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Short communication

[Dmt[1], D-1-Nal[3]]morphiceptin, a novel opioid peptide analog with high analgesic activity

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ABSTRACT

The morphiceptin-derived peptide [Dmt[1], D-1-Nal[3]]morphiceptin, labeled μ-opioid receptor (MOP) with very high affinity and selectivity in the receptor binding assays. In the mouse hot plate test, [Dmt[1], D-1-Nal[3]]morphiceptin given intracerebroventricularly (i.c.v.) produced profound supraspinal analgesia, being approximately 100-fold more potent than the endogenous MOP receptor ligand, endomorphin-2. The antinociceptive effect of this new analog lasted up to 120 min. Thus, [Dmt[1], D-1-Nal[3]]morphiceptin is an interesting and extraordinarily potent analgesic, raising the possibility of novel approaches in the design of clinically useful drugs for pain treatment.

1. Introduction

Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2, EM-1) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2, EM-2) are two endogenous opioid peptides isolated from the mammalian brain which show remarkably high selectivity for the μ-opioid receptor (MOP receptor) [28]. Endomorphins exhibit pharmacological properties similar to those of morphine and also produce morphine-like undesirable side effects upon exogenous administration (for review see [10]).

In efforts to develop new candidate drugs with antinociceptive activity, numerous chemical modifications have been performed in order to improve the pharmacological profile of endomorphins [3,13,16,17,27,29]. Structurally related to EM-2 is morphiceptin (Tyr-Pro-Phe-Pro-NH_2), which does not occur in neuronal tissue but, like EM-2, displays morphine-like physiological activity and is very selective for the MOP receptor [4]. The two aromatic amino acids in EM-2 and morphiceptin, Tyr^1 and Phe^3, are important structural elements in the interaction with the receptor. Hansen et al. first showed that replacing Tyr^1 in the cyclic opioid peptide H-Tyr-c[D-Pen-Gly-Phe-D-Pen]NH_2 (DPDPE) with the more hydrophobic 2′,6′-dimethyltyrosine (Dmt) residue resulted in a compound with greatly increased MOP and DOP receptor binding affinities and much improved antinociceptive potency [12]. Subsequently, it was reported that substitution of Dmt for Tyr^1 in various MOP receptor agonist peptides or mixed MOP receptor agonist/DOP receptor antagonist peptides also greatly enhanced MOP receptor agonist potency and antinociceptive activity [2,5,11,19,22–26]. Furthermore, we showed that introduction of a D-1-naphthylalanine (D-1-Nal) residue in place of Phe^3 in

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morphiceptin increased MOP receptor binding affinity and associated functional bioactivity 40- and 30-fold, respectively, and elevated the *in vivo* activity in mice, as measured by the classical hot plate test of analgesia [6].

In the present study, [D-1-Nal[3]]analogs of morphiceptin and EM-2 were synthesized and further modified by substitution of a Dmt residue in position 1 in place of Tyr. The pharmacological profiles of the new analogs were determined in *in vitro* and *in vivo* assays.

2. Materials and methods

2.1. Materials

Morphiceptin, EM-2, and their analogs were synthesized by standard solid phase procedures as described before [8]. [^{3}H]DAMGO and [^{3}H]naltrindole were purchased from PerkinElmer-NEN Life Science Products (Paris, France).

2.2. Methods

2.2.1. Opioid receptor binding assays

The MOP and DOP receptor binding studies were performed according to the modified method described earlier [9]. Crude membrane preparations, isolated from Wistar rat brains, were incubated at 25 °C for 120 min with appropriate concentration of a tested peptide in the presence of either 0.5 nM [^{3}H]DAMGO or 0.5 nM [^{3}H]naltrindole in a total volume of 0.5 ml of 50 mM Tris–HCl (pH 7.4), containing $MgCl_2$ (5 mM), BSA (1 mg/ml), bacitracin (50 μg/ml), bestatin (30 μg/ml), and captopril (10 μM). Non-specific binding was determined in the presence of 10 μM naloxone. Incubations were terminated by rapid filtration through Whatman GF/B (Brentford, UK) glass fiber strips, which had been presoaked for 2 h in 0.5% polyethylamine, using Sampling Manifold (Millipore, Billerica, MA, USA). The filters were washed three times with 4 ml of ice-cold Tris buffer solution. The bound radioactivity was measured in a Tri–Carb 2100 TR liquid scintillation counter (Packard, Ramsey, MN, USA) after overnight extraction of the filters in 4 ml of Ultima Gold scintillation fluid (PerkinElmer, Wellesley, MA, USA). Three independent experiments for each assay were carried out in duplicate.

