IR (neat, cm^{-1}): 1659; ^{1}H NMR ($CDCl_3$): δ 7.50–7.33 (m, 3H), 7.26–7.18 (m, 2H), 3.60 (t, J = 6.8 Hz, 2H), 3.30 (s, 3H), 2.65 (t, J = 6.8 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 169.99, 143.28, 129.91, 128.10, 127.35, 37.32, 37.07, 27.47. Anal. Calcd for $C_{10}H_{12}BrNO$: C, 49.61; H, 5.00; N, 5.79. Found: C, 49.75; H, 5.21; N, 5.63.

5.2.1.4. 3-Bromo-*N*-ethyl-*N*-phenylpropanamide (4). Colorless oil (70%); IR (neat, cm^{-1}): 1658; ^{1}H NMR ($CDCl_3$): δ 7.50–7.34 (m, 3H), 7.20–7.13 (m, 2H), 3.78 (q, J = 7.2 Hz, 2H), 3.59 (t, J = 7.0 Hz, 2H), 2.60 (t, J = 7.0 Hz, 2H), 1.13 (t, J = 7.2 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 170.04, 141.53, 129.80, 128.42, 128.19, 44.09, 37.35, 27.59, 27.95. Anal. Calcd for $C_{11}H_{14}BrNO$: C, 51.58; H, 5.51; N, 5.47. Found: C, 51.71; H, 5.53; N, 5.35.

5.2.1.5. *N*-Benzyl-3-bromopropanamide (5). Colorless oil (70%); IR (neat, cm^{-1}): 1639; ^{1}H NMR ($CDCl_3$): δ 7.32–7.25 (m, 5H), 6.30 (s, 1H, exchangeable in D_2O), 4.42 (d, 2H), 3.61 (t, J = 6.6 Hz, 2H), 2.75 (t, J = 6.6 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 169.60, 137.80, 128.64, 127.71, 127.49, 43.63, 39.41, 27.48. Anal. Calcd for $C_{10}H_{12}BrNO$: C, 49.61; H, 5.00; N, 5.79. Found: C, 49.78; H, 5.54; N, 5.67.

5.2.1.6. 3-Bromo-*N*-(cyclohexylmethyl)propanamide (6). Colorless oil (53%); IR (neat, cm^{-1}): 1640; ^{1}H NMR ($CDCl_3$): δ 5.88 (s, 1H, exchangeable in D_2O), 3.65 (t, J = 6.6 Hz, 2H), 3.15–3.13 (m, 2H), 2.76 (t, J = 6.6 Hz, 2H), 1.76–1.63 (m, 5H), 133–1.11 (m, 3H), 1.57–1.39 (m, 1H), 1.01–0.84 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 169.63, 45.86, 39.84, 37.81, 30.73, 27.75, 26.29, 25.73. Anal. Calcd for $C_{10}H_{18}BrNO$: C, 48.40; H, 7.31; N, 5.64. Found: C, 48.61; H, 7.44; N, 5.32.

5.2.1.7. 3-Bromo-*N*-(*tert*-butyl)propanamide (7). Colorless oil (40%); IR (neat, cm^{-1}): 1648; ^{1}H NMR ($CDCl_3$): δ 5.40 (s, 1H, exchangeable in D_2O), 3.63 (t, J = 6.6 Hz, 2H), 2.67 (t, J = 6.6 Hz, 2H), 1.38 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 168.23, 51.61, 40.42, 28.71, 27.90. Anal. Calcd for $C_7H_{14}BrNO$: C, 40.40; H, 6.78; N, 6.73. Found: C, 40.44; H, 6.69; N, 6.65.

5.2.2. General procedure for the preparation of 2-chloro-*N*-substituted- and 2-chloro-*N*,*N*-disubstituted acetamide derivatives (8–11)

By employing the previously-described procedure for the propanamide derivatives, the following compounds were also prepared.

