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Effect of 2′,6′-dimethyl-L-tyrosine (Dmt) on pharmacological activity of cyclic endomorphin-2 and morphiceptin analogs

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ABSTRACT

This study reports the synthesis and biological evaluation of a series of new side-chain-to-side-chain cyclized endomorphin-2 (EM-2) and morphiceptin analogs of a general structure Tyr-c(Xaa-Phe-Phe-Yaa)NH_2 or Tyr-c(Xaa-Phe-D-Pro-Yaa)NH_2, respectively, where Xaa and Yaa were L/D Asp or L/D Lys. Further modification of these analogs was achieved by introduction of 2′,6′-dimethyl-L-tyrosine (Dmt) instead of Tyr in position 1. Peptides were synthesized by solid phase method and cleaved from the resin by a microwave-assisted procedure.

Dmt[1]-substituted analogs displayed high affinity at the μ-opioid receptors, remained intact after incubation with the rat brain homogenate and showed remarkable, long-lasting μ-opioid receptor-mediated antinociceptive activity after central, but not peripheral administration.

Our results demonstrate that cyclization is a promising strategy in the development of new opioid analgesics, but further modifications are necessary to enhance the blood–brain barrier permeability.

1. Introduction

Cyclization is a well recognized powerful tool in peptide chemistry for generating analogs with improved bioactivity. Cyclic peptides, compared to linear peptides, have been considered to have greater potential as therapeutic agents due to their increased chemical and enzymatic stability, receptor selectivity, and improved pharmacodynamic properties.[1] Cyclization can be achieved through various bridging bonds between peptide ends and/or side chains.[2] Cyclic peptides adopt conformations that are better defined than those of their linear counterparts, which often exist in a conformational equilibrium. Indeed, cyclization reduces the molecular conformational freedom, which is responsible for the contemporary activation of different receptors, increases metabolic stability and may increase lipophilicity, which often improves the blood–brain barrier (BBB) permeability of peptides.

Cyclization has been successfully used in the field of opioid peptides. A great number of cyclic analogs of δ-selective enkephalins and deltorphins[3–8] and κ-selective dynorphins[9,10] have been synthesized over the years through a variety of strategies. The endogenous μ-opioid receptor selective endomorphins are more difficult to cyclize due to their short, only tetrapeptide sequence and the lack of the reactive side-chain groups. Therefore, to obtain cyclic analogs of endomorphins different approaches have been used, such as extension of a peptide chain by an additional amino acid or/and functionalization of amino acid side-chains.

Cardillo et al. synthesized a library of cyclic analogs of endomorphin-1 (EM-1) containing a Gly^5 bridge between Tyr^1 and Phe^4 .[11] From this library, the cyclopeptide c[Tyr-D-Pro-D-Trp-Phe-Gly] produced significant antinociception in a visceral pain model.[12]

Purington et al. synthesized a series of cyclic pentapeptides of a general structure Tyr-c[D-Cys-Xaa-Yaa-Cys]-NH_2, cyclized via a disulfide bridge, which displayed μ-opioid receptor agonist and δ-opioid receptor partial agonist/antagonist properties.[13]

In our earlier papers we have described the synthesis and antinociceptive activity of a series of endomorphin-2 (EM-2) and morphiceptin analogs obtained by cyclization through an amide bond between the side-chain amino and carboxy groups of diamino and dicarboxy amino acids introduced into the peptide chain into positions 2 and 5.[14,15]

In this report we describe further modifications of these cyclic peptides, in particular introduction of highly lipophilic unnatural

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amino acid 2′,6′-dimethyl-L-tyrosine (Dmt) into position 1. Replacement of Tyr in opioid peptides by Dmt paved the way for achieving increased bioactivities. Introduction of methyl groups into the aromatic ring of Tyr influences the conformation of the analogs because of the increased bulkiness of the side-chain. The methyl groups on the aromatic ring of Dmt undoubtedly play a dominant role in the interaction within the opioid binding domain by either direct interaction with hydrophobic side-chains of receptor residues in order to align the critical OH group, or by stabilization of a favored *cis*-conformer prior to and during binding.[16] However, Dmt enhances affinities relative to Tyr cognates for both, μ- and δ-receptors, which results in decreased selectivity of Dmt[1] analogs.[17–19] Here we wanted to study the impact of the incorporation of Dmt[1] into the sequence of cyclic opioid peptides on their binding affinity and antinociceptive activity.

2. Material and methods

2.1. General

All reagents, unless otherwise stated, were purchased from Sigma–Aldrich (Poznan, Poland). *t*-Butyloxycarbonyl (Boc)-protected amino acids and *p*-methylbenzhydrylamine (MBHA) resin were purchased from Bachem AG (Bubendorf, Switzerland).

