Acta Physiologica Hungarica, Volume 99 (3), pp. 353–363 (2012)
DOI: 10.1556/APhysiol.99.2012.3.12

Characterization of antinociceptive potency of endomorphin-2 derivatives with unnatural amino acids in rats

Gy Kovács[1], Z Petrovszki[2], JR Mallareddy[3], G Tóth[3], Gy Benedek[2], Gy Horváth[2]

[1]Department of Orthopedics, József Hollós County Hospital, Kecskemét, Hungary
[2]Department of Physiology, Faculty of Medicine, Szeged, Hungary
[3]Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

Received: January 25, 2012
Accepted after revision: March 9, 2012

This study reports on the *in vivo* effects of four endomorphin-2 (EM-2) derivatives (EMD1-4) containing unnatural amino acids, i.e. 2-aminocyclohexanecarboxylic acid (Achc[2]), para-fluorophenylalanine (pFPhe[4]), β-methyl-phenylalanine (βMePhe[4]) and/or 2',6'-dimethyltyrosine (Dmt[1]). After induction of osteoarthritis by monosodium iodoacetate into the ankle joint of male Wistar rats, a chronic intrathecal catheter was inserted for spinal drug delivery. The mechanical threshold was assessed by a dynamic aesthesiometer. Intrathecal injection of the original EM-2 and the ligands (0.3–10 μg) caused dose-dependent antiallodynic effects. The comparison of the different substances revealed that EMD3 and EMD4 showed more prolonged antinociception than EM-2, and the effects of the highest dose of EMD4 were comparable to morphine, while EMD3 caused paralysis at this dose. The potency of the different ligands did not differ from EM-2. The results show that the derivatives of EM-2 have similar *in vivo* potency to the original ligand, but their effects were more prolonged suggesting that these structural modifications may play a role in the development of novel endomorphin analogues with increased therapeutic potential.

Keywords: opioid peptide, endomorphin, pain, spinal, intrathecal, osteoarthritis C

Morphine and related compounds that are clinically valuable for pain relief, act primarily at the μ-opioid receptor (MOR), a member of the G-protein-coupled receptor superfamily (39). A major goal in opioid peptide research is the development of novel analgesics that could substitute for morphine without its well-known side effects of dependence, tolerance, respiratory depression and reward-seeking behavior (25). The study of naturally occurring peptides provides a rational and powerful approach in the design of peptide medications. Endomorphin-1 (EM-1, Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (EM-2, Tyr-Pro-Phe-Phe-NH_2) are high-affinity, MOR-selective endogenous opioids which also inhibit nociception similarly to opiates of plant origin in both acute and chronic pain models (10, 31, 44). The exogenous application of EMs encounters serious limitations, including a short duration of action, a lack of activity after oral administration and poor metabolic stability (23, 34, 37). Aminopeptidases play a key role in the biodegradation of EMs, during which the main cleavage occurs at the Pro^2-Trp^3 and Pro^2-Phe^3 peptide bonds. For their consideration as actual therapeutic drugs it is essential to enhance their resistance to enzymatic degradation (12).

Corresponding author: Gyöngyi Horváth
Department of Physiology, Faculty of Medicine, University of Szeged,
P. O. Box 427, H-6701 Szeged, Hungary
Phone: +36-62-544971; Fax: +36-62-545842; E-mail: horvath.gyongyi@med.u-szeged.hu

Such objectives may possibly be achieved through systematic modification of the peptide sequence (9, 11, 15).

The solution structures of the EMs and their analogues have been investigated in detail in relation to their bioactivity (15). In a recent paper, the synthesis and structure-activity study of new analogues with unnatural amino acids were reported (22). The systematic replacement of natural amino acids by 2',6'-dimethyltyrosine (Dmt^1), 2-amino-cyclohexanecarboxylic acid [cis-(1S,2R)$Achc^2$/cis-(1R,2S)$Achc^2$], β-methylphenylalanine [(2R,3R)β$MePhe^4$/(2S,3S)β$MePhe^4$] and para-fluorophenylalanine ($pFPhe^4$) in different positions resulted in proteolytically stable compounds with high MOR affinity in some cases (22). Thus, it was found that the analogues carrying Dmt^1 and $Achc^2$ residues displayed the highest MOR affinities, depending upon the configuration of the incorporated $Achc^2$. Combination of such derivatives with $pFPhe^4$ or β$MePhe^4$ yielded compounds with high binding potency, while their efficacy did not differ from the parent ligand. Several earlier studies investigated the *in vivo* activities of different endomorphin derivatives in acute heat or chemical pain models (2, 17, 24, 27, 45). The goal of this study was to investigate the antiallodynic effects of the recently synthetized and most potent EM-2 derivatives (EMD1-4, Table I) at spinal level, in a chronic joint pain model.

Materials and Methods

Animals

After institutional ethical approval had been obtained (Institutional Animal Care Committee of the Faculty of Medicine at the University of Szeged), male Wistar rats (Charles River strain, Bioplan, Budapest, Hungary; 334 ± 3.8 g; n = 7–14 / group) were housed in groups of 5–6 per cage, with free access to food and water, and with a natural light/dark cycle.