5.2.2.1. 2-Chloro-*N*-phenylacetamide (8). White solid (96%); mp: 136–138 °C; IR (KBr, cm^{-1}): 1669; ^{1}H NMR ($CDCl_3$): δ 8.44 (s, 1H, exchangeable in D_2O), 7.55–7.54 (m, 2H), 7.50–7.27 (m, 2H), 7.16–7.09 (m, 1H), 4.16 (s, 2H). ^{13}C NMR ($CDCl_3$): δ 164.00, 136.86, 129.02, 125.09, 120.10, 42.91. Anal. Calcd for C_8H_8ClNO: C, 56.65; H, 4.75; N, 8.26. Found: C, 56.74; H, 4.91; N, 8.17.

5.2.2.2. 2-Chloro-*N*-cyclohexylacetamide (9). White solid (87%); mp: 113–115 °C; IR (KBr, cm^{-1}): 1648; ^{1}H NMR ($CDCl_3$): δ 6.40 (s, 1H, exchangeable in D_2O), 3.96 (s, 2H), 3.76–3.67 (m, 1H), 1.88–1.82 (m, 2H), 1.72–1.52 (m, 3H), 1.42–1.04 (m, 5H). ^{13}C NMR ($CDCl_3$): δ 164.77, 48.57, 42.66, 32.70, 25.33, 24.63. Anal. Calcd for $C_8H_{14}ClNO$: C, 54.70; H, 8.03; N, 7.97. Found: C, 54.85; H, 8.21; N, 7.78.

5.2.2.3. 2-Chloro-*N*-methyl-*N*-phenylacetamide (10). White solid (90%); mp: 77–79 °C; IR (KBr, cm^{-1}): 1680; ^{1}H NMR ($CDCl_3$): δ 7.47–7.42 (m, 3H), 7.28–7.23 (m, 2H), 3.86 (s, 2H), 3.32 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 166.16, 142.57, 129.98, 128.49, 126.96, 41.45, 37.89. Anal. Calcd for $C_9H_{10}ClNO$: C, 58.86; H, 5.49; N, 7.63. Found: C, 58.92; H, 5.55; N, 7.54.

5.2.2.4. 2-Chloro-*N*-ethyl-*N*-phenylacetamide (11). Colorless oil (93%); IR (neat, cm^{-1}): 1669; ^{1}H NMR ($CDCl_3$): δ 7.51–7.41 (m, 3H), 7.28–7.21 (m, 2H), 3.81 (s, 2H), 3.79 (q, J = 7.2 Hz, 2H), 1.15 (t, J = 7.2 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 165.59, 140.84, 129.91, 128.61, 128.11, 44.81, 41.91, 12.68. Anal. Calcd for $C_{10}H_{12}ClNO$: C, 60.76; H, 6.12; N, 7.09. Found: C, 60.82; H, 6.21; N, 7.00.

5.2.3. General procedure for the preparation of *N*-substituted *cis*-(−)-*N*-normetazocine derivatives (12–22)

A mixture of *cis*-(−)-(1*R*,5*R*,9*R*)-*N*-normetazocine (500 mg, 2.3 mmol), the appropriate amide (**1–11**, 3.45 mmol), $NaHCO_3$ (289.83 mg, 3.45 mmol) and a catalytic amount of KI was stirred in DMF (10 mL) at 50 °C for 4 h. After cooling, the reaction mixture was diluted with AcOEt (200 mL) and H_2O (30 mL). The organic layer was separated, washed with brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was purified by flash chromatography using $CHCl_3$ and CH_3OH as solvents. Compounds **12–22** were recrystallized from absolute ethanol to give white solids.

5.2.3.1. 3-[(2*R*,6*R*,11*R*)-8-Hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2*H*)-yl]-*N*-phenylpropanamide (12). White solid (86%); mp: 173–174 °C; $[\alpha]_D^{20}$ −50° (*c* 1.0, MeOH); ^{1}H NMR ($CDCl_3$): δ 11.27 (s, 1H), 7.58–7.54 (m, 2H), 7.38–7.26 (m, 2H), 7.13–7.10 (m, 1H), 6.97–6.93 (m, 1H), 6.78–6.77 (m, 1H), 6.69–6.63 (m, 1H), 3.07–2.53 (m, 6H), 2.51–2.49 (m, 2H), 2.27–2.13 (m, 1H), 1.97–1.77 (m, 2H), 1.40 (s, 3H), 1.50–1.25 (m, 1H), 0.89 (d, *J* = 5 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 170.67, 154.95, 142.08, 138.53, 129.00, 128.32, 126.41, 123.88, 119.75, 113.51, 112.39, 57.79, 50.31, 44.73, 41.40, 36.14, 32.52, 29.51, 25.21, 23.60, 14.01. Anal. Calcd for $C_{23}H_{28}N_2O_2$: C, 75.79; H, 7.74; N, 7.69. Found: C, 75.44; H, 7.56; N, 7.55.