2.2.2. In vitro bioassays

The GPI and MVD assays were performed as described previously [23].

2.2.3. Antinociception studies

The hot plate test in mice was performed as described previously [6], according to the European Communities Council Directive from 24 November 1986 (86/609/EEC) and were conducted by authorized investigators.

Table 1 – Physicochemical data of morphiceptin and endomorphin-2 analogs

No.	Sequence	HPLC t_r[a] (min)	FAB-MS			Purity (%)
			Formula	MW	$[M + H]^+$	
1	Tyr-Pro-Phe-Pro-NH_2 (morphiceptin)	8.15	$C_{28}H_{35}N_5O_5$	521	522	98
2	Tyr-Pro-D-1-Nal-Pro-NH_2	11.36	$C_{32}H_{37}N_5O_5$	571	572	97
3	Dmt-Pro-D-1-Nal-Pro-NH_2	14.86	$C_{34}H_{41}N_5O_5$	599	600	97
4	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)	12.51	$C_{32}H_{37}N_5O_5$	571	572	98
5	Tyr-Pro-D-1-Nal-Phe-NH_2	16.05	$C_{36}H_{39}N_5O_5$	621	622	98
6	Dmt-Pro-D-1-Nal-Phe-NH_2	17.06	$C_{38}H_{43}N_5O_5$	649	650	97

[a] HPLC elution on a Vydac C_{18} column (0.46 cm × 25 cm, 5 μm) using the solvent system of 0.1% TFA in water (A)/80% acetonitrile in water containing 0.1% TFA (B) and a linear gradient of 20–80% solvent B over 25 min at flow rate of 1 ml/min.

Table 2 – Competitive binding and functional assay data obtained with morphiceptin and endomorphin-2 analogs

No.	Sequence	IC_{50} (nM)				
		MOP[a]	DOP[b]	DOP/MOP	GPI	MVD
1	Tyr-Pro-Phe-Pro-NH_2 (morphiceptin)	19.8 ± 0.9	>1000	>50.5	318 ± 22[c]	4800 ± 440[c]
2	Tyr-Pro-D-1-Nal-Pro-NH_2	0.47 ± 0.05	>1000	>2127	9.57 ± 1.01[c]	35.4 ± 2.3[c]
3	Dmt-Pro-D-1-Nal-Pro-NH_2	0.000549 ± 0.000145	132 ± 31	>13000	0.45 ± 0.06	0.64 ± 0.06
4	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)	0.79 ± 0.05	>1000	>1265	7.71 ± 1.47	15.3 ± 1.8
5	Tyr-Pro-D-1-Nal-Phe-NH_2	22.3 ± 0.9	>1000	>44.8	872 ± 90	2040 ± 290
6	Dmt-Pro-D-1-Nal-Phe-NH_2	0.93 ± 0.13	>1000	>1075	1.53 ± 0.27	1.63 ± 0.09
	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 (deltorphin-II)	–	0.56 ± 0.05	–	–	–
	[Leu[5]]enkephalin	–	–	–	246 ± 39	11.4 ± 1.1

All values are expressed as mean ± S.E.M. of three to six determinations.
[a] Determined against [^{3}H]DAMGO.
[b] Determined against [^{3}H]naltrindole.
[c] Data from Ref. [6].

