


# Low-molecular synthetic peptides with non-narcotic type of analgesia: comparative study and mechanism of analgesic activity

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## Abstract

The group of synthetic low-molecular peptides exhibiting profound analgesic activity was developed by modifying the salmon calcitonin molecule fragment sCT<sub>16-21</sub>, which retains the previously reported analgesic activity of the full-sized molecule. The mechanism of analgesic action of these synthetic oligopeptides has been investigated and their analgesic effect was compared with analgesic activity of ketorolac tromethamine, one of the strongest non-steroidal anti-inflammatory drug painkiller. It was demonstrated that the analgesic effect of the developed synthetic oligopeptides was associated with the specific binding of the clathrin heavy chain. It is postulated that inhibition of clathrin-mediated endocytosis of pain receptors in the postsynaptic vesicular cycle causes is more efficient analgesia than inhibition of those receptors on plasma membranes that may allow to replace opioid and non-steroidal anti-inflammatory drug's analgesics with a much less toxic low-molecular synthetic peptides with non-narcotic type of analgesia.

## Keywords

Analgesia, calcitonin, clathrin, short peptides

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## Introduction

Currently, opioids and non-steroidal anti-inflammatory drugs (NSAIDs) account for more than half of the global pain management therapeutics market (Pain Therapeutics Market—Growth, Future Prospects and Competitive Analysis, 2017–2025). Because of deficiencies, they do not meet the demand for nociceptive pain therapy, which is growing due to an increase in the number of diseases such as diabetes and cancer. The disadvantage of opioid analgesics (morphine and structures close to it, as well as opioid peptides) is a drug addiction realized through the same opioid receptors of various types as anesthesia.<sup>1</sup> In addition, opioid analgesics are not effective for all pain syndromes.<sup>2</sup>

The mechanism of action of NSAIDs (such as ibuprofen, ketorolac) is associated with non-selective inhibition of the activity of the isoforms of the enzyme cyclooxygenase COX-1 and COX-2, which catalyzes the formation of prostaglandins from arachidonic acid, which play an important role in the pathogenesis of pain,

inflammation and fever. All of these compounds, unlike opioid analgesics, are not addictive but toxic. For example, in the United States, 43% of all hospitalizations associated with medicine side effects are in NSAIDs.<sup>3</sup> Numerous cardiovascular disorders caused by this class of compounds are associated with the suppression of cyclooxygenases.<sup>4</sup>

Recent advances in the use of anticonvulsant drugs for the treatment of neuropathic pain do not solve the problem of deficiency of relatively inexpensive, safe, and effective non-narcotic pain medications for patients suffering from nociceptive pain.<sup>5</sup>

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In this regard, synthetic peptide analgesics with a non-narcotic type of anesthesia, primarily calcitonins, whose analgesic effect is realized through specific calcitonin receptors and the serotonergic system of the brain, are attracting attention.<sup>6</sup> The most active of all known calcitonins is the full-length salmon calcitonin molecule. However, the use of a full-sized salmon calcitonin molecule for analgesia is limited due to its hormonal and antigenic properties<sup>7,8</sup> and relatively high cost.

The authors have developed synthetic analogs of the salmon calcitonin molecule fragment sCT<sub>16-21</sub> (Leu-His-Lys-Leu-Gln-Thr), which retains the analgesic activity of the full-sized molecule and do not have the above-mentioned disadvantages of a full-sized salmon calcitonin molecule. The whole family of synthetic low-molecular peptides, characterized by greater analgesic stability and effectiveness than the natural sequence, was produced by modifying the natural sequence sCT<sub>16-21</sub>.<sup>9</sup>

This article presents the results of a study of the mechanism of analgesic action of the developed peptides and comparative study of their analgesic activity and that of ketorolac tromethamine. The latter is comparable to morphine in the effect of analgesic activity and is much superior to other NSAIDs.<sup>10</sup> It was also taken into account in the comparison drug selection that ketorolac tromethamine is available in the form of nasal spray, since intranasal administration is of greatest interest in view of the central activity and the use of small doses which are typical for the studied synthetic peptides.<sup>11</sup>

As the peptides developed are synthetic analogues of the salmon calcitonin molecular fragment, it was natural to assume that their analgesic action mechanism is similar to the non-narcotic analgesic action mechanism of the full-sized salmon calcitonin molecule. The primary thing in the analgesic action of calcitonin is interaction with specific central and peripheral calcitonin receptors.<sup>12</sup> Derivatives of calcitonin fragments are also bound to calcitonin receptors;<sup>13,14</sup> for instance, the carboxybenzoyl derivative of the sCT<sub>16-21</sub> sequence was found to be the minimal sequence which interacts with the calcitonin receptor.<sup>14</sup>