5.2.3.2. *N*-Cyclohexyl-3-[(2*R*,6*R*,11*R*)-8-hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2*H*)-yl]propanamide (13). White solid (73%); mp: 152-154 °C; $[\alpha]_D^{20}$ −52° (*c* 1.0, MeOH); ^{1}H NMR ($CDCl_3$): δ 7.49 (s, 1H), 6.97–6.92 (m, 1H), 6.73–6.69 (m, 2H), 3.86–3.73 (m, 1H), 3.33–2.88 (m, 6H), 2.85–2.79 (m, 2H), 2.50–2.07 (m, 3H), 1.91–1.86 (m, 2H), 1.69–1.58 (m, 3H), 1.38 (s, 3H), 1.48–1.17 (m, 6H), 0.88 (d, *J* = 5 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 169.83, 155.68, 140.97, 128.49, 124.35, 114.15, 112.44, 59.23, 50.59, 48.39, 45.85, 39.27, 35.56, 32.85, 32.79, 31.76, 25.42, 24.73, 24.63, 23.63, 13.67. Anal. Calcd for $C_{23}H_{34}N_2O_2$: C, 74.56; H, 9.25; N, 7.56. Found: C, 74.88; H, 9.04; N, 7.54.

5.2.3.3. 3-[(2*R*,6*R*,11*R*)-8-Hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2*H*)-yl]-*N*-methyl-*N*-phenylpropanamide (14). White solid (82%); mp: 169–171 °C; $[\alpha]_D^{20}$ −56° (*c* 1.0, MeOH); ^{1}H NMR ($CDCl_3$): δ 7.44–7.31 (m, 3H), 7.17–7.14 (m, 2H), 6.89–6.85 (m, 1H), 6.68–6.56 (m, 2H), 3.26 (s, 3H), 2.99–2.53 (m, 5H), 2.36–2.32 (m, 3H), 2.05–1.87 (m, 1H), 1.86–1.67 (m, 2H), 1.27 (s, 3H), 1.21–1.17 (m, 1H), 0.78 (d, *J* = 5 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 172.04, 154.57, 143.66, 142.73, 129.81, 128.10, 127.88, 127.18, 126.40, 113.18, 112.23, 58.27, 51.10, 45.65, 41.44, 40.92, 37.39, 36.09, 32.25, 25.23, 23.58, 14.00. Anal. Calcd for $C_{24}H_{30}N_2O_2$: C, 76.16; H, 7.99; N, 7.40. Found: C, 76.45; H, 8.09; N, 7.65.

5.2.3.4. 3-[(2*R*,6*R*,11*R*)-8-Hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2*H*)-yl]-*N*-ethyl-*N*-phenylpropanamide (15). White solid (78%); mp: 156–158 °C; $[\alpha]_D^{20}$ −58° (*c* 1.0, MeOH); ^{1}H NMR ($CDCl_3$): δ 7.45–7.33 (m, 3H), 7.17–7.12 (m, 2H), 6.86–6.67 (m, 1H), 6.61–6.57 (m, 2H), 3.73 (q, *J* = 7.0 Hz, 2H), 2.90–2.56 (m, 5H), 2.31–2.16 (m, 3H), 2.05–1.78 (m, 3H), 1.28 (s, 3H), 1.21–1.18 (m, 1H), 1.11 (t, *J* = 7.0 Hz, 3H), 0.77 (d, *J* = 5 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 171.04, 154.89, 142.29, 141.76, 129.85, 128.27, 128.17, 125.99, 113.45, 112.31, 58.50, 50.93, 45.80, 44.25, 40.95, 40.43, 35.96, 32.12, 29.68, 25.09, 23.60, 13.94, 12.98. Anal. Calcd for $C_{25}H_{32}N_2O_2$: C, 76.50; H, 8.22; N, 7.14. Found: C, 76.40; H, 8.54; N, 7.34.