Drugs

The following drugs were purchased: ketamine hydrochloride (Calypsol, Richter Gedeon RT, Budapest, Hungary), xylazine hydrochloride (Rompun, Bayer, Leverkusen, Germany), Gentamycin (Sanofi-Aventis, Budapest, Hungary), monosodium iodoacetate (MIA; Sigma-Aldrich Ltd. Budapest, Hungary) and morphine hydrochloride (Hungaropharma, Budapest, Hungary). EM-2 and the derivatives (Table I) were synthesized as was described earlier (22). All substances were dissolved in saline. Intrathecally (i.t.) administered drugs were injected over 120 s in a volume of 10 µl, followed by a 10 µl flush of physiological saline.

Abbreviations

Achc = 2-aminocyclohexanecarboxylic acid
ANOVA = analysis of variance
AUC = area under the curve
βMePhe = β-methylphenylalanine
CI = 95% confidence intervals
Dmt = 2',6'-dimethyltyrosine
ED_{25} = 25% effective dose
EM-1 = endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2)
EM-2 = endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2)
EMD1 = Tyr-(1S,2R)Achc-Phe-pFPhe-NH_2
EMD2 = Tyr-(1S,2R)Achc-Phe-(2S,3S)βMePhe-NH_2
EMD3 = Dmt-(1S,2R)Achc-Phe-pFPhe-NH_2
EMD4 = Dmt-(1S,2R)Achc-Phe-(2S,3S)βMePhe-NH_2
HP = hot-plate
i.t = intrathecally
i.c.v. = intracerebroventrical
MOR = µ-opioid receptor
%MPE = % maximum possible effect
pFPhe = para-fluorophenylalanine
TF = tail-flick

Monosodium iodoacetate-induced inflammation

Osteoarthritis was induced by injecting MIA (1 mg/30 µl) into the tibiotarsal joint of the right hind leg on two consecutive days. All treatments were given to gently restrained conscious animals, using a 27-gauge needle, without anaesthesia so as to exclude any drug interaction. These injections did not elicit signs of major distress. Animals were allowed to recover for 14 days, which has consistently been shown to cause severe end-stage cartilage destruction resulting in osteoarthritis-like joint pain (3, 13).

To determine the changes in the size of the inflamed joint, we measured the anteroposterior and mediolateral diameter of the paw at the level of ankle joint with a digital caliper. The cross-section area was calculated with the formula $a \times b \times \pi$, where a and b are the radii in the two aspects.

Intrathecal catheterization

Two weeks after MIA administration rats were anaesthetized with a mixture of ketamine hydrochloride and xylazine (72 and 8 mg/kg intraperitoneally: i.p., respectively). An i.t. catheter (PE-10 tubing; Intramedic, Clay Adams; Becton Dickinson; Parsippany, NJ; I.D. 0.28 mm; O.D. 0.61 mm) was inserted via the cisterna magna and passed 8.5 cm caudally into the subarachnoid space (42), which served to place the catheter tip between Th12 and L2 vertebrae, corresponding to the spinal segments that innervate the hindpaws (6). After surgery, rats were housed individually and had free access to food and water. Animals exhibiting postoperative neurologic deficits (about 10%), and also those that did not show paralysis of one of the hindpaws after the administration of 100 µg lidocaine (about 0.5%) were excluded (6). After the surgery, animals were administered antibiotics (13 mg/kg gentamicin, subcutaneously) to prevent infection. The rats were allowed to recover for at least four days before testing, and were assigned randomly to the treatment groups. The observer was blind to the treatment administered. Repeated intrathecal injections in the same animals were separated by 5–7 days.

Behavioral nociceptive testing

Mechanical allodynia was determined using a dynamic plantar aesthesiometer (Ugo Basile, Comerio, Italy). Prior to baseline testing, each rat was habituated to a testing box with a wire-mesh grid floor for at least 20 min. Measurements were done with a straight metal filament that exerts an increasing upward force at a constant rate (4.25 g/s) with a maximum cut-off force of 50 g. The filament was placed under the plantar surface of the hind paw. Measurement was stopped when the paw was withdrawn, and results were expressed as paw withdrawal thresholds in grams.

Experimental protocol

After baseline determination of tibiotarsal joint diameter and mechanical paw withdrawal threshold (pre-MIA baseline values at 1st day), MIA was injected. These measurements were repeated 7 and 14 days later, and then the intrathecal catheterization was performed. One week later the non-paralysed rats were selected and after the post-MIA baseline value determination, the different ligands were administered (Table I). The control group received physiological saline. In the positive control group, animals were treated with 10 µg morphine. The pain thresholds were registered 10, 20, 30, 45, 60, 75, 90, 105 and 120 min after the intrathecal injection, and the mean of the values obtained between 10–30, 45–75 and 90–120 min were analyzed.

