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ORIGINAL ARTICLE

Synthesis and analgesic effects of novel β^2-tryptophan hexapeptide analogs

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Abstract Aiming to develop more potent analgesic substances a new series of hexapeptides containing β^2-tryptophan analogues was synthesized. The Trp in position 4 and 5, respectively in Ac-Arg-Phe-Met-Trp-Met-Lys-NH_2 (opioid receptor antagonist) and Ac-Arg-Tyr-Tyr-Arg-Trp-Lys-NH_2 (highly potent and selective NOP-receptor agonist) was substituted by the (*S*)-2-(1-methyl-1*H*-indol-3-yl)propionic residue or the (*S*)-2-(5-methoxy-1*H*-indol-3-yl)propionic residue. The analgesic effect of the four newly synthesized compounds has been evaluated in male Wistar rats by PP- and HP tests and compared to the native templates. Further estimation of the mechanisms of action of each compound was achieved using specific antagonists—naloxone for opioid and JTC801 for the NOP receptor. Replacement of Trp with β^2-tryptophan analogues in 4th position (Ac-Arg-Phe-Met-Trp-Met-Lys-NH_2) led to increased and longer lasting analgesic effect. The results obtained permit us to assume that both opioid and NOP receptors take part in the newly synthesized compounds analgesic effects.

Keywords Nociceptin analogue · NOP receptor · SPPS · β-Tryptophan analogues · Nociception · PP-test · HP-latency

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Introduction

Bioactive peptides are important starting structures for the development of potential therapeutic agents. They bind to different receptors (opioid, non-opioid or both) and are involved in the physiological control of various functions among which nociception is particularly emphasized.

The heptadecapeptide nociceptin/orphanin FQ (N/OFQ) is an endogenous ligand for the G(i)-protein-coupled N/OFQ receptor (NOP), structurally and functionally related to the classical opioid receptors. The N/OFQ and its NOP receptor modulate a variety of biological functions, both at central and peripheral levels. Development of selective and highly potent peptide and non-peptide agonist and antagonist ligands is of highly importance for the understanding of the role of N/OFQ/NOP system. Among them are N/OFQ related peptides (Guerrini et al. 2000; Naydenova et al. 2010; Ambo et al. 2007), small peptides, identified by screening of peptide combinatorial libraries (Dooley et al. 1993, 1997) and non-peptide ligands such as Ro 64–6,198 and JTC–801 (Zaveri 2003). In recent years, considerable progress has been made in the development of selective NOP receptor peptide ligands having agonist or antagonist properties (Naydenova and Todorov 2011). The ligands were obtained using various design strategies, including substitution of natural and non-proteinogenic amino acids, conformational restriction, and the bivalent ligand approach (Wollemann et al. 1993). The synthesis of new NOP receptor ligands with higher affinity and lower enzymatic degradation is a primary goal of many research teams. Dooley et al. (1993, 1997) screened a synthetic peptide combinatorial library, and isolated and characterized several hexapeptides, including Ac-Arg-Tyr-Tyr-Arg-Trp-Lys-NH_2 that has been identified as the shortest peptide sequence with high NOP receptor affinity, selectivity

and marked analgesic effect. It was found that these peptides act as partial or full agonists or antagonists of NOP receptor in different in vivo and in vitro systems. Based on the templates Ac-Arg-Tyr-Tyr-Arg-Trp-Lys-NH_2 and Ac-Arg-Tyr-Tyr-Arg-lle-Lys-NH_2 new compounds were recently synthesized by our group substituting ornithine (Orn), diaminobutanoic (Dab) and diaminopropanoic (Dap) acids for lysine (Lys) (Vezenkov et al. 2006; Kasakov et al. 2010).