Analytical and semi-preparative RP-HPLC used were Waters Breeze (Milford, MA, USA) with Vydac C_{18} column (5 μm, 4.6 × 250 mm) and Vydac C_{18} column (10 μm, 22 × 250 mm), respectively. Mass spectra of peptides were recorded on Bruker Apex Ultra 7T FT-ICR mass spectrometer with electrospray ionization (ESI-MS; Billerica, MA, USA). ^{1}H NMR (δ) spectra were recorded in DMSO-d_6 solution on Bruker Avance II+ 700 MHz spectrometer with TMS as an internal standard. *J* values are given in Hz.

2.2. Peptide synthesis

Peptides were synthesized by manual solid-phase procedure as described before, using techniques for Boc-protected amino acids on MBHA resin (100–200 mesh, 0.8 mM/g, Novabiochem).[14] N^{ε}-amino group of Lys and D-Lys was protected by 9-fluorenylmethyloxycarbonyl (Fmoc), β-carboxy group of Asp and D-Asp by fluorenylmethyl ester (OFm), and hydroxy group of Tyr by 2-bromo-benzyloxycarbonyl (2-Br-Z). 50% trifluoroacetic acid (TFA) in dichloromethane (DCM) was used for deprotection of Boc-groups and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) was employed to facilitate coupling. Fully assembled Boc-protected peptides were treated with 20% piperidine in dimethylformamide (DMF) to remove base-labile groups (Fmoc and OFm), followed by cyclization (TBTU).

Simultaneous deprotection and cleavage of peptides from the resin was accomplished using TFA in a microwave-assisted procedure, described elsewhere.[20] Shortly, a sample of the resin (50 mg) in a 2 ml polypropylene syringe reactor was treated with TFA/TIS/H_2O (95:2.5:2.5; 1 ml) for 15 min at room temperature. The reactor was then transferred into the microwave synthesizer and irradiated with gas cooling at 30 W with magnetic stirring and temperature limit of 50 °C for 30 s at a time (total irradiation time 32.5 min). Between irradiations, the resin was cooled in an ice-water bath for 2 min. Every 10 cycles the solution was removed, the resin washed with TFA, and a new portion of cleavage mixture added to the syringe. The combined filtrates were evaporated in a stream of nitrogen.

Crude peptides were purified by RP-HPLC 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 0–100% B over 15 min. The purity of peptides was verified by analytical RP-HPLC, employing the solvent system of 0.1% TFA in water (A) and 80% acetonitrile in water containing 0.1% TFA (B). A linear gradient of 0–100% of solvent B over 25 min was used.

2.3. Peptide characterization

2.3.1. Tyr-c(D-Lys-Phe-Phe-Asp)-NH_2 (2)

R_T: 20.50 min. ESI-MS: calcd for $C_{37}H_{45}N_7O_7$ 699, found $[M+H]^+$ 700. ^{1}H NMR (DMSO-d_6) δ 0.75–0.81 (m, 1H), 1.01–1.09 (m, 1H), 1.21–1.34 (m, 4H), 2.34–2.44 (m, 2H), 2.78–2.82 (m, 1H), 2.92–3.15 (m, 7H), 3.85–3.88 (m, 1H), 3.90–3.95 (m, 1H), 4.15–4.19 (m, 1H), 4.32–4.36 (m, 1H), 4.46–4.50 (m, 1H), 6.62 (d, *J* = 8.5, 2H), 7.01–7.29 (m, 12H), 7.64 (t, *J* = 5.5, 1H), 7.75 (d, *J* = 6.5, 1H), 7.79 (d, *J* = 8.5, 1H), 7.92 (d, *J* = 6, 1H), 7.98 (d, *J* = 8.5, 1H), 8.49 (br s, 2H), 9.19 (s, 1H).

2.3.2. Dmt-c(D-Lys-Phe-Phe-Asp)-NH_2 (3)

R_T: 14.59 min. ESI-MS: calcd for $C_{39}H_{49}N_7O_7$ 728, found $[M+H]^+$ 729. ^{1}H NMR (DMSO-d_6) δ 0.70–0.76 (m, 1H), 0.92–1.00 (m, 1H), 1.20–1.31 (m, 4H), 2.19 (s, 6H), 2.36–2.47 (m, 2H), 2.75–2.79 (m, 1H), 2.87–3.07 (m, 7H), 3.82–3.85 (m, 1H), 3.94–3.99 (m, 1H), 4.14–4.18 (m, 1H), 4.38–4.42 (m, 1H), 4.47–4.51 (m, 1H), 6.43 (s, 2H), 7.05–7.27 (m, 10H), 7.67 (t, *J* = 5.5, 1H), 7.78 (d, *J* = 6.5, 1H), 7.84 (d, *J* = 8.5, 1H), 7.88 (d, *J* = 6.0, 1H), 8.01 (d, *J* = 8.5, 1H), 8.33 (br s, 2H), 9.11 (s, 1H).

