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Novel highly potent μ-opioid receptor antagonist based on endomorphin-2 structure

Jakub Fichna,[a] Jean-Claude do-Rego,[b] Tomasz Janecki,[c] Renata Staniszewska,[a] Jeroen Poels,[d] Jozef Vanden Broeck,[d] Jean Costentin,[b] Peter W. Schiller[e] and Anna Janecka[a,*]

[a]*Laboratory of Biomolecular Chemistry, Institute of Biomedicinal Chemistry, Medical University of Lodz, Mazowiecka 6/8, 92-215 Lodz, PL, Poland*
[b]*Laboratoire de Neuropsychopharmacologie Expérimentale, CNRS-FRE 2735, IFRMP 23, Université de Rouen, Rouen, France*
[c]*Institute of Organic Chemistry, Technical University of Lodz, Lodz, Poland*
[d]*Laboratory for Developmental Physiology, Genomics and Proteomics, Zoological Institute, Catholic University, Leuven, Belgium*
[e]*Laboratory of Chemical Biology and Peptide Research, Clinical Research Institute of Montreal, Montreal, Canada*

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Abstract—The μ-opioid agonists endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2) exhibit an extremely high selectivity for the μ-opioid receptor and thus represent a potential framework for modification into μ-antagonists. Here we report on the synthesis and biological evaluation of novel [D-2-Nal[4]]endomorphin-2 analogs, [Sar[2],D-2-Nal[4]]endomorphin-2 and [Dmt[1],Sar[2],D-2-Nal[4]]endomorphin-2 (Dmt = 2′6′-dimethyltyrosine; Sar = *N*-methylglycine, sarcosine; D-2-Nal = 3-(2-naphthyl)-D-alanine). [Dmt[1],Sar[2],D-2-Nal[4]]endomorphin-2 possessed very high affinity for the μ-opioid receptor (IC_{50} = 0.01 ± 0.001 nM) and turned out to be a potent and extremely selective μ-opioid receptor antagonist, as judged by the in vitro aequorin luminescence-based calcium assay (pA_2 = 9.19). However, in the in vivo hot plate test in mice this analog was less potent than our earlier μ-opioid receptor antagonist, [Dmt[1],D-2-Nal[4]]endomorphin-2 (antanal-2). The exceptional μ-opioid receptor in vitro activity and selectivity of [Dmt[1], Sar[2],D-2-Nal[4]]endomorphin-2 makes this analog a valuable pharmacological tool, but further modifications are needed to improve its in vivo profile.

The development of selective and potent opioid peptide antagonists for the major receptor types (μ, δ, and κ) continues to be a key objective in pharmacological research. In particular, μ-opioid receptor antagonists are of interest both, as pharmacological tools and as potential therapeutic agents in the treatment of opioid abuse and overdose. μ-Antagonists of alkaloid structure, such as naloxone and naltrexone, lack a high degree of selectivity,[1] while β-funaltrexamine, which is μ-opioid receptor selective, behaves as an irreversible antagonist.[2] For that reason, the search for potent and selective μ-opioid antagonists of a peptide structure is a challenge for peptide chemists. The μ-opioid agonists, endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2), exhibit an extremely high selectivity for the μ-opioid receptor,[3] and thus represent a potential framework for modification into μ-antagonists.[4,5]

In our earlier studies we have shown that the introduction of 3-(2-naphthyl)-D-alanine (D-2-Nal) into position 4 of endomorphins resulted in obtaining analogs with weak antagonist activity.[6] Further modification of these analogs, involving the replacement of Tyr[1] by 2′6′-dimethyltyrosine (Dmt), resulted in obtaining two analogs, antanal-1 ([Dmt[1],D-2-Nal[4]]endomorphin-1) and antanal-2 ([Dmt[1],D-2-Nal[4]]endomorphin-2), which displayed a high degree of μ-antagonist potency, both in vitro and in vivo.[7]

In this study we described the synthesis and biological activities of two novel [D-2-Nal[4]]endomorphin-2 analogs: [Sar[2],D-2-Nal[4]]endomorphin-2 and [Dmt[1],Sar[2],D-2-Nal[4]]endomorphin-2, in which Pro in position 2 was

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* Corresponding author. Tel.: +48 42 679 04 50x259; fax: +48 42 678 42 77; e-mail: ajanecka@zdn.am.lodz.pl

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replaced by *N*-methylglycine (sarcosine, Sar). The rationale for this modification was based on our earlier observations that Sar[2] increased the μ-receptor affinity and the metabolic stability, when introduced into the sequence of endomorphin-2.[8,9]

Peptides were prepared by manual solid phase synthesis, using Rink Amide resin and Fmoc protection, according to a protocol published earlier.[7] After cleavage from the resin by TFA/TIS treatment, the crude peptides were purified by preparative RP HPLC. Their purity and structural identity were established by analytical RP HPLC and FAB-MS (Table 1). The ^{1}H NMR data for analog **5** are given in Ref. 10. ^{1}H NMR spectrum revealed the 3:7 ratio of stereoisomers derived from a conformational equilibrium around the Dmt-Sar amide bond.