The β^2-tryptophan analogues represent non-natural amino acids that are important building blocks for the synthesis of peptidomimetics, natural products and biologically active compounds (Miller and Martin 2007). Another important property of tryptophan and tryptophan analogues is the fluorescence of the indole ring that can be used to study conformational changes in protein and in protein-membrane interactions (Chen and Barkley 1998). These β^2-tryptophan derivatives incorporated into peptides and proteins will result in biologically active materials with enhanced resistance to enzymatic degradation and relevant roles in medicinal chemistry.

With a view to developing ligands with more potent analgesic activity and lower enzymatic degradation, a new series of hexapeptides containing β^2-tryptophan analogues in position 4 and 5, based on the templates Ac-Arg-Phe-Met-Trp-Met-Lys-NH_2 (opioid receptor antagonist) and Ac-Arg-Tyr-Tyr-Arg-Trp-Lys-NH_2 (highly potent and selective NOP-receptor agonist) have been synthesized and evaluated.

Herein we report the analgesic effects of the four newly synthesized hexapeptides and compare them to the native templates. Further evaluation of the mechanisms of peptides' action was attempted using naloxone (an opioid antagonist) and JTC-801 (a NOP receptor antagonist).

Materials and methods

Synthesis

The protected amino acids and Fmoc-Rink Amide MBHA Resin were purchased from IrisBiotech (Germany). All other reagents and solvents were analytical or HPLC grade and were bought from Merck (Germany).

The solid-phase peptide synthesis by Fmoc (9-fluorenylmethoxycarbonyl) strategy was used to obtain modified hexapeptide containing β^2-tryptophan analogues. Rink-amide resin was used as a solid-phase carrier, and 2-(1H-benzotriazole-1-yl)1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) was used as a coupling reagent. The three functionalized amino acids were embedded as follows: Arg—as Fmoc-Arg(Pbf)-OH, Lys—as Fmoc-Lys(Boc)-OH, Trp—as Fmoc-Trp(Boc)-OH, Tyr—as Fmoc-Tyr(tBu)-OH. The coupling and deprotection reactions were performed using standard protocol and were checked by the Kaiser test. The two N-Fmoc β^2-tryptophan analogues (Pavlov et al. 2011) ((*S*)-3-(9-fluorenylmethoxycarbonylamino)-2-(1-methyl-1*H*-indol-3-yl)propionic acid and (*S*)-3-(9-fluorenylmethoxycarbonylamino)-2-(5-methoxy-1*H*-indol-3-yl)propionic acid) (three equiv) were coupled to the growing peptide chain by using TBTU/HOBt/DIEA, a molar ratio of 3/3/6, the coupling reaction time being 4 h. The cleavage of the synthesized peptides from the resin was done, using a mixture of 95 % trifluoroacetic acid (TFA), 2.5 % triisopropylsilane (TIS), and 2.5 % water. After filtration of the exhausted resin, the solvent was concentrated in vacuum and the residue triturated with cool ether. The purity of the peptides was checked by HPLC analysis, column: SymmetryShield™ RP-18, 3.5 μ (50 × 4.6 mm), flow: 1 ml/min, H_2O (0.1 % TFA)/CH_3CN (0.1 % TFA), gradient 0 → 100 % (15 min) and 100 % (4 min). The crude peptides were purified using semi-preparative HPLC, column XBridge™ Prep C18 10 μm (10 × 250 mm), flow: 5 ml/min, H_2O (0.1 % TFA)/CH_3CN (0.1 % TFA), gradient 20 → 70 % (20 min). The ESI mass spectra were recorded with a platform II quadrupole mass spectrometer fitted with an electrospray source. Optical rotations were measured with a Perkin Elmer 341 polarimeter. The analytical data for the synthetic peptides prepared were as follows: compound **1** t_R 7.15 min, >98 % pure, 1,025.6 calculated (MH^+), 1,025.9 observed (MH^+); compound **2** t_R 9.11 min, >99 % pure, 952.5 calculated (MH^+), 953.0 observed (MH^+); compound **3** t_R 11.45 min, >99 % pure, 1,042.1 calculated (MH^+), 1,042.6 observed (MH^+); compound **4** t_R 10.80 min, >99 % pure, 968.9 calculated (MH^+), 969.1 observed (MH^+).