At the end of the experiment, the joint diameters were measured again. We did not examine the motor behavior systematically, nor did we quantify it, but the animals' behaviors were observed, and in most animals there were no signs of altered behavior (immobility, flaccidity, excitation or motor weakness), except for the administration of 10 μg EMD3 which produced paralysis. Animal suffering and the number of animals per group were kept at a minimum.

Statistical analysis

Data are presented as means ± SEM. Paw withdrawal thresholds on the inflamed side were transformed to % maximum possible effect (%MPE) by the following formula:
%MPE = (observed threshold – post-MIA baseline threshold) / (50 – post-MIA baseline threshold) × 100.

Therefore, 100% MPE means perfect relief of allodynia (equivalent to cut-off value [50 g] for all measurements), while 0% MPE means that the observed threshold is equivalent to the post-MIA baseline value. The time-course data sets were examined by two-way analysis of variance. The significance of differences between the different groups was calculated using the Fisher LSD test for post hoc comparison (p value < 0.05 was considered significant).

Area under the curve (AUC) values were obtained by calculating the area between 0 and 120 min to construct dose response curves for different doses of ligands. AUC 5500 (AUC_{max}) value would mean the complete relief of hyperalgesia (50 g) during the whole period. We observed almost no effects regarding the AUC values after saline treatment (AUC_{min} = 2813 ± 227). The mean AUC values were used for linear regression analysis (least square method) to determine the ED_{25} values with 95% confidence intervals (CI). The 25% effective dose (ED_{25}) means the dose that yielded 25 % increase in the PWD latency for the whole period (32).

The AUC data sets were examined by one-way analysis of variance (ANOVA), and the time-course curves were analyzed by repeated measurement of ANOVA. Post hoc comparisons were carried out with the Fisher's LSD test. Statistical analysis was performed with the STATISTICA for Windows (Statistica Inc., Tulsa, Oklahoma, USA) and GraphPad Prism 4.0 (GraphPad Software Inc. La Jolla, CA, US) softwares.

Results

Joint edema

The MIA injection caused a permanent increase in joint cross-section area compared with the contralateral side (48.4 ± 0.37 vs. 38.3 ± 0.15 mm^2, $p < 0.01$). This conspicuous increase in joint size was a result of edema formation, confirming that the MIA treatment resulted in an inflammatory reaction (1). None of the treatments influenced the degree of edema; the cross section of the ankle was 49.5 ± 0.53 mm^2 at the end of the experiments, which did not differ from the post-MIA baseline value.

Mechanosensitivity

Basal mechanical withdrawal threshold was 41 ± 0.6 g, and MIA caused a significant decrease in paw withdrawal threshold on the inflamed side. This threshold was lowest after 1 week of MIA (15 ± 0.6 g), and later it stabilized at 24 ± 0.5 g. There was no significant difference between before and after intrathecal catheterization (p = 0.62), suggesting that the catheterization did not change the inflammatory pain sensitivity. MIA did not have a

significant influence on the non-inflamed side (43 ± 0.5 g). None of the treatments changed the mechanosensitivity on the non-inflamed side; therefore, results were analyzed only on the inflamed paws.

All the drugs had antinoallodynic potency therefore, we compared the effects of different doses of the analogues with EM-2. As for the lowest dose (0.3 μg), ANOVA with repeated measurements showed significant effects of time ($F_{2,104} = 9.6$, $p < 0.001$) and interaction ($F_{10,104} = 2.4$, $p < 0.05$). The post hoc comparison revealed that EMD3 and EMD4 produced significant antinociception, while EM-2 and the other two ligands were ineffective in this dose (Fig. 1).

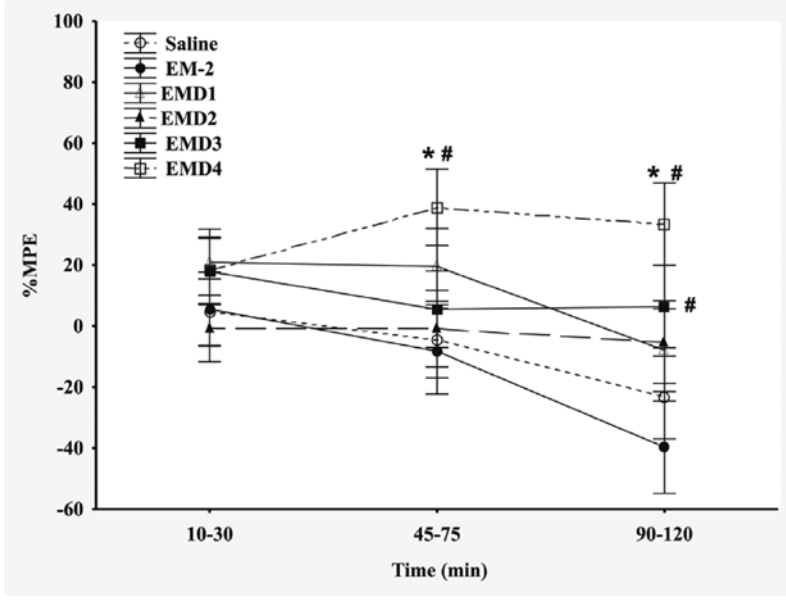

Fig. 1. Time-course effects of EM2 and the derivatives at 0.3 μg dose.
Each point signifies the mean ± SEM of the results. * indicates a significant ($p < 0.05$) difference as compared with the saline-treated group. # denotes a significant difference as compared with the EM-2-treated group