Affinities for the μ- and δ-opioid receptors were determined in rat brain membrane binding assays, as described elsewhere.[11] The results in Table 2 show that changing Pro with Sar in **4** caused a total loss of binding affinity (IC_{50} = 1560 ± 160 and 15800 ± 3200 nM, for the μ- and δ-opioid receptors, respectively). An outstanding increase of the μ-opioid affinity and selectivity in comparison with [Dmt[1],D-2-Nal[4]]endomorphin-2 (antanal-2, **3**) and the parent peptide, endomorphin-2, was observed for **5** (IC_{50} = 0.01 ± 0.001, 1.52 ± 0.38, and 0.79 ± 0.05 nM, respectively).

For the determination of the in vitro opioid activities, compounds were tested in aequorin luminescence-based calcium assay.[7] Recombinant mammalian cell lines, CHO-MOR-Aeq and CHO-DOR-Aeq, expressing, respectively, the μ- or δ-opioid receptors and apoaequorin, were used to study the agonist-induced bioluminescent responses caused by the release of intracellular Ca^{2+} ions. The EC_{50} and pA_2 values and maximal calcium rise for the μ- and δ-mediated intracellular calcium responses induced by endomorphin-2 and its analogs are shown in Table 3. Endomorphin-2 produced a potent agonist effect in the CHO-MOR-Aeq cells, with the EC_{50} value of 0.040 ± 0.001 nM and a maximal Ca^{2+} rise of 85.89 ± 0.30%. For comparison, deltorphin-II was used as a potent δ-agonist in the CHO-DOR-Aeq cell line (EC_{50} value <0.01 nM and a maximal Ca^{2+} stimulation of 86.59 ± 0.04%). Both analogs **4** and **5** did not produce any significant calcium responses in either cell line.

In our earlier studies we have shown that [D-2-Nal[4]]endomorphin-2 (**2**) and [Dmt[1],D-2-Nal[4]]endomorphin-2 (antanal-2, **3**) were μ-opioid receptor antagonists with pA_2 values of 6.42 and 8.89, respectively.[7] In order to examine the μ-antagonist properties of the peptide analogs **4** and **5** in the aequorin luminescence-based calcium assay, competition experiments against the μ-selective agonist endomorphin-2 were performed. The pA_2 values were then calculated and compared with the value for a well-known universal opioid antagonist of the alkaloid structure, naloxone. Analog **5** produced the concentration-dependent rightward shift of the concentration–response curve of endomorphin-2 (Fig. 1), with the pA_2 value of 9.19 and was slightly less potent than naloxone (pA_2 = 9.66). This analog did not produce any antagonist effect at the

Table 1. Physicochemical data of endomorphin-2 analogs

Compound	Sequence	HPLCt_r[a] (min)	FAB-MS			Purity (%)
			Formula	MW	$[M+H]^+$	
1	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)	12.51	$C_{32}H_{37}N_5O_5$	571	572	98
2	Tyr-Pro-Phe-D-2-Nal-NH_2	10.43	$C_{36}H_{39}N_5O_5$	621	622	97
3	Dmt-Pro-Phe-D-2-Nal-NH_2 (antanal-2)	16.60	$C_{38}H_{43}N_5O_5$	649	650	98
4	Tyr-Sar-Phe-D-2-Nal-NH_2	14.73	$C_{34}H_{37}N_5O_5$	595	596	98
5	Dmt-Sar-Phe-D-2-Nal-NH_2	15.06	$C_{36}H_{41}N_5O_5$	623	624	98