2.3.3. Tyr-c(D-Lys-Phe-D-Pro-Asp)-NH_2 (5)

R_T: 18.35 min. ESI-MS: calcd for $C_{33}H_{43}N_7O_7$ 649, found $[M+H]^+$ 650. ^{1}H NMR (DMSO-d_6) δ 0.71–1.86 (m, 10H), 2.23–2.30 (m, 1H), 2.66–2.71 (m, 1H), 2.81–3.05 (m, 3H), 3.13–3.18 (m, 2H), 3.23–3.28 (m, 1H), 3.54–3.58 (m, 1H), 3.75–3.79 (m, 1H), 4.12–4.16 (m, 1H), 4.30–4.34 (m, 1H), 4.44–4.59 (m, 3H), 6.74 (d, *J* = 8.5, 2H), 7.02–7.29 (m, 8H), 7.54 (t, *J* = 5.0, 1H), 7.64 (d, *J* = 6.0, 1H), 7.73 (d, *J* = 5.6, 1H), 8.42 (br s, 2H), 9.37 (s, 1H).

2.3.4. Dmt-c(D-Lys-Phe-D-Pro-Asp)-NH_2 (6)

R_T: 12.69 min. ESI-MS: calcd for $C_{35}H_{47}N_7O_7$ 678, found $[M+H]^+$ 679. ^{1}H NMR (DMSO-d_6) δ 0.65–1.85 (m, 10H), 2.18 (s, 6H), 2.27–2.31 (m, 1H), 2.66–2.70 (m, 1H), 2.79–3.05 (m, 3H), 3.13–3.19 (m, 2H), 3.23–3.28 (m, 1H), 3.55–3.59 (m, 1H), 3.76–3.81 (m, 1H), 4.12–4.16 (m, 1H), 4.31–4.36 (m, 1H), 4.44–4.58 (m, 3H), 6.44 (s, 2H), 7.08 (d, *J* = 7.5, 1H), 7.13–7.32 (m, 5H), 7.61 (t, *J* = 5.0, 1H), 7.68 (d, *J* = 6.3, 1H), 7.74 (d, *J* = 5.6, 1H), 8.42 (br s, 2H), 9.18 (s, 1H).

2.3.5. Tyr-c(D-Asp-Phe-D-Pro-Lys)-NH_2 (7)

R_T: 16.25 min. ESI-MS: calcd for $C_{33}H_{43}N_7O_7$ 649, found $[M+H]^+$ 650. ^{1}H NMR (DMSO-d_6) δ 1.08–1.73 (m, 10H), 2.13–2.17 (m, 1H), 2.65–2.70 (m, 1H), 2.83–3.02 (m, 4H), 3.15–3.20 (m, 1H), 3.20–3.24 (m, 1H), 3.58–3.61 (m, 1H), 3.77–3.80 (m, 1H), 4.00–4.08 (m, 2H), 4.31–4.35 (m, 1H), 4.41–4.46 (m, 1H), 4.58–4.62 (m, 1H), 6.64 (d, *J* = 8.5, 2H), 6.75 (d, *J* = 7.7, 1H), 6.98 (d, *J* = 8.5, 2H), 7.18–7.27 (m, 5H), 7.56 (d, *J* = 5.0, 1H), 7.73 (t, *J* = 5.6, 1H), 8.09 (d, *J* = 9.0, 1H), 8.56 (br s, 2H), 9.10 (s, 1H).

2.3.6. Dmt-c(D-Asp-Phe-D-Pro-Lys)-NH_2 (8)

R_T: 12.44 min. ESI-MS: calcd for $C_{35}H_{47}N_7O_7$ 678, found $[M+H]^+$ 679. ^{1}H NMR (DMSO-d_6) δ 1.03–1.67 (m, 10H), 2.09–2.13 (m, 1H), 2.18 (s, 6H), 2.67–2.71 (m, 1H), 2.85–3.04 (m, 4H), 3.14–3.18 (m, 1H), 3.26 (dd, *J* = 6.3, *J* = 10.5, 1H) 3.55–3.58 (m, 1H), 3.78–3.81 (m, 1H), 4.01–4.10 (m, 2H), 4.31–4.35 (m, 1H), 4.44–4.49 (m, 1H), 4.53–4.58 (m, 1H), 6.42 (s, 2H), 6.68 (d, *J* = 7.7, 1H), 7.17–7.27 (m, 5H), 7.58 (d, *J* = 5.0, 1H), 7.70 (t, *J* = 5.6, 1H), 8.11 (d, *J* = 9.0, 1H), 8.44 (br s, 2H), 9.11 (s, 1H).