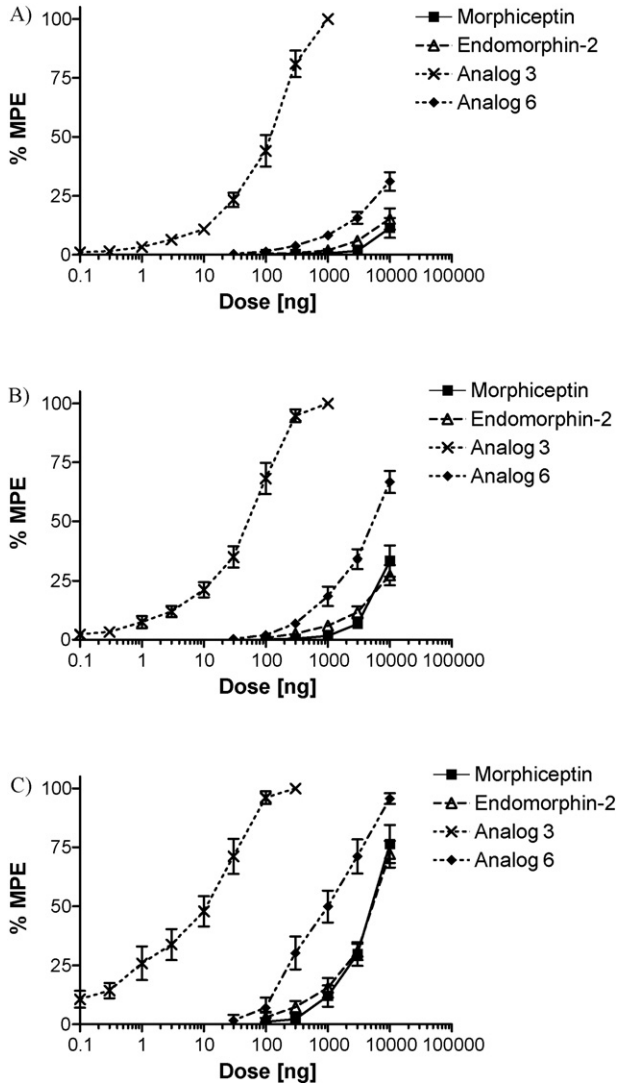

Fig. 1 – Dose–response curves determined in the hot plate test for the inhibition of paw licking (A), rearing (B), and jumping (C) induced by i.c.v. injection of morphiceptin, EM-2, [Dmt^1, D-1-Nal^3]morphiceptin (analog 3), and [Dmt^1, D-1-Nal^3]EM-2 (analog 6). The data represent the mean ± S.E.M. of 10 mice per group.

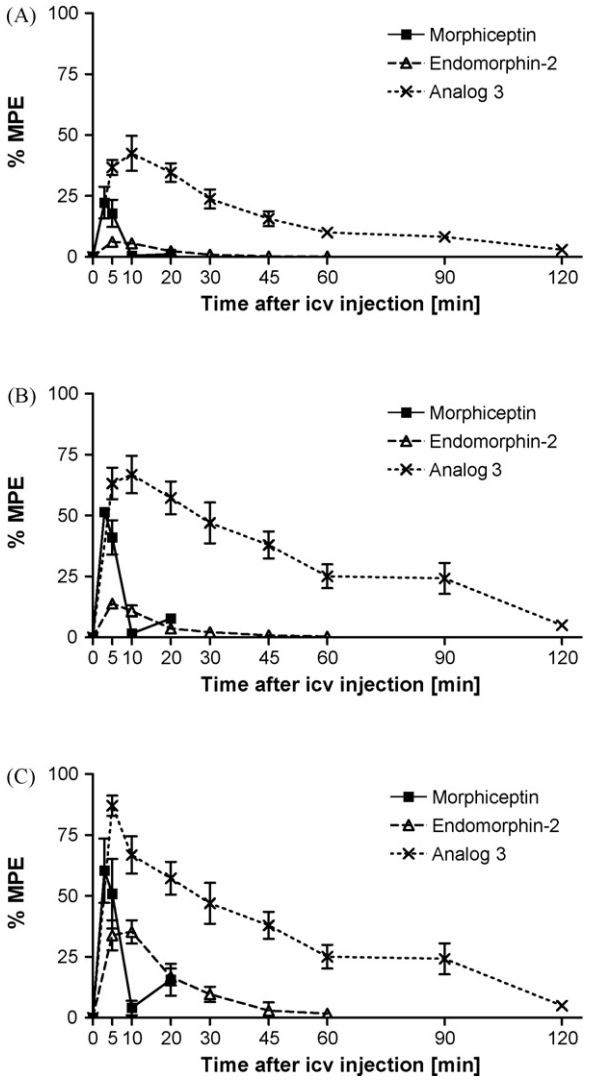

Fig. 2 – Time-course of the changes in inhibition of paw licking (A), rearing (B), and jumping (C) induced by i.c.v. injection of morphiceptin (10 μg), EM-2 (3 μg), and [Dmt^1, D-1-Nal^3]morphiceptin (analog 3, 0.1 μg), determined in the hot plate assay.

Table 3 – Antinociceptive effect of morphiceptin and endomorphin-2 analogs in the mouse hot plate test after i.c.v. injection (10 μg/animal)

No.	Sequence	Latencies (%MPE) to			ED_{50} (jumping) (μg)
		Paw licking	Rearing	Jumping	
1	Tyr-Pro-Phe-Pro-NH_2 (morphiceptin)[a]	11.52 ± 4.19	33.08 ± 6.54	75.12 ± 8.48	1.75
2	Tyr-Pro-D-1-Nal-Pro-NH_2[a]	44.48 ± 8.63	75.84 ± 6.63	92.72 ± 5.57	0.50
3	Dmt-Pro-D-1-Nal-Pro-NH_2	100%[c]	100%[c]	100%[c]	0.006
4	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)[b]	13.70 ± 2.30	24.80 ± 3.00	64.70 ± 8.00	1.83
5	Tyr-Pro-D-1-Nal-Phe-NH_2[b]	6.88 ± 1.21	15.60 ± 3.37	40.12 ± 6.06	8.32
6	Dmt-Pro-D-1-Nal-Phe-NH_2	31.09 ± 3.91	66.70 ± 4.67	95.69 ± 2.27	0.85

All values are expressed as mean ± S.E.M. (n = 10).
[a] Data from Ref. [6].
[b] Data from Ref. [7].
[c] Percentage of mice with latency ≥240 s (cut-off time).

