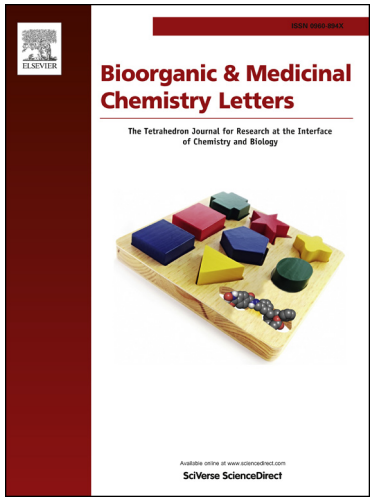

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Synthesis and changes in affinity for NOP- and opioid receptors of novel hexapeptides containing β^2-tryptophan analogues

Rositsa Zamfirova [a], Nikola Pavlov [b], Petar Todorov [b], Polina Mateeva [a], Jean Martinez [c], Monique Calmès [c], Emilia Naydenova [b,*]

[a] *Institute of Neurobiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*

[b] *Department of Organic Chemistry, University of Chemical Technologies and Metallurgy, Sofia, Bulgaria, e-mail: e_naydenova@abv.bg, tel. +35928163425*

[c] *Institut des Biomolécules Max Mousseron (IBMM) UMR 5247 CNRS-Université Montpellier 1 et Université Montpellier 2, France*

ABSTRACT

We report the synthesis and the biological activity of new analogues of Ac-RFMWMK-NH_2 and Ac-RYYRWK-NH_2, modified in position 4 and 5, respectively, with incorporation of newly synthesized β^2-tryptophan analogues. Trp was substituted by the (*S*)-2-(1-methyl-1*H*-indol-3-yl)propionic residue or by (*S*)-2-(5-methoxy-1*H*-indol-3-yl)propionic residue. The biological activity (pEC_{50} and E_{max}) of these compounds was tested on electrically stimulated preparations of rat vas deferens. The 5-methoxy β-tryptophan group reverses the affinity of the compounds.

Keywords: Nociceptin analogue, Nociceptin/orphanin FQ - NOP receptor, β-Tryptophan analogues, Rat vas deferens

The numerous endogenous opioid peptides (nociceptin, enkephalins, endomorphines etc.) exert their effects by activating *μ, δ, κ* and *ε*.receptors. The opioids have numerous pharmacological effects. Both receptor-specific opioid agonists and antagonists are useful pharmacological tools and have potential as therapeutic agents.

Nociceptin (N/OFQ) is an endogenous hexadecapeptide[1,2] that belongs to the family of opioids, but it does not interact with the classical μ, δ and κ receptors.[3,4] There are close structural similarities between N/OFQ and opioid peptides (especially with dynorphyn A[5,6]), as well as between nociceptin receptor (NOP) and opioid receptors.[7-9] Moreover, activating

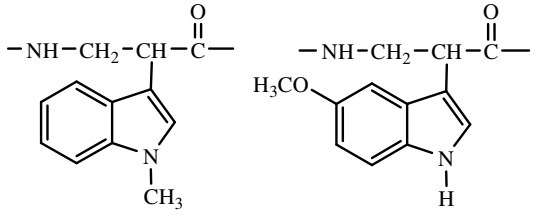
NOP receptor (coupled to the same G-protein-mediated second messenger system, as the opioids), decreases cAMP production,[1,2] inhibits voltage-sensitive calcium channels,[10] and activates the inward potassium conductance,[11] intracellular processes that result in inhibition of cellular excitability. Despite these similarities, there are a lot of pharmacological and functional differences between N/OFQ/NOP and the classical opioid systems.[12,13] The N/OFQ and its NOP receptor modulate a variety of biological functions, both at central and peripheral level. The understanding of its role depends upon the development of selective and highly potent peptide and non-peptide agonists and antagonists. The ligands for NOP receptors could be summarized in the following groups: a) non-peptide ligands like J-113397[14], SB-612111, and Ro 64-6198[15]; b) nociceptin-related peptides identified by structure-activity studies[16] and N/OFQ(1-13)NH_2[17]; c) hexapeptides. The latter are small synthetic peptides with general formula Ac-RYY-R/K-W/I-R/K-NH_2, isolated and characterized by Dooley et al. in 1997 by combinatorial chemistry.[18] They are selective agonists and antagonists with a very high affinity to NOP receptors. In other study, the same group[19] identified other hexapeptides, strong inhibitors of μ- and κ-opioid receptors, named acetalins.