2.4. Opioid receptor binding assays

Receptor-binding assays were performed as described previously.[15] Briefly, binding affinities for μ- and δ-opioid receptors were determined during incubation with crude membrane preparation

isolated from Wistar rat brains, in the presence of [^{3}H]DAMGO or [^{3}H][Ile^{5,6}]deltorphin-2, used as the μ- and δ-selective radioligands, respectively. Non-specific binding was determined in the presence of 1 μM naloxone. Incubations were terminated by rapid filtration through GF/B Whatman glass fiber strips, soaked in 0.5% polyethylamine, using Sampling Manifold (Millipore, Billerica, MA, USA). The filters were washed twice with ice-cold TrisHCl buffer. The bound radioactivity was measured in Packard Tri-Carb 2100 TR liquid scintillation counter (Ramsey, MN, USA) after overnight extraction of the filters in 4 ml of Perkin Elmer Ultima Gold scintillation fluid (Wellesley, MA, USA). Three independent experiments for each assay were carried out in duplicate.

2.5. Metabolic stability

Enzymatic degradation studies of endomorphin-2, morphiceptin and their cyclic analogs were performed using rat brain homogenate, following a protocol reported previously.[15] Briefly, rat brains were isolated, pooled and homogenized in a Polytron with 20 volumes of Tris–HCl (50 mM, pH 7.4), and the homogenate was then stored at −80 °C. The aliquots (100 μl, 10 mg protein/ml) were incubated with 100 μl of a peptide (0.5 mM) for 0, 7.5, 15, 22.5, 30 and 60 min at 37 °C in a final volume of 200 μl. The reaction was stopped at the required time by placing the tube on ice and acidifying the content with 20 μl of aqueous HCl (1 M). The aliquots were centrifuged at 20,000g for 10 min at 4 °C. The obtained supernatants were filtered over Millex-GV syringe filters (Millipore, Billerica, MA, USA) and analyzed by RP-HPLC on a Vydac C_{18} column (5 μm, 4.6 × 250 mm), using the solvent system of 0.1% TFA in water (A) and 80% acetonitrile in water containing 0.1% TFA (B) and a linear gradient of 0–100% B over 25 min. Three independent experiments for each assay were carried out in duplicate. The rate constants of degradation (k) were obtained by a least square linear regression analysis of logarithmic peak areas [ln(A/AD)], where A is the amount of the remaining peptide and AD is the initial amount of peptide, versus time courses. Degradation half-lives (t0.5) were calculated from the rate constants as ln2/k.

2.6. Animals

Male Swiss albino mice (CD1 IFFA-CREDO/Charles River, France), weighing 20–26 g, were used for the study. The animals were housed at a constant temperature (22 °C) and maintained under a 12-h light/dark cycle in sawdust coated plastic cages with access to standard laboratory chow and tap water ad libitum.

2.7. Assessment of antinociception

The procedures used in this study were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), and were approved by the Local Ethical Committee for Animal Research with the following numbers: N/10-04-04-12 and N/12-04-04-14.

Peptides or vehicle, in a final volume of 10 μl, were administrated intracerebroventricularly (i.c.v.) in the left brain ventricle of manually immobilized mice, using a Hamilton microsyringe (50 μl, Hamilton Company, Remo, NV, USA) connected to a needle (diameter 0.5 mm). In dose–response experiments, the i.c.v. injections were performed 5 min before the beginning of the test. All compounds were dissolved in DMSO and further diluted in saline to desired concentration. Vehicle alone did not influence the observed parameters.

The analgesic activity was assessed in the hot plate test in mice, as described earlier.[15] A transparent plastic cylinder (14 cm diameter, 20 cm height) was used to confine the mouse on the heated (55 ± 0.5 °C) surface of the plate. The animals were placed on the hot plate and the latencies to paw licking, rearing and jumping were measured. A cut-off time of 240 s was used to avoid tissue injury. The percentage of the maximal possible effect (%MPE) was calculated as: $\%MPE = (t_1 - t_0)/(t_2 - t_0) \times 100$, where t_0–control latency, t_1–test latency, and t_2–cut-off time. The median antinociceptive dose (ED_{50}) was calculated as described earlier.[15]

To determine the in vivo blood–brain barrier permeability, morphiceptin and analogs (0.01 and 1 mg/kg) were injected intravenously (iv) 30 min before the experiment and the hot plate test was performed as described above.

2.8. Statistical analysis

Statistical and curve-fitting analyses were performed using Prism 5.0 (GraphPad Software Inc.). The data are expressed as means ± SEM. Differences between groups were assessed by one-way analysis of variance (ANOVA), followed by a post-hoc multiple comparison Student–Newman–Keuls test. Antagonist effects in the combination experiments were analyzed using two-way analysis of variance (ANOVA) and a post-hoc multiple comparison Student-Newman-Keuls test was used for multiple comparisons between groups. A probability level of 0.05 or lower was considered as statistically significant.