5.2.3.5. *N*-Benzyl-3-[(2*R*,6*R*,11*R*)-8-hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2*H*)-yl]propanamide (16). White solid (84%); mp: 162–164 °C; $[\alpha]_D^{20}$ −50° (*c* 1.0, MeOH); ^{1}H NMR ($CDCl_3$): δ 9.21 (s, 1H), 7.39–7.26 (m, 5H), 6.89–6.85 (m, 1H), 6.69–6.60 (m, 2H), 4.43 (q, *J* = 14.4 Hz, 1H), 4.40 (q, *J* = 14.4 Hz, 1H), 2.83–2.66 (m, 5H), 2.59–2.44 (m, 3H), 2.12–1.99 (m, 1H), 1.54–1.21 (m, 3H), 1.18 (s, 3H), 0.69 (d, *J* = 5 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 172.53, 154.73, 142.37, 138.29, 128.69, 128.14, 128.05, 127.47, 126.85, 113.25, 112.28, 57.70, 50.43, 44.39, 43.63, 41.44, 41.13, 36.02, 31.59, 25.02, 23.39, 13.89. Anal. Calcd for $C_{24}H_{30}N_2O_2$: C, 76.16; H, 7.99; N, 7.40. Found: C, 76.32; H, 7.67; N, 7.54.

5.2.3.6. *N*-(Cyclohexylmethyl)-3-[(2*R*,6*R*,11*R*)-8-hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2*H*)-yl]propanamide (17). White solid (77%); mp: 163–164 °C; $[\alpha]_D^{20}$ −56° (*c* 1.0, MeOH); ^{1}H NMR ($CDCl_3$): δ 8.76 (s, 1H), 6.94–6.89 (m, 1H), 6.76–6.75 (m, 1H), 6.68–6.63 (m, 1H), 3.15–3.09 (m, 2H), 3.08–2.57 (m, 6H), 2.43–2.40 (m, 2H), 2.19–2.05 (m, 1H), 1.77–1.73 (m, 7H), 1.43–1.18 (m, 4H), 1.35 (s, 3H), 1.12–0.85 (m, 3H), 0.87 (d, *J* = 5 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 172.99, 154.68, 142.45, 128.15, 127.15, 113.25, 112.30, 57.59, 50.62, 45.40, 44.58, 42.02, 41.93, 37.88, 36.31, 31.74, 30.96, 26.35, 25.85, 25.37, 23.33, 14.01. Anal. Calcd for $C_{24}H_{36}N_2O_2$: C, 74.96; H, 9.44; N, 7.28. Found: C, 75.03; H, 9.51; N, 7.47.

5.2.3.7. *N*-(*tert*-Butyl)-3-[(2*R*,6*R*,11*R*)-8-hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2*H*)-yl]propanamide (18). White solid (67%); mp: 157–159 °C; $[\alpha]_D^{20}$ −50° (*c* 1.0, MeOH); ^{1}H NMR ($CDCl_3$): δ 7.28 (s, 1H), 6.93–6.89 (m, 1H), 6.79–6.74 (m, 2H), 3.34–3.20 (m, 3H), 3.00–2.90 (m, 3H), 2.80–2.75 (m, 2H), 2.43–2.30 (m, 2H), 2.20–2.07 (m, 1H), 1.36, (s, 12H), 1.46–1.25 (m, 1H), 0.86 (d, *J* = 5 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 169.88, 155.79, 140.86, 128.55, 124.14, 114.26, 112.47, 59.46, 51.50, 50.60, 45.98, 39.53, 38.98, 35.55, 32.49, 28.68, 24.61, 23.77, 13.68. Anal. Calcd for $C_{21}H_{32}N_2O_2$: C, 73.22; H, 9.36; N, 8.13. Found: C, 73.11; H, 9.43; N, 8.31.