Biological tests

Animals

All studies were performed on adult (60–90 days old) male rats from the Wistar strain (*Rattus norvegicus*), weighting 180–200 g. They were housed in colony room at 22 ± 1 °C temperature, where light was maintained on a 12-h light 12-h dark cycle. Food and water were provided ad libitum. All animals were allowed a minimum of 2 weeks for acclimatization to the test environment before any experimental manipulations were performed.

Each group included 8–10 animals. All procedures were approved by the Animal Care and Use Committee of the Medical University of Sofia and BFSA.

Paw-pressure test (Randall-Selitto test)

The changes in the mechanical nociceptive threshold of the rats were measured by an analgesimeter (Ugo Basile). Pressure was applied to the hind-paw and the pressure

(g) required eliciting a nociceptive response such as squeak or struggle was taken as the mechanical nociceptive threshold. A cut-off value of 500 g was used to prevent damage of the paw.

Hot plate test

The latency of response to pain was measured from the moment an animal was placed on a metal plate (heated to 55 ± 0.5 °C) to the first signs of pain (paw licking, jumping). A cut-off time of 30 s was observed.

For biological tests of nociception two referent nociceptin analogues (Ref. 1 and Ref. 2) and four β^2-tryptophan modified nociceptin analogues (1, 2, 3, and 4) were adopted, all obtained from the Department of Organic Chemistry of the University of Chemical Technologies and Metallurgy of Sofia, Bulgaria. All the substances at a dose of 10 μg/kg were injected intraperitoneally (i.p.) after being dissolved in 0.9 % NaCl solution.

Further explanation of biological mechanisms of action of novel peptides was made by applying JTC-801, N-(4-amino-2-methylquinolin-6-yl)-2-(4-ethylphenoxymethyl)benzamide monohydrochloride, a NOP receptor antagonist, at a dose of 0,5 mg/kg, i.p., 10 min before the injection of newly synthesized peptides.

Additional explanation of nociceptive mechanisms of the investigated peptides used naloxone (Nal), an opioid receptor antagonist, at a dose of 1 mg/kg, i.p. Nal-pretreatment occurred 20 min before injection of referent compounds or analogues respectively.

Results and discussion

Chemistry

The novel hexapeptides containing β^2-tryptophan analogues, as NOP receptor ligands, have the following sequences (Fig. 1).

These novel hexapeptides have been synthesized including β^2-tryptophan analogues in position 4 and 5 respectively, using SPPS by Fmoc (9-fluorenylmethoxycarbonyl) chemistry. In order to estimate the 'structure–activity' relation, we prepared the compounds **1–4** 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 (Gademann et al. 1999) and they have often-relevant roles in medicinal chemistry (Cole 1994; Juaristi 1997; Kuhl et al. 2005).

The N-Fmoc β^2-tryptophan analogues: (*S*)-3-(9-fluorenylmethoxy carbonylamino)-2-(1-methyl-1*H*-indol-3-yl) propionic acid (**IVa**) and (*S*)-3-(9-fluorenylmethoxycarbonylamino)-2-(5-methoxy-1*H*-indol-3-yl)propionic acid (**IVb**) was prepared following the previously described procedure (Scheme 1) (Pavlov et al. 2011). An asymmetric Friedel–Crafts alkylation involving the enantiopure chiral auxiliary **I** (benzyl-4-(3-(3-nitroacryloyloxy)-4,4-dimethyl-2-oxopyrrolidin-1-yl)benzoate) (Calmès et al. 2005) and differently substituted indoles **II** in presence of a catalyst (copper triflate) was used as key step for the preparation of the precursor compound **III**. After reduction of the nitro group of compound **III** using H_2/Pd/AcOH/H_2O 5 % and Fmoc protection, hydrolysis with trimethyltin hydroxide (Me_3S-nOH) in 1,2-DCE yielded the desired enantiopur N-(*S*)-Fmoc-β-tryptophan analogues (*S*)-**IVa-b** in good yield. The use of Me_3SnOH allowed to remove the chiral auxiliary as well as to avoid the undesired epimerization observed when using classical LiOH conditions (Pavlov et al. 2011). Application of this procedure (Scheme 1) yielded the desired β^2-Tryptophan analogues in their N-Fmoc-protected form, ready to use in the solid-phase peptide synthesis of the selected hexapeptides.