3. Results and discussion

EM-2 and morphiceptin were used as parent compounds for the design of new cyclic pentapeptides of a general structure: Xaa-c(Yaa-Phe-Phe-Zaa)-NH_2 (Xaa = Tyr or Dmt, Yaa = D-Lys or D-Asp and Zaa = Asp or Lys) or Xaa-c(Yaa-Phe-D-Pro-Zaa)-NH_2 (Xaa = Tyr or Dmt, Yaa = D-Lys or D-Asp and Zaa = Asp or Lys). Peptides were synthesized using standard Boc/Fmoc orthogonal protection on p-methylbenzhydrylamine (MBHA) resin. Cyclization was carried out between the side-chain amino and carboxy groups of diamino and dicarboxy amino acids introduced at positions 2 and 5 of the sequence, when a protected peptide was still bound to the resin. Cyclic peptides were cleaved from the resin by mixture of TFA/TIS/H_2O (95:2.5:2.5:1), using a microwave-assisted procedure.

The microwave-assisted cleavage of peptides was used earlier exclusively for analytical purposes.[20] Here we demonstrated that this procedure can be successfully applied also for the cleavage of cyclic peptides from the MBHA resin in quantities sufficient for biological experiments. Since cleavage yields in this novel procedure were about 25% lower than those in traditional methods, additional optimization of cleavage conditions is necessary.

Opioid receptor binding affinities of new analogs at μ- and δ-receptors were determined in rat brain membrane preparations in vitro against [^{3}H]DAMGO and [^{3}H][Ile^{5,6}]deltorphin-2, respectively and are reported in Table 1. The affinities of the cyclic analogs at the μ-opioid receptors were in general higher than those of parent compounds, EM-2 and morphiceptin. The introduction of Dmt in position 1 resulted in further significant enhancement of the μ-opioid receptor affinity, which was more pronounced for morphiceptin analogs. Additionally, cyclic analogs acquired some affinity at the δ-opioid receptors.

The resistance of cyclic analogs against proteolytic degradation was evaluated in vitro, using the rat brain homogenate. The calculated half-lives are summarized in Table 2. The cyclic analogs were more resistant to enzymatic degradation than their parent compounds, which are particularly vulnerable to proteolysis. Analogs **3**, **6** and **8**, with Dmt in position 1, remained practically intact after the incubation with the homogenate.

The antinociceptive effect of peptides was assessed in the hot plate test in mice (supraspinally-mediated analgesia). The i.c.v. injection of all tested analogs produced a dose-dependent analgesic

Table 1
Receptor binding affinities of endomorphin-2 (EM-2), morphiceptin and their cyclic analogs at μ- and δ-receptors in rat brain homogenate

No.	Sequence	IC_{50} ± SEM (nM)		
		μ[a]	δ[b]	δ/μ
1	Tyr-Pro-Phe-Phe-NH_2 (EM-2)[c]	0.99 ± 0.08	>1000	>1000
2	Tyr-c(D-Lys-Phe-Phe-Asp)-NH_2[c]	0.56 ± 0.03	279 ± 21	498
3	Dmt-c(D-Lys-Phe-Phe-Asp)-NH_2	0.26 ± 0.02	400 ± 10	>1000
4	Tyr-Pro-Phe-Pro-NH_2 (morphiceptin)	56.6 ± 4.5	>1000	>17.7
5	Tyr-c(D-Lys-Phe-D-Pro-Asp)-NH_2	89.5 ± 7.2	>1000	>11.2
6	Dmt-c(D-Lys-Phe-D-Pro-Asp)-NH_2	5.60 ± 0.90	132 ± 9	23.5
7	Tyr-c(D-Asp-Phe-D-Pro-Lys)-NH_2	15.3 ± 0.9	>1000	>65.3
8	Dmt-c(D-Asp-Phe-D-Pro-Lys)-NH_2	4.40 ± 0.60	58.3 ± 5.0	13.2
	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 (DLT)	–	0.080 ± 0.004	–

All values are mean ± SEM of three independent experiments performed in duplicate.
[a] Displacement of [^{3}H]DAMGO.
[b] Displacement of [^{3}H][Ile^{5,6}]deltorphin-2.
[c] Data from Ref. 15.

action, significantly stronger than that of EM-2 and morphiceptin (Fig. 1). Exceptionally strong antinociceptive effect was observed for analogs **6** and **8**, with the ED_{50} values lower than 0.01 μg/animal (i.c.v.) (Table 3).