Based on these findings, we report in the present study the synthesis and the biological screening of new analogues of Ac-RFMWMK-NH_2 and Ac-RYYRWK-NH_2, modified in position 4 and 5, respectively, with incorporation of newly synthesized β^2-tryptophan analogues:

Ac-Arg-Tyr-Tyr-Arg-**X**-Lys-NH_2

Ac-Arg-Phe-Met-**X**-Met-Lys-NH_2

Where ***X*** is:

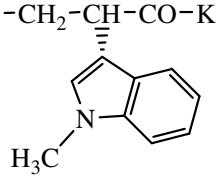
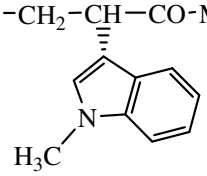
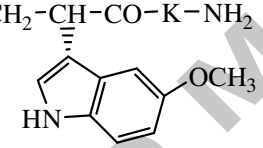
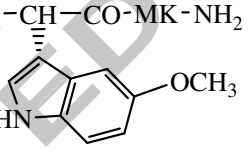


HP3 - HP4 **HP5 - HP6**

These novel hexapeptides have been synthesized by including β^2-tryptophan analogues[20] in position 4 and 5, respectively, using SPPS by Fmoc (9-fluorenylmethoxy-carbonyl) chemistry (see Supplementary data). The analytical data of the synthetic peptides are shown in Table 1. In order to estimate the relationship, we prepared the compounds HP3-HP6 by replacement of the natural tryptophan with an enantiopure β^2-tryptophan analogue. In

general, β-amino acids are more resistant than α-amino acids to enzymatic degradation[21] and they have often-relevant roles in medicinal chemistry.[22-24]

Table 1. Analytical data of synthetic peptides.

№	Peptides	$[\alpha]_D^a$ (°)	$^b t_{R,\,min}$	^{c} ESI MS: $(MH)^+$	
				calculated	found
HP1	Ac-RYYRWK-NH_2	-23	4.90	1011.5	1012.2
HP2	Ac-RFMWMK-NH_2	-18	5.20	938.5	939.6
HP3	Ac−RYYR−NH−CH_2−CH−CO−K−NH_2 	-21	7.15	1025.6	1025.9
HP4	Ac−RFM-NH−CH_2−CH−CO-MK-NH_2 	-19	9.11	952.5	953.0
HP5	Ac−RYYR−NH−CH_2−CH−CO−K−NH_2 	-25	11.45	1042.1	1042.6
HP6	Ac−RFM-NH−CH_2−CH−CO-MK-NH_2 	-28	10.80	968.9	969.1

[a] Optical rotation in methanol (c = 1) at 20 °C; [b] t_R is the retention time determined by HPLC; [c] The mass ion (MH^+) was obtained by electrospray mass spectrometry.

HP1 (Ac-RYYRWK-NH_2) is a well-known hexapeptide, isolated and characterized by Dooley et al.[18] It was found to be a short-chain peptide ligand with a very high affinity for NOP receptors. In our experiments, it was synthesized and used like a reference compound and a template for hexapeptides HP3 and HP5. Tested on rat vas deferens (rvd), HP1 exerted effects similar to those reported previously by us[25] and: by Ho et al.[26] on musculus anococcygeus. The onset of peptide-evoked inhibition of ES-induced smooth-muscle contractions occurs in a concentration of $1x10^{-11}$M, which is lower than that of N/OFQ(1-13)-NH_2. Compared to the latter, the maximal effect of HP1 was reached at the same concentration ($1x10^{-6}$M), but it evoked only about 40% inhibition of the contractions (Fig. 1A, Table 2). The effect was not influenced by naloxone (Nal, a blocker of opioid receptors) and

was completely prevented by naloxone benzoylhydrazone (Nal-B, a blocker of opioid and NOP receptors), confirming that HP1 interacts with NOP receptors only.

Table 2. Effects of the newly synthesized peptides on LFES-Evoked Contractions of Rat Vas Deferens.