5.2.3.8. 2-[(2*R*,6*R*,11*R*)-8-Hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2*H*)-yl]-*N*-phenylacetamide (19). White solid (92%); mp: 186–188 °C; $[\alpha]_D^{20}$ −64° (*c* 1.0, MeOH); ^{1}H NMR ($CDCl_3$): δ 9.52 (s, 1H), 7.62–7.57 (m, 2H), 7.40–7.26 (m, 2H), 7.16–7.08 (m, 1H), 6.97–6.93 (m, 1H), 6.76–6.75 (m, 1H), 6.68–6.63 (m, 1H), 3.37 (d, *J* = 17 Hz, 1H), 3.12 (d, *J* = 17 Hz, 1H), 2.91–2.81 (m, 3H), 2.51–2.26 (m, 2H), 1.98–1.72 (m, 2H), 1.41–1.40 (m, 1H), 1.37 (s, 3H), 0.86 (d, *J* = 5 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 169.43, 154.34, 142.64, 137.54, 129.05, 128.33, 127.66, 124.24, 119.42, 113.25, 112.34, 59.46, 59.05, 46.31, 42.35, 42.24, 35.89, 26.08, 25.36, 14.04. Anal. Calcd for $C_{22}H_{26}N_2O_2$: C, 75.40; H, 7.48; N, 7.99. Found: C, 75.32; H, 7.12; N, 8.06.

5.2.3.9. *N*-Cyclohexyl-2-[(2*R*,6*R*,11*R*)-8-hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2*H*)-yl]acetamide (20). White solid (76%); mp: 156–158 °C; $[\alpha]_D^{20}$ −65° (*c* 1.0, MeOH); ^{1}H NMR ($CDCl_3$): δ 7.48 (d, 1H), 6.92–6.88 (m, 1H), 6.74–6.60 (m, 2H), 3.83–3.78 (m, 1H), 3.21 (d, *J* = 16.6 Hz, 1H), 2.97 (d, *J* = 16.6 Hz, 1H), 2.97–2.74 (m, 4H), 2.36–2.10 (m, 2H), 2.03–1.65 (m, 6H), 1.36 (s, 3H), 1.49–1.26 (m, 6H), 0.83 (d, *J* = 5 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 170.34, 154.51, 142.57, 128.17, 127.53, 113.26, 112.34, 58.86, 58.79, 47.26, 46.13, 42.27, 42.08, 35.92, 32.91, 25.71, 25.48, 25.33, 24.55, 14.16. Anal. Calcd for $C_{22}H_{32}N_2O_2$: C, 74.12; H, 9.05; N, 7.86. Found: C, 74.33; H, 9.01; N, 7.95.

5.2.3.10. 2-[(2*R*,6*R*,11*R*)-8-Hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2*H*)-yl]-*N*-methyl-*N*-phenylacetamide (21). White solid (90%); mp: 166–168 °C; $[\alpha]_D^{20}$ −63° (*c* 1.0, MeOH); ^{1}H NMR ($CDCl_3$): δ 7.46–7.30 (m, 3H), 7.27–7.21 (m, 2H), 6.82–6.78 (m, 1H), 6.70–6.68 (m, 1H), 6.61–6.56 (m, 1H), 3.28 (s, 3H), 3.96 (d, *J* = 14.8 Hz, 1H), 3.14 (d, *J* = 14.8 Hz, 1H), 2.62–2.54 (m, 3H), 2.44–2.38 (m, 1H), 2.05–1.93 (m, 1H), 1.79–1.64 (m, 2H), 1.26 (s, 3H), 1.21–1.14 (m, 1H), 0.72 (d, *J* = 5 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 170.71, 154.34, 143.75, 143.11, 129.41, 127.93, 127.83, 127.60, 127.18, 112.85, 112.24, 57.65, 57.01, 45.86, 41.82, 41.18, 37.65, 35.90, 25.31, 23.94, 13.93. Anal. Calcd for $C_{23}H_{28}N_2O_2$: C, 75.79; H, 7.74; N, 7.69. Found: C, 75.67; H, 7.66; N, 7.58.