The antinociception produced by the cyclic analogs was further investigated in the time-course experiments. The maximal analgesic action of analogs **6** and **8** (both 10 ng/animals, i.c.v.) was observed up to 30 min after administration, compared to 5–10 min for morphiceptin (10 μg/animal, i.c.v.) and EM-2 (3 μg/animal, i.c.v.), and the effect lasted up to 90 min (Fig. 2).

To characterize the involvement of opioid receptors in the analgesic action of analogs **6** and **8**, co-administration studies with opioid receptor antagonists were performed. The antinociceptive effect of both analogs (3 ng/animal, i.c.v.) was blocked by β-funaltrexamine (β-FNA, 1 μg/animal), showing the involvement of the μ-opioid receptors (Fig. 3). δ-Opioid receptor antagonist naltrindole (NTL, 1 μg/animal, i.c.v.) and κ-opioid receptor antagonist norbinaltorphimine (nor-BNI, 5 μg/animal, i.c.v.) did not influence the analgesic action of analogs **6** and **8** (Fig. 3).

The hot plate test in mice was then used to examine the permeability of analogs **6** and **8** through BBB after peripheral administration. However, intravenous (iv) injection of these peptides (0.01 and 1 mg/kg) did not produce any significant central effects, indicating that they did not cross the BBB.

In conclusion, this is the first report on the synthesis of a new series of cyclic analogs based on EM-2 and morphiceptin structure, incorporating Dmt in position 1. We also demonstrated that the cyclic peptides can be cleaved from the resin using the microwave-assisted procedure.

Table 2
Degradation rates and half-lives of endomorphin-2 (EM-2), morphiceptin and their cyclic analogs

No.	Sequence	Brain homogenate	
		$100 \times k$ (per min)	$t_{1/2}$ (min)
1	Tyr-Pro-Trp-Phe-NH_2 (EM-2)	4.83 ± 0.37	14.3 ± 1.1
2	Tyr-c(D-Lys-Phe-Phe-Asp)-NH_2	0.19 ± 0.01	363 ± 22
3	Dmt-c(D-Lys-Phe-Phe-Asp)-NH_2	0.06 ± 0.01	>1000
4	Tyr-Pro-Phe-Pro-NH_2 (morphiceptin)	4.13 ± 0.27	16.7 ± 2.2
5	Tyr-c(D-Lys-Phe-D-Pro-Asp)-NH_2	0.60 ± 0.05	117 ± 12
6	Dmt-c(D-Lys-Phe-D-Pro-Asp)-NH_2	0.020 ± 0.001	>1000
7	Tyr-c(D-Asp-Phe-D-Pro-Lys)-NH_2	1.00 ± 0.03	63.3 ± 2.5
8	Dmt-c(D-Asp-Phe-D-Pro-Lys)-NH_2	0.11 ± 0.01	627 ± 38

All values are mean ± SEM of three independent experiments performed in duplicate.
RP-HPLC elution on a Vydac C_{18} column (5 μm, 4.6 × 250 mm) 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 0–100% solvent B over 25 min at flow rate of 1 ml/min.

