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Bifunctional Opioid/Melanocortin Peptidomimetics for Use in Neuropathic Pain

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Introduction

Neuropathic pain results from a damage of the nervous system. and belongs to the category of chronic pain. The use of conventional opioid agonists for the treatment of chronic pain with neuropathic component is limited due to their weaker analgesic effects and the potential occurrence of undesirable side effects such as constipation, respiratory depression, tolerance and dependence. After the first post-injury period, when the endogenous antinociceptive (opioid) system is activated, other endogenous systems come into play as counteractants of the elicited opioid activity. These systems generate pronociceptive compounds that exert their effect through non-opioid receptors and weaken the effect of analgesics [1].

One of the most promising strategies in the search for new analgesics for this type of pain is to design compounds that activate the opioid system and simultaneously block the pronociceptive systems. A number of such endogenous pain-enhancing systems have been described [2], among them the melanocortin system (MC), particularly the melanocortin 4 (MC4) receptor, is of great interest [3,4].

Based on the above strategy, we have designed and evaluated for biological activity several bifunctional peptidomimetics [5-7] which consist of an opioid agonist (fragment of enkephalin [8]) and a melanocortin-4 (MC4) receptor antagonist (fragment of SHU9119 [9]), joined by linkers of varying length and rigidity. Three of them containing flexible linkers, namely one or two 6- aminohexanoic acid (Ahx) residues, were found to be very effective at low doses in reducing allodynia and hyperalgesia (von Frey and Cold Plate, respectively) that characterize neuropathic pain, while showing little activity in acute pain. An important element of preclinical studies of bifunctional peptidomimetics was the demonstration that the compounds we synthesized were much more potent than the mixture of parent compounds. Moreover, looking for an effect in neuropathic pain, we compared different forms (acetate and trifluoroacetate) and routes of administration of selected peptidomimetics in a neuropathic pain model.

The structure and possible application(s) of our compounds are subject to intellectual property rights protection, including but not limited to patent protection (US11041010) and pending patent protection (PL422093, PCT/IB2018/054925).

We have now started developing a larger-scale synthesis of our compounds and a series of analytical, physicochemical, biological, and biochemical tests necessary to begin preclinical studies on these compounds. In this publication we present several aspects of our research.

Results and Discussion

The three most efficient peptidomimetics mentioned above NOM1 (Tyr^1-D-Ala^2-Gly^3-Phe^4-**Ahx**^{5}-Nle^6-c[Asp^7-His^8-D-Nal(2')^9-Arg^{10}-Trp^{11}-Lys^{12}]-NH_2), NOM2 (Tyr^1-D-Ala^2-Gly^3-Phe^4-**Ahx**^{5}-**Ahx**^{6}-Nle^7-c[Asp^8-His^9-D-Nal(2')^{10}-Arg^{11}-Trp^{12}-Lys^{13}]-NH_2) and NOM3 (Dmt^1-D-Ala^2-Gly^3-Phe^4-**Ahx**^{5}-Nle^6-c[Asp^7-His^8-D-Nal(2')^9-Arg^{10}-Trp^{11}-Lys^{12}]-NH_2) were synthetized on a Rink amide AM resin (IRIS, 0,35 mmol/g) using the standard Fmoc strategy and carbodiimide (DIC) as a coupling reagent. The final crude peptides were purified by RP-HPLC and characterized by ESI MS method. The exchange of trifluoroacetates with acetates was performed using AG 1-X2 (acetate), 200-400 mesh ion exchange resin (BioRad). Before the upscaling of the synthesis, it was checked which step was the

most advantageous to carry out the cyclization (the formation of the lactam bond). For that purpose, three syntheses of the NOM3 were performed. In the first synthesis, cyclization was performed after attachment of Asp^7 residue, in the second - after attachment of Nle^6 residue and in the third - after the peptide chain has been assembled. After removing the peptides from the resin, the crude products were analysed by HPLC and the chromatograms were compared. The best result was achieved in the case of heptapeptide cyclization (the second synthesis)- 58% of NOM3 (HPLC). In the other two syntheses - the first and the third (cyclization of hexapeptide and dodecapeptide), 21% of NOM3 (HPLC) and a mixture of products were obtained, respectively.

Preclinical studies were performed in a model of neuropathic pain after chronic constriction injury to the sciatic nerve (CCI) in mice. The pain sensitivity characteristic of neuropathic pain was measured 7-14 days after nerve injury using two tests: von Frey filaments (tactile stimulus) and cold plate (thermal stimulus). Importantly, the behavioural tests have shown that the new bifunctional structure causes a stronger analgesic effect than the mixture of its components, i.e. the opioid and the MC4 antagonist. Figure 1A shows a comparison of the effect provided by the mixture of parent compounds, NOM1 and NOM2, in the cold plate test. As can be seen, the analgesic effect of NOM1 and NOM2 is significantly stronger. Moreover, the effect delivered by these peptidomimetics lasts much longer than the effect provided by the parent compounds and their mixture, what is clearly visible in Figure 1B when the the areas under the curve (AUC) are compared.