5.2.3.11. 2-[(2*R*,6*R*,11*R*)-8-Hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2*H*)-yl]-*N*-ethyl-*N*-phenylacetamide (22). White solid (94%); mp: 152–154 °C; $[\alpha]_D^{20}$ −62° (*c* 1.0, MeOH); ^{1}H NMR ($CDCl_3$): δ 7.47–7.35 (m, 3H), 7.22–7.17 (m, 2H), 6.82–6.78 (m, 1H), 6.68–6.67 (m, 1H), 6.60–6.55 (m, 1H), 3.74 (q, *J* = 7.0 Hz, 2H), 3.09 (d, *J* = 14.8 Hz, 1H), 2.90 (d, *J* = 14.8 Hz, 1H), 2.60–2.53 (m, 3H), 2.44–2.38 (m, 1H), 2.02–1.91

(m, 1H), 1.79–1.65 (m, 2H), 1.26 (s, 3H), 1.21–1.18 (m, 1H), 1.11 (t, J = 7.0 Hz, 3H), 0.72 (d, J = 5 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 170.04, 154.18, 143.23, 142.00, 129.35, 128.39, 128.08, 127.99, 127.76, 112.83, 112.24, 57.68, 57.35, 45.88, 44.41, 41.88, 41.21, 35.95, 25.36, 23.99, 13.95, 12.99. Anal. Calcd for $C_{24}H_{30}N_2O_2$: C, 76.16; H, 7.99; N, 7.40. Found: C, 76.34; H, 8.13; N, 7.55.

5.3. Radioligand binding assay

Membranes were prepared from male Hartley guinea pigs (250–300 g) and Sprague–Dawley rats (200–250 g) (Morini, S. Polo d'Enza, RE, Italy). Binding to the μ and δ opioid receptors was carried out on rat membranes obtained from whole brain minus the cerebellum, as previously reported.[22] The μ binding assay was carried out as reported by Furness et al.[23] Briefly, membranes (1 mg/mL) were incubated in 50 mM Tris–HCl, pH 7.4, containing 100 mM choline chloride, 3 mM $MnCl_2$, 100 nM DPDPE to eliminate binding to δ opioid receptors and a protease inhibitor cocktail (100 μg/mL bacitracin, 10 μg/mL bestatin, 4 μg/mL leupeptin, and 2 μg/mL chymostatin). Incubation proceeded for 2 h at 25 °C with 2 nM [^{3}H]DAMGO. Nonspecific binding was defined in the presence of 1 μM unlabeled DAMGO. The δ binding assay was performed with 2 nM [^{3}H]DPDPE and rat brain membranes as reported above. Aliquots of the homogenates (1 mg/mL) were incubated under the same conditions with the exception of DAMGO in place of DPDPE to eliminate binding to the μ opioid receptor. Nonspecific binding was defined in the presence of 20 μM unlabeled DPDPE. The κ binding assay was performed on membranes obtained from guinea pig cerebella, prepared as previously described.[6] Aliquots of homogenates (1 mg/mL) were incubated in 50 mM Tris–HCl, pH 7.4, containing a protease inhibitor cocktail, 100 nM DPDPE and 100 nM DAMGO, with the latter two to eliminate binding to δ and μ opioid receptors, respectively. Incubation proceeded for 1 h at 25 °C with 2 nM [^{3}H]U69,593. Nonspecific binding was defined in the presence of 10 μM U50,488. The incubation was terminated by rapid filtration through Whatman GF/C glass filters presoaked in 0.1% polyethyleneimine solution for 1 h. The filters were washed twice with 4 mL of ice-cold 50 mM Tris–HCl buffer. After overnight incubation in 4 mL of Filter Count Cocktail (Packard), radioactivity retained on the filters was measured by liquid scintillation spectrometry using a 1414 Winspectral Perkin-Elmer Wallac. Competition inhibition constant (K_i) values were calculated with the EBDA/LIGAND program (Elsevier/Biosoft).[24] All values are presented as means ± SEM of three separate experiments, each carried out in duplicate.

5.3.1. cAMP accumulation assay

5.3.1.1. Cell culture. HEK293 cells stably expressing either the EE-tagged μ or δ opioid receptor were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin under 5% CO_2 at 37 °C.[25,26]

5.3.1.2. Measurements of cAMP accumulation. Measurements of adenylyl cyclase activity were performed as described by Merkouris et al.[27] Briefly, HEK293 cells stably expressing the μ or the δ opioid receptor were seeded in 12-well plates and incubated for 24 h in a medium containing [^{3}H]adenine (1.5 μCi/well). The generation of [^{3}H]cAMP was assessed in response to treatment of the cells with various concentrations of the appropriate ligand (1 pM–10 μM) using 50 μM forskolin at 37 °C for 30 min. Results were calculated as the ratio of levels of [^{3}H]cAMP to total [^{3}H]adenine nucleotides (×1000), and the data are presented as a percentage of forskolin-stimulated cAMP accumulation upon agonist treatment. The radioactivity was measured by liquid scintillation counting (Liquid Scintillation Analyzer, Packard). Values are means ± SD of triplicate determinations from four independent experiments. Analysis of the data was performed using Origin 7.5 software (OriginLab Corporation, Northampton, USA).

