Hybrid peptides endomophin-2/DAMGO: Design, synthesis and biological evaluation

ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · AUGUST 2013

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2013.07.044 · Source: PubMed

9 AUTHORS, INCLUDING:

Roberto Costante
Università degli Studi G. d'Annunzio Chieti …
23 PUBLICATIONS **71** CITATIONS
SEE PROFILE

Grazia Luisi
Università degli Studi G. d'Annunzio Chieti …
34 PUBLICATIONS **274** CITATIONS
SEE PROFILE

Stefano Pieretti
Istituto Superiore di Sanità
100 PUBLICATIONS **1,050** CITATIONS
SEE PROFILE

Sándor Benyhe
Hungarian Academy of Sciences
133 PUBLICATIONS **1,550** CITATIONS
SEE PROFILE

European Journal of Medicinal Chemistry 68 (2013) 167–177

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Hybrid peptides endomophin-2/DAMGO: Design, synthesis and biological evaluation

Adriano Mollica [a,*], Roberto Costante [a], Azzurra Stefanucci [a], Francesco Pinnen [a], Grazia Luisi [a], Stefano Pieretti [b], Anna Borsodi [c], Engin Bojnik [c], Sándor Benyhe [c]

[a] Dipartimento di Farmacia, Università degli Studi "G. d'Annunzio" di Chieti-Pescara, Via dei Vestini 31, 66100 Chieti, Italy
[b] Istituto superiore di Sanità, Dipartimento del Farmaco, Viale Regina Elena 299, 00161 Rome, Italy
[c] Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences, Temesvari krt 62, 6726 Szeged, Hungary

ARTICLE INFO

Article history:
Received 15 March 2013
Received in revised form
3 July 2013
Accepted 5 July 2013
Available online 11 August 2013

Keywords:
Analgesics
DAMGO
Endomorphins
Hot plate
Nociception
Opioid
Tail flick

ABSTRACT

Endomorphin-2 [Tyr-Pro-Phe-Phe-NH_2] and DAMGO [Tyr-D-Ala-Gly-(*N*–Me)Phe-Gly-ol] are natural (EM2) and synthetic (DAMGO) opioid peptides both selective for μ opioid receptor with high analgesic activity. In this work we report synthesis, *in vitro* and *in vivo* biological evaluation of five new hybrid EM2/DAMGO analogues, with the aim to obtain compounds with high affinity at μ-opioid receptor, high activity in animal nociception tests (hot plate and tail flick) and improved enzymatic stability. Double *N*-methylation on both Phe residues and C-terminal ethanolamide led to analogue **6e**, which possesses a good *in vitro* μ affinity (K_i^{μ} = 34 nM), combined with a remarkable *in vivo* antinociceptive activity.

1. Introduction

Morphine and other opioid drugs are the most used analgesics for the relief of moderate and severe pain [1]. However, prolonged use of these compounds leads to a well-known range of side-effects, including tolerance, physical dependence and respiratory depression [2,3].

Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2, EM1) and Endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2, EM2) are endogenous μ-selective opioid peptides originally isolated from bovine brain [4] and subsequently from human brain cortex [5] by Zadina and Hackler. Endomorphins (EMs) present a unique *N*-terminal sequence Tyr-Pro-Trp/Phe structurally related to opioid peptide morphiceptin [6,7], different from the typical *N*-terminal sequence Tyr-Gly-Gly-Phe of other opioid peptides, such as enkephalins and dynorphins [8]. This structural features deeply influence the activity, hence EMs label μ opioid receptor (MOR) with a high affinity and selectivity compared to a poor affinity for δ and κ receptors [2,9–12]. Endomorphins exhibit potent *in vivo* antinociceptive activity, which duration depends on the species, pain tests applied, and administration route [13–16]. In contrast to other opioids, including morphine, EMs could also be effective in treatment of neuropathic pain [17,18].

According to the *message/address* concept [19], endomorphins' structure can be divided into two parts: the *N*-terminal sequence Tyr-Pro-Trp/Phe, which represents the *message* sequence and provides the correct conformation in the receptor binding and the Phe^4-NH_2 moiety, which is the *address* sequence which contributes to peptide stability and selectivity [20–22]; the Pro^2 residue acts as stereochemical spacer, inducing the correct orientation of the other residues necessary for receptor–ligand interaction [23–26]. The Tyr^1-Pro^2 amide bond exists in an equilibrium mixture of *cis*/*trans* conformations [27]; the isomerization around this bond seems to be a crucial factor to control π–π interaction between the three aromatic rings, determining the preferred EM conformation [23,28,29]. Keller et al. demonstrated that the bioactive conformation is *cis* [30], after estimation of *cis*/*trans* EMs ratios in various conditions [20–23,25].

* Corresponding author. Tel.: +39 08713554476; fax: +39 08713554470.
E-mail address: a.mollica@unich.it (A. Mollica).

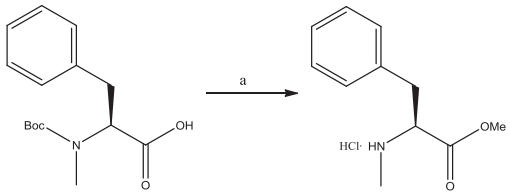
0223-5234/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejmech.2013.07.044

Endomorphin-related ligands show favorable profiles in terms of analgesia and tolerance/dependence, and seem to produce less respiratory depression than other agonists [31], nevertheless the susceptibility to enzymatic degradation [32,33] and a limited uptake in the central nervous system of opioid peptides strongly reduces a possible therapeutic application [34].

To obtain more stable and efficacious μ selective agonists, several chemical modifications of EMs were performed, and a large number of products were synthesized [16,35–46].

DAMGO [Tyr-D-Ala-Gly-(*N*-Me)Phe-Gly-ol] is a highly μ selective opioid peptide, synthetized for the first time in 1980 [47]. Its structural features are different from those of endomorphins being more similar to enkephalins, in fact Tyr^1 is conserved, $D\text{-}Ala^2$ was introduced in place of Gly, while *N*-methyl Phe in position 3 and the *C*-terminus ethanolamine represent the distinctive features. In *mouse writhing nociception test*, DAMGO was more than 100-fold potent than morphine [47]. In addition, DAMGO displays an increased enzymatic stability compared to other opioid peptides. These characteristics make DAMGO excellent as radiolabeled ligand for μ receptors for *in vitro* binding assays, used as a standard [48,49].

In this work we report synthesis, *in vitro* and *in vivo* biological evaluation of five new hybrid EM2/DAMGO analogues (Fig. 1). The aim of this project is to combine the distinctive features of EMs and DAMGO in order to obtain compounds with a remarkable affinity at μ-opoid receptor, high activity in animal nociception tests (hot plate and tail flick), together with an improved resistance toward enzymatic degradation due to the presence of *N*-methylated residues as previously reported [50–52]. In this paper the typical EM Pro^2 residues have been maintained, as well as the DAMGO *C*-terminal ethanolamine. *N*-methylation was performed on the Phe^3 and Phe^4 residues, with the exception for compound **6d**, in which Sarcosine (*N*–Me Gly) was used in place of Phe^3.



1a

Scheme 1. Synthesis of intermediate **1a**. Reagents and conditions: (a) $SOCl_2$, MeOH.

2. Chemistry

All products were synthesized in solution phase [53]. For compounds **6a** and **6e**, HCl·(*N*–Me)Phe-OMe was obtained starting from commercially available Boc-(*N*–Me)Phe-OH by treatment with $SOCl_2$ in MeOH for 3 h at r.t. (Scheme 1) [54]. Coupling reactions were performed using EDC/HOBt/DIPEA in DMF, with the exception of compound **6e**, for which Bop-Cl/NMM in DMF were used in the first two couplings. Deprotection of N^{α}-*tert*-butyloxycarbonyl group was performed using 1:1 TFA/CH_2Cl_2 mixture for 1 h, under N_2 atmosphere (Scheme 2).

3. Results and discussion

The *in vitro* biological evaluation of novel EM2-DAMGO analogues **6a**–**e** was performed as previously described [55–57] and results are shown in Table 1. The binding affinity of the hybrid peptides **6a**–**e** toward rat δ- and rat μ-opioid receptors was determined by competitive binding against [^{3}H]Ile^{5,6}deltorphin-II [58] and [^{3}H]DAMGO [49] respectively. For functional characterization