A common feature for calcitonin receptors (as well as for other receptors which contain G-protein) is that they are internalized into the cell mainly using the mechanism of clathrin-dependent endocytosis (CME) after stimulation by the respective ligand.<sup>15</sup> The ligand bound to the receptor enters the cell together with it. It is obvious that the ligand of calcitonin receptors, when entering the cell, may have other intracellular targets, different from the receptor.

We should note a special role of CME in the synaptic pain transmission in the nervous system.<sup>16</sup> The signal-transmitting (presynaptic) region of the nerve cell and

the signal-receiving (postsynaptic) region of the other cell are separated by a narrow synaptic cleft. When a nerve impulse is received, the vesicles filled with a neurotransmitter, the main component of which is clathrin, merge with the presynaptic membrane (exocytosis), releasing the neurotransmitter into the synaptic cleft. When the neurotransmitter interacts with the receptor on the postsynaptic membrane, there is an electric signal, the value of which determines the excitation in the postsynaptic cell. A rapid decrease in the vesicle number in the nerve end with a continuous nerve impulse is compensated by generation of new vesicles using the mechanism of CME, which are filled with a neurotransmitter and lined up for repeated exocytosis. The combination of these processes is called presynaptic vesicular cycle.

In addition to this recycling, there is a postsynaptic vesicular cycle in which some of the receptors are removed from the membrane surface also using the CME mechanism. This process is stimulated by interaction between the neurotransmitter and a specific receptor.<sup>17</sup> After that, some receptors return to the cell surface, also using the CME mechanism. Lowered number of neurotransmitter-specific receptors reduces the postsynaptic membrane sensitivity to this ligand and leads to a decrease in the amplitude of postsynaptic signals (analgesia).

Inhibition of CME leads to irregular recycling of pain receptors in the postsynaptic vesicular cycle, which results in the analgesic effect.

The recycling deceleration mechanism is universal and functions in numerous clinically significant receptors of nerve pathways and synapses which transmit a nociceptive signal from the periphery to the brain and are targets of various painkillers.<sup>18</sup> For instance, pharmacological or genetic disorder of clathrin, dynamine, or beta-acepine functions blocks induced by substance P (SP-induced) endocytosis of type 1 neurokinin receptors (NK 1 R) and prevents the sustained excitation of neurons in spinal cord sections in vitro more effectively than simple inhibition of NK 1 R receptor by antagonists on the cell membrane.<sup>19</sup> It is also shown that the specific inhibition of receptors in endosomes, in conjunction with the CME inhibition, provides a more effective and stable analgesia than just the use of pain receptor antagonists on the plasma membrane.<sup>20</sup>

Based on the central activity of the developed synthetic peptides, the authors, when investigating the mechanism of the analgesic action, were focused on searching for possible intracellular targets of the synthetic analogs of the salmon calcitonin molecular fragment, the interaction which results in analgesia.

The "Formalin test"<sup>21,22</sup> and the "Haffner Tailset Pinch Test"<sup>23,24</sup> were selected as somatic pain models for the purposes of the study, which together provide

the most complete information about specific (analgesic) activity of the studied drugs both in case of a moderate, continuous pain caused by formaldehyde administration and on the mechanical somatic pain model.

The advantage of the “Formalin Test” is the ability to identify various aspects of the analgesic activity of the compounds under study.<sup>21</sup> Two pain phases develop in response to the formalin administration. The first phase which is characterized by acute pain lasts for 3 to 5 min from the moment of injection and is related, in particular, to the stimulation of TRPA1 nociceptors and activation of C-fibers. Moreover, it is shown that the TRPA1 cationic channels are activated directly by formalin in the pain model specified.<sup>25</sup> Then, there is actually no pain response for 10 to 15 min.