Administration of these two forms (acetate and trifluoroacetate) of peptidomimetics in a mouse model of neuropathic pain showed similar potency after intraperitoneal administration. Figure 2 shows the analgesic effects provided by two forms of NOM3. The analgesic effects provided by the two forms, as observed at different time points, are not significantly different, which indicates the possibility of using the form recommended in preclinical studies.

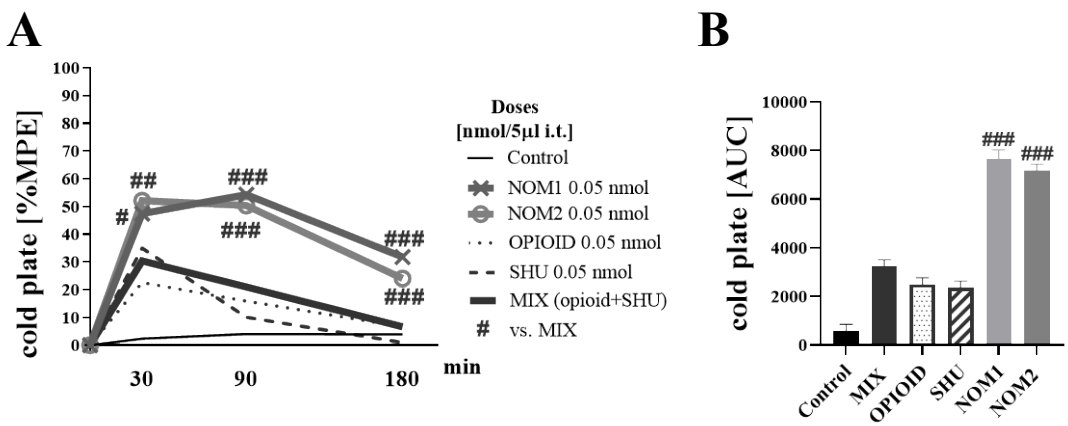

Fig. 1. Comparison of effects of hybrid structure NOM1 and NOM2 (0.05 nmol) with mixture of parent compounds (MIX = 0.05 nmol opioid + 0.05 nmol SHU9119) on: (A) hypersensitivity to thermal stimuli (cold plate), and (B) the effect by the area under the curve (AUC), as measured 30, 90, and 180 min after administration in CCI-exposed mice.

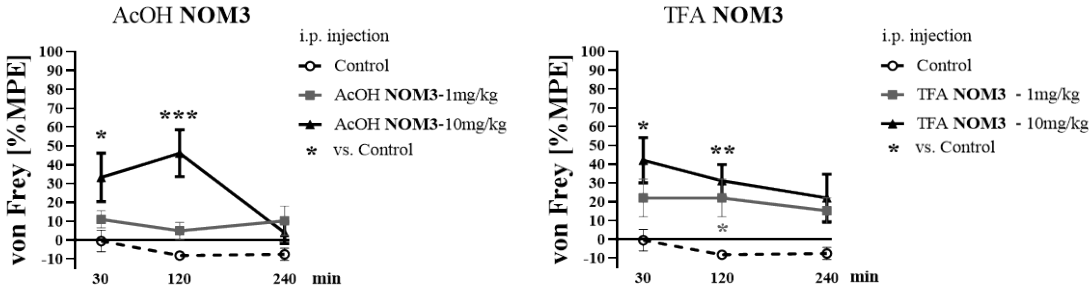

Fig. 2. Analgesic effect of NOM3 in two forms (acetate and trifluoroacetate) after intraperitoneal injection in mouse neuropathic pain model (chronic constriction injury to the sciatic nerve – CCI).

A comparison of the effects of peptidomimetics following intraspinal (by lumbar puncture), intravenous and intraperitoneal administration in a mouse model of neuropathic pain was also performed. The results of these studies are shown in Figure 3.