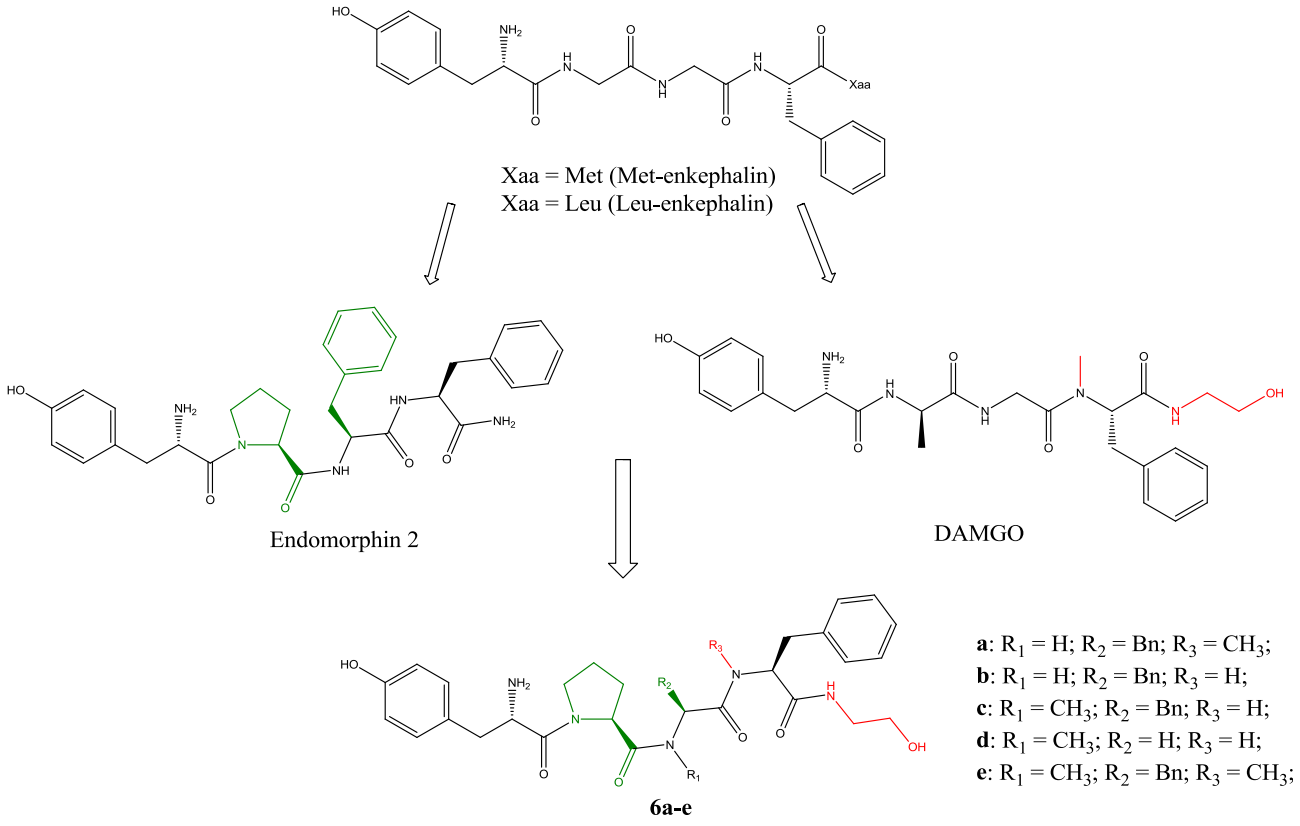
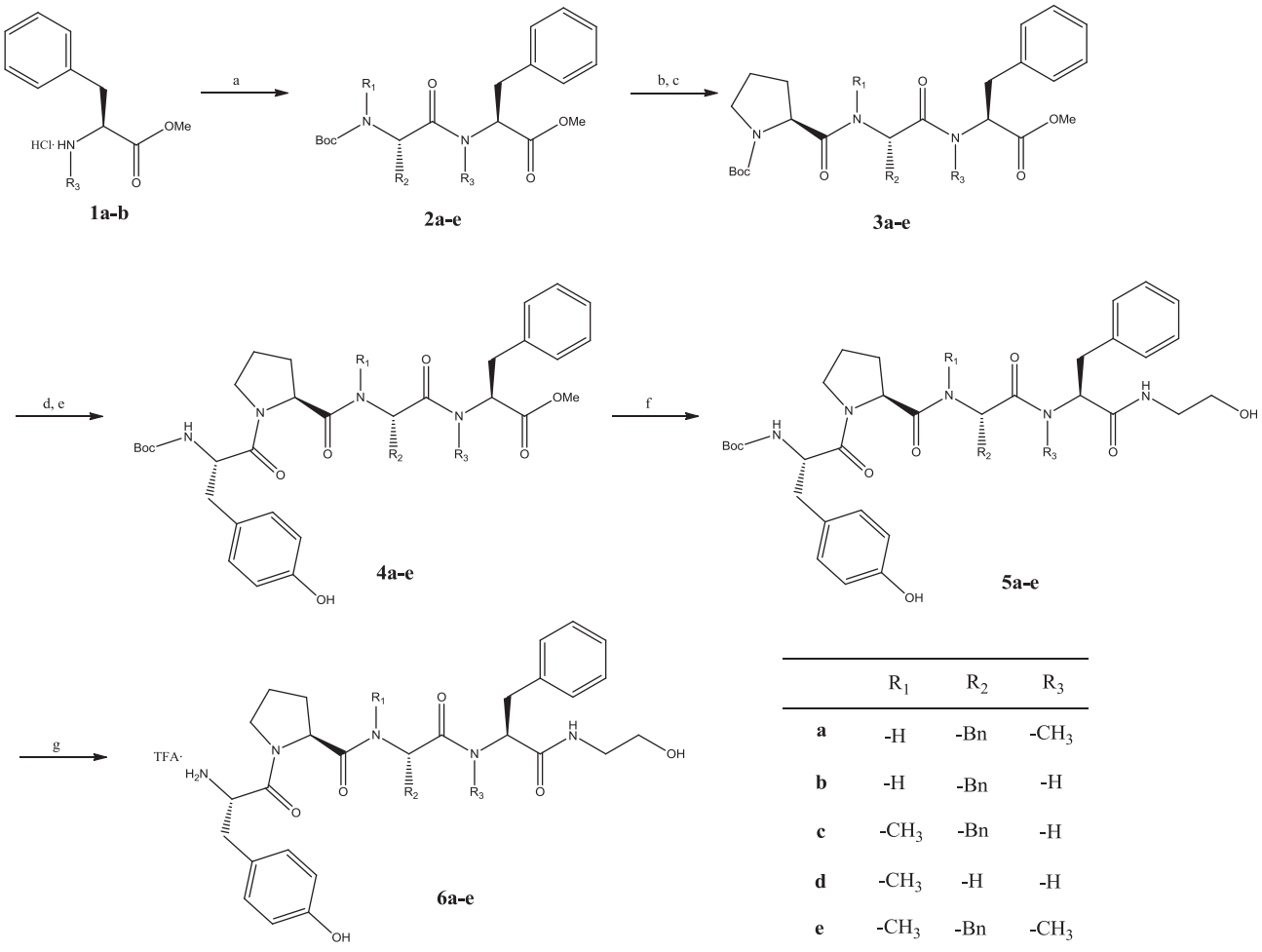


Fig. 1. Structures of Met/Leu-enkephalins, Endomorphin-2, DAMGO and analogues **6a**–**e**.



	R_1	R_2	R_3
a	-H	-Bn	$-CH_3$
b	-H	-Bn	-H
c	$-CH_3$	-Bn	-H
d	$-CH_3$	-H	-H
e	$-CH_3$	-Bn	$-CH_3$

Scheme 2. Synthesis of products **6a**–**e**. Reagents and conditions: (a) Boc-Phe-OH for compounds **2a**–**b**; Boc-*N*(Me)Phe-OH for compounds **2c** and **2e**; Boc-Sar-OH for compound **2d**; EDC, HOBt·H_2O, DIPEA, DMF for compounds **2a**–**d**; Bop-Cl, NMM, CH_2Cl_2 for compound **2e**. (b) TFA 50% in CH_2Cl_2. (c) Boc-Pro-OH, EDC, HOBt·H_2O, DIPEA, DMF for compounds **3a**–**d**; Boc-Pro-OH, Bop-Cl, NMM, CH_2Cl_2 for compound **3e**. (d) TFA 50% in CH_2Cl_2. (e) Boc-Tyr-OH, EDC, HOBt·H_2O, DIPEA, DMF for compounds **4a**–**d**; Boc-Tyr-OH, Bop-Cl, NMM, CH_2Cl_2 for compound **4e**. (f) 2-aminoethanol, 60 °C. (g) TFA 50% in CH_2Cl_2.

of the compounds, the dose-effect response for DOR and MOR activation was evaluated by MVD and GPI assays respectively. As shown in Table 1, compound **6d** which presents sarcosine at position 3, shows no affinity for both μ and δ receptors, and no activity in MDV/GPI bioassays. This data is not surprising, being in full accordance with a previous study showing that modifications of the aromatic moiety in position 3 may deeply influence the activity of EM2 derivatives [39]. Compounds **6a** and **6c**, with a single *N*-MePhe

Table 1
Binding affinity and *in vitro* functional bioactivity of compounds **6a**–**e**.

Compound	rMOR,[a] [^{3}H]DAMGO		rDOR,[a] [^{3}H]Ile^{5,6}-Deltorphin-II		Selectivity	Functional bioactivity	
	IC_{50}[b]	K_i^{μ} (nM)[c]	IC_{50}[b]	K_i^{δ} (nM)[c]	K_i^{δ}/K_i^{μ}	MVD (IC_{50}[d,e])	GPI (IC_{50}[d,e])
EM2	8.33 ± 0.06	3.56 ± 0.3	NA[f]	>10,000	–	510 ± 35	15 ± 2
DAMGO	8.62 ± 0.04	1.7 ± 0.03	NA[f]	>10,000	–	–	–
[^{3}H]Ile^{5,6} Deltorphin-II	NA[f]	>10,000	8.51 ± 0.06	1.6 ± 0.02	–	–	–
6a	6.54 ± 0.04	224 ± 18	5.28 ± 0.04	3864 ± 180	17.2	>10,000	717 ± 58
6b	6.94 ± 0.03	90 ± 4	5.97 ± 0.06	799 ± 35	8.9	1909 ± 353	165 ± 14
6c	6.71 ± 0.03	155 ± 10	NA[f]	>10,000	–	>10,000	503 ± 26
6d	NA[f]	>10,000	NA[f]	>10,000	–	>10,000	>10,000
6e	7.35 ± 0.04	34 ± 2	5.23 ± 0.02	6401 ± 122	188.3	3641 ± 470	74 ± 9

[a] Displacement of [^{3}H]Ile^{5,6}-Deltorphin-2 (δ-selective) and [^{3}H]DAMGO (μ-selective) from rat brain membrane binding site.
[b] The logIC_{50} ± standard error are expressed as logarithmic values determined from the nonlinear regression analysis of data collected from at least two independent experiments performed in duplicate.
[c] The K_i values are calculated using the Cheng and Prusoff equation to correct for the concentration of the radioligand used in the assay.
[d] Concentration at 50% inhibition of muscle contraction at electrically stimulated isolated tissues ($n = 4$).
[e] ±S.E.M.
[f] NA = not binding affinity.

residue, have a low affinity (>100) but a comparable activity with EM2 at μ-receptor; no substantial affinity and activity at δ receptor was detected. Compound **6b**, with unsubstituted NH-amide bond, shows a good affinity, with K_i^μ of 90 nM and the highest K_i^δ of the series, correlated with a slight activity in MVD assay. Analogue **6e**, with two *N*–Me residues, displays the highest affinity (34 nM) and selectivity (188-fold) toward MOR, with a great activity in GPI bioassay. As previously reported, *N*-methylation of EM2 is significantly associated with the bioactive conformation of the modified peptides and their interaction with the μ-opioid receptor [50]. None of analogues **6a**–**e** reached the affinity of parent compounds and unexpectedly, the combination of structural features of two selective μ agonists, such as EM2 and DAMGO, provided compounds with residual δ affinity (e.g. compound **6b**, K_i^δ = 799 nM). The μ binding affinity and selectivity increase with grade of *N*-methylation, confirming that introduction of *N*-methylated amino acids, in addition to well-known protection against proteolysis [44], produces an increment in MOR binding in contrast to DOR, due to different conformational requirement of these receptors.

It is worth to note that the presence of a single methyl on the nitrogen in position 3 and 4 respectively (**6a** and **6c**), does not produce some changes in activity and receptor selectivity, while their simultaneous presence determines an increase of potency and selectivity toward μ receptor.

Compounds **6a**–**e** were evaluated through *in vivo* tail flick and hot plate tests, as previously reported [59]. Mice response to heat was recorded after intracerebroventricular administration of EM2 and compounds **6a**–**e** (7 nmol). Maximum effects for each peptide (efficacy) are shown in Figs. 2 and 3. Table 2 shows also the dose that produces 50% of the maximum antinociceptive effect (potency).

Analogue **6e**, resulted the most potent compound in eliciting antinociception in both the tests according to μ-binding and GPI assays data. In the hot plate test **6e** reaches an MPE of 74%, lower than EM2 (92%), but clearly higher than other analogues. These data are further confirmed by tail flick nociception test, in which peptide **6e** shows a more comparable efficacy respect to EM2. AD_{50} values indicate that, in addition to the compound **6e**, analogues **6b** and **6c** have a discrete potency, in particular in the tail flick assay, with 3.44 and 3.03 nmol respectively. Compound **6a** has the lowest

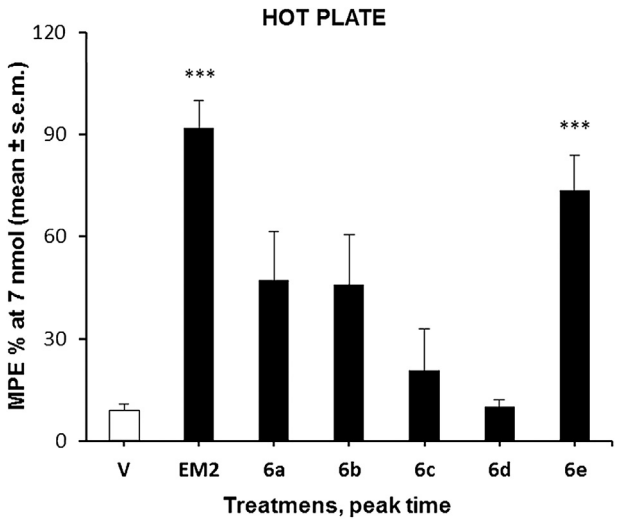


Fig. 2. Antinociceptive effect (peak time) of EM2 and EM2 analogues **6a**–**e** at the dose of 7 nmol after i.c.v administration in the hot plate test. The antinociceptive activity is expressed as percentage of the maximum possible effect (% MPE.) ± s.e.m. *** is for $P < 0.001$ *vs* V (vehicle-treated animals). $N = 8–11$.