According to modern concepts, the existence and duration of the rest period between the two peaks of the pain response to the formalin administration characterize the intensity of accumulation of endogenous analgesic metabolites and is mainly related to the central activity of the stimulant.<sup>26,27</sup>

The second phase begins 15 to 20 min after injection, lasts 20 to 40 min, and enables to evaluate the tonic pain related to the inflammatory reaction.<sup>28</sup> It is important that both pain phases are mutually independent and develop through different mechanisms in the “Formalin Test.”<sup>29</sup> Opioid analgesics are active in both phases. NSAIDs suppress the second phase, and local anesthetics suppress the first one only.<sup>30</sup> The “Formalin Test” records the number and frequency of pain reactions, with flinching being considered a more reliable indicator of pain response than licking or drawn up paw.<sup>31</sup> The study of the effect of the active compound on the number and frequency of pain reactions in each of the phases of the formalin test allows us to establish the direction of its action.

## Materials and methods

### Peptide synthesis

The Leu-D-His-Lys-Leu-Gln-Thr-NH<sub>2</sub> hexapeptide synthesis was carried out by automatic solid-phase Fmoc synthesis on the Rink polymer (Rink Amide Resin, 0.6 mmol of amino-groups per 1 g of polymer) using the DCC/HOBt (N,N'-dicyclohexylcarbodiimide/1-hydroxybenzotriazole) amino-acid activation method. Deblocking was carried out by treatment with piperidine/DMF (piperidine/N,N-dimethylformamide) (1:4) solution during 7 min. The side chain groups were protected by the following groups: tBu (tert-butyl ether) for tyrosine, threonine, Trt (trityl or triphenylmethyl) for glutamine and histidine, Boc (t-butyloxycarbonyl) for lysine. The peptides were removed from the polymer and deblocked by the TFA/H<sub>2</sub>O/EDT (trifluoroacetic

acid/water/1,2-ethanediol) (90:5:5) mixture. The peptide was purified by reverse-phase high-performance liquid chromatography (HPLC) (C18 column), eluent—acetonitrile-water (0.1 M potassium dihydrophosphate) at a ratio of 6:4. The peptide was described by mass spectrometer and HPLC—Waters DeltaPak C18 3.9 × 150 mm 5 μm 100 column; solution A: 0.1% TFA in 100% water/MeCN—with a flow rate of 1 ml/min and detection wave length of 230 nm.

### Drugs for the study of specific activity

The synthetic peptide-based drug was prepared in two concentrations: 20 μg/ml (corresponds to the single dose, recalculated taking into account the interspecific dose transfer) and 100 μg/ml (corresponds to the maximum daily dose for humans, recalculated taking into account the interspecific dose transfer) using the following excipients: benzalkonium chloride; sodium chloride; hydrochloric acid (to bring the pH to 5.0 ± 0.1); distilled water. The use of peptide in doses exceeding the maximum daily dose does not increase its relative bioavailability, as in the case of the full-sized salmon calcitonin molecule.<sup>32</sup>

The ketorolac tromethamine-based comparison drug was prepared in a concentration of 21.4 mg/ml using the following excipients: EDTA, monobasic potassium phosphate, sodium hydroxide, and distilled water. The choice of the comparison drug dose was based on the recommended maximum daily dose for humans with intranasal administration (weblinks: <https://www.sprix.com/>), recalculated taking into account the interspecific dose transfer.<sup>33</sup>

### Animals for the study of specific activity

Sixty-four white outbred male rats aged about 3 months weighing 180 to 200 g, purposefully bred and previously not involved in the study, were used in the experiment (Animal manufacturer: “Andreevka”, branch of the Scientific Center of Biomedical Technologies of the Russian Academy of Medical Sciences, Russia). The rats were kept in premises with controlled conditions at an air temperature of 20°C–26°C and a relative humidity of 30%–70%, with a regular change of the light cycle (12-hours light/12-hours darkness) with water and food ad libitum.

All animals underwent the 30-day quarantine before being sent and the 7-day adaptation after being delivered to vivaria. During the adaptation period, the clinical health signs (overall health, liveliness, cleanliness, fatness, appetite, absence of symptoms) were visually controlled in animals. Before beginning the study, the animals that met the criteria for inclusion in the experiment (species, gender, body weight, clinical health) were

distributed into groups of eight animals. The distribution was made so that the average weights of animal groups of a single species differed by no more than 10%. Each animal was assigned with a unique number.

The animal experiments were carried out in compliance with the legal and ethical standards for animal treatment in accordance with the regulations adopted by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS 123, Strasbourg, 1986).

#### *Comparative study of analgesic activity of low-molecular synthetic peptides with non-narcotic type of analgesia and ketorolac tromethamine on the model of somatic pain caused by the algogene ("Formalin test")*

Thirty minutes before the formalin administration into the paw, the male rats were intranasally injected with prepared solutions containing analgesics or the saline solution for the control group, at the doses indicated in Table 1.