Peptide	Peptide alone		Peptide + Nal	
	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)
HP 1	7.80±0.38	-36±6	8.21±0.44	-31 ± 6
HP 2	5.99±0.24	-52±9	5.94±0.17	-14 ±17
HP 3	6.50±0.24	-36±7	6.21±0.24	-17 ± 9
HP 4	5.91±0.18	-50±10	5.79±0.10	-15 ± 5
HP 5	5.85±0.50	-52±11	5.78±0.22	-12 ± 12
HP 6	5.78±0.58	-34±5	6.26±0.40	-14 ± 9

The maximal inhibitory effect of HP1 is reached at a concentration of $1x10^{-6}$M. Compared to HP1, the other tested peptides have shown lower activity. With them, the initial effect, as well as the maximal one, developed at higher concentrations. Therefore, the presented pEC_{50} and E_{max} (compounds HP2 – HP6) are calculated for concentration $1x10^{-5}$M. Data represent mean values ±S.E.M. of 6 separate experiments.

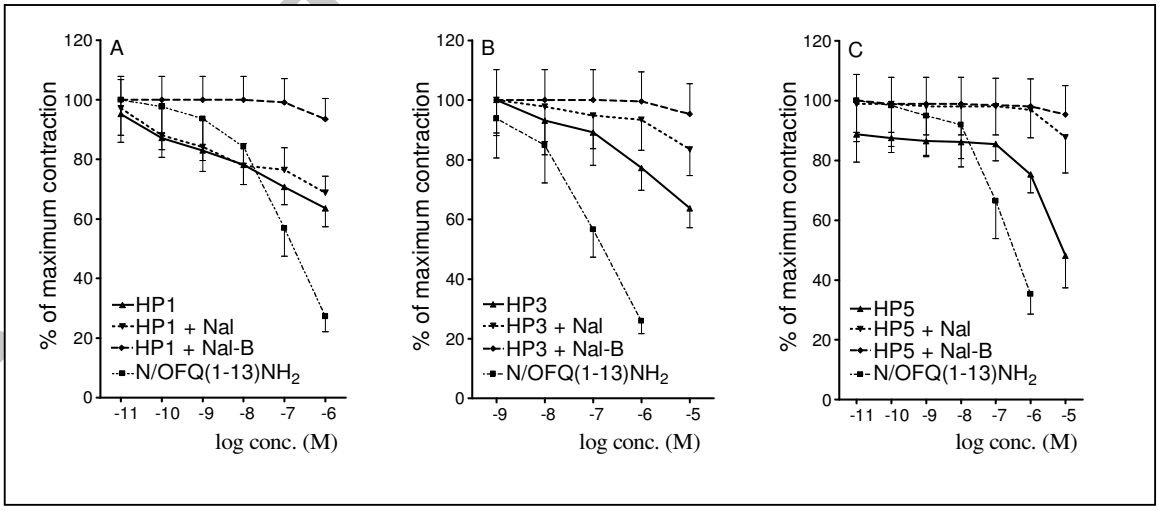


Figure 1. Rat vas deferens. Concentration-response curve of **$N/OFQ(1-13)NH_2$, HP1 (A), HP3 (B) and HP5 (C)** on the contractions induced by LFES before and after naloxone or naloxone benzoylhydrazone. The data are means ± SEM of six experiments.

In our experiments, HP1 induced a strong tachyphylaxis, as reported by Dooley et al.[18] Because of this, each dose-response curve (without or after blockade of opioid or NOP receptors) was created on a separate preparation (see Supplementary data).

HP3, in which Trp in position 5 is substituted by (*S*)-2-(1-methyl-1*H*-indol-3-yl)propionic residue exhibited lower affinity (pEC_{50}=6.5), compared to the parent molecule. The maximal inhibitory effect (E_{max}), almost the same as that of HP1, was produced by the peptide in a concentration of $1x10^{-5}$M. The effect of HP3 was strongly reduced by pre-incubation of smooth muscles with Nal and completely prevented by Nal-B (Fig. 1B). It is evident, that inclusion of N-methyl β^2-tryptophan analogue in position 5 results in a peptide (HP3) that possesses affinity for both opioid- and NOP receptors.