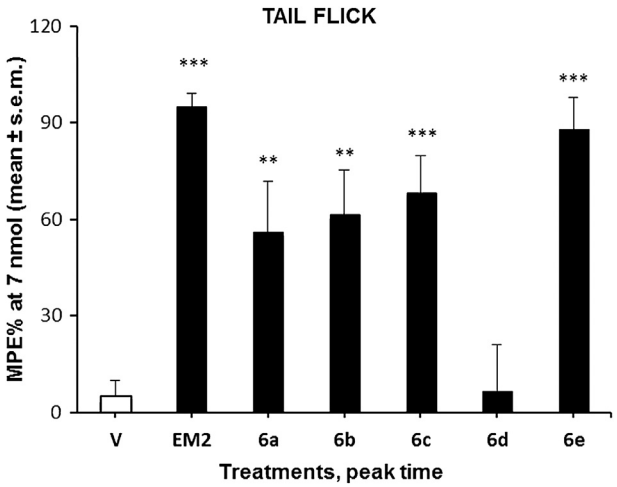


Fig. 3. Antinociceptive effect (peak time) of EM2 and EM2 analogues **6a**–**e** at the dose of 7 nmol after i.c.v administration in the tail flick test. The antinociceptive activity is expressed as percentage of the maximum possible effect (% MPE.) ± s.e.m. ** is for $P < 0.01$ and *** is for $P < 0.001$ *vs* V (vehicle-treated animals). $N = 8–11$.

antinociceptive efficacy in the tail flick test and the lowest potency, as demonstrated by the AD_{50} value, while in the hot plate test **6c** proves to be the less efficacious compound. This effect was previously observed by Goldber and Tseng [9,14] in their studies on C-terminal amide to alcohol conversion, in which high-affinity and low intrinsic efficiency agonists were reported. Compound **6d** was confirmed to be devoid of activity.

By comparing *in vivo* and *in vitro* activities with *in vitro* receptor affinity, it should be observed that, despite a generally low affinity toward ORs if compared with parent peptides, novel hybrid EM2/DAMGO peptides, with the exception of compound **6d**, have a good antinociceptive profile. In particular, tail flick graphic (Fig. 3) shows that peptides **6a**–**c** produce moderate analgesia, over 50% of the maximum possible effect, and **6e** almost reaches the level of EM2, although it has a lower MOR affinity. This result can be explained considering the better enzymatic stability of product **6e** that partially compensates the lower binding affinity. In order to better evaluate the effects of the N-methylation and C-terminal ethanolamide conversion, the plasma stability of product **6e** was tested and compared to the parent peptide EM2 (Fig. 4).

4. Conclusion

Systematic investigation of the effect of *N*-methylation joined to *C*-terminal modification was performed in this study.

We synthetized and tested five new peptides with common features of μ selective agonists EM2 and DAMGO. Compounds **6a**–**c**

Table 2
In vivo antinociceptive activity of EM2 and compounds **6a**–**e**.

Cmpd.	Hot plate, AD_{50},[a] (95% confidence limits),[b] nmol	Tail flick, AD_{50},[a] (95% confidence limits),[b] nmol
EM2	2.29 (1.20–4.37)	1.49 (0.86–2.57)
6a	9.44(5.44–16.38)	5.56 (2.36–13.12)
6b	6.66 (3.08–14.36)	3.44 (1.59–7.41)
6c	9.60 (3.14–29.35)	3.03 (0.80–11.41)
6d	NA[c]	NA[c]
6e	3.77 (1.75–8.07)	2.65 (1.41–4.96)

[a] AD_{50} = dose that produces 50% of maximum antinociceptive effect.
[b] The AD_{50} values and their 95% confidence limits were determined by using the graded dose–response procedure described by Tallarida and Murray [60].
[c] Not activity.

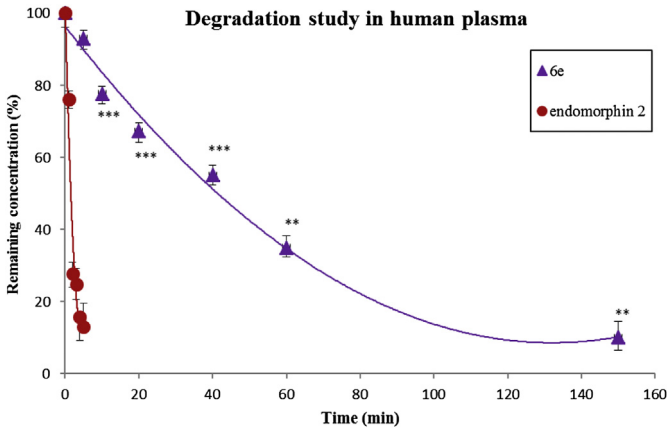


Fig. 4. Stability curves of EM2 (red line) and compound **6e** (purple line) in human plasma. The samples were tested in three independent experiments ($n = 3$) and represent the mean ± SEM. The significance among groups was evaluated with the analysis of variance (two-way ANOVA test) followed by Bonferroni's posthoc comparisons between compound **6e** and EM2 using the statistical software GraphPad Prism v.4. Statistical significance was $P < 0.05$ ($^{**}P < 0.01$; $^{***}P < 0.001$); times from t_0 to t_5 present no statistical significance ($P > 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

display a moderate *in vivo* antinociceptive activity; compound **6d** was inactive at both *in vitro* and *in vivo* tests; conversely peptide **6e** showed the greatest analgesic potency, with μ-receptor affinity and selectivity comparable to EM2. Structure–activity relationships concluded that *N*-methylation is significantly associate with the bioactive conformation of EM2 analogues and their interaction with opioid receptors. In addition, the *C*-terminal amide conversion to alcohol leads to high-affinity but low intrinsic efficiency agonists.

Metabolic stability studies, carried out on product **6e**, confirmed that multi-*N*-methylation and C-terminal modification with ethanolamide moiety confer to the derivative **6e** a better plasma stability than parent peptide, with half life around 40 min, and could be a useful tools to develop stable candidates as opioid drugs. In conclusion, this work allowed to obtain compounds with prolonged persistence on the opioid receptors. Nevertheless the two different key features of EM2 and DAMGO were found to be not synergic for binding, hence no enhancement of potency or efficacy have been recorded. *N*-methylated analogues of endomorphin-2-ol described in this paper can be viewed as interesting models for the study of ligand–receptor interactions.

5. Experimental

5.1. General

All the starting materials and reagents were purchased from Sigma–Aldrich (Sigma Aldrich Corp., Saint Louis, MO, USA). ^{1}H NMR spectra were determined in $CDCl_3$ or DMSO-d_6 solution with a Varian 300 MHz (Varian Medical Systems, Inc., Palo Alto, CA, USA), and chemical shifts were referred to TMS. The mass spectra were performed on a TOF-ESI spectrometer. Thin layer chromatography was performed on silica gel Merck (Merck & Co., Inc., Whitehouse Station, NJ, USA) 60 F_{254} plates.

5.2. General coupling procedure (*A*)

The N^{α} terminal Boc-protected peptides were all deprotected by a mixture of TFA in CH_2Cl_2 1:1 at r.t. The intermediate TFA salts were used for subsequent reactions without further purification. The N^{α} Boc-protected amino acid (1.1 eq) was dissolved in DMF, then EDC (1.1 eq.), HOBt (1.1 eq.) and DIPEA (1.65 eq.) were added at 0 °C. After 10 min the amino acid or the intermediate peptide as TFA salt (1 eq.) was added together with DIPEA (1.65 eq.). The reaction was stirred for additional 10 min at 0 °C, allowed to warm at r.t. and stirred overnight. The solvent was evaporated under reduced pressure, the residue was suspended in EtOAc and washed with three portions of citric acid 5%, $NaHCO_3$ s.s., and brine. The organic layers were combined, dried under Na_2SO_4, filtered and evaporated under reduced pressure to give a crude solid.

5.3. General N-terminal ethanolamide introduction procedure (*B*)

The *N*-Boc protected tetrapeptide was dissolved in a large excess of ethanolamine and the reaction mixture was stirred at 60 °C for 12 h. The ethanolamine was evaporated under reduced pressure, and the residue was extracted with two portions of EtOAc. The organic layers were combined, dried under Na_2SO_4, filtered and evaporated under vacuum to give a crude solid.

5.4. HCl·(*N*–Me)Phe-OMe (*1a*)

A solution of Boc-(*N*–Me)-Phe-OH (1 eq.) was dissolved in MeOH, then $SOCl_2$ (2.2 eq.) was added dropwise at 0 °C. The reaction mixture was stirred at r.t. for 3 h, then it was concentrated and treated several times with Et_2O. Finally it was evaporated under vacuum until to obtain the final product (quantitative). Rf = 0.63 (CH_2Cl_2/MeOH 9:1). ^{1}HNMR (DMSO-d_6) δ: 2.95–3.02 (2H, m, β-CH_2), 3.30 (3H, s, *N*–CH_3), 3.75 (3H, s, $COOCH_3$), 4.82 (1H, m, α-CH), 7.05–7.41 (5H, m, Ar), 9.36 (3H, s, NH_3^+). ESI-HRMS calcd for $C_{12}H_{17}ClO_2$ *m*/*z*: 229.0995 $[M + H]^+$; found 229.0992.

5.5. N-Boc-Phe-(*N*–Me)Phe-OMe (*2a*)

Coupling reaction was performed between Boc-Phe-OH and HCl·(*N*–Me)-Phe-OMe (**1a**) according to general procedure A. The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 9:1 to CH_2Cl_2/EtOAc 7:3) to obtain the pure product (58%). Rf = 0.63 (CH_2Cl_2/EtOAc 9:1). ^{1}H NMR ($CDCl_3$) δ: 1.37 (9H, s, Boc), 2.80–3.38 (5H, m, Phe^2 β-CH_2 and Phe^2 *N*–CH_3), 3.44–3.56 (2H, m, Phe^1 β-CH_2), 3.69 (3H, s, $COOCH_3$), 4.70 (1H, m, Phe^1 α-CH), 5.05 (1H, d, Phe^1 NH), 5.22 (1H, m, Phe^2 α-CH), 7.10–7.35 (10H, m, Ar). ESI-HRMS calcd for $C_{25}H_{32}N_2O_5$ *m*/*z*: 441.2389 $[M + H]^+$; found 441.2393.

5.6. N-Boc-Pro-Phe-(*N*–Me)Phe-OMe (*3a*)

N-Boc deprotection and coupling reaction between Boc-Pro-OH and TFA·Phe-(*N*–Me)-Phe-OMe were performed according to general procedure A. The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 9:1 to CH_2Cl_2/EtOAc 6:4) to obtain the pure product (32%). Rf = 0.26 (CH_2Cl_2/EtOAc 8:2). ^{1}H NMR (DMSO-d_6) δ: 1.35 (9H, s, Boc), 1.67 (2H, m, Pro γ-CH_2), 1.95 (2H, m, Pro β-CH_2), 2.78–2.98 (4H, m, Phe^2 β-CH_2 and Phe^3 β-CH_2), 3.10 (3H, s, Phe^3 *N*–CH_3), 3.24 (2H, m, Pro δ-CH_2), 3.60 (3H, s, $COOCH_3$), 4.05 (1H, m, Pro α-CH), 4.90 (2H, m, Phe^2 α-CH and Phe^3 α-CH), 7.00–7.35 (10H, m, Ar), 7.77 (1H, d, Phe^2 NH). ESI-HRMS calcd for $C_{30}H_{39}N_3O_6$ *m*/*z*: 538.2917 $[M + H]^+$; found 538.2919.