5.3.2. Antinociception

Male Sprague–Dawley rats (Morini, S. Polo d'Enza, RE, Italy) weighing 180–200 g were used. The animals were kept at a constant room temperature (25 ± 1 °C) under a 12:12 h light and dark cycle, with free access to food and water. Each rat was used for only one experiment. The antinociceptive response was evaluated by recording the latency with the radiant heat tail-flick test.[28] Briefly, it consisted of an irradiation of the lower third of tail with an IR source (Ugo Basile, Comerio, Italy). The day before the experiment, rats were habituated to the procedure for measuring the nociception threshold. The basal pre-drug latency was established between 3 and 4 s, and was calculated as the average of the first three measurements, which were performed at 5 min intervals. A cut-off latency of 10 s was established to minimize damage to the tail. Post-treatment tail flick latencies (TFLs) were determined at 20, 40, 60, 80, and 100 min after sc injection. The behavioral tests were conducted by researchers blinded to the treatment group. Data were expressed as means ± SE of the values recorded in animals of the same group. Intergroup comparisons were assessed using an initial two-way analysis of variance (ANOVA) followed by Duncan's multiple range post-hoc test. Differences were considered significant when $^*p < 0.05$.

The ED_{50} values were calculated from linear regression of the dose–effect functions, and means of the 95% confidence interval were obtained. Linear regression was determined using the GraphPad Prism program (GraphPad, San Diego, CA, USA). Experimental procedures were approved by the local ethical committee I.A.C.U.C. (Institutional Animal Care and Use Committee) and were conducted in accordance with International Guidelines as well as European Communities Council Directive and National Regulations (EEC Council 86/609 and DL 116/92).

Acknowledgments

This work was supported by a FP6 European Grant (LSHC-CT-2006-037733) and by the Italian MIUR (COFIN and FIRB Programs). The authors gratefully acknowledge Fabbrica Italiana Sintetici (Italy) for providing *cis*-(±)-*N*-normetazocine.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.005.

MOL files

The following ZIP file contains the MOL files of the most important compounds referred to in this article.

References and notes

1. Pan, H. L.; Wu, Z. Z.; Zhou, H. Y.; Chen, S. R.; Zhang, H. M.; Li, D. P. *Pharmacol. Ther.* **2008**, *117*, 141.
2. Geppetti, P.; Benemei, S. *Clin. Drug Invest.* **2009**, *29*, 3.
3. Trescot, A. M.; Datta, S.; Lee, M.; Hansen, H. *Pain Physician* **2008**, *11*, S133.
4. Waldhoer, M.; Bartlett, S. E.; Whistler, J. L. *Annu. Rev. Biochem.* **2004**, *73*, 953.
5. Aldrich, J. V.; Vigil-Cruz, S. C.. In *Burger's Medicinal Chemistry and Drug Discovery*; Abraham, D. J., Ed.; John Wiley & Sons: New York, 2003; Vol. 6, pp 329–481.
6. Ronsisvalle, G.; Pasquinucci, L.; Pappalardo, M. S.; Vittorio, F.; Fronza, G.; Romagnoli, C.; Pistacchio, E.; Spampinato, S.; Ferri, S. *J. Med. Chem.* **1993**, *36*, 1860.
7. May, E. L.; Aceto, M. D.; Bowman, E. R.; Bentley, C.; Martin, B. R.; Harris, L. S.; Medzihradsky, F.; Mattson, M. V.; Jacobson, A. E. *J. Med. Chem.* **1994**, *37*, 3408.