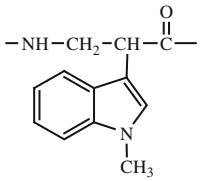
Ac-Arg-Tyr-Tyr-Arg-**Trp**-Lys-NH_2 - Referent compound ***1***

Ac-Arg-Phe-Met-**Trp**-Met-Lys-NH_2 - Referent compound ***2***

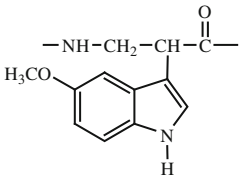
Ac-Arg-Tyr-Tyr-Arg-**X**-Lys-NH_2

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

Where ***X*** is:



or



In analogues ***1*** and ***2*** In analogues ***3*** and ***4***

Fig. 1 New hexapeptides containing β-tryptophan analogues

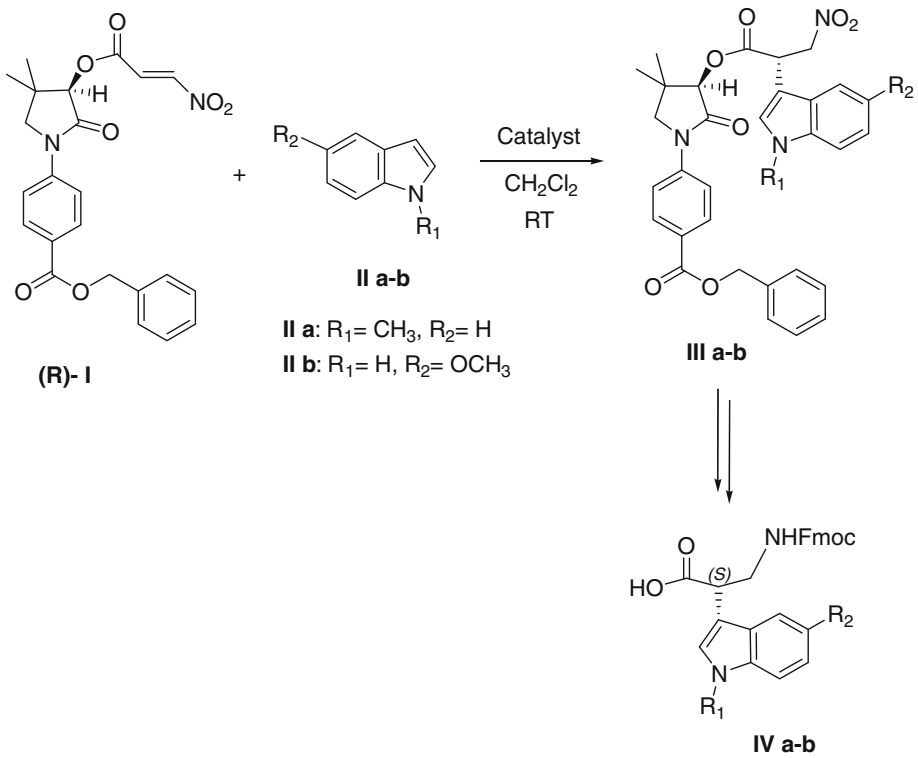
The crude hexapeptides were purified on a reversed-phase high-performance liquid chromatography (HPLC) and the molecular weights were determined, using electrospray ionisation mass-spectrometry. The analytical data are shown in the "Materials and methods".