5.7. N-Boc-Tyr-Pro-Phe-(*N*–Me)Phe-OMe (*4a*)

N-Boc deprotection and coupling reaction between Boc-Tyr-OH and TFA·Pro-Phe-(*N*–Me)-Phe-OMe were performed according to general procedure A. The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 6:4 to CH_2Cl_2/EtOAc 1:1) to obtain the pure product (72%). Rf = 0.25 (CH_2Cl_2/EtOAc 1:1). ^{1}H NMR

($CDCl_3$) δ: 1.31 (9H, s, Boc), 1.80 (2H, m, Pro β-CH_2), 2.00 (2H, m, Pro γ-CH_2), 2.71–2.80 (2H, m, Tyr β-CH_2), 2.77–3.02 (5H, m, Phe^3 β-CH_2 and Phe^4 *N*–CH_3), 2.90–3.30 (2H, m, Phe^4 β-CH_2), 3.50 (2H, m, Pro δ-CH_2), 3.58 (3H, s, $COOCH_3$), 4.38 (1H, m, Pro α-CH), 4.47 (1H, m, Tyr α-CH), 4.92 (1H, m, Phe^3 α-CH), 5.20 (2H, m, Phe^4 α-CH and Tyr NH), 6.88 (1H, d, Phe^3 NH), 6.90–7.30 (14H, m, Ar), 8.15 (1H, s, Tyr OH). ESI-HRMS calcd for $C_{39}H_{48}N_4O_8$ *m/z*: 701.3550 $[M + H]^+$; found 701.3548.

5.8. *N*-Boc-Tyr-Pro-Phe-(N–Me)Phe-NH(CH_2)$_2$OH (**5a**)

Reaction was performed according to procedure B. The product was purified by silica gel column chromatography (EtOAc/MeOH 98:2 to EtOAc/MeOH 94:6) to obtain the pure product (41%). Rf = 0.46 (EtOAc/MeOH 9:1). ^{1}H NMR (DMSO-d_6) δ: 1.35 (9H, s, Boc), 1.80 (2H, m, Pro γ-CH_2), 2.65 (3H, s, Phe^4 *N*–CH_3), 2.68–2.72 (2H, m, Tyr β-CH_2), 2.76–3.36 (12H, m, Phe^3 β-CH_2, Phe^4 β-CH_2, Pro β-CH_2, ethanolamine 2 × CH_2 and Pro δ-CH_2), 4.24 (1H, m, Tyr α-CH), 4.36 (1H, s, ethanolamine OH), 4.64 (2H, m, Pro α-CH and Phe^3 α-CH), 4.80 (1H, m, Phe^4 α-CH), 5.40 (1H, m, Tyr NH), 6.50–7.30 (15H, m, Ar and Phe^4 NH), 8.00 (1H, t, ethanolamine NH), 9.2 (1H, s, Tyr OH). ESI-HRMS calcd for $C_{40}H_{51}N_5O_8$ *m/z*: 730.3816 $[M + H]^+$; found 730.3820.

5.9. TFA·Tyr-Pro-Phe-(N–Me)Phe-NH(CH_2)$_2$OH (**6a**)

Deprotection of *N*-Boc-Tyr-Pro-Phe-(*N*–Me)Phe-NH(CH_2)$_2$OH (**5a**) was performed by a mixture of TFA in CH_2Cl_2 1:1 to give the final product (quantitative). ^{1}H NMR (DMSO-d_6) δ: 1.23 (2H, m, Pro γ-CH_2), 2.28 (2H, m, Tyr β-CH_2), 2.44 (2H, m, Pro β-CH_2), 2.62–3.47 (13H, m, Phe^3 β-CH_2, Phe^3 *N*–CH_3, Phe^4 β-CH_2 Pro δ-CH_2 and ethanolamine 2 × CH_2), 4.31 (1H, m, Pro α-CH), 4.53–4.60 (2H, m, Tyr α-CH and Phe^2 α-CH), 4.66 (1H, s, ethanolamine OH), 4.88 (1H, m, Phe^4 α-CH), 6.68–7.23 (16H, m, Ar), 7.58 (1H, t, ethanolamine NH), 7.99 (2H, m, Phe^3 NH), 9.35 (3H, s, Tyr NH_3^+), 9.56 (1H, s, Tyr OH). ESI-HRMS calcd for $C_{37}H_{44}F_3N_5O_8$ *m/z*: 744.3220 $[M + H]^+$; found 744.3224.

5.10. *N*-Boc-Phe-Phe-OMe (**2b**)

Coupling reaction was performed between Boc-Phe-OH and HCl·Phe-OMe (**1b**) according to general procedure A, obtaining the pure product (quantitative). Rf = 0.59 (CH_2Cl_2/EtOAc 9:1). ^{1}H NMR ($CDCl_3$) δ: 1.39 (9H, s, Boc), 3.04 (4H, m, Phe^1 β-CH_2 and Phe^2 β-CH_2), 3.66 (3H, s, $COOCH_3$), 4.30 (1H, m, Phe^2 α-CH), 4.70 (1H, m, Phe^1 α-CH), 4.90 (1H, m, Phe^2 NH), 6.25 (1H, d, Phe^2 NH), 6.90–7.30 (10H, m, Ar). ESI-HRMS calcd for $C_{24}H_{30}N_2O_5$ *m/z*: 427.2233 $[M + H]^+$; found 427.2230.

5.11. *N*-Boc-Pro-Phe-Phe-OMe (**3b**)

N-Boc deprotection and coupling reaction between Boc-Pro-OH and TFA·Phe-Phe-OMe were performed according to general procedure A, obtaining the pure product (quantitative). Rf = 0.57 (CH_2Cl_2/EtOAc 1:1). ^{1}H NMR ($CDCl_3$) δ: 1.37 (9H, s, Boc), 1.83 (2H, m, Pro γ-CH_2), 2.10 (2H, m, Pro β-CH_2), 2.80–3.10 (4H, m, Phe^2 β-CH_2 and Phe^3 β-CH_2), 3.16 (2H, m, Pro δ-CH_2), 3.61 (3H, s, $COOCH_3$), 4.10 (1H, m, Pro α-CH), 4.12 (1H, m, Phe^2 α-CH), 4.58 (1H, m, Phe^3 α-CH), 6.11 (1H, d, Phe^3 NH), 6.58 (1H, d, Phe^2 NH), 6.90–7.30 (10H, m, Ar). ESI-HRMS calcd for $C_{29}H_{37}N_3O_6$ *m/z*: 524.2761 $[M + H]^+$; found 524.2765.

5.12. *N*-Boc-Tyr-Pro-Phe-Phe-OMe (**4b**)

N-Boc deprotection and coupling reaction between Boc-Tyr-OH and TFA·Pro-Phe-Phe-OMe were performed according to general procedure A. The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 8:2 to CH_2Cl_2/EtOAc 3:7) to obtain the pure product (10%). Rf = 0.39 (CH_2Cl_2/EtOAc 1:1). ^{1}H NMR (DMSO-d_6) δ: 1.32 (9H, s, Boc), 1.90 (2H, m, Pro γ-CH_2), 2.70–2.80 (2H, m, Tyr β-CH_2), 2.89 (2H, m, Pro β-CH_2), 2.90–3.10 (6H, m, Pro δ-CH_2, Phe^3 β-CH_2 e Phe^4 β-CH_2), 3.60 (3H, s, $COOCH_3$), 4.12 (1H, m, Tyr α-CH), 4.52 (2H, m, Pro α-CH e Phe^4 α-CH), 4.73 (1H, m, Phe^3 α-CH), 5.16 (1H, d, Tyr NH), 6.50 (1H, d, Phe^3 NH), 6.65–7.30 (15H, m, Phe^4 NH e Ar), 9.18 (1H, s, Tyr OH). ESI-HRMS calcd for $C_{38}H_{46}N_4O_7$ *m/z*: 671.3445 $[M + H]^+$; found 671.3441.

5.13. *N*-Boc-Tyr-Pro-Phe-Phe-NH(CH_2)$_2$OH (**5b**)

Reaction was performed according to procedure B. The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 3:7 to EtOAc/MeOH 92:8) to obtain the pure product (38%). Rf = 0.39 (EtOAc/MeOH 9:1). ^{1}H NMR (DMSO-d_6) δ: 1.39 (9H, s, Boc), 1.60 (2H, m, Pro γ-CH_2), 2.63 (2H, m, Tyr β-CH_2), 2.90 (2H, m, Pro β-CH_2), 2.95–3.10 (8H, m, Phe^3 β-CH_2, Phe^4 β-CH_2 and ethanolamine 2 × CH_2), 3.40 (2H, m, Pro δ-CH_2), 4.20 (1H, m, Pro α-CH), 4.31 (2H, m, Tyr α-CH and Phe^2 α-CH), 4.45 (1H, s, ethanolamine OH), 4.68 (1H, m, Phe^4 α-CH), 5.03 (1H, d, Tyr NH), 6.60–7.30 (16H, m, Phe^3 NH, Phe^4 NH and Ar), 7.60 (1H, t, etanolamine NH), 9.22 (1H, s, Tyr OH). ESI-HRMS calcd for $C_{39}H_{49}N_5O_7$ *m/z*: 700.3710 $[M + H]^+$; found 700.3711.

5.14. TFA·Tyr-Pro-Phe-Phe-NH(CH_2)$_2$OH (**6b**)

Deprotection of *N*-Boc-Tyr-Pro-Phe-Phe-NH(CH_2)$_2$OH (**5b**) was performed by a mixture of TFA in CH_2Cl_2 1:1 to give the final product (quantitative). ^{1}H NMR (DMSO-d_6) δ: 1.28 (2H, m, Pro γ-CH_2), 2.28 (2H, m, Tyr β-CH_2), 2.44 (2H, m, Pro β-CH_2), 2.73-3.24 (10H, m, Phe^3 β-CH_2, Phe^4 β-CH_2 Pro δ-CH_2 and ethanolamine 2 × CH_2), 4.13 (1H, m, Pro α-CH), 4.50–4.53 (2H, m, Tyr α-CH and Phe^2 α-CH), 4.65 (1H, s, ethanolamine OH), 4.67 (1H, m, Phe^4 α-CH), 6.68–7.25 (16H, m, Ar), 7.69 (1H, t, ethanolamine NH), 7.90–8.13 (2H, m, Phe^3 NH and Phe^4 NH), 9.36 (3H, s, Tyr NH_3^+), 9.43 (1H, s, Tyr OH). ESI-HRMS calcd for $C_{36}H_{42}F_3N_5O_8$ *m/z*: 730.3064 $[M + H]^+$; found 730.3065.