8. May, E. L.; Jacobson, A. E.; Mattson, M. V.; Traynor, J. R.; Woods, J. H.; Harris, L. S.; Bowman, E. R.; Aceto, M. D. *J. Med. Chem.* **2000**, *43*, 5030.
9. Aceto, M. D.; May, E. L.; Harris, L. S.; Bowman, E. R.; Cook, C. D. *Eur. J. Pharmacol.* **2007**, *566*, 88.
10. Metcalf, M. D.; Aceto, M. D.; Harris, L. S.; Woods, J. H.; Traynor, J. R.; Coop, A.; May, E. L. *Bioorg. Med. Chem.* **2008**, *16*, 869.
11. May, E. L.; Jacobson, A. E.; Mattson, M. V.; Coop, A.; Aceto, M. D.; Bowman, E. R.; Traynor, J. R.; Woods, J. H.; Harris, L. S. *Med. Chem. Res.* **1998**, *8*, 311.
12. Fichna, J.; do-Rego, J. C.; Chung, N. N.; Lemieux, C.; Schiller, P. W.; Poels, J.; Broeck, J. V.; Costentin, J.; Janecka, A. *J. Med. Chem.* **2007**, *50*, 512.
13. Li, T.; Shiotani, K.; Miyazaki, A.; Tsuda, Y.; Ambo, A.; Sasaki, Y.; Jinsmaa, Y.; Marezak, E.; Bryant, S. D.; Lazarus, H. L.; Okada, Y. *J. Med. Chem.* **2007**, *50*, 2753.
14. Purington, L. C.; Pogozheva, I. D.; Traynor, J. R.; Mosberg, H. I. *J. Med. Chem.* **2009**, *52*, 7724.
15. Ballet, S.; Feytens, D.; Wachter, R. D.; Vlaeminck, M. D.; Marczak, E. D.; Salvadori, S.; Graaf, C.; Rognan, D.; Negri, L.; Lattanzi, R.; Lazarus, L. H.; Tourwé, D.; Balboni, G. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 433.
16. Brine, G. A.; Berrang, B.; Hayes, J. P.; Carroll, F. I. *J. Heterocycl. Chem.* **1990**, *27*, 2139.
17. Ronsisvalle, G.; Marrazzo, A.; Prezzavento, O.; Pasquinucci, L.; Vittorio, F.; Pittalà, V.; Pappalardo, M. S.; Cacciaguerra, S.; Spampinato, S. *J. Med. Chem.* **1998**, *41*, 1574.
18. Megaritis, G.; Merkouris, M.; Georgoussi, Z. *Receptors Channels* **2000**, *7*, 199.
19. Negus, S. S.; Bear, A. E.; Folk, J. E.; Rice, K. C. *Eur. J. Pharmacol.* **2009**, *602*, 92.
20. Ossipov, M. H.; Lai, J.; King, T.; Vanderah, T. W.; Porreca, F. *Biopolymers* **2005**, *80*, 319.
21. Bailey, C. P.; Connor, M. *Curr. Opin. Pharmacol.* **2005**, *5*, 60.
22. Ronsisvalle, G.; Pasquinucci, L.; Pittalà, V.; Marrazzo, A.; Prezzavento, O.; Di Toro, R.; Falcucci, B.; Spampinato, S. *J. Med. Chem.* **2000**, *43*, 2992.
23. Furness, M. S.; Zhang, X.; Coop, A.; Jacobson, A. E.; Rothman, R. B.; Dersch, C. M.; Xu, H.; Porreca, F.; Rice, K. C. *J. Med. Chem.* **2000**, *43*, 3193.
24. McPherson, G. A. *J. Pharmacol. Methods* **1985**, *14*, 213.
25. Morou, E.; Georgoussi, Z. *J. Pharmacol. Exp. Ther.* **2005**, *315*, 1368.
26. Douris, V.; Swevers, L.; Labropoulou, V.; Andronopoulou, E.; Georgoussi, Z.; Iatrou, K. *Adv. Virus Res.* **2006**, *68*, 113.
27. Merkouris, M.; Mullaney, I.; Georgoussi, Z. *J. Neurochem.* **1997**, *69*, 2115.
28. Scoto, G. M.; Aricò, G.; Ronsisvalle, S.; Parenti, C. *Peptides* **2007**, *28*, 1441.