Rats were administered an aqueous formalin solution in a 1:50 dilution in a volume of 50  $\mu$ l subplantarily (under plantar aponeurosis) of the right hind paw.

After administration of formalin solution, an animal was immediately placed in a transparent cylinder to register pain reactions. All animal experiments were performed in double-blind in accordance with Good Laboratory Practice (GLP) principles.

The duration of the first phase of the pain reaction in minutes and the number of pain signs (drawn up paw, flinching, licking) during the first phase were estimated, then the rest period duration and the second pain phase duration in minutes and the number of pain signs in the second phase (drawn up paw, flinching, and licking) were estimated.

The statistically significant decrease in the frequency of pain symptoms and increase in the rest period in the experimental animal group after the drug administration

compared to the control group were considered as the criterion of analgesic effect.

#### *Comparative study of analgesic activity of low-molecular synthetic peptides with non-narcotic type of analgesia and ketorolac tromethamine on mechanical somatic pain model ("Haffner Tailset Pinch Test")*

The study of analgesic activity on mechanical somatic pain model with dosed mechanical stimulation of the rats' tailsets using the clip ("Haffner Tailset Pinch Test") is based on the stimulation of low threshold nociceptors.

Thirty minutes before mechanical stimulation, male rats were intranasally administered prepared solutions with analgesics, or the saline solution for the control group at the same doses as indicated in Table 1. Then, the animals were placed on a horizontal surface and the tailset was pinched with a special clip. All animal experiments were performed in double-blind in accordance with GLP principles.

The rats' innate defensive reaction to the tailset pinching was evaluated on a 4-point scale: 0—no reaction; 1—flinching and vocalization; 2—running forward; 3—twisting to the tailset and biting the clip. The statistically significant decrease in the intensity of pain reactions estimated in points compared to the control group was considered as a criterion of analgesic effect.

#### *Study of the possible mechanism of analgesic action of the peptides*

The experiment was based on the affine chromatography, where a peptide fixed on a special matrix serves as a stationary phase. Rat brain homogenate represents a mobile phase. The fraction, which contains proteins being specifically bound to the peptide under study, undergoes further fractioning by electrophoresis and direct detection of individual protein molecules.

A "spacer" had to be injected to plant the peptide on the matrix. The purpose of the "spacer" is to separate

**Table 1.** Study design for different experimental animal groups.

Group name	Route of administration	Analgesic dosage and type	Number of animals in each test
K	Intranasal, once daily, saline solution, 2 drops of 50 $\mu$ l, 1 drop per nostril	—	8
T	Intranasal, once daily, 2 drops of 50 $\mu$ l, 1 drop per nostril	Synthetic peptide 2 $\mu$ g per animal (1 $\mu$ g per nostril)	8
5T	Intranasal, once daily, 2 drops of 50 $\mu$ l, 1 drop per nostril	Synthetic peptide 10 $\mu$ g per animal (5 $\mu$ g per nostril)	8
R	Intranasal, once daily, 2 drops of 50 $\mu$ l, 1 drop per nostril	Ketorolac tromethamine 2.14 mg per animal (1.07 mg per nostril)	8

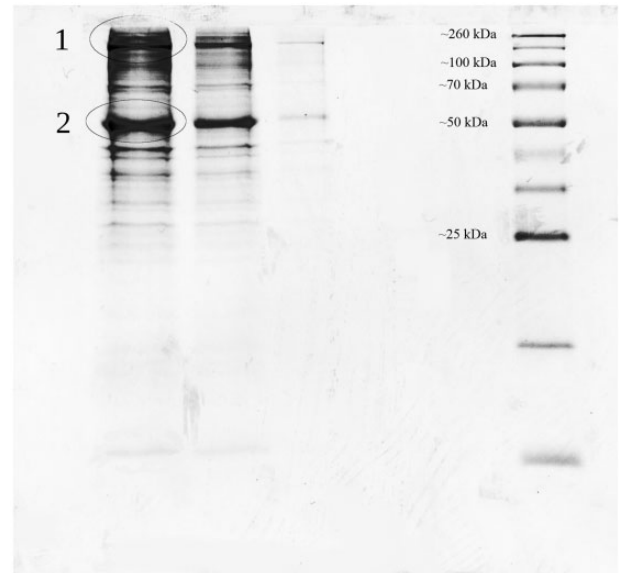
the matrix and the peptide itself spatially to create the conditions for interaction of the peptide and brain homogenate proteins. Such use of “spacer” is quite common for affine sorption. The nature of “spacers” is very diverse.<sup>34–36</sup> We used the “spacer” consisting of two amino-acid residues (Asp-Asp). The introduction of the dipeptide occurred in phosphate buffer (pH 7.8–8.0). To exclude the possible effect of the “spacer” on the experimental results, it was decided to synthesize the “reverse” peptide sequence (NH<sub>2</sub>-Thr-Gln-Leu-Lys-D-His-Leu-Asp-Asp) with the same molecular weight as an additional control.