5.15. *N*-Boc-(N–Me)Phe-Phe-OMe (**2c**)

Coupling reaction was performed between Boc-(*N*–Me)-Phe-OH and HCl·Phe-OMe (**1b**) according to general procedure A, obtaining the pure product (90%). Rf = 0.59 (CH_2Cl_2/EtOAc 9:1). ^{1}H NMR ($CDCl_3$) δ: 1.28 (9H, s, Boc), 2.42 (3H, s, Phe^1 *N*–CH_3), 2.70–3.30 (4H, m, Phe^1 β-CH_2 and Phe^2 β-CH_2), 3.63 (3H, s, $COOCH_3$), 4.74–4.85 (2H, m, Phe^1 α-CH and Phe^2 α-CH), 6.34 (2H, d, Phe^2 NH), 7.00–7.26 (10H, m, Ar). ESI-HRMS calcd for $C_{25}H_{32}N_2O_5$ *m/z*: 441.2389 $[M + H]^+$; found 441.2386.

5.16. *N*-Boc-Pro-(N–Me)Phe-Phe-OMe (**3c**)

N-Boc deprotection and coupling reaction between Boc-Pro-OH and TFA·(*N*–Me)Phe-Phe-OMe were performed according to general procedure A. The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 9:1 to CH_2Cl_2/EtOAc 1:1) to obtain the pure product (32%). Rf = 0.77 (CH_2Cl_2/EtOAc 8:2). ^{1}H NMR ($CDCl_3$) δ: 1.44 (9H, s, Boc), 1.85 (2H, m, Pro γ-CH_2), 2.38 (3H, s, Phe^2 *N*–CH_3), 2.80–3.26 (6H, m, Phe^2 β-CH_2, Phe^3 β-CH_2 and Pro β-CH_2), 3.45 (2H, m, Pro δ-CH_2), 3.67 (3H, s, $COOCH_3$), 4.20 (1H, m, Pro α-CH), 4.80 (2H, m, Phe^2 α-CH and Phe^3 α-CH), 7.05–7.35 (10H, m, Ar), 8.70 (1H, d, Phe^3 NH). ESI-HRMS calcd for $C_{30}H_{39}N_3O_6$ *m/z*: 538.2917 $[M + H]^+$; found 538.2918.

5.17. N-Boc-Tyr-Pro-(N–Me)Phe-Phe-OMe (**4c**)

N-Boc deprotection and coupling reaction between Boc-Tyr-OH and TFA·Pro-(*N*–Me)Phe-Phe-OMe were performed according to general procedure A. The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 9:1 to CH_2Cl_2/EtOAc 6:4) to obtain the pure product (77%). Rf = 0.67 (CH_2Cl_2/EtOAc 1:1). ^{1}H NMR ($CDCl_3$) δ: 1.39 (9H, s, Boc), 1.85 (2H, m, Pro γ-CH_2), 2.51 (3H, s, Phe^3 *N*–CH_3), 2.68–3.18 (8H, m, Tyr β-CH_2, Pro β-CH_2, Phe^3 β-CH_2 e Phe^4 β-CH_2), 3.45 (2H, m, Pro δ-CH_2), 3.71 (3H, s, $COOCH_3$), 4.22 (1H, m, Pro α-CH), 4.55 (1H, m, Tyr α-CH), 4.72 (1H, m, Phe^4 α-CH), 4.81 (1H, m, Phe^3 α-CH), 5.20 (1H, d, Tyr NH), 6.70–7.30 (14H, m, Ar), 8.15 (1H, s, Tyr OH), 8.75 (1H, d, Phe^4 NH). ESI-HRMS calcd for $C_{39}H_{48}N_4O_8$ *m*/*z*: 701.3550 $[M + H]^+$; found 701.3552.

5.18. N-Boc-Tyr-Pro-(N–Me)Phe-Phe-NH(CH$_2$)$_2$OH (**5c**)

Reaction was performed according to procedure B. The product was purified by silica gel column chromatography (EtOAc/MeOH 98:2 to EtOAc/MeOH 94:6) to obtain the pure product (20%). Rf = 0.65 (EtOAc/MeOH 9:1). ^{1}H NMR ($CDCl_3$) δ: 1.35 (9H, s, Boc), 1.70 (2H, m, Pro γ-CH_2), 2.52 (3H, s, Phe^3 *N*–CH_3), 2.55–3.24 (12H, m Tyr β-CH_2, Pro β-CH_2, Phe^3 β-CH_2, Phe^4 β-CH_2 and ethanolamine 2 × CH_2), 3.50 (2H, m, Pro δ-CH_2), 4.16 (1H, m, Pro α-CH), 4.36 (1H, m, Phe^3 α-CH), 4.40 (1H, m, Tyr α-CH), 4.56 (1H, s, ethanolamine OH), 4.80 (1H, m, Phe^4 α-CH), 5.61 (1H, s, Tyr NH), 6.65–7.26 (15H, m, ethanolamine NH and Ar), 8.05 (1H, d, Phe^4 NH), 8.24 (1H, s, Tyr OH). ESI-HRMS calcd for $C_{40}H_{51}N_5O_8$ *m*/*z*: 730.3816 $[M + H]^+$; found 730.3819.

5.19. TFA·Tyr-Pro-Phe-(N–Me)Phe-NH(CH$_2$)$_2$OH (**6c**)

Deprotection of *N*-Boc-Tyr-Pro-Phe-(*N*–Me)Phe-NH(CH_2)$_2$OH (**5c**) was performed by a mixture of TFA in CH_2Cl_2 1:1 to give the final product (quantitative). ^{1}H NMR (DMSO-d_6) δ: 1.24 (2H, m, Pro γ-CH_2), 2.26 (2H, m, Tyr β-CH_2), 2.46 (2H, m, Pro β-CH_2), 2.62–3.22 (13H, m, Phe^3 β-CH_2, Phe^4 *N*–CH_3, Phe^4 β-CH_2 Pro δ-CH_2 and ethanolamine 2 × CH_2), 4.29 (1H, m, Pro α-CH), 4.51–4.58 (2H, m, Tyr α-CH and Phe^2 α-CH), 4.66 (1H, s, ethanolamine OH), 4.88 (1H, m, Phe^4 α-CH), 6.68–7.28 (16H, m, Ar), 7.66 (1H, t, ethanolamine NH), 7.92–8.15 (2H, m, Phe^3 NH and Phe^4 NH), 9.38 (3H, s, Tyr NH_3^+), 9.53 (1H, s, Tyr OH). ESI-HRMS calcd for $C_{37}H_{44}F_3N_5O_8$ *m*/*z*: 744.3220 $[M + H]^+$; found 744.3222.

5.20. N-Boc-Sar-Phe-OMe (**2d**)

Coupling reaction was performed between Boc-Sar-OH and HCl·Phe-OMe (**1b**) according to general procedure A, obtaining the pure product (99%). Rf = 0.67 (CH_2Cl_2/EtOAc 1:1). ^{1}H NMR ($CDCl_3$) δ: 1.27 (9H, s, Boc), 2.82 (3H, m, Sar *N*–CH_3), 3.04–3.20 (2H, m, Phe β-CH_2), 3.72 (3H, s, $COOCH_3$), 3.83 (2H, m, Sar CH_2), 4.90 (1H, m, Phe α-CH_2), 6.36–6.57 (1H, d, Phe NH), 7.00–7.35 (5H, m, Ar). ESI-HRMS calcd for $C_{18}H_{26}N_2O_5$ *m*/*z*: 351.31920 $[M + H]^+$; found 351.1923.

5.21. N-Boc-Pro-Sar-Phe-OMe (**3d**)

N-Boc deprotection and coupling reaction between Boc-Pro-OH and TFA·Sar-Phe-OMe were performed according to general procedure A. The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 2:8 to CH_2Cl_2/EtOAc 1:9) to obtain the pure product (76%). Rf = 0.35 (EtOAc). ^{1}H NMR ($CDCl_3$) δ: 1.35 (9H, s Boc), 1.80 (2H, m, Pro γ-CH_2), 2.10 (2H, m, Pro β-CH_2), 2.90–3.30 (5H, m, Phe β-CH_2–Sar *N*–CH_3), 3.40 (2H, m, Pro δ-CH_2), 3.50 (2H, m, Sar CH_2), 3.60 (3H, s, $COOCH_3$), 4.50 (1H, m, Pro α-CH), 4.90 (1H, m, Phe α-CH_2), 7.00–7.35 (5H, m, Ar), 8.50 (1H, d, Phe NH). ESI-HRMS calcd for $C_{23}H_{33}N_3O_6$ *m*/*z*: 448.2448 $[M + H]^+$; found 448.2447.

5.22. N-Boc-Tyr-Pro-Sar-Phe-OMe (**4d**)

N-Boc deprotection and coupling reaction between Boc-Tyr-OH and TFA·Pro-Sar-Phe-OMe were performed according to general procedure A. The product was purified by silica gel column chromatography (EtOAc/MeOH 98:2 to EtOAc/MeOH 94:6) to obtain the pure product (56%). Rf = 0.52 (EtOAc/MeOH 9:1). ^{1}H NMR ($CDCl_3$) δ: 1.35 (9H, s, Boc), 1.89 (2H, m, Pro γ-CH_2), 2.10 (2H, m, Pro β-CH_2), 2.70–3.25 (7H, m, Tyr β-CH_2, Phe β-CH_2, Sar *N*–CH_3), 3.42 and 2.67 (2H, m, Pro δ-CH_2), 3.55 (3H, s $COOCH_3$), 3.65 (2H, m, Sar CH_2), 4.46 (1H, m, Tyr α-CH), 4.68 (1H, m, Pro α-CH), 5.03 (1H, m, Phe α-CH), 6.65–7.30 (9H, m, Ar), 7.45 (1H, d, Tyr NH), 7.90 (1H, s, Tyr OH), 8.42 (1H, d, Phe NH). ESI-HRMS calcd for $C_{32}H_{42}N_4O_8$ *m*/*z*: 611.3081 $[M + H]^+$; found 611.3078.

5.23. N-Boc-Tyr-Pro-Sar-Phe-NH(CH$_2$)$_2$OH (**5d**)

Reaction was performed according to procedure B. The product was purified by silica gel column chromatography (EtOAc/MeOH 95:5 to EtOAc/MeOH 9:1) to obtain the pure product (48%). Rf = 0.52 (EtOAc/MeOH 9:1). ^{1}H NMR ($CDCl_3$) δ: 1.35 (9H, s, Boc), 1.90 (2H, m, Pro γ-CH_2), 2.14 (2H, m, Pro β-CH_2), 2.93–3.50 (11H, m, Tyr β-CH_2, Phe β-CH_2, Sar *N*–CH_3 and ethanolamine 2 × CH_2), 3.75 (2H, m, Sar CH_2), 3.76 (2H, m, Pro δ-CH_2), 4.60 (1H, m, Tyr α-CH), 4.63 (1H, s, ethanolamine OH), 4.70 (2H, m, Pro α-CH and Phe α-CH), 5.28 (1H, d, Tyr NH), 6.65–7.35 (10H, m, ethanolamine NH and Ar), 7.76 (1H, d, Phe NH), 8.38 (1H, s, Tyr OH). ESI-HRMS calcd for $C_{33}H_{45}N_5O_8$ *m*/*z*: 640.3346 $[M + H]^+$; found 640.3342.