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

Table 2. Opioid receptor binding assays of endomorphin-2 analogs

Compound	Sequence	IC_{50} (nM)		
		μ	*δ*	*δ*/*μ*
1	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)	0.79 ± 0.05[a]	>10,000[c]	>10,000
2	Tyr-Pro-Phe-D-2-Nal-NH_2	19.5 ± 2.10[b]	ND	—
3	Dmt-Pro-Phe-D-2-Nal-NH_2 (antanal-2)	1.52 ± 0.38[a]	7.74 ± 0.22[d]	5.09
4	Tyr-Sar-Phe-D-2-Nal-NH_2	1560 ± 160[a]	>10,000[c]	>6.41
5	Dmt-Sar-Phe-D-2-Nal-NH_2	0.01 ± 0.001[a]	>10,000[c]	>10,000
	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 (deltorphin-II)	—	0.56 ± 0.05[c]	—

All values are expressed as means ± SEM of three to six determinations.
[a] Determined against [^{3}H]DAMGO.
[b] Determined against [^{3}H]naloxone.
[c] Determined against [^{3}H]naltrindole.
[d] Determined against [^{3}H]DSLET. Data for **2** from Ref. 6 data for **3** from Ref. 7. ND, no data.

Table 3. The EC_{50} and pA_2 values and maximal calcium rise for the μ- and δ-mediated intracellular calcium response induced by endomorphin analogues

Compound	Sequence	CHO-MOR-Aeq			CHO-DOR-Aeq		
		EC_{50} ± SEM (nM)	pA_2[a]	Max Ca^{2+}-rise (%)	EC_{50} ± SEM (nM)	pA_2[b]	Max Ca^{2+}-rise (%)
1	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)	0.040 ± 0.001	—	85.89 ± 0.30	>1000	—	5.75 ± 0.11
2	Tyr-Pro-Phe-D-2-Nal-NH_2	—	6.42	35.75 ± 1.66	>1000	—	42.62 ± 0.60
3	Dmt-Pro-Phe-D-2-Nal-NH_2 (antanal-2)	—	8.89	22.87 ± 0.02	15.5 ± 0.44	—	55.71 ± 0.58
4	Tyr-Sar-Phe-D-2-Nal-NH_2	—	7.50	0.03 ± 0.01	>1000	—	0.18 ± 0.11
5	Dmt-Sar-Phe-D-2-Nal-NH_2	—	9.19	26.77 ± 4.40	>1000	—	7.49 ± 0.13
	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 (deltorphin-II)	—	—	—	<0.01	—	86.59 ± 0.04
	Naloxone	—	9.66	0.10 ± 0.01	—	—	—

All values are expressed as means ± SEM of three independent experiments performed in duplicate.
[a] Determined against endomorphin-2.
[b] Determined against deltorphin-II. Data for **2** and **3** from Ref. 7.

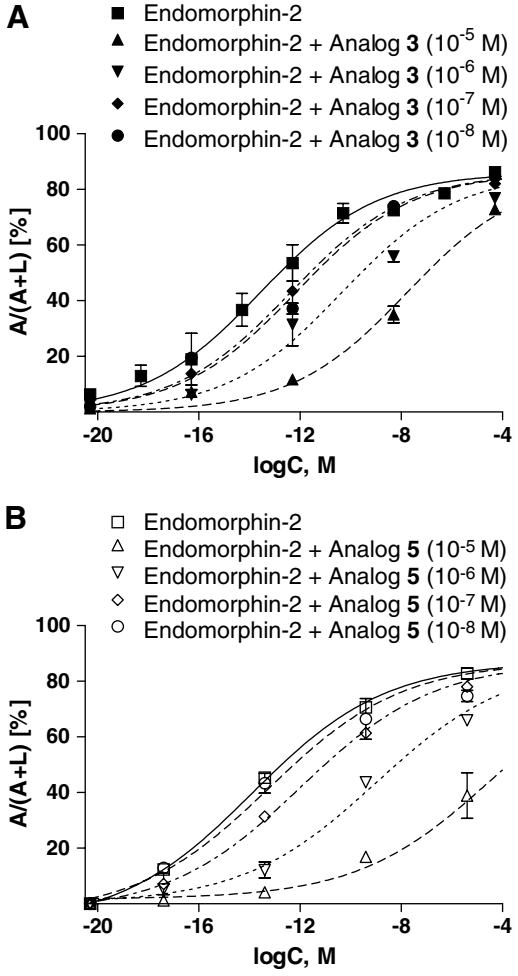

Figure 1. Concentration-dependent effect of (A) [Dmt^1, D-2-Nal^4]endomorphin-2 (antanal-2, **3**) and (B) [Dmt^1, Sar^2, D-2-Nal^4]endomorphin-2 (**5**) on the concentration–response curve for the calcium rise induced by endomorphin-2 in the CHO-MOR-Aeq cells. The data represent means ± SEM of three independent experiments carried out in duplicate. Results are expressed as the fractional luminescence, that is, the ratio of the agonist generated signal (A) and the total luminescence (agonist + lysed cells, A + L), thereby correcting for potential well-to-well variation in the number of injected cells. Calcium (100%) rise corresponds to the amount of calcium ions released during cell lysis.