**Affine sorbent obtaining.** Cyanogen bromide-activated 4B sepharose (Sigma-Aldrich) dissolved in 1 mM hydrochloric acid (HCl) was used as the basis for the affine sorbent. The peptide under study was dissolved in bidistilled water and added under strict pH control at a ratio of 5 mg peptide per 1 g activated sepharose. “Cross-link” was carried out at a temperature of 4°C during 12 h. When the “cross-linking” was over, the unbound product was thoroughly washed with an acetate buffer pH 5.0 and a bicarbonate pH 9.0 buffer.

**Obtaining cerebral cortex preparations.** To obtain the rat cerebral cortex preparations, the Wistar male rats weighing about 300 g were taken (Rappolovo nursery). The animals were decapitated, with their cerebral cortex extracted in the cold. The resulting samples were placed in liquid nitrogen, where they were ground to a fine powder using MPBiomed the FastPrep 24. Then, the sample was transferred to 10 mM TRIS pH 7.6 buffer with 0.3 M sucrose (Helicon), 1 mM CaCl<sub>2</sub> (Sigma-Aldrich) and 10 mM MgCl<sub>2</sub> (Sigma-Aldrich). The resulting homogenate was then centrifuged in the cold at 15,000 r/min for 20 min. The centrifugation conditions were chosen so as to deplete the content of membrane fragments of brain cells in the homogenate.

Animal experiments were carried out in compliance with the regulations and ethical standards for animal treatment in accordance with the rules adopted by the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (ETS 123, Strasbourg, 1986).

**Affine sorption.** The supernatant obtained after centrifugation of rat cerebral cortex preparations was incubated with the affine sorbent on a shaker for 24 h at 4°C. The sorbent was then transferred to the column (Bio-Rad) and placed into the BioRad NGC Discovery 10 chromatograph, then washed from a non-specifically bound protein. Washing was carried out with 10 mM TRIS buffer (pH 7.6). The protein elution was controlled by a through-flow photometric detector at two wavelengths of  $\lambda = 280$  nm and  $\lambda = 205$  nm, with flow rates of 0.1 ml/