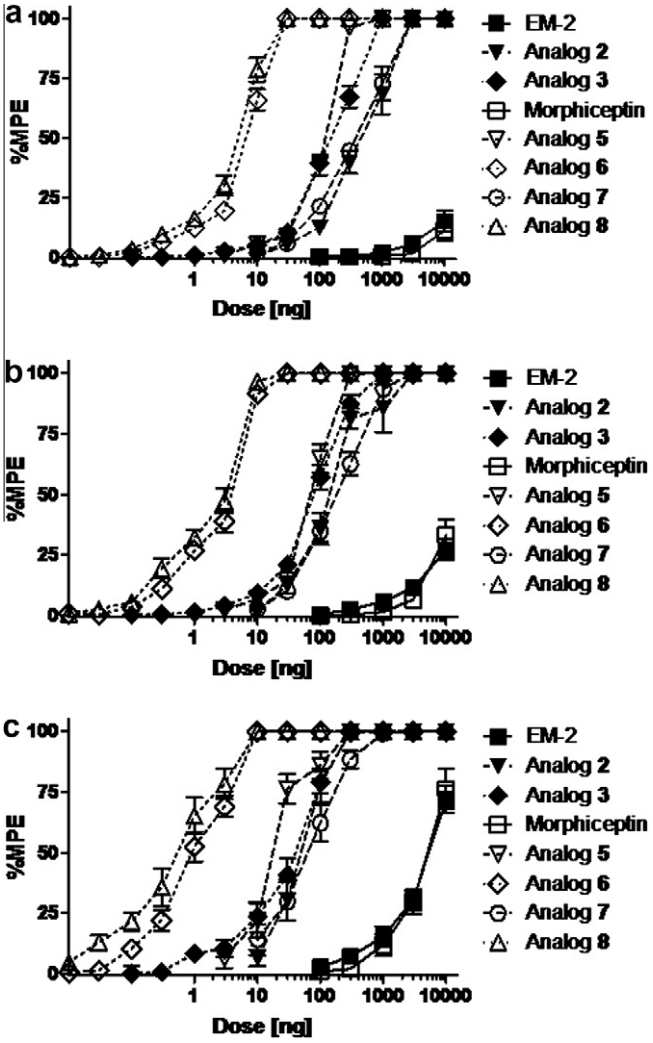

Figure 1. Dose–response curves for the inhibition of paw licking (a), rearing (b), and jumping (c) induced by i.c.v. injection of endomorphin-2 (EM-2), morphiceptin and their cyclic analogs determined in the hot plate test in mice. Data are mean ± SEM of 10 animals per group.

Biological evaluation of the new analogs revealed that the cyclization improves the μ-opioid receptor binding affinity and significantly enhances stability against enzymatic degradation, which is in line with earlier observations of our group, as well as others.[12,14,15]

Table 3
ED_{50} values for endomorphin-2 (EM-2), morphiceptin and their cyclic analogs in the hot plate test in mice

No.	Sequence	ED_{50} (jumping) (μg/animal)
1	Tyr-Pro-Phe-Phe-NH_2 (EM-2)	5.48 ± 0.61
2	Tyr-c(D-Lys-Phe-Phe-Asp)-NH_2	0.05 ± 0.01
3	Dmt-c(D-Lys-Phe-Phe-Asp)-NH_2	0.020 ± 0.003
4	Tyr-Pro-Phe-Pro-NH_2 (morphiceptin)	5.11 ± 0.42
5	Tyr-c(D-Lys-Phe-D-Pro-Asp)-NH_2	0.020 ± 0.002
6	Dmt-c(D-Lys-Phe-D-Pro-Asp)-NH_2	<0.01
7	Tyr-c(D-Asp-Phe-D-Pro-Lys)-NH_2	0.07 ± 0.01
8	Dmt-c(D-Asp-Phe-D-Pro-Lys)-NH_2	<0.01

Data are mean ± SEM of 10 animals per group.

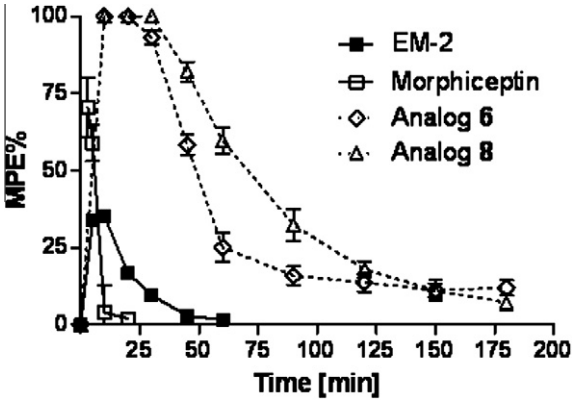

Figure 2. Time-course of the changes in inhibition of jumping induced by i.c.v. injection of endomorphin-2 (EM-2, 3 μg/animal), morphiceptin (10 μg/animal) and analogs **6** and **8** (both 10 ng/animal), determined in the hot plate test in mice. Data are mean ± SEM of 10 animals per group.

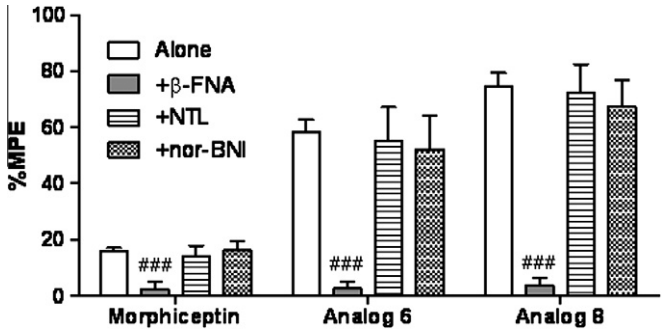

Figure 3. Antagonist effect of β-funaltrexamine (β-FNA, 1 μg/animal, i.c.v.), naltrindole (NTL, 1 μg/animal, i.c.v.) and nor-binaltorphimine (nor-BNI, 5 μg/animal, i.c.v.), on the inhibition of jumping induced by morphiceptin (10 μg/animal, i.c.v.) and analogs **6** and **8** (both 3 ng/animal) determined in the hot plate test in mice. Data are mean ± SEM of 10 animals per group. ###p <0.001 for agonist + β-FNA versus agonist alone using two-way ANOVA, followed by the Student–Newman–Keuls test.