δ-opioid receptor, thus being extremely μ-selective. It is interesting to notice that the replacement of Tyr^1 by Dmt in this analog did not result in a loss of μ-opioid receptor selectivity, as usually observed with this unnatural amino acid.[12,13]

The in vivo experiments for analog **5**, using the hot plate test in mice, were performed as described previously.[7] Analog **5** alone, at the dose of 10 μg/animal (icv), did not produce any analgesic effect (Table 4). The co-administration of **5** reversed the antinociception induced by endomorphin-2 (Table 5), but surprisingly, the observed effect was weaker than expected. Judging from the results in Tables 2 and 5, it seems that higher μ-opioid receptor selectivity results in lower in vivo antagonist activity. However, the number of compared analogs is too low to draw more general conclusions.

In summary, further modification of [Dmt^1,D-2-Nal^4]endomorphin-2 (antanal-2), involving the replacement of Pro^2 by Sar, resulted in a great increase of the μ-opioid receptor affinity and selectivity, as well as the μ-antagonist potency, as judged by the in vitro tests. However, in vivo antanal-2 remained the most potent μ-opioid receptor antagonist among [D-2-Nal^4]endomorphin-2 analogs.

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Table 4. Antinociceptive effect of endomorphin-2 analogs in the mouse hot plate test after intracerebroventricular injection (10 μg/animal)

Compound	Sequence	Latencies (%MPE) to		
		Paw licking	Rearing	Jumping
1	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)	13.70 ± 2.30	24.80 ± 3.00	64.70 ± 8.00
3	Dmt-Pro-Phe-D-2-Nal-NH_2 (antanal-2)[a]	0.09 ± 0.03	0.18 ± 0.07	1.54 ± 0.32
5	Dmt-Sar-Phe-D-2-Nal-NH_2	0.04 ± 0.42	0.09 ± 0.53	1.42 ± 2.55

All values are expressed as means ± SEM ($n = 10$).

[a]Data from Ref. 7. To evaluate the hot plate test responses, the control latencies (t_0) and test latencies (t_1) were determined after injection of saline and a peptide, respectively. The percentage of maximal possible effect (%MPE) was calculated as $\%MPE = (t_1 - t_0)/(t_2 - t_0) \times 100$, where the cutoff time (t_2) was 240 s.

Table 5. Determination of antagonist activity of endomorphin-2 analogs in the mouse hot plate test after intracerebroventricular injection

Sequence	Latencies (%MPE) to		
	Paw licking	Rearing	Jumping
Endomorphin-2[a]	5.25 ± 0.90	12.0 ± 2.0	34.7 ± 4.7
Endomorphin-2 + naloxone[b]	0.34 ± 0.04	0.22 ± 0.09	2.19 ± 0.26
Endomorphin-2 + Tyr-Pro-Phe-D-2-Nal-NH_2 (**2**)[c]	2.94 ± 0.06	5.26 ± 0.10	17.9 ± 0.2
Endomorphin-2 + Dmt-Pro-Phe-D-2-Nal-NH_2 (**3**)[c]	0.26 ± 0.15	0.13 ± 0.62	2.26 ± 3.25
Endomorphin-2 + Dmt-Sar-Phe-D-2-Nal-NH_2 (**5**)[c]	1.89 ± 0.92	3.63 ± 1.83	11.5 ± 5.7

To evaluate the hot plate test responses, the control latencies (t_0) and test latencies (t_1) were determined after injection of saline and a peptide, respectively. The percentage of maximal possible effect (%MPE) was calculated as $\%MPE = (t_1 - t_0)/(t_2 - t_0) \times 100$, where the cutoff time (t_2) was 240 s.

[a] Endomorphin-2 was injected at the dose of 3 μg/animal.

[b] Endomorphin-2 and naloxone were injected at the dose of 3 and 5 μg/animal, respectively.

[c] Endomorphin-2 and each analog were injected at the dose of 3 and 10 μg/animal, respectively. Data for **2** from Ref. 6, data for **3** from Ref. 7.

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