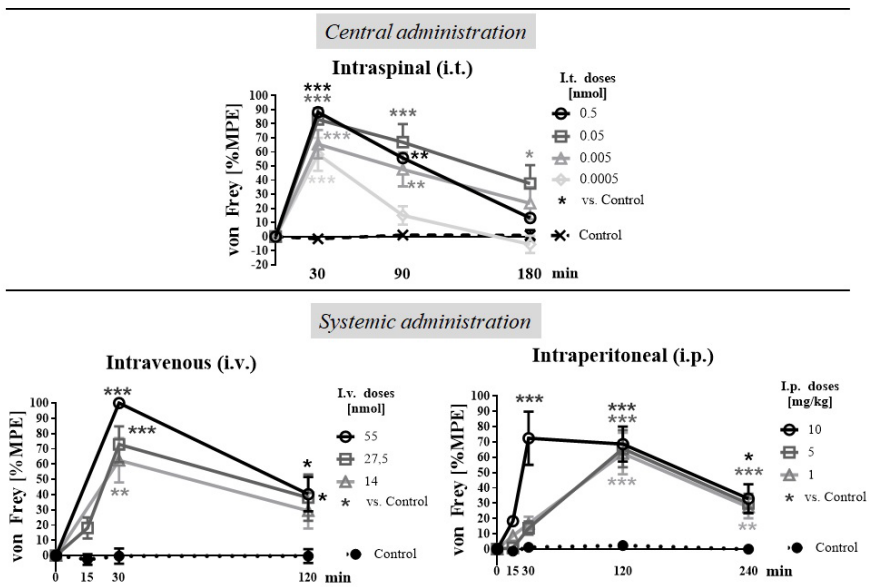

Fig. 3. Analgesic effect of NOM1 in different administration routes (Intraspinal – i.t., intravenous – i.v., and intraperitoneal – i.p.) in mouse neuropathic pain model.

Peptides intended for preclinical research must not contain trifluoroacetate ions, due to their harmful properties. Therefore, the replacement of these counterions with other ones is necessary. The HPLC-MS/MS method was developed to determine the concentration of TFA in synthetic samples. The multiple reaction monitoring (MRM) method in negative ion mode was used to follow the precursor-fragment ion transitions presented on the figure below (Figure 4). The calibration curve was prepared based on five different standard sample solutions. The representative peak area of the main MS2 transition was tracked in the test sample and compared with calibration linear equation resulting in TFA concentration in the test sample. The LC-MS/MS system consisted of LC-2050C 3D Liquid

Chromatograph and triple quadrupole mass spectrometer LCMS-8045 (Shimadzu), equipped with an ESI source and the polar type of column (30 mm × 2.1 mm) at 40 ºC were used in the study. The water-based and organic mobile phases were used for mixture separation: (A) ammonium acetate/acetic acid water solution and (B) a mixture of AcCN:MeOH:HCOOH. The 3.5-minute-long gradient program at a flow rate of 0.8 ml/min was used. The electrospray (ESI) parameters were optimized to collect the most ion transitions *via* multiple reaction monitoring (MRM) method in negative ion mode.

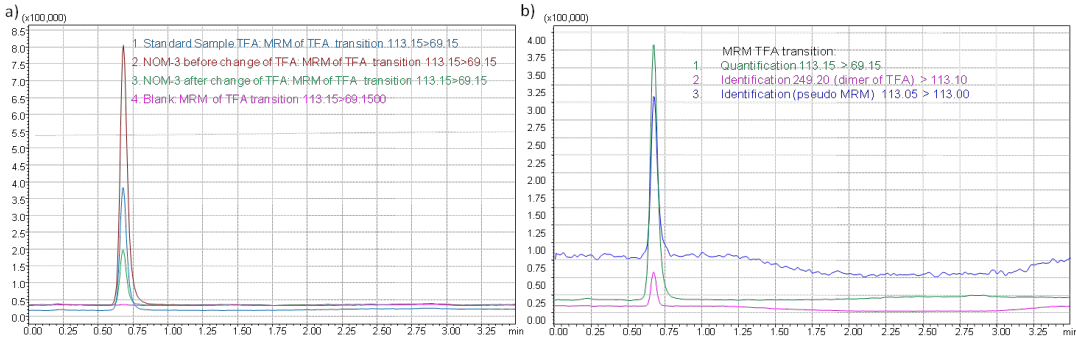

Fig. 4. Overlay TFA MRM transitions recorded for: a) 1. standard sample, 2. compound NOM3 before exchange of TFA to AcOH, 3. NOM3 after exchange of TFA to AcOH, 4. blank. b) MRM TFA transitions 1. Quantification 113.15/69.15, 2. Identification 249.20 (dimer of TFA)/113.10 3. Identification (pseudo MRM) 113.05/113.00.

Our studies showed that tested bifunctional peptidomimetics exhibit significantly greater analgesic activity compared to the action of the mixture of their components (opioid agonist and MC4 receptor antagonist). The best efficiency of peptidomometics was observed after spinal administration, but a similar effect was also obtained after intravenous administration, which is the basis for the selection of a drug formulation for use in the treatment of neuropathic pain. Moreover, the tested compounds show antinociceptive activity in both forms (acetate and trifluoroacetate) in rodents, which indicates the possibility of using the form recommended in preclinical studies.

The results of our research on bifunctional peptidomimetics offer hope for the development of new potential drugs for the treatment of neuropathic pain.

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