Concentrations higher than $1x10^{-5}$M (data not shown), markedly decreased vas deferens contractions, which is not due to specific interactions with NOP or opioid receptors. The effect of HP5 could be divided in 2 components. In low concentrations ($1x10^{-11}$M), the compound weakly decreased the electrically evoked contractions, expressing a plateau at concentrations $1x10^{-10}$M – $1x10^{-7}$M. Applied in higher concentrations ($1x10^{-6}$M – $1x10^{-5}$M), HP5 strongly and concentration-dependently depressed the evoked muscle contractions, the maximal inhibitory effect being almost equal (but in 10-fold higher concentration) to that of N/OFQ(1-13)NH_2 (pEC50= 7.29, Emax= -73%). When the peptide was applied after Nal-blockade of opioid receptors, this inhibitory effect was not observed, suggesting that HP5 is an opioid agonist (Fig. 1C).

The opioid antagonist HP2 (Ac-RFMWMK-NH_2) and its derivative HP4 showed dynamic properties similar to those of HP5 (Fig. 2A, Fig. 2B).

Low concentrations of the peptides ($1x10^{-11}$M) moderately diminished the smooth-muscle contractions. The higher concentrations did not change ($1x10^{-10}$M -$1x10^{-9}$M) or even slightly enhanced ($1x10^{-8}$M-$1x10^{-7}$M) the contractions. The blockade of opioid receptors by $1x10^{-6}$M Nal completely prevented the effects of HP2 and HP4 in the concentration range $1x10^{-11}$M-$1x10^{-6}$M, showing that their effects are a result of interaction with μ-receptors. Only in very high concentrations, it is possible to presume also an activation of NOP receptors, as far as Nal-B is more effective than Nal in preventing the inhibitory action of HP2 and HP4 ($1x10^{-6}$M-$1x10^{-5}$M). Both peptides showed similar affinity and efficacy (Table 2).

The effects of low concentrations of HP6 differ from that of HP2 and HP4, displaying a slight inhibition of smooth muscle contractions, due probably to interaction with NOP receptors (Fig. 2C). This effect (up to concentration of $1x10^{-5}$M) was blocked by Nal-B only. It appears that the insertion of (*S*)-2-(5-methoxy-1*H*-indol-3-yl)propionic residue in position 4

reversed the affinity of the original peptide Ac-RFMWMK-NH_2 from μ- to NOP receptors. In the concentration range $1x10^{-6}$M-$1x10^{-5}$ M, HP6 most probably activates opioid receptor, too.

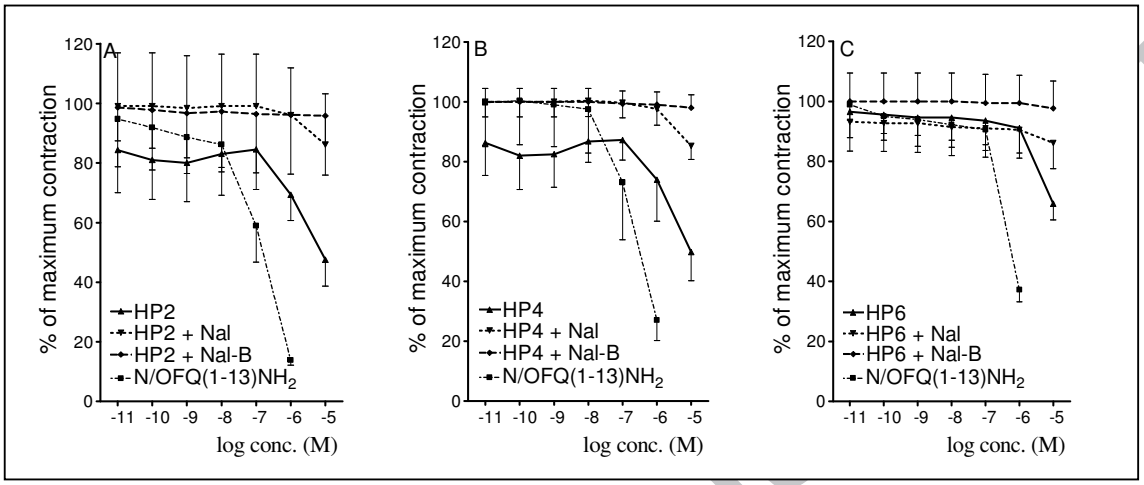


Figure 2. Rat vas deferens. Concentration-response curve of **N/OFQ(1-13)NH_2 , HP2 (A), HP4 (B) and HP6 (C)** on the contractions induced by LFES before and after naloxone or naloxone benzoylhydrazone. The data are means ± SEM of six experiments.