5.24. TFA·Tyr-Pro-Sar-Phe-NH(CH$_2$)$_2$OH (**6d**)

Deprotection of *N*-Boc-Tyr-Pro-Sar-Phe-NH(CH_2)$_2$OH (**5d**) was performed by a mixture of TFA in CH_2Cl_2 1:1 to give the final product (quantitative). ^{1}H NMR (DMSO-d_6) δ: 1.77 (2H, m, Pro γ-CH_2), 2.25 (2H, m, Pro β-CH_2), 2.92–3.46 (11H, m, Tyr β-CH_2, Phe β-CH_2, Sar *N*–CH_3 and ethanolamine 2 × CH_2), 3.72 (2H, m, Sar CH_2), 3.78 (2H, m, Pro δ-CH_2), 4.57 (1H, m, Tyr α-CH), 4.64 (1H, s, ethanolamine OH), 4.74 (2H, m, Pro α-CH and Phe α-CH), 5.28 (1H, d, Tyr NH), 6.65–7.26 (10H, m, ethanolamine NH and Ar), 7.96 (1H, d, Phe NH), 8.13 (1H, s, Tyr OH), 9.37 (3H, s, Tyr NH_3^+), 9.56 (1H, s, Tyr OH). ESI-HRMS calcd for $C_{30}H_{38}N_3F_5O_8$ *m*/*z*: 654.2751 $[M + H]^+$; found 654.2748.

5.25. N-Boc-(N–Me)Phe-(N–Me)Phe-OMe (**2e**)

Boc-(*N*–Me)Phe-OH (1.1 eq) was dissolved in CH_2Cl_2, than Bop·Cl (1.1 eq.), and DIPEA (1.65 eq.) were added at −15 °C. After 30 min. HCl·(*N*–Me)-Phe-OMe (**1a**, 1 eq.) was added together with DIPEA (1.65 eq.). The reaction was stirred at r.t. overnight. The solvent was evaporated under reduced pressure; the residue was suspended in EtOAc and washed with three portions of citric acid 5%, $NaHCO_3$ s.s., and brine. The organic layers were combined, dried under Na_2SO_4, filtered and evaporated under reduced pressure to give a crude solid. The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 95:5 to EtOAc/MeOH 9:1) to obtain the pure product (42%). Rf = 0.39 (CH_2Cl_2/EtOAc 95:5). ^{1}H NMR ($CDCl_3$) δ: 1.38 (9H, s, Boc), 2.36 (3H, s, Phe^2 *N*–CH_3), 2.42 (3H, s, Phe^1 *N*–CH_3), 2.70–3.40 (4H, m, Phe^1 β-CH_2 and Phe^2 β-CH_2), 3.66 (3H, s, $COOCH_3$), 4.37 and 5.20 (1H, m, Phe^2 α-CH), 4.80 and 5.20 (1H, m, Phe^1 α-CH), 6.80–7.35 (10H, m, Ar). ESI-HRMS calcd for $C_{26}H_{34}N_2O_5$ *m*/*z*: 455.2546 $[M + H]^+$; found 455.2547.

5.26. N-Boc-Pro-(N–Me)Phe-(N–Me)Phe-OMe (**3e**)

N-Boc-(*N*–Me)Phe-(*N*–Me)Phe-OMe (**2e**) was deprotected by a mixture of TFA in CH_2Cl_2 1:1 at r.t. The intermediate TFA salt was used for subsequent reaction without further purification. Boc-Pro-OH (1.1 eq.) was dissolved in CH_2Cl_2, then Bop·Cl (1.1 eq.), and DIPEA (1.65 eq.) were added at −15 °C. After 30 min. TFA·(*N*–Me)Phe-(*N*–Me)Phe-OMe (1 eq.) was added together with DIPEA (1.65 eq.). The reaction was stirred at r.t. overnight. The solvent was evaporated under reduced pressure, the residue was suspended in EtOAc and washed with three portions of citric acid 5%, $NaHCO_3$ s.s., and brine. The organic layers were combined, dried under Na_2SO_4, filtered, and evaporated under reduced pressure to give a crude solid. The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 9:1 to EtOAc/MeOH 1:1) to obtain the pure product (34%). Rf = 0.67 (CH_2Cl_2/EtOAc 1:1). ^{1}H NMR ($CDCl_3$) δ: 1.37 (9H, s, Boc), 1.60 (2H, m, Pro γ-CH_2), 2.01 (2H, m, Pro β-CH_2), 2.52 (3H, s, Phe^3 *N*–CH_3), 2.55 (3H, s, Phe^2 *N*–CH_3), 2.90–3.20 (4H, m, Phe^2 β-CH_2 and Phe^3 β-CH_2), 3.50 (2H, m, Pro δ-CH_2), 3.67 (3H, s, $COOCH_3$), 4.51 (1H, m, Pro α-CH), 4.70 (1H, m, Phe^3 α-CH), 5.60 (1H, m, Phe^2 α-CH), 6.80–7.37 (10H, m, Ar). ESI-HRMS calcd for $C_{31}H_{41}N_3O_6$ *m*/*z*: 552.3074 $[M + H]^+$; found 552.3077.

5.27. N-Boc-Tyr-Pro-(N–Me)Phe-(N–Me)Phe-OMe (**4e**)

N-Boc deprotection and coupling reaction between Boc-Tyr-OH and TFA·Pro-(*N*–Me)Phe-(*N*–Me)Phe-OMe were performed according to general procedure A. The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 1:1) to obtain the pure product (45%). Rf = 0.53 (CH_2Cl_2/EtOAc 7:3). ^{1}H NMR ($CDCl_3$) δ: 1.36 (9H, s, Boc), 1.95 (2H, m, Pro γ-CH_2), 2.001 (2H, m, Pro β-CH_2), 2.53 (3H, s, Phe^4 *N*–CH_3), 2.75 (3H, s, Phe^3 *N*–CH_3), 2.80–3.40 (4H, m, Phe^3 β-CH_2 and Phe^4 β-CH_2), 2.81 and 3.97 (2H, m, Tyr β-CH_2), 3.50 (2H, m, Pro δ-CH_2), 3.69 (3H, s, $COOCH_3$), 4.60 (1H, m, Tyr α-CH), 4.74 (1H, m, Pro α-CH), 5.14 (1H, m, Phe^3 α-CH), 5.15 (1H, d, Tyr NH), 5.53 (1H, m, Phe^4 α-CH), 6.58–7.40 (14H, m, Ar), 8.06 (1H, s, Tyr OH). ESI-HRMS calcd for $C_{40}H_{50}N_4O_8$ *m*/*z*: 715.3707 $[M + H]^+$; found 715.3705.

5.28. N-Boc-Tyr-Pro-(N–Me)Phe-(N–Me)Phe-NH(CH$_2$)$_2$OH (**5e**)

Reaction was performed according to procedure B, obtaining the pure product (96%). Rf = 0.64 (EtOAc/MeOH 9:1). ^{1}H NMR ($CDCl_3$) δ: 1.53 (9H, s, Boc), 1.85 (2H, m, Pro γ-CH_2), 2.07 (2H, m, Pro β-CH_2), 2.37 (2H, m, ethanolamine α-CH_2), 2.50 (3H, s, Phe^4 *N*–CH_3), 2.60 (3H, s, Phe^3, *N*–CH_3), 2.70–3.30 (10H, m, Tyr β-CH_2, Pro δ-CH_2, Phe^3 β-CH_2, Phe^4 β-CH_2, ethanolamine β-CH_2), 4.39 (1H, s, ethanolamine OH), 4.60 (1H, m, Tyr α-CH), 4.85 (1H, m, Pro α-CH), 5.10 (1H, m, Phe^3 α-CH), 5.20 (1H, d, Tyr NH), 5.40 (2H, m, Phe^4 α-CH and ethanolamine NH), 6.50–7.45 (14H, m, Ar), 8.17 (1H, s, Tyr OH). ESI-HRMS calcd for $C_{41}H_{53}N_5O_8$ *m*/*z*: 744.3972 $[M + H]^+$; found 744.3969.

5.29. TFA·Tyr-Pro-(N–Me)Phe-(N–Me)Phe-NH(CH$_2$)$_2$OH (**6e**)

Deprotection of *N*-Boc-Tyr-Pro-(*N*–Me)Phe-(*N*–Me)Phe-NH(CH_2)$_2$OH (**6e**) was performed by a mixture of TFA in CH_2Cl_2 1:1 to give the final product (quantitative). ^{1}H NMR (DMSO-d_6) δ: 1.28 (2H, m, Pro γ-CH_2), 2.28 (2H, m, Tyr β-CH_2), 2.44 (2H, m, Pro β-CH_2), 2.53 (3H, s, Phe^4 *N*–CH_3), 2.65 (3H, s, Phe^3, *N*–CH_3), 2.73–3.24 (10H, m, Phe^3 β-CH_2, Phe^4 β-CH_2 Pro δ-CH_2 and ethanolamine 2 × CH_2), 4.13 (1H, m, Pro α-CH), 4.50–4.53 (2H, m, Tyr α-CH and Phe^2 α-CH), 4.65 (1H, s, ethanolamine OH), 4.67 (1H, m, Phe^4 α-CH), 6.68–7.25 (16H, m, Ar), 7.69 (1H, *t*, ethanolamine NH), 9.36 (3H, s, Tyr NH_3^+), 9.43 (1H, s, Tyr OH). ESI-HRMS calcd for $C_{38}H_{46}F_3N_5O_8$ *m*/*z*: 758.3377 $[M + H]^+$; found 758.3380.

6. *In vitro* biological assays

6.1. Chemicals and radioligands

[^{3}H]DAMGO ([D-Ala^2, NMePhe^4, Gly^5-ol]enkephalin; 41 Ci/mmol) and [^{3}H]Ile^{5,6}deltorphin-2; 48 Ci/mmol) were radiolabeled in the Isotope Laboratory of BRC (Radiolab), Szeged as described previously [58]. Tris(hydroxymethyl)amino-methane (Tris), and the peptidase inhibitors bestatin and phosphoramidon were purchased from Sigma–Aldrich (St. Louis, MO, USA). Captopril was obtained from The Squibb Institute for Medical Research (Princeton, NJ).

6.2. Animals

Inbred Wistar rats (250–300 g body weight) were housed in the local animal house of the Biological Research Center (BRC, Szeged, Hungary). Animals were kept in groups of four, allowed free access to standard food and tap water and maintained on a 12:12-h light/dark cycle until the time of sacrifice. Animals were handled according to the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§). Accordingly, the number of animals and their suffering were minimized.

6.3. Rat brain membrane preparations

Crude membrane fractions from brains of Wistar rats and guinea pigs were prepared as described earlier [61]. Animals were decapitated and the brains without cerebellum were quickly removed and washed several times to remove any unwanted blood or tissue particles with chilled 50 mM Tris–HCl (pH 7.4) buffer. The brains were blotted dry, weighed and suspended in 5 volumes/weight of the original brain tissue with ice-cold 50 mM Tris–HCl (pH 7.4) buffer. The brains were than homogenized at 1000× rpm with an electrically driven Braun Teflon-glass rota-homogenizer at 4 °C, using 10–15 strokes of the homogenizer. The final volume of the homogenate was made up to 30 volume/weight of the brain and filtered through four layers of gauze to remove any larger aggregates. After centrifugation with a Sorvall RC5C centrifuge at 40,000× *g* (18,000× rpm) for 20 min at 4 °C, the resulting pellet was resuspended in fresh buffer (30 volumes/weight) by using a vortex. The suspension was incubated for 30 min at 37 °C to remove any endogenous opioids. Centrifugation was repeated under the same conditions as described above, and final pellet was resuspended in 5 volumes of 50 mM Tris–HCl (pH 7.4) buffer containing 0.32 M sucrose to give a final concentration of 3–4 mg/ml protein. The presence of sucrose is necessary for stabilization of proteins for storage. The membranes were kept in 5 ml aliquots at −70 °C until use. The binding activity of the protein remained stable for at least two months. Membranes were thawed and resuspended in 50 mM Tris–HCl (pH 7.4) buffer and centrifuged at 40,000× g for 20 min at 4 °C to remove the sucrose. The resulting pellets were taken up in appropriate fresh buffer and immediately used in binding assays.