Biological activity

The evaluation of pain threshold and HP-latency started 10 min after i.p. injection of reference or analogue peptides (Fig. 2).

The results showed that on the 10th min of the experiment both the reference peptides had an analgesic effect compared to controls ($p < 0.001$), with Ref. ***2***—animals having higher pain thresholds in comparison to Ref. ***1***. Among the analogues examined compound ***2*** and ***4*** showed

Scheme 1 Synthetic pathway for preparation of N-Fmoc β-tryptophan analogues



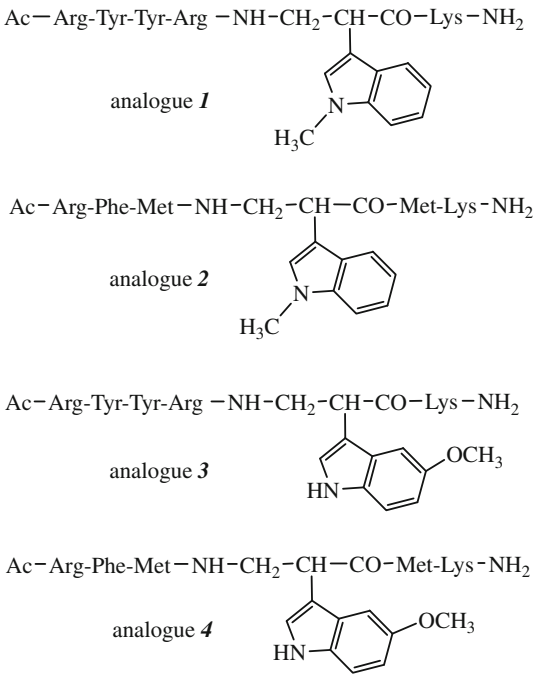


Fig. 2 Structures of hexapeptides modified with β^2-tryptophan analogues

a statistically relevant analgesic effect compared to control ($p < 0.001$) and referent ***2*** ($p < 0.001$). The effect was short lasting and on the 20th min only compound ***2*** maintained an analgesic effect relevantly higher ($p < 0.001$) than the controls and Ref. ***2*** peptide. Compound ***2*** maintained increased pain thresholds as compared to control ($p < 0.001$) and referent ***2*** ($p < 0.01$) even on the 30th min of the time estimated (Fig. 3).

HP-latencies showed similar results with analogues ***2*** and ***4*** having the more pronounced analgesic effect (results not shown).

In a second experimental series an inhibitor of nociceptin receptor antagonist was applied 10 min before each of the referent substances and the analogues estimated. Injection of JTC801 antagonized the analgesic effect of both the referent substances, as well as analogues ***1***, ***2***, and ***4***. All the pain thresholds detected were comparable to the controls, except for analogue ***3*** which showed a statistically relevant analgesic effect compared to controls ($p < 0.001$) and both the referents ($p < 0.05$) (Fig. 4).

Measured by Hot-plate test the nociception was increased for both the referent substances and all the analogues compared to the controls. HP-latencies showed a statistically relevant decrease ($p < 0.001$), with a tendency toward hyperalgesia, compared to controls. Yet analogue ***3*** showed a statistically higher HP-latency as compared to Ref. ***1*** and ***2*** ($p < 0.001$), as well as analogues ***1***, ***2***, and ***4*** (Fig. 5).