At 3 μg, all of the ligands produced antiallodynia. ANOVA with repeated measurements showed significant effects of treatment ($F_{5,55} = 5.4$, $p < 0.001$) and time ($F_{2,110} = 5.6$, $p < 0.01$). Post hoc comparison showed that EMD3 was more effective than EM-2 during the last investigated interval (75–120 min, Fig. 2).

Regarding the highest dose, 10 μg of EMD3 caused prolonged paralysis of the animals, therefore, we could not analyze their data on the pain test. ANOVA with repeated measurements showed significant effects of treatment ($F_{5,50} = 8.4$, $p < 0.001$) and time ($F_{2,100} = 19.8$, $p < 0.001$). Post hoc comparison showed that all the drugs were effective compared to the control group at the first and second investigation period, however, only EMD4 was effective during the whole period compared to both control and EM-2 treated groups (Fig. 3). Morphine, as a positive control, produced long-lasting and highly effective antinociception. EM-2, EMD1 and EMD2 were effective as morphine only at the first investigated period (10–30 min), while the effect of EMD4 did not differ significantly from morphine during the whole period.

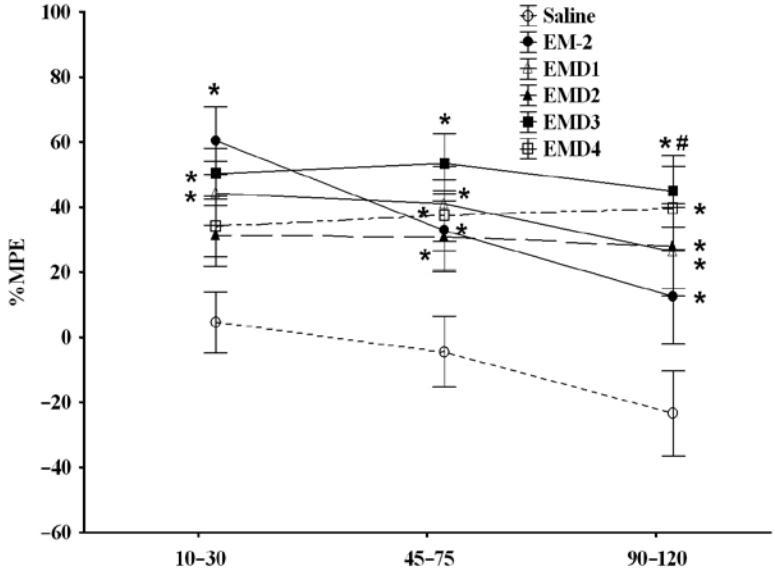

Fig. 2. Time-course effects of EM2 and the derivatives at 3 μg dose.
Each point signifies the mean ± SEM of the results. * indicates a significant ($p < 0.05$) difference as compared with the saline-treated group. # denotes a significant difference as compared with the EM-2-treated group

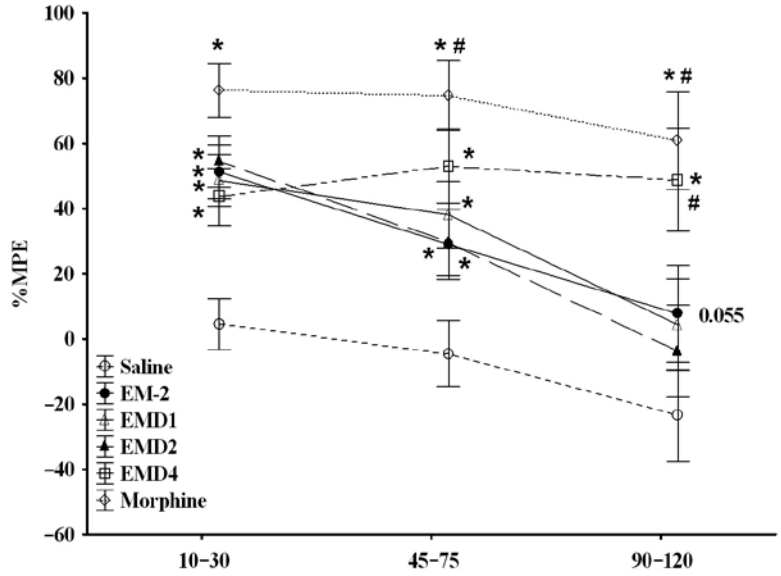

Fig. 3. Time-course effects of EM2, morphine and the derivatives at 10 μg dose.
Each point signifies the mean ± SEM of the results. * indicates a significant ($p < 0.05$) difference as compared with the saline-treated group. # denotes a significant difference as compared with the EM-2-treated group