6.4. Receptor binding assays

All binding assays were performed at 25 °C for 30 min in 50 mM Tris–HCl buffer (pH 7.4) in a final volume of 1 ml, containing 1 mg BSA and 0.2–0.4 mg/ml membrane protein. Rat brain membranes were incubated with the type-specific radioprobes, such as the

selective μ receptor agonist [^{3}H]DAMGO (0.9–1.2 nM) and δ receptor selective agonist [^{3}H]Ile^{5,6}deltorphin-2 (0.8–1.3 nM) in the presence of unlabeled test ligands (their concentrations ranged from 10−5 to 10−11 M). Conditions for incubations are given in the figure legends. Non-specific binding was determined in the presence of 10 μM naloxone. Three peptidase inhibitors, 1 μM captopril, 1 μM bestatin and 1 μM phosphoramidon were included in the assay buffer to prevent metabolic inactivation of the compounds tested [62]. Reaction was terminated and bound and free radioligands were separated by rapid filtration under vacuum through Whatman GF/C (radiolabeled peptides) filters by using Brandel M24R Cell Harvester. Filters were washed three times with 5 ml ice-cold 50 mM Tris–HCl (pH 7.4) buffer. After filtration and separation procedure had been completed, fiber-disks were dried under an infrared lamp, and then removed from the filter-sheet by tweezers. Each disk was inserted into UltimaGold™ environment friendly, non-volatile, toluene-free scintillation cocktail, and placed into individual sample vials (transparent glass, Packard). Bound radioactivity was determined in Packard Tricarb 2300TR liquid scintillation analyzer. Receptor binding experiments were performed in duplicate and repeated at least three times. Experimental data were analyzed and graphically processed by GraphPad Prism research software package with standard office computers.

6.5. GPI and MVD in vitro bioassays

Electrically induced smooth muscle contractions of mouse vas deferens and strips of guinea pig ileum longitudinal muscle myenteric plexus were used as bioassays and performed as described previously [63,64]. Tissues came from male ICR mice weighing 25–30 g and from male Hartley guinea pigs weighing 150–400 g. The tissues were first tied to gold chains with suture silk, suspended in 20 mL baths containing 37 °C oxygenated (95% O_2, 5% CO_2), Krebs bicarbonate solution (magnesium-free for the MVD), and allowed to equilibrate for 15 min. Tissues were then stretched to optimal length previously determined to be 1 g tension, allowed to equilibrate for 15 min. The tissues were stimulated transmurally between platinum plate electrodes at 0.1 Hz, 0.4 ms pulses (2.0 ms pulses for MVD) at supramaximal voltage. Endomorphin-2 and analogues **1**–**5** at five to seven different concentrations were added to the baths in 20–60 μL volumes to produce cumulative dose–response curves. Percent inhibition was calculated by using an average contraction height for 1 min preceding the addition of the peptide divided by contraction height 3 min after the exposure to the peptide. IC_{50} values are the mean of not less than four separate assays. IC_{50} estimates and their associated standard errors were determined by fitting the mean data to the Hill equation using a computerized least-squares method.

7. *In vivo* nociception tests

7.1. Animals

Male CD-1 mice weighing 25–30 g and male guinea-pigs weighing 300–400 g (Harlan, Milan, Italy) were used for the experiments. Animals were housed for at least 1 week before experimental sessions in colony cages (7 mice in each cage; 4 guinea-pigs in each cage) under standard light (light on from 7.00 a.m. to 7.00 p.m.), temperature (21 ± 1 °C), relative humidity (60 ± 10%) with food and water available ad libitum. The experiments were conformed to the guidelines for pain research with laboratory animals. The research protocol was approved by the Service for Biotechnology and Animal Welfare of the Istituto Superiore di Sanità and authorized by the Italian Ministry of Health, according to Legislative Decree 116/92, which implemented the European Directive 86/609/EEC on laboratory animal protection in Italy. Animal welfare was routinely checked by veterinarians from the Service for Biotechnology and Animal Welfare.

7.2. Nociception assay

Antinociceptive responses were determined with the hot plate test and the tail flick test as previously reported [60]. In the case of hot plate test, the thermal nociception was assessed with a commercially available apparatus consisting in a metal plate 25 × 25 cm (Ugo Basile, Italy) heated to a constant temperature of 55.0 ± 0.1 °C, on which a plastic cylinder (20 cm diameter, 18 cm high) was placed. The time of latency (s) was recorded from the moment the animal was inserted inside the cylinder up to when it licked its paws or jumped off the hot plate; the latency exceeded the cut-off time of 60 s. The baseline was calculated as mean of three readings recorded before testing at intervals of 15 min. The time course of latency was then determined at 5, 10, 15, 20 and 30 min after treatment. The tail flick latency was obtained using a commercial unit (Ugo Basile, Italy), consisting of an infrared radiant light source (100 W, 15 V bulb) focused onto a photocell utilizing an aluminum parabolic mirror. During the trials the mice were gently hand-restrained with a glove. Radiant heat was focused 3–4 cm from the tip of the tail, and the latency (s) of the tail withdrawal recorded. The measurement was interrupted if the latency exceeded the cut off time (15 s at 15 V). Also in this case, the baseline was calculated as mean of three readings recorded before testing at intervals of 15 min and the time course of latency determined at 5, 10, 15, 20 and 30 min after treatment. In both the hot plate and tail flick tests, data were expressed as time course of the percentage of maximum effect (% MPE) = (post drug latency − baseline latency)/(cut-off time − baseline latency) × 100. Various doses of endomorphin-2 and analogues **6a**–**d** and **6e** were injected i.c.v according to the following procedure: mice were lightly anesthetized with isoflurane, and an incision was made in the scalp. Injections were performed using a 10 μl Hamilton microsyringe at a point 2-mm caudal and 2-mm lateral from the bregma. Compounds were injected at a depth of 3 mm in a volume of 5 μL. Complete dose–responses curves were then established for the analysis of 50% antinociceptive dose (AD_{50}) values and slope function. The AD_{50} values and their 95% confidence limits were determined by using the graded dose–response procedure described by Tallarida and Murray [60].

7.3. Metabolic stability in human plasma

The evaluation of the metabolic stability of product **6e** compared to that of EM2 was carried out following a previously reported method [65].

495 μL of thawed human plasma (previously frozen at −70 °C) was spiked with 5 μL EM2 and analogue **6e** in DMSO to achieve a final concentration of 100 μg/mL, and then incubated at 37 °C (±1 °C).

Prepared samples were removed at several designated time points and incubation was stopped by adding an equal volume of the blocking solution 5% aqueous $ZnSO_4$ solution, MeOH, and ACN (5:3:2) which precipitated proteins. The mixture was vortexed and centrifuged at 12,000 × *g* for 5 min, then 50 μL of clear supernatant was directly injected into the HPLC system (Waters model 600 solvent pump and 2996 photodiode array detector, with XBridge BEH 130 C18, 4.6 × 250 mm, 5 μm). Empower v.2 Software (Waters Spa, Milford, MA, USA) was used for data acquisition and elaboration. The samples were tested in three independents experiments (n = 3) and reported values represent the mean ± the Standard Error (SEM). The significance among groups was evaluated with the

analysis of variance (two-way ANOVA test) followed by Bonferroni's post-hoc comparisons using the statistical software GraphPad Prism v.4. Statistical significance was assumed at $P < 0.05$ ($^{**}P < 0.01$; $^{***}P < 0.001$); times from t_0 to t_5 produced no statistical significance ($P > 0.05$).

The chemical standards stock solutions were made at the concentration of 1 μg/mL in a final volume of 10 mL of DMSO. Five calibration standards were injected into the HPLC-UV/Vis system.

A reversed-phase packing column (XBridge BEH 130C18, 4.6 × 250 mm, 5 μm; Waters Spa, Milford, MA, USA) was employed for the separation and the column was held at room temperature (25 ± 1 °C) using a Jetstream2 Plus column oven.

EM2 and compound **6e** remaining concentrations at different times are reported in Fig. 4. For quantitative analyses, selective detection was performed at 275 nm for EM2 and 254 nm for **6e**. Elution was performed through a linear gradient using as mobile phase starting from 95:5 water–acetonitrile (0.1% TFA) to 100% acetonitrile over 40 min.

Calibration curves from 10 to 100 μg/mL were calculated by analyzing five non-zero concentration standards prepared in freshly spiked plasma solution in triplicate and extracted.

All quantitative analyses were performed at 275 nm for EM2 and 254 nm for **6e**. Calibration curves were linear with r^2 values always greater than 0.9927 ($n = 3$). LOD can be set, under previously reported conditions, at 4 μg/mL, while LOQ was set at 10 μg/mL. Variability of EM2 and **6e** quality control samples was less than 7%. Thus, the lower limit of quantification was at least 10 μg/mL (10% of the starting concentration). Recovery for the precipitation procedure was quantitatively.

After establishing the linearity and recovery of the assay, stability tests were performed without calibration curves, as long as the signal of the starting concentration ($t = 0$) was in agreement with values determined during the validation.

Acknowledgments

We are grateful to "Laboratorio Analisi Cliniche SO.PRE.MA. srl" of Montesilvano (Pe), Italy, for providing human plasma free of charge.

Abbreviations

AD_{50}	dose that produces 50% of maximum antinociceptive effect
BOP-Cl	bis(2-oxo-3-oxazolidinyl)phosphinic chloride
Boc	*tert*-butyloxycarbonyl
DAMGO	Tyr-D-Ala-Gly-(*N*–Me)Phe-Gly-Ol
DIPEA	*N*,*N*-diisopropylethylamine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
DOR	δ-opioid receptor
EDC	*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride
EM1	endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2)
EM2	endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2)
GPI	Guinea Pig Ileum
HOBt	hydroxybenzotriazole
MOR	μ-opioid receptor
MPE	percentage of maximum effect
MVD	Mouse vas Deferens
NMM	4-methylmorpholine
TFA	trifluoroacetic acid
Tris	tris(hydroxymethyl)amino-methane