An additional third experimental series was performed with application of opioid receptor antagonist naloxone 20 min before referents and analogues estimated. Injection

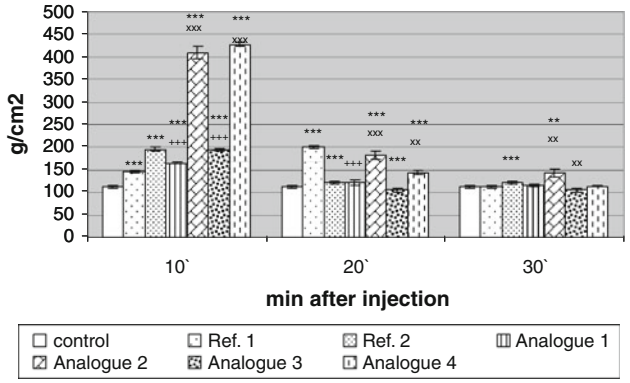


Fig. 3 Effects of the novel hexapeptides containing β^2-tryptophan analogues and referent compounds on nociception estimated by PP-test in rats. Main values ± SEM are presented. $^{***}p < 0.001$ vs. control; $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. referent substance **1**; $^{x}p < 0.05$, $^{xx}p < 0.01$, $^{xxx}p < 0.001$ vs. referent substance **2**

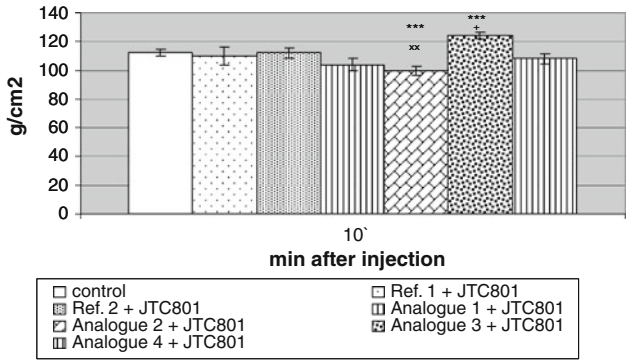


Fig. 4 Effects of the novel hexapeptides containing β^2-tryptophan analogues and referent compounds after JTC801 injection on nociception estimated by PP-test in rats. Main values ± S.E.M. are presented. $^{***}p < 0.001$ vs. control; $^{+}p < 0.051$ vs. referent substance **1**; $^{x}p < 0.05$, $^{xx}p < 0.01$ vs. referent substance **2**

of naloxone decreased pain thresholds of Ref. **2** substance and analogues **1, 2**, and **3** to the control value. Ref. **1** and analogue **4** showed a statistically lower pain threshold compared both to controls ($p < 0.001$) and Ref. 2 ($p < 0.001$) (Fig. 6).

A statistically relevant decrease ($p < 0.001$) in HP-latencies compared to controls was detected for both referent substances, and the analogues estimated. Analogue **4** showed the shortest HP-latency compared to controls ($p < 0.001$), both the referent substances ($p < 0.001$) and analogues **1, 2**, and **3** (Fig. 7).

We have described the synthesis and analgesic investigation of novel hexapeptides containing β^2-tryptophan analogues. The newly synthesized compounds were obtained by solid-phase peptide synthesis—Fmoc-strategy. All the peptides were tested for analgesic effects. The results showed that substitution in 4th position of Ac-Arg-Phe-Met-Trp-Met-Lys-NH_2 both with (*S*)-2-(1-methyl-1*H*-indol-3-yl)propionic and (*S*)-2-(5-methoxy-1*H*-indol-3-

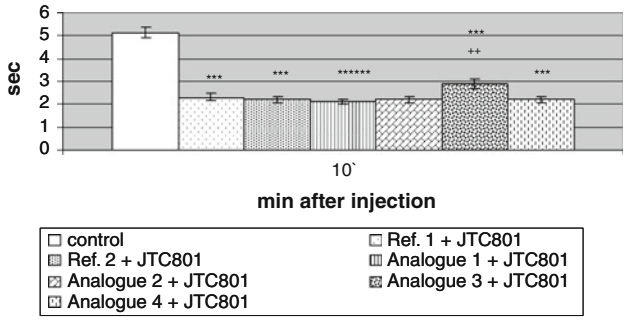


Fig. 5 Effects of the novel hexapeptides containing β^2-tryptophan analogues and the referent compounds on nociception estimated by HP-test in rats. Main values ± SEM are presented. $^{***}p < 0.001$ vs. control; $^{++}p < 0.01$ vs. referent substance **1**; $^{xxx}p < 0.001$ vs. referent substance **2**