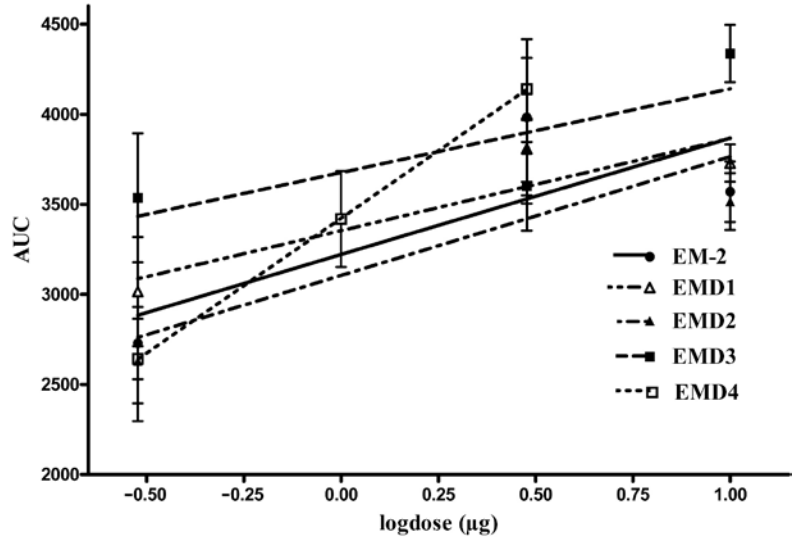

Fig. 4. The dose-dependent effects of EM and derivatives (AUC values between 0–120 min)

As for the dose-response curves of the ligands, we observed that in the case of EMD3 the steepness of the curve is higher, and the curve of EMD4 is also slightly left-shifted (Fig. 4). However, the ED25 values did not reveal significant difference between these ligands (Table I).

Table I. The applied drugs and their dosage, the number of animals in each group and *in vivo* potency

	Doses (µg) and number of animals					
	0	**0.3**	**1**	**3**	**10**	**ED_{25} (CI) (µg)**
Saline	8					
EM-2: Tyr-Pro-Phe-Phe-NH2		8		8	9	6.3 [1.30–10.00]
EMD1: Tyr-(1S,2R)Achc-Phe-pFPhe-NH_2		10		9	10	5.3 [1.73–8.85]
EMD2: Tyr-(1S,2R)Achc-Phe-(2S,3S)βMePhe-NH_2		10		10	10	7.2 [2.95–10.00]
EMD3: Dmt-(1S,2R)Achc-Phe-pFPhe-NH_2		10	9	14		1.6 [1.02–2.11]
EMD4: Dmt-(1S,2R)Achc-Phe-(2S,3S)βMePhe-NH_2		10		10	8	2.6 [0.31–4.81]
Morphine					9	

Abbreviations: ED_{25}: 25% effective dose after intrathecal administration. CI: confidence interval

Discussion

We found that new EM-2 analogues with unnatural amino acids produced dose-dependent antinociception. In agreement with the *in vitro* results, the ligands with high potency at MOR and long half-life (EMD3 and EMD4) were the most effective in the *in vivo* tests (22). To our knowledge, we are also the first to prove the antinociceptive effects of intrathecally administered morphine and EM-2 in a MIA-induced osteoarthritis model. These data are in agreement with some earlier studies showing the antinociceptive effect of systemically administered morphine in this model (5, 40).

The modification of different opioid ligands is a well-known method for the enhancement of their antinociceptive potency. An earlier study showed that replacement of Tyr^1 by Dmt resulted in marked increases in receptor-binding affinity and bioactivity in numerous opioid peptide agonists and antagonists (4). Replacement of Pro^2 by alicyclic β-amino acids, pseudoprolines or piperidine-2-, -3- and -4-carboxylic acids resulted in increased affinity for the MOR and enhanced proteolytic stability (7, 14, 16, 35). The insertion of pFPhe in place of the Phe^4 in enkephalin or endomorphins resulted in increased potency in functional assays (22, 38). Regarding the in vivo antinociceptive potency of endomorphin derivatives, several studies investigated the effects of different analogues after systemic or intracerebroventrical (i.c.v.) administration in acute pain tests, while only a few studies are available on the effects of derivatives at spinal level. Furthermore, no data were available about their effects in chronic pain models. Thus, different cyclic analogues of EM-2 induced more potent and/or prolonged antinociception in the hot-plate (HP) test after i.c.v. administration in mice compared to the parent ligand (17, 27, 29). EM analogues containing D-amino acids also induced effective antinociception in mice assessed in HP or tail-flick (TF) test after i.c.v. administration (21, 28). EM analogues containing other natural (e.g. arginine) or non-natural aminoacids (e.g. phenylglycine or homophenylalanine) had more prolonged and/or more potent antinociception in acute heat pain tests after i.c.v. administration in mice (8, 41, 43). A number of studies proved that in contrast to the parent ligands, some analogues could produce antinociception after peripheral administration, too, which suggests that these substances can pass thorugh the blood-brain barrier (27). A few studies found that analogues of EMs could antagonise opioid-induced antinociception after i.t. or i.c.v. administration in HP or TF tests in mice (33).