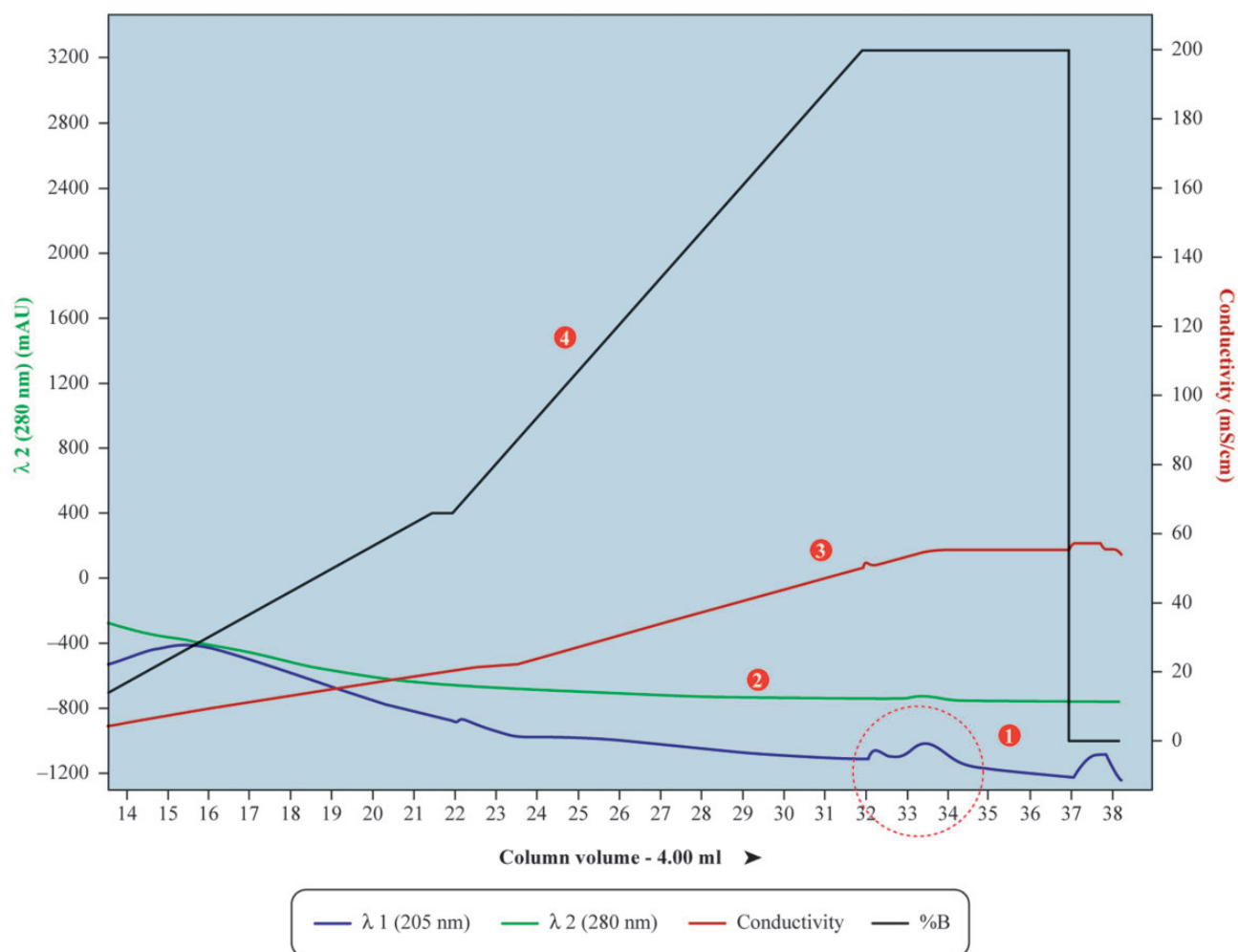


**Figure 1.** Electrophoresis of a concentrated protein fraction obtained by affinity chromatography. On the right, there is a Spectra marker with mass values (kDa), on the left—the studied fraction. The upper major spot corresponding to a mass of ~190 kDa was identified as a clathrin heavy chain by 13 peptide sequences. The second major spot corresponding to a mass of ~55 kDa was identified as different tubulin chains by 22 peptide sequences.

min. The protein was then washed off with a NaCl gradient of 0.05 M to 0.5 M in a 10 mM TRIS buffer (pH 7.6). The chromatogram (Figures 1 and 2) shows two peaks from 32nd to 34th min which indicate the presence of a protein fraction being specifically bound to a peptide molecule. Protein fractions, which had been specifically bound to the peptide under study, were further concentrated by means of concentrators (Vivaspin 3 kDa), then electrophoresis was performed.

**Electrophoresis.** Electrophoresis of a concentrated fraction (in quantities of 1, 5 and 10  $\mu$ l) was carried out in polyacrylamide gel (PAAG-gel) (sodium dodecyl sulfate (SDS–electrophoresis) by Laemli).<sup>37</sup> PAAG gel was prepared immediately before the experiment using the acrylamide (Sigma-Aldrich) and bisacrylamide (Sigma-Aldrich) solutions. Ammonium persulfate (Sigma-Aldrich) was used as a polymerization initiator and tetramethylethylenediamine (Sigma-Aldrich) as a catalyst. We used the Spectra (Spectra™ Multicolor Broad Range Protein Ladder) marker. Figure 1 shows clearly two major protein compounds. Two strips corresponding to the marker scale in the range of 140 to 260 and 50 kDa were cut out for identification.