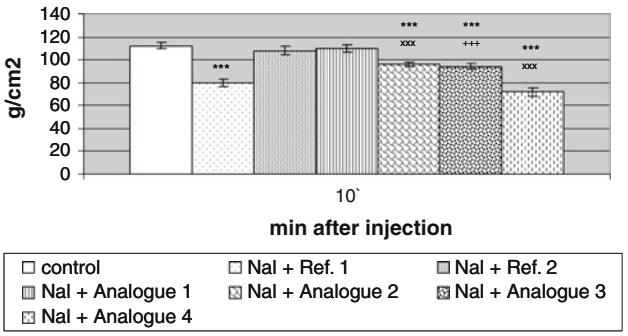


Fig. 6 Effects of the novel hexapeptides containing β^2-tryptophan analogues and the referent compounds after naloxone injection on nociception estimated by PP-test in rats. Main values ± SEM are presented. $^{***}p < 0.001$ vs. control; $^{+++}p < 0.001$ vs. referent substance **1**; $^{xxx}p < 0.001$ vs. referent substance **2**

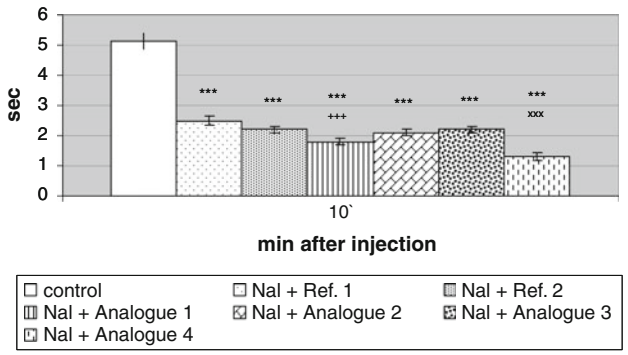


Fig. 7 Effects of the novel hexapeptides containing β^2-tryptophan analogues and the referent compounds after naloxone injection on nociception estimated by HP-test in rats. Main values ± SEM are presented. $^{***}p < 0.001$ vs. control; $^{+++}p < 0.001$ vs. referent substance **1**; $^{xxx}p < 0.001$ vs. referent substance **2**

yl)propionic residues led to an early analgesic effect stronger and longer lasting compared to the native substance and compared to substitution in 5th position of Ac-

Arg-Tyr-Tyr-Arg-Trp-Lys-NH_2 with the same residues. Substitution with (*S*)-2-(1-methyl-1*H*-indol-3-yl)propionic residue had the more pronounced analgesic effect compared to (*S*)-2-(5-methoxy-1*H*-indol-3-yl)propionic residue.

JTC801 injection led to a significant decrease the pain thresholds of referent substances and analogues ***1***, ***2*** and ***4*** and a significant decrease in HP-latencies of all the investigated compounds. Interestingly, analogue ***3*** that represents a substitution in 5th position of a template known to be a NOP-receptors agonist, maintained on the 10th min a still manifest analgesic effect compared to controls and both the referents estimated by PP- but not the HP-test.

Naloxone injection abolished the analgesic effect of all the substances investigated established by both PP- and HP- tests. Interestingly, the more pronounced decrease both in pain threshold and HP-latency was observed after application of analogue ***4*** that represents a substitution in a template known to be a μ-receptor antagonist.