EM-2 analogoues containing N-methylated amino acids consecutively in each position showed the strongest analgesic effect when administered centrally in the HP test in mice (18). An earlier study showed that a dimethyl-analogue of EM-2 (Dmt^1-EM-2) produced antinociception after i.t. injection in formalin test (rat) (19). The effect evoked by Dmt^1-EM-2 was similar to the antinociceptive effect of EM-2 in the first phase but it was much stronger in the second phase. As for our results, we found that following the modification of the parent ligand at the 1^{st}, 2^{nd} and 4^{th} positions by unnatural aminoacids the antinociceptive potency of these analogues remained. EMD1 and EMD2 which was modified in the 2^{nd} and 4^{th} positions had similar effects as EM-2, and this is in agreement with their K_i values for MOR, too (22). The insertion of 3 amino acids at the 1^{st}, 2^{nd} and 4^{th} positions (EMD3 and EMD4) caused a considerable potency increase at MOR *in vitro,* and these ligands had also long half-life in a crude rat brain membrane homogenate (22). Therefore, the activation of the MOR and their high metabolic stability could have led to prolonged antinociception in our model.

All of the above-mentioned studies applied acute heat or chemical pain models. However, osteoarthritis, a widespread condition, affects several million in the World accompanied by chronic pain. Intra-articular injection of MIA in the joint of rats disrupts

chondrocyte metabolism resulting in cartilage degeneration and subsequent nociceptive behavior that has been described as a model of osteoarthritis pain (3). An earlier study showed that systemic administration of morphine reversed the hind limb weight bearing decrease in this model (30). A recent study proved that MIA-induced joint pain was associated with significant changes in the spinal cord, too, that is associated with increased phosphorylation of mitogen activated protein kinases, and it was suggested that these changes were involved in nociceptie behaviors (20). Our study showed that intrathecally applied morphine, EM-2 and derivatives can decrease the MIA-induced mechanical allodynia, supporting the role of the opioid receptors in the spinal cord in this type of pain as well.

To our knowledge, our results are the first to demonstrate that complex modification of endomorphins by introduction of Dmt, alicyclic β-amino acids, βMePhe, and pFPhe in the EM-2 can induce effective and prolonged antinociception in a chronic arthritis model. It is very important that the antinociceptive effects are in agreement with the binding experiments, that is, the ligands with high potency at MOR with long half-life (EMD3 and EMD4) were the most effective in the *in vivo* tests, too. These structural modifications of EM-2 might be a promising strategy to enhance bioavailability of peptides and may serve a role in the development of novel endomorphin analogues with increased therapeutic potential. Further studies are required to clarify the possible side-effects of these ligands.

Acknowledgements

This work was supported by a grant of TAMOP 4.2.2.-08/01-2008-0002, Hungarian Research Grants (OTKA: K83810) and "Normolife" European Grant (LSHC-CT-2006-037733). The authors wish to thank Agnes Tandari and Eva Papp for her technical assistance.

REFERENCES

1. Bar-Yehuda S, Rath-Wolfson L, Del Valle L, Ochaion A, Cohen S, Patoka R, Zozulya G, Barer F, Atar E, Pina-Oviedo S, Perez-Liz G, Castel D, Fishman P: Induction of an antiinflammatory effect and prevention of cartilage damage in rat knee osteoarthritis by CF101 treatment. Arthritis Rheum. 60, 3061–3071 (2009)
2. Bedini A, Baiula M, Gentilucci L, Tolomelli A, De Marco R, Spampinato S: Peripheral antinociceptive effects of the cyclic endomorphin-1 analog c[YpwFG] in a mouse visceral pain model. Peptides 31, 2135–2140 (2010)
3. Bove SE, Calcaterra SL, Brooker RM, Huber CM, Guzman RE, Juneau PL, Schrier DJ, Kilgore KS: Weight bearing as a measure of disease progression and efficacy of anti-inflammatory compounds in a model of monosodium iodoacetate-induced osteoarthritis. Osteoarthritis Cartilage 11, 821–830 (2003)
4. Bryant SD, Jinsmaa Y, Salvadori S, Okada Y, Lazarus LH: Dmt and opioid peptides: a potent alliance. Biopolymers 71, 86–102 (2003)
5. Chandran P, Pai M, Blomme EA, Hsieh GC, Decker MW, Honore P: Pharmacological modulation of movement-evoked pain in a rat model of osteoarthritis. Eur. J. Pharmacol. 613, 39–45 (2009)
6. Dobos I, Toth K, Kekesi G, Joo G, Csullog E, Klimscha W, Benedek G, Horvath G: The significance of intrathecal catheter location in rats. Anesth. Analg. 96, 487–492 (2003)
7. Fichna J, Gach K, Perlikowska R, Cravezic A, Bonnet JJ, do-Rego JC, Janecka A, Storr MA: Novel endomorphin analogues with antagonist activity at the mu-opioid receptor in the gastrointestinal tract. Regul. Pept. 162, 109–114 (2010)
8. Gao Y, Liu X, Liu W, Qi Y, Liu X, Zhou Y, Wang R: Opioid receptor binding and antinociceptive activity of the analogues of endomorphin-2 and morphiceptin with phenylalanine mimics in the position 3 or 4. Bioorg. Med. Chem. Lett. 16, 3688–3692 (2006)
9. Gentilucci L, Tolomelli A: Recent advances in the investigation of the bioactive conformation of peptides active at the mu-opioid receptor. Conformational analysis of endomorphins. Curr. Top. Med. Chem. 4, 105–121 (2004)