**Mass-spectrometric protein analysis.** Foretic spots were identified using mass spectrometry. The “bottom-up”



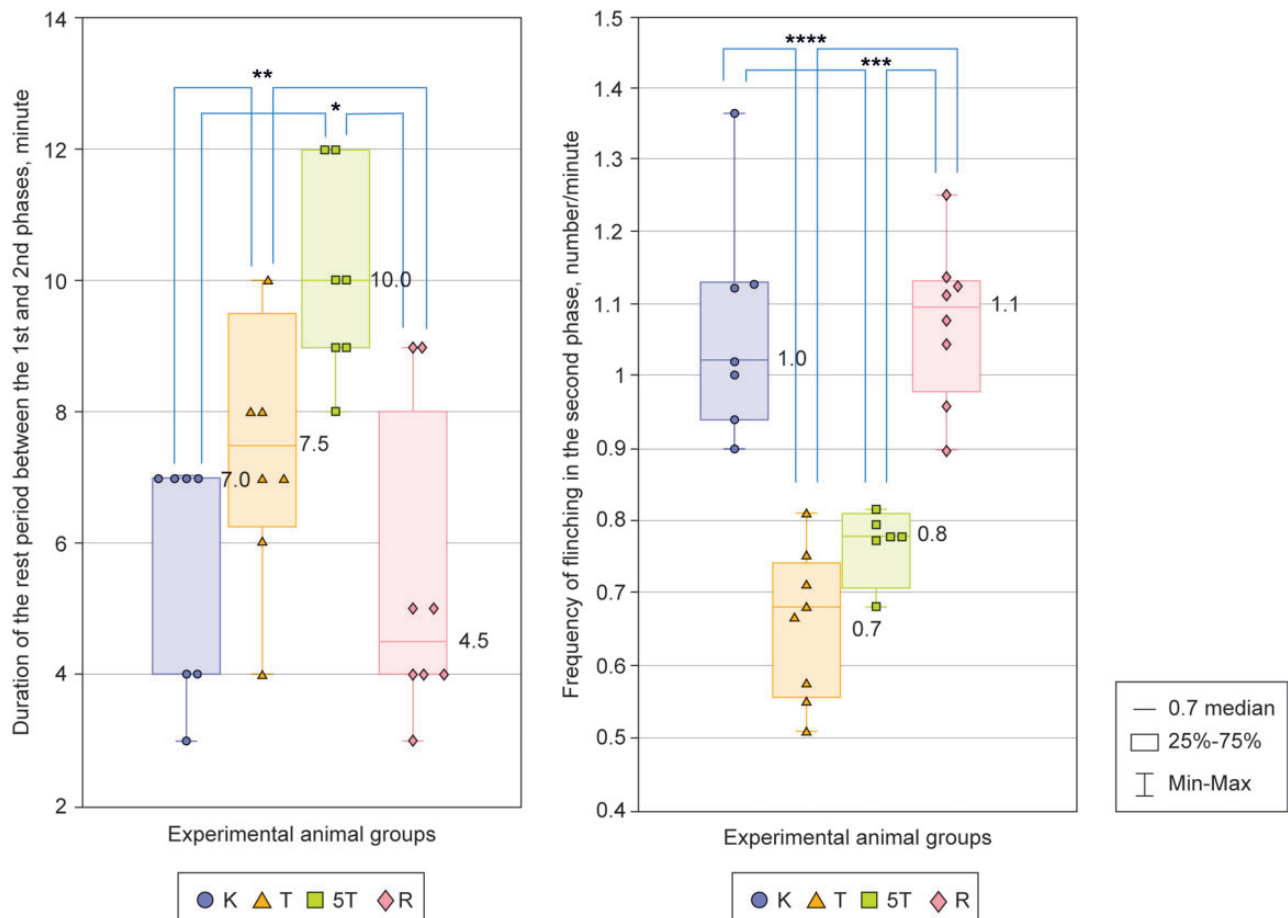
**Figure 2.** Protein elution. Detection was carried out at a wavelength of  $\lambda = 280$  nm and  $\lambda = 205$  nm, with flow rates of 0.1 ml/min. 1—elution detection at 205 nm, 2—elution detection at 280 nm, 3—conductivity values (mS/cm), 4—changes in the concentration of the mobile phase “B” (NaCl). The selected area shows two peaks from 32nd to 34th min which indicate the presence of a protein fraction being specifically bound to a peptide molecule.

approach was used to identify proteins in target electrophoretic signals.<sup>38</sup> After two-dimensional electrophoresis, the gel was colored with a Coomassie G250 (Diaem) alcoholic solution. The gel plots containing target proteins were then cut out and chopped with a scalpel. To wash out the coloring agent and SDS, the gel pieces were trice washed with 50% acetonitrile (HPLC-grade; LiChrosolv)/30 mM Tris (Sigma), pH 8.2 solution for 15 min at room temperature and constantly stirred. Once the solution was removed, the gel pieces were dehydrated in 100% acetonitrile. To remove completely the acetonitrile, the samples were dried in the CentriVap (Labronco) vacuum concentrator at 4°C for 40 min. The dried samples were added with a bovine trypsin (20 ng/ml) solution and incubated on ice for an hour until complete gel rehydration. After that, the excess trypsin was removed, and the samples were added with