The introduction of Dmt into position 1 increased peptide affinity at the μ-opioid receptors, drastically increased stability in vitro and antinociceptive activity, therefore produced similar effects as shown in linear peptides.[21–23] Selective opioid antagonists confirmed that the long-lasting analgesia after central administration was mediated exclusively by the μ-opioid receptors.

The extra-ordinary antinociceptive activity of cyclic analogs incorporating Dmt[1] confirms their potential as useful tools for the pharmacological studies of the μ-opioid receptors.

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References and notes

1. Katsara, M.; Tselios, T.; Deraos, S.; Deraos, G.; Matsoukas, M. T.; Lazoura, E.; Matsoukas, J.; Apostolopoulos, V. *Curr. Med. Chem.* **2006**, *13*, 2221.
2. Janecka, A.; Kruszynski, R. *Curr. Med. Chem.* **2005**, *12*, 471.
3. Shreder, K.; Zhang, L.; Dang, T.; Yaksh, T. L.; Umeno, H.; DeHaven, R.; Daubert, J.; Goodman, M. *J. Med. Chem.* **1998**, *41*, 2631.
4. Bilsky, E. J.; Qian, X.; Hruby, V. J.; Porreca, F. *J. Pharmacol. Exp. Ther.* **2000**, *293*, 151.
5. Pawlak, D.; Oleszczuk, M.; Wojcik, J.; Pachulska, M.; Chung, N. N.; Schiller, P. W.; Izdebski, J. *J. Pept. Sci.* **2001**, *7*, 128.
6. Rew, Y.; Malkmus, S.; Svensson, C.; Yaksh, T. L.; Chung, N. N.; Schiller, P. W.; Cassel, J. A.; DeHaven, R. N.; Taulane, J. P.; Goodman, M. *J. Med. Chem.* **2002**, *45*, 3746.
7. Rodziewicz-Motowidlo, S.; Czaplewski, C.; Luczak, S.; Ciarkowski, J. *J. Pept. Sci.* **2008**, *14*, 898.
8. Ciszewska, M.; Ruszczynska, K.; Oleszczuk, M.; Chung, N. N.; Witkowska, E.; Schiller, P. W.; Wojcik, J.; Izdebski, J. *Acta Biochim. Pol.* **2011**, *58*, 225.
9. Vig, B. S.; Murray, T. F.; Aldrich, J. V. *Biopolymers* **2003**, *71*, 620.
10. Vig, B. S.; Murray, T. F.; Aldrich, J. V. *J. Med. Chem.* **2004**, *47*, 446.
11. Cardillo, G.; Gentilucci, L.; Tolomelli, A.; Spinosa, R.; Calienni, M.; Qasem, A. R.; Spampinato, S. *J. Med. Chem.* **2004**, *47*, 5198.
12. Bedini, A.; Baiula, M.; Gentilucci, L.; Tolomelli, A.; De, M. R.; Spampinato, S. *Peptides* **2010**, *31*, 2135.
13. Purington, L. C.; Pogozheva, I. D.; Traynor, J. R.; Mosberg, H. I. *J. Med. Chem.* **2009**, *52*, 7724.
14. Janecka, A.; Fichna, J.; Kruszynski, R.; Sasaki, Y.; Ambo, A.; Costentin, J.; do-Rego, J. C. *Biochem. Pharmacol.* **2005**, *71*, 188.
15. Perlikowska, R.; do-Rego, J. C.; Cravezic, A.; Fichna, J.; Wyrebska, A.; Toth, G.; Janecka, A. *Peptides* **2010**, *31*, 339.
16. Janecka, A.; Staniszewska, R.; Fichna, J. *Curr. Med. Chem.* **2007**, *14*, 3201.
17. Schiller, P. W.; Fundytus, M. E.; Merovitz, L.; Weltrowska, G.; Nguyen, T. M.; Lemieux, C.; Chung, N. N.; Coderre, T. J. *J. Med. Chem.* **1999**, *42*, 3520.
18. Sasaki, Y.; Suto, T.; Ambo, A.; Ouchi, H.; Yamamoto, Y. *Chem. Pharm. Bull. (Tokyo)* **1999**, *47*, 1506.
19. Narita, M.; Funada, M.; Suzuki, T. *Pharmacol. Ther.* **2001**, *89*, 1.
20. Kluczyk, A.; Rudowska, M.; Stefanowicz, P.; Szewczuk, Z. *J. Pept. Sci.* **2010**, *16*, 31.
21. 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.
22. Fichna, J.; do-Rego, J. C.; Chung, N. N.; Costentin, J.; Schiller, P. W.; Janecka, A. *Peptides* **2008**, *29*, 633.
23. Fichna, J.; do-Rego, J. C.; Janecki, T.; Staniszewska, R.; Poels, J.; Broeck, J. V.; Costentin, J.; Schiller, P. W.; Janecka, A. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1350.