10. Horvath G: Endomorphin-1 and endomorphin-2: pharmacology of the selective endogenous mu-opioid receptor agonists. Pharmacol. Ther. 88, 437–463 (2000)
11. Janecka A, Kruszynski R: Conformationally restricted peptides as tools in opioid receptor studies. Curr. Med. Chem. 12, 471–481 (2005)
12. Janecka A, Staniszewska R, Gach K, Fichna J: Enzymatic degradation of endomorphins. Peptides 29, 2066–2073 (2008)
13. Kalbhen DA: Chemical-model of osteoarthritis – a pharmacological evaluation. J. Rheumatol. 14, 130–131 (1987)
14. Keller M, Boissard C, Patiny L, Chung NN, Lemieux C, Mutter M, Schiller PW: Pseudoproline-containing analogues of morphiceptin and endomorphin-2: evidence for a cis Tyr-Pro amide bond in the bioactive conformation. J. Med. Chem. 44, 3896–3903 (2001)
15. Keresztes A, Borics A, Toth G: Recent advances in endomorphin engineering. Chem. Med. Chem. 5, 1176–1196 (2010)
16. Keresztes A, Szucs M, Borics A, Kover KE, Forro E, Fulop F, Tomboly C, Peter A, Pahi A, Fabian G, Muranyi M, Toth G: New endomorphin analogues containing alicyclic beta-amino acids: influence on bioactive conformation and pharmacological profile. J. Med. Chem. 51, 4270–4279 (2008)
17. Kruszynski R, Fichna J, do-Rego JC, Chung NN, Schiller PW, Kosson P, Costentin J, Janecka A: Novel endomorphin-2 analogs with mu-opioid receptor antagonist activity. J. Pept. Res. 66, 125–131 (2005)
18. Kruszynski R, Fichna J, do-Rego JC, Janecki T, Kosson P, Pakulska W, Costentin J, Janecka A: Synthesis and biological activity of N-methylated analogs of endomorphin-2. Bioorg. Med. Chem. 13, 6713–6717 (2005)
19. Labuz D, Chocyk A, Wedzony K, Toth G, Przewlocka B: Endomorphin-2, deltorphin II and their analogs supress formalin-induced nociception and c-Fos expression in the rat spinal cord. Life Sci. 73, 403–412 (2003)
20. Lee Y, Pai M, Brederson JD, Wilcox D, Hsieh G, Jarvis M, Bitner R: Monosodium iodoacetate-induced joint pain is associated with increased phosphorylation of mitogen activated protein kinases in the rat spinal cord. Mol. Pain 7, 39 (2011)
21. Liu H, Zhang B, Liu X, Wang C, Ni J, Wang R: Endomorphin-1 analogs with enhanced metabolic stability and systemic analgesic activity: design, synthesis, and pharmacological characterization. Bioorg. Med. Chem. 15, 1694–1702 (2007)
22. Mallareddy JR, Borics A, Keresztes A, Kover KE, Tourwe D, Toth G: Design, synthesis, pharmacological evaluation, and structure-activity study of novel endomorphin analogues with multiple structural modifications. J. Med. Chem. 54, 1462–1472 (2011)
23. Mentlein R: Dipeptidyl-peptidase IV (CD26)-role in the inactivation of regulatory peptides. Regul. Pept. 85, 9–24 (1999)
24. Mizoguchi H, Nakayama D, Watanabe H, Ito K, Sakurada W, Sawai T, Fujimura T, Sato T, Sakurada T, Sakurada S: Involvement of spinal mu1-opioid receptors on the Tyr-d-Arg-Phe-sarcosine-induced antinociception. Eur. J. Pharmacol. 540, 67–72 (2006)
25. Olson GA, Olson RD, Vaccarino AL, Kastin AJ: Endogenous opiates: 1997. Peptides 19, 1791–1843 (1998)
26. Paterlini MG, Avitabile F, Ostrowski BG, Ferguson DM, Portoghese PS: Stereochemical requirements for receptor recognition of the mu-opioid peptide endomorphin-1. Biophys. J. 78, 590–599 (2000)
27. Perlikowska R, do-Rego JC, Cravezic A, Fichna J, Wyrebska A, Toth G, Janecka A: Synthesis and biological evaluation of cyclic endomorphin-2 analogs. Peptides 31, 339–345 (2010)
28. Perlikowska R, Fichna J, Wyrebska A, Poels J, Vanden Broeck J, Toth G, Storr M, Do Rego JC, Janecka A: Design, synthesis and pharmacological characterization of endomorphin analogues with non-cyclic amino acid residues in position 2. Basic Clin. Pharmacol. Toxicol. 106, 106–113 (2010)
29. Perlikowska R, Gach K, Fichna J, Toth G, Walkowiak B, do-Rego JC, Janecka A: Biological activity of endomorphin and [Dmt1]endomorphin analogs with six-membered proline surrogates in position 2. Bioorg. Med. Chem. 17, 3789–3794 (2009)
30. Pomonis JD, Boulet JM, Gottshall SL, Phillips S, Sellers R, Bunton T, Walker K: Development and pharmacological characterization of a rat model of osteoarthritis pain. Pain 114, 339–346 (2005)
31. Przewlocka B, Mika J, Labuz D, Toth G, Przewlocki R: Spinal analgesic action of endomorphins in acute, inflammatory and neuropathic pain in rats. Eur. J. Pharmacol. 367, 189–196 (1999)
32. Raffa RB, Friderichs E, Reimann W, Shank RP, Codd EE, Vaught JL, Jacoby HI, Selve N: Complementary and synergistic antinociceptive interaction between the enantiomers of tramadol. J. Pharmacol. Exp. Ther. 267, 331–340 (1993)