In conclusion it may be stated that hexapeptides containing β^2-tryptophan analogues expressed analgesic activity through both NOP- and opioid receptors. Additional investigation of mechanisms of action would be interesting to ascertain the biological activity in respect to structural changes in the native templates.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Ambo A, Kohara H, Kawano S, Sasaki Y (2007) Opioid receptor-like 1 (ORL1) receptor binding and the biological properties of Ac-Arg-Tyr-Tyr-Arg-Ile-Arg-NH_2 and its analogs. J Pept Sci 13:672

Calmès M, Didierjean C, Martinez J, Songis O (2005) (R)-(4-(Benzyloxycarbonylphenyl)-3-hydroxy-4,4-dimethyl-2-pyrrolidinone)acrylate derivative as a chiral dienophile for the synthesis of enantiopure 2-aminocyclohexane carboxylic acids. Tetrahedron: Asymm 15:2173–2178

Chen Y, Barkley MD (1998) Toward understanding tryptophan fluorescence in proteins. Biochem 37:9976–9982

Cole CD (1994) Recent stereoselective synthetic approaches to β-amino acids. Tetrahedron 50:9517–9958

Dooley CT, Chung NN, Shiller PW, Houghten RA (1993) Acetalins: opioid receptor antagonists determined through the use of synthetic peptide combinatorial libraries. Proc Natl Acad Sci 90:10911

Dooley CT, Spaeth CG, Berzetei-Gurske IP, Craymer K, Adapa ID, Brandt SR, Houghten RA, Toll L (1997) Binding and in vitro activities of peptides with high affinity for the nociceptin/Orphanin FQ receptor, ORL1. J Pharmacol Exp Ther 283:735–741

Gademann K, Hintermann T, Scheiber JV (1999) Beta-peptides: twisting and turning. Curr Med Chem 6:905–925

Guerrini R, Calo' G, Bigoni R, Rizzi A, Varani K, Toth G, Gessi S, Hashiba E, Hashimoto Y, Lambert DG, Borea PA, Tomatis R, Salvadori S, Regoli D (2000) Further studies on nociceptin-related peptides: discovery of a new chemical template with antagonist activity on the nociceptin receptor. J Med Chem 43:2805–2813

Juaristi E (1997) Enantioselective synthesis of β-aminoacids. Wiley-VLH John Wiley and Sons, New-York, pp 1–66

Kasakov L, Nashar M, Naydenova E, Vezenkov L, Vlaskovska M (2010) In vitro studies of the activity of newly sinthesized nociceptin/orphanin FQ receptor ligand analogues. Protein Pept Lett 17:616–620

Kuhl A, Hahn MG, Dumic M, Mittendorf J (2005) Alicyclic beta-amino acids in medicinal chemistry. Amino Acids 29:89–100

Miller A, Martin SF (2007) Concise, enantioselective total synthesis of (-)-alstonerine. Org Lett 9:1113–1116

Naydenova E, Todorov P (2011) Nociceptin/Orphanin (N/OFQ) analogues: chemistry and biology. J UCTM 46:333–348

Naydenova E, Todorov P, Mateeva P, Zamfirova R, Pavlov N, Todorov S (2010) Synthesis and biological activity of novel small peptides with aminophosphonates moiety as NOP receptor ligands. Amino Acids 39:1537–1543

Pavlov N, Gilles P, Didierjean C, Wenger E, Naydenova E, Martinez J, Calmès M (2011) Asymmetric synthesis of β^2-tryptophan analogues via Friedel-Crafts alkylation of indoles with a chiral nitroacrylate. J Org Chem 76:6116–6124

Vezenkov L, Zhivkova V, Naydenova E (2006) Desin and synthesis of new analogues of the nociceptin/orphanin FQ receptor ligands Ac-Arg-Tyr-Tyr-Arg-Trp-Lys-NH_2 and Ac-Arg-Tyr-Tyr-Arg-lle-Lys-NH_2. Bull Chem Comm 38:64–66

Wollemann M, Benyhe S, Simon J (1993) The kappa-opioid receptor: evidence for the different subtypes. Life Sci 52:599–611

Zaveri N (2003) Peptide and nonpeptide ligands for the nociceptin/orphanin FQ receptor ORL1: research tools and potential therapeutic agents. Life Sci 73:663–678