In the present paper, we studied the changes in peptide profile evoked by structural modification in position 5 of the hexapeptide HP1, an agonist of NOP receptors, and in position 4 of the hexapeptide HP2, an antagonist of opioid receptors.

There are, but only a few data in the literature, concerning the role of particular amino acids and their position in biologically active hexapeptides, activating NOP-receptors. At present, the research efforts of several groups have proved some structure requirements for biological activity of hexapeptide ligands with affinity to NOP receptors.

It was found that acetyl group at N-terminal of hexapeptides is required for high affinity to NOP receptors and also that presence of positively charged Arg in position 1,[27,28] as well as Arg in position 4,[29] could be decisive for short-chain peptide affinity and biological activity.

Binding studies[29] on N-terminally modified hexapeptides reveal that such compounds possess very high receptor affinity, but changed intrinsic activity. Similar data have been established about acetelins; Arg in position 1 is a prerequisite[19] for their activity, while the presence of N-terminal acetyl group does not change their affinity. This gave us reason to suggest, that the remaining part of the hexapeptide molecule, has a role for the selective binding to NOP- or μ-receptors. Studying the structure-activity requirements for NOP-activating hexapeptides, Ambo et al.[30] have found that the amino acid residue in position 5

plays a key role in agonist/antagonist activity. In some respect, our study extends these findings showing that replacement of Trp in position 5 by β^2-tryptophan analogs also affects the selectivity of hexapeptides.

Based on the test of HP2 (Ac-RFMWMK-NH_2) on guinea-pig ileum, Dooley et al.[18] described this hexapeptide like a potent and selective opioid antagonist, binding with a high affinity to κ_3 and μ-receptors. They also found, that the effects of HP2 depend on the species and it is relatively weak antagonist in mouse vas deferens. On rat vas deferens used in our experiments, the short-chain peptide in low concentrations decreased (by about 20%) the smooth-muscle contractions, suggesting that in this case HP2 is rather weak agonist, than antagonist of opioid receptors. Since rat vas deferens is a μ-specific tissue[30], it could be concluded that HP2 is an agonist of μ-receptors in this tissue. Moreover, it has been suggested that rat vas deferens is a good model "to assess the μ-receptor efficacy of opioid agonists proving a more physiological environment for a ligand-receptor interaction than other efficacy measuring methods".[31]

Our present results showed, that the presence of N-methyl β^2-tryptophan residue in position 5 in Ac-RYYRWK-NH_2, modified the selectivity of the referent peptide – HP3 interact with both NOP- and opioid-receptors. The same group in position 4 did not change the properties of Ac-RFMWMK-NH_2 - HP4 possess the same affinity and efficacy as the parent substance. The 5-methoxy β^2-tryptophan residue in position 5 in Ac-RYYRWK-NH_2 (HP5) or in position 4 of Ac-RFMWMK-NH_2 (HP6) reverse the affinity of the compounds – HP5, a derivative of NOP-agonist apparently has low affinity to NOP-receptors and activates opioid receptors, while HP6, a derivative of μ-receptor antagonist, exhibits properties of a weak NOP-receptor agonist. These data give us ground to suggest that in this case not only the position of modification, but also the nature of the incorporated group, lead to significant changes in peptide's selectivity and affinity.

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Graphical abstract

Ac-Arg-Tyr-Tyr-Arg-**X**-Lys-NH_2

Ac-Arg-Phe-Met-**X**-Met-Lys-NH_2

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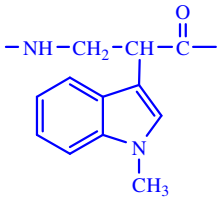
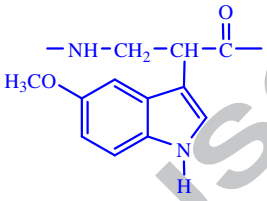


HP3 - HP4



HP5 -HP 6