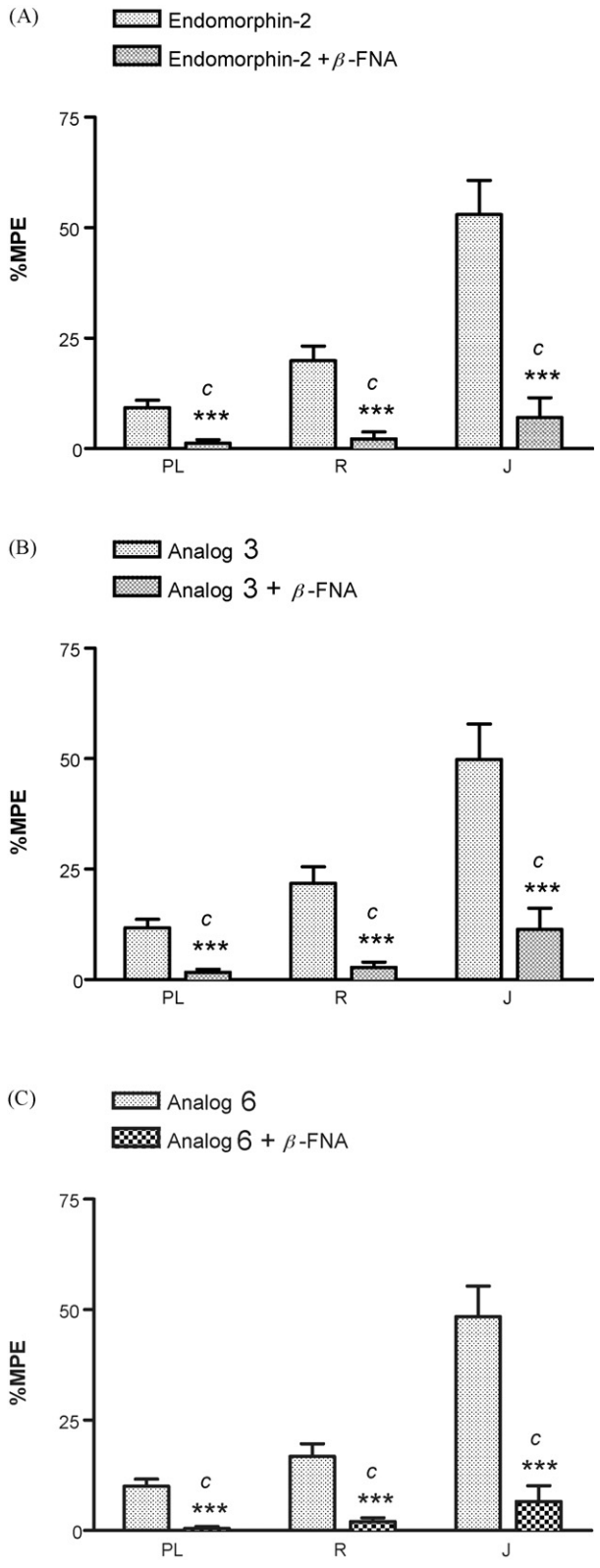

Fig. 3 – Antagonist effect of β-funaltrexamine (β-FNA, 1 μg) on the hot plate inhibition of paw licking (PL), rearing (R), and jumping (J) induced by i.c.v. injection of A) EM-2 (3 μg), B) [Dmt[1], D-1-Nal[3]]morphiceptin (analog 3, 0.01 μg), and C) [Dmt[1], D-1-Nal[3]]EM-2 (analog 6, 1 μg), determined in the hot plate assay. The data represent the mean ± S.E.M. of 10 mice per group. A two-way ANOVA analysis revealed a significant interaction between β-funaltrexamine and endomorphin-2: $F(1,36) = 17.743$; $^{c}p < 0.001$ (for paw licking), $F(1,36) = 22.957$; $^{c}p < 0.001$ (for rearing), $F(1,36) = 23.959$; $^{c}p < 0.001$ (for jumping); between β-funaltrexamine and [Dmt[1], D-1-Nal[3]]morphiceptin (analog 3): $F(1,36) = 23.162$; $^{c}p < 0.001$ (for paw licking), $F(1,36) = 22.811$; $^{c}p < 0.001$ (for rearing), $F(1,36) = 15.709$; $^{c}p < 0.001$ (for jumping); between β-funaltrexamine and [Dmt[1], D-1-Nal[3]]EM-2 (analog 6): $F(1,36) = 29.823$; $^{c}p < 0.001$ (for paw licking), $F(1,36) = 22.326$; $^{c}p < 0.001$ (for rearing), $F(1,36) = 25.147$; $^{c}p < 0.001$ (for jumping).