33. Sakurada S, Watanabe H, Hayashi T, Yuhki M, Fujimura T, Murayama K, Sakurada C, Sakurada T: Endomorphin analogues containing D-Pro2 discriminate different mu-opioid receptor mediated antinociception in mice. Br. J. Pharmacol. 137, 1143–1146 (2002)
34. Shane R, Wilk S, Bodnar RJ: Modulation of endomorphin-2-induced analgesia by dipeptidyl peptidase IV. Brain Res. 815, 278–286 (1999)
35. Staniszewska R, Fichna J, Gach K, Toth G, Poels J, Broeck JV, Janecka A: Synthesis and biological activity of endomorphin-2 analogs incorporating piperidine-2-, 3- or 4-carboxylic acids instead of proline in position 2. Chem. Biol. Drug Des. 72, 91–94 (2008)
36. Tomboly C, Kover KE, Peter A, Tourwe D, Biyashev D, Benyhe S, Borsodi A, Al Khrasani M, Ronai AZ, Toth G: Structure-activity study on the Phe side chain arrangement of endomorphins using conformationally constrained analogues. J. Med. Chem. 47, 735–743 (2004)
37. Tomboly C, Peter A, Toth G: In vitro quantitative study of the degradation of endomorphins. Peptides 23 (9), 1573–1580 (2002)
38. Toth G, Kramer TH, Knapp R, Lui G, Davis P, Burks TF, Yamamura HI, Hruby VJ: [D-Pen2,D-Pen5]Enkephalin analogs with increased affinity and selectivity for delta-opioid receptors. J. Med. Chem. 33, 249–253 (1990)
39. Vaccarino AL, Kastin AJ: Endogenous opiates: 2000. Peptides 22, 2257–2328 (2001)
40. Vonsy JL, Ghandehari J, Dickenson AH: Differential analgesic effects of morphine and gabapentin on behavioural measures of pain and disability in a model of osteoarthritis pain in rats. Eur. J. Pain 13, 786–793
41. Wang Cl, Guo C, Wang Yq, Zhou Y, Li Q, Ni J, Wang R: Synthesis and antinociceptive effects of endomorphin-1 analogs with C-terminal linked by oligoarginine. Peptides 32, 293–299 (2011)
42. Yaksh TL, Rudy TA: Chronic catheterization of the spinal subarachnoid space. Physiol. Behav. 17, 1031–1036 (1976)
43. Yu Y, Shao X, Wang Cl, Liu HM, Cui Y, Fan YZ, Liu J, Wang R: In vitro and in vivo characterization of opioid activities of endomorphins analogs with novel constrained C-terminus: evidence for the important role of proper spatial disposition of the third aromatic ring. Peptides 28, 859–870 (2007)
44. Zadina JE, Hackler L, Ge LJ, Kastin AJ: A potent and selective endogenous agonist for the mu-opiate receptor. Nature 386, 499–502 (1997)
45. Zhao QY, Chen Q, Yang DJ, Feng Y, Long Y, Wang P, Wang R: Endomorphin 1[[psi]] and endomorphin 2[[psi]], endomorphins analogues containing a reduced (CH2NH) amide bond between Tyr1 and Pro2, display partial agonist potency but significant antinociception. Life Sci. 77, 1155–1165 (2005)