References

[1] A.D. Corbett, G. Henderson, A.T. McKnight, S.J. Paterson, Br. J. Pharmacol. 147 (2006) S153–S162.
[2] G.A. Olson, R.D. Olson, A.L. Vaccarino, A.J. Kastin, Peptides 19 (1998) 1791–1843.
[3] M.M. Morgan, M.J. Christie, Br. J. Pharmacol. 164 (2011) 1322–1334.
[4] J.E. Zadina, L. Hackler, L.J. Ge, A.J. Kastin, Nature 386 (1997) 499–502.
[5] L. Hackler, J.E. Zadina, L.J. Ge, A.J. Kastin, Peptides 18 (1997) 1635–1639.
[6] K.J. Chang, A. Lillian, E. Hazum, P. Cuatrecasas, J.K. Chang, Science 212 (1981) 75–77.
[7] T. Yamazaki, S. Ro, M. Goodman, N.N. Chung, P.W. Schiller, J. Med. Chem. 36 (1993) 708–719.
[8] Y. Gao, X. Liu, W. Liu, Y. Qi, X. Liu, Y. Zhou, R. Wang, Bioorg. Med. Chem. Lett. 16 (2006) 3688–3692.
[9] I.E. Goldberg, G.C. Rossi, S.R. Letchworth, J.P. Mathis, J. Ryan-Moro, L. Leventhal, W. Su, D. Emmel, E.A. Bolan, G.W. Pasternak, J. Pharmacol. Exp. Ther. 286 (1998) 1007–1013.
[10] L.J. Sim, Q. Liu, S.R. Childers, D.E. Selley, J. Neurochem. 70 (1998) 1567–1576.
[11] J. Fichna, A. Janecka, J. Costentin, J.C. Do Rego, Pharmacol. Rev. 59 (2007) 88–123.
[12] K. Monory, M.C. Bourin, M. Spetea, C. Tömböly, G. Tóth, H.W. Matthes, B.L. Kieffer, J. Hanoune, A. Borsodi, Eur. J. Neurosci. 12 (2000) 577–584.
[13] G. Horvath, Pharmacol. Ther. 88 (2000) 437–463.
[14] L.F. Tseng, M. Narita, C. Suganuma, H. Mizoguchi, M. Ohsawa, H. Nagase, J.P. Kampine, J. Pharmacol. Exp. Ther. 292 (2000) 576–583.
[15] R. Przewłocki, D. Labuz, J. Mika, B. Przewłocka, C. Tomboly, G. Toth, Ann. N.Y. Acad. Sci. 897 (1999) 154–164.
[16] V.S. Hau, J.D. Huber, C.R. Campos, A.W. Lipkowski, A. Misicka, T.P. Davis, J. Pharm. Sci. 91 (2002) 2140–2149.
[17] B. Przewłocka, J. Mika, D. Labuz, G. Toth, R. Przewłocki, Eur. J. Pharmacol. 367 (1999) 189–196.
[18] X. Liu, Y. Wang, Y. Xing, J. Yu, H. Ji, M. Kai, Z. Wang, D. Wang, Y. Zhang, D. Zhao, R. Wang, J. Med. Chem. 56 (2013) 3102–3114.
[19] R. Schwyzer, Ann. N.Y. Acad. Sci. 297 (1977) 3–26.
[20] Y. In, K. Minoura, H. Ohishi, H. Minakata, M. Kamigauchi, M. Sugiura, T. Ishida, J. Pept. Res. 58 (2001) 399–412.
[21] B. Leitgeb, Chem. Biodivers. 4 (2007) 2703–2724.
[22] Y. Wang, Y. Xing, X. Liu, H. Ji, M. Kai, Z. Chen, J. Yu, D. Zhao, H. Ren, R. Wang, J. Med. Chem. 55 (2012) 6224–6236.
[23] B.L. Podlogar, M.G. Paterlini, D.M. Ferguson, G.C. Leo, D.A. Demeter, F.K. Brown, A.B. Reitz, FEBS Lett. 439 (1998) 13–20.
[24] M.G. Paterlini, F. Avitabile, B.G. Ostrowski, D.M. Ferguson, P.S. Portoghese, Biophys. J. 78 (2000) 590–599.
[25] S. Fiori, C. Renner, J. Cramer, S. Pegoraro, L. Moroder, J. Mol. Biol. 291 (1999) 163–175.
[26] A. Mollica, F. Pinnen, A. Stefanucci, L. Mannina, A.P. Sobolev, G. Lucente, P. Davis, J. Lai, S.W. Ma, F. Porreca, V.J. Hruby, J. Med. Chem. 55 (2012) 8477–8482.
[27] U. Reimer, G. Scherer, M. Drewello, S. Kruber, M. Schutkowski, G. Fischer, J. Mol. Biol. 279 (1998) 449–460.
[28] Y. Okada, Y. Fujita, T. Motoyama, Y. Tsuda, T. Yokoi, T. Li, Y. Sasaki, A. Ambo, Y. Jinsmaa, S.D. Bryant, L.H. Lazarus, Bioorg. Med. Chem. 11 (2003) 1983–1994.
[29] A. Mollica, F. Pinnen, A. Stefanucci, F. Feliciani, C. Campestre, L. Mannina, A.P. Sobolev, G. Lucente, P. Davis, J. Lai, S.W. Ma, F. Porreca, V.J. Hruby, J. Med. Chem. 55 (2012) 3027–3035.
[30] M. Keller, C. Boissard, L. Patiny, N.N. Chung, C. Lemieux, M. Mutter, P.W. Schiller, J. Med. Chem. 44 (2001) 3896–3903.
[31] G. Rivero, J. Llorente, J. McPherson, A. Cooke, S.J. Mundell, C.A. McArdle, E.M. Rosethorne, S.J. Charlton, C. Krasel, C.P. Bailey, G. Henderson, E. Kelly, Mol. Pharmacol. 82 (2012) 178–188.
[32] C. Tömböly, A. Péter, G. Tóth, Peptides 23 (2002) 1573–1580.
[33] A. Péter, G. Tóth, C. Tömböly, G. Laus, D. Tourwè, J. Chromatogr. A 846 (1999) 39–48.
[34] R.D. Egleton, T.J. Abbruscato, S.A. Thomas, T.P. Davis, J. Pharm. Sci. 87 (1998) 1433–1439.
[35] G. Cardillo, L. Gentilucci, A.R. Qasem, F. Sgarzi, S. Spampinato, J. Med. Chem. 45 (2002) 2571–2578.
[36] C. Giordano, A. Sansone, A. Masi, G. Lucente, P. Punzi, A. Mollica, F. Pinnen, F. Feliciani, I. Cacciatore, P. Davis, J. Lai, S.W. Ma, F. Porreca, V.J. Hruby, Eur. J. Med. Chem. 45 (2010) 4594–4600.
[37] A. Janecka, J. Fichna, R. Kruszynski, Y. Sasaki, A. Ambo, J. Costentin, J.C. do-Rego, Biochem. Pharmacol. 71 (2005) 188–195.
[38] D. Torino, A. Mollica, F. Pinnen, G. Lucente, F. Feliciani, P. Davis, J. Lai, S.W. Ma, F. Porreca, V.J. Hruby, Bioorg. Med. Chem. Lett. 19 (2009) 4115–4118.
[39] D. Torino, A. Mollica, F. Pinnen, F. Feliciani, G. Lucente, G. Fabrizi, G. Portalone, P. Davis, J. Lai, S.W. Ma, F. Porreca, V.J. Hruby, J. Med. Chem. 53 (2010) 4550–4554.
[40] Y. Sasaki, A. Sasaki, H. Niizuma, H. Goto, A. Ambo, Bioorg. Med. Chem. 11 (2003) 675–678.
[41] C.L. Wang, J.L. Yao, Y. Yu, X. Shao, Y. Cui, H.M. Liu, L.H. Lai, R. Wang, Bioorg. Med. Chem. 16 (2008) 6415–6422.
[42] J.R. Mallareddy, A. Borics, A. Keresztes, K.E. Kövér, D. Tourwé, G. Tóth, J. Med. Chem. 54 (2011) 1462–1472.
[43] G. Tóth, A. Keresztes, Cs. Tömböly, A. Péter, F. Fülöp, D. Tourwé, E. Navratilova, É. Varga, W.R. Roeske, H.I. Yamamura, M. Szűcs, A. Borsodi, Pure Appl. Chem. 76 (2004) 951–957.

[44] C. Tömböly, K.E. Kövér, A. Péter, D. Tourwé, D. Biyashev, S. Benyhe, A. Borsodi, M. Al-Khrasani, A.Z. Rónai, G. Tóth, J. Med. Chem. 47 (2004) 735–743.
[45] M. Al-Khrasani, G. Orosz, L. Kocsis, V. Farkas, A. Magyar, I. Lengyel, S. Benyhe, A. Borsodi, A.Z. Rónai, Eur. J. Pharmacol. 421 (2001) 61–67.
[46] W.X. Liu, R. Wang, Med. Res. Rev. 32 (2012) 536–580.
[47] B.K. Handa, A.C. Land, J.A. Lord, B.A. Morgan, M.J. Rance, C.F. Smith, Eur. J. Pharmacol. 70 (1981) 531–540.
[48] T. Onogi, M. Minami, Y. Katao, T. Nakagawa, Y. Aoki, T. Toya, S. Katsumata, M. Satoh, FEBS Lett. 357 (1995) 93–97.
[49] T. Seki, M. Minami, T. Nakagawa, Y. Ienaga, A. Morisada, M. Satoh, Eur. J. Pharmacol. 350 (1998) 301–310.
[50] R. Kruszynski, J. Fichna, J.C. do-Rego, T. Janecki, P. Kosson, W. Pakulska, J. Costentin, A. Janecka, Bioorg. Med. Chem. 13 (2005) 6713–6717.
[51] I. Lengyel, G. Orosz, D. Biyashev, L. Kocsis, M. Al-Khrasani, A. Rónai, C. Tömböly, Z. Fürst, G. Tóth, A. Borsodi, Biochem. Biophys. Res. Commun. 290 (2002) 153–161.
[52] A. Janecka, R. Kruszynski, J. Fichna, P. Kosson, T. Janecki, Peptides 27 (2006) 131–135.
[53] A. Mollica, F. Pinnen, F. Feliciani, A. Stefanucci, G. Lucente, P. Davis, F. Porreca, S.W. Ma, J. Lai, V.J. Hruby, Amino Acids 40 (2011) 1503–1511.
[54] A. Mollica, F. Feliciani, A. Stefanucci, R. Costante, G. Lucente, F. Pinnen, D. Notaristefano, S. Spisani, J. Pept. Sci. 18 (2012) 418–426.
[55] A. Mollica, P. Davis, S.W. Ma, J. Lai, F. Porreca, V.J. Hruby, Bioorg. Med. Chem. Lett. 15 (2005) 2471–2475.
[56] G. Lesma, S. Salvadori, F. Airaghi, E. Bojnik, A. Borsodi, T. Recca, A. Sacchetti, G. Balboni, A. Silvani, Mol. Divers. 17 (2013) 19–31.
[57] Z. Gyulai, A. Udvardy, A. Cs Benyei, J. Fichna, K. Gach, M. Storr, G. Toth, S. Antus, S. Berenyi, A. Janecka, A. Sipos, Med. Chem. 9 (2013) 1–10.
[58] S.T. Nevin, L. Kabasakal, F. Ötvös, G. Tóth, A. Borsodi, Neuropeptides 26 (1994) 261–265.
[59] A. Mollica, R. Costante, A. Stefanucci, F. Pinnen, G. Lucente, S. Fidanza, S. Pieretti, J. Pept. Sci. 19 (2013) 233–239.
[60] J. Tallarida, R.B. Murray, J. Pharm. Sci. 77 (1988) 284.
[61] E. Bojnik, J. Farkas, A. Magyar, C. Tömböly, U. Güçlü, O. Gündüz, A. Borsodi, M. Corbani, S. Benyhe, Neurochem. Int. 55 (2009) 458–466.
[62] T. Hiranuma, K. Iwao, K. Kitamura, T. Matsumiya, T. Oka, J. Pharmacol. Exp. Ther. 281 (1997) 769–774.
[63] R.L. Polt, F. Porreca, L.Z. Szabo, E.J. Bilsky, P. Davis, T.J. Abbruscato, T.P. Davis, R. Horvath, H.I. Yamamura, V.J. Hruby, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 7114–7118.
[64] M. Colucci, M. Mastriota, F. Maione, A. Di Giannuario, N. Mascolo, M. Palmery, C. Severini, M. Perretti, S. Pieretti, Peptides 32 (2011) 266–271.
[65] A. Mollica, F. Pinnen, R. Costante, M. Locatelli, A. Stefanucci, S. Pieretti, P. Davis, J. Lai, D. Rankin, F. Porreca, V.J. Hruby, J. Med. Chem. 56 (2013) 3419–3423.