3. Results and discussion

3.1. Structure-activity relationship studies

In our previous studies it was demonstrated that the introduction of naphtylalanine residues into the sequence of various MOP receptor-selective peptide ligands was advantageous for their activity [5,6,15]. Here we report on the synthesis and pharmacological characterization of morphiceptin and EM-2 analogs with D-1-Nal substituted for Phe in position 3 and containing Dmt in place of Tyr[1] (Table 1). Binding affinities of the new analogs for the MOP and DOP receptors in comparison with the parent peptides are listed in Table 2. In the series of morphiceptin analogs, replacement of Phe[3] by D-1-Nal led to **2** with a 40-fold enhanced μ-affinity. The same replacement of Phe[3] in EM-2 resulted in an approximately 30-fold drop in MOP receptor binding affinity, indicating that the Phe[3] residue is more critical in EM-2 than in morphiceptin. Introduction of Dmt in position 1 resulted in a drastic (about two orders of magnitude) increase in MOP and, to a lesser extent, in DOP receptor binding affinity. The effect of Dmt[1] replacement is not surprising, since it was expected from the results of other Dmt[1]-containing opioid peptide analogs [2,18,19,20,23,24]. The *in vitro* opioid agonist potencies were determined using the isolated guinea-pig ileum (GPI) assay for MOP receptors and the mouse vas deferens (MVD) assay for DOP receptors (Table 2). The results obtained with these functional assays were in agreement with the receptor binding affinities measured in the binding assays. Morphiceptin exhibited 40-fold lower GPI potency than EM-2 (IC_{50} = 318 and 7.71 nM, respectively). D-1-Nal substitution in positions 3 of morphiceptin resulted in an enormous increase in the MOP receptor agonist potency, while in EM-2 that same substitution produced a drastic potency decrease (IC_{50} = 9.57 and 872 nM, respectively). Substitution of Dmt for Tyr[1] in the latter two analogs greatly increased potency in the GPI and MVD assays.

3.2. Antinociceptive activity studies

It is well known that even a small change in the hydrophobicity and spatial conformation of an opioid ligand may not only affect its receptor affinity [1,7,21,24], but also its biological efficacy as an antinociceptive agent *in vivo* [7,14,26]. In that regard, the methyl groups on the phenolic ring in Dmt undoubtedly play a role in strengthening

receptor binding, presumably by engaging in additional hydrophobic interactions.

The antinociceptive activity of the analogs was determined using the hot plate test (supraspinally mediated analgesia) after intracerebroventricular (i.c.v.) administration, in comparison with the effect produced by morphiceptin and EM-2. The obtained results show that a similar analgesic effect was produced by morphiceptin and EM-2 (Table 3). Much stronger activities were observed with analogs **2**, **3**, and **6**. For the two most potent analogs **3** and **6** dose–response curves were obtained (Fig. 1). In the case of compound **3**, the antinociceptive effect was observed at a dose as low as 0.1 ng, and for **6** around 10 ng, whereas in the case of both parent compounds much higher doses were required (100 ng per animal). In a time-course study of the antinociceptive effect, compound **3**, morphiceptin, and EM-2 showed maximal response within 10 min after i.c.v. injection. The duration of the effect was about 20 min for morphiceptin, 60 min for EM-2, and 120 min for **3** (Fig. 2). The MOP receptor selective antagonist β-funaltrexamine (β-FNA) effectively reversed the analgesic effect of all tested compounds, indicating that their action was mediated by MOP receptor (Fig. 3).

4. Conclusions

In conclusion, the novel analog [Dmt^1, D-1-Nal^3]morphiceptin showed very high binding affinity for the MOP receptor and was a very potent MOP receptor agonist in the GPI functional assay. The exceptionally strong analgesic effect of this analog and its greatly increased duration of action in comparison with EM-2 place [Dmt^1, D-1-Nal^3]morphiceptin among the most potent MOP receptor ligands published so far. The study demonstrated that D-1-Nal might be useful as a Phe surrogate in the design of the opioid analogs with unique biological activity.

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