50  $\mu$ l 30 mM TRIS buffer, pH 8.2 and incubated at 37°C for 16 to 18 h. The tryptic peptides were extracted from the gel with 50% acetonitrile acidified with 0.1% formic acid (Sigma). The resulting solution was dried in a CentriVap (Labronco) vacuum concentrator at 4°C and dissolved at phase A for subsequent chromatography. Mass-spectrometric analysis of tryptic peptides was carried out on a quadrupole time-of-flight Agilent ESI-Q-TOF 6538 UHD (Agilent Technologies) mass spectrometer combined with a high-performance liquid Agilent 1260 (Agilent Technologies) chromatograph. Chromatographic separation was carried out in the water-acetonitrile system in the presence of 0.1% formic acid (phase A—5% acetonitrile, 0.1% formic acid; phase B—90% acetonitrile, 0.1% formic acid) in the acetonitrile gradient (5% to 60% phase B in 25 min and up to 100% phase B in 5 min) on the Zorbax 300 sb-

C18 column (Agilent Technologies; grain 3.5  $\mu\text{m}$ , inner diameter 0.10 mm, length 150 mm) at a flow rate of 15  $\mu\text{l}/\text{min}$ . The chromatographic system was washed for 5 min after each sample. The ion analysis was carried out at 3 spectra per second in the mode of automatic tandem mass spectrometry (MS)/MS analysis with the parent ions 2+, 3+, n+, with automatic calculation of the collision cell energy depending on the m/z of the parent ion. The analysis of mass spectrometric data was carried out in the Spectrum Mill MS Proteomic Workbench program (Rev B.04.00.127; Agilent Technologies) with the UniProt database search in the identification mode, taking into account that a possible error in determining the mass of parent ions would be at most 20 ppm. Data validation was carried out using the auto-validation procedure at the threshold of false discovery rate of 1.2%. The consistency of the identified protein with its real position on the gel was checked after identification.

**Matrix-assisted laser desorption/ionization.** In addition to the standard mass spectrometric analysis of the gel strip, qualitative analysis by matrix-assisted laser desorption/ionization (MALDI) using UltrafleXtreme mass spectrometer (Bruker Daltonics) was used to identify proteins. The concentrated sample was added with 5  $\mu\text{l}$  trypsin solution (10 ng/ml) and incubated at 37°C for 12 h. Trypsin was inactivated by adding 0.5  $\mu\text{l}$  10% trifluoroacetic acid. The sample was centrifuged for 30 min (20,000 g, 4°C), dried under vacuum with subsequent addition of 5  $\mu\text{l}$  0.1% trifluoroacetic acid, and placed in a 384-well plate (MTP AnchorChip™ 800/384, Bruker Daltonics). Proteins were identified by means of UltrafleXtreme spectrometer, using  $\alpha$ -cyano-4-hydroxycinnamic acid as a matrix. FlexAnalysis 3.2 software (Bruker Daltonics) was used to analyze the ion peaks. Protein identification was carried out using the Mascot 2.4.2 program (Matrix Science, <http://www.matrixscience.com>).



**Figure 3.** Duration of the rest period between the first and second phases and frequency of flinching in the second phase in the “Formalin Test” for the various experimental animal groups (K—control, T—animals that received the synthetic peptide 2  $\mu\text{g}$ , 5T—animals that received the synthetic peptide 10  $\mu\text{g}$ , R—animals that received ketorolac tromethamine 2.14 mg). \* $P < 0.002$  significantly different from groups K, R. \*\* $P < 0.05$  significantly different from groups K, R. \*\*\* $P < 0.005$  significantly different from groups K, R. \*\*\*\* $P < 0.001$  significantly different from groups K, R.

science.com) in the UniProt databases (Uniprot, <http://www.uniprot.org/>). We used the “weight tolerance” parameters (precursor weight tolerance of 100 ppm, fragment weight tolerance of 0.9 Da). Cysteine carboxymethylation and partial methionine oxidation were considered as acceptable modification. One missed trypsinolysis site was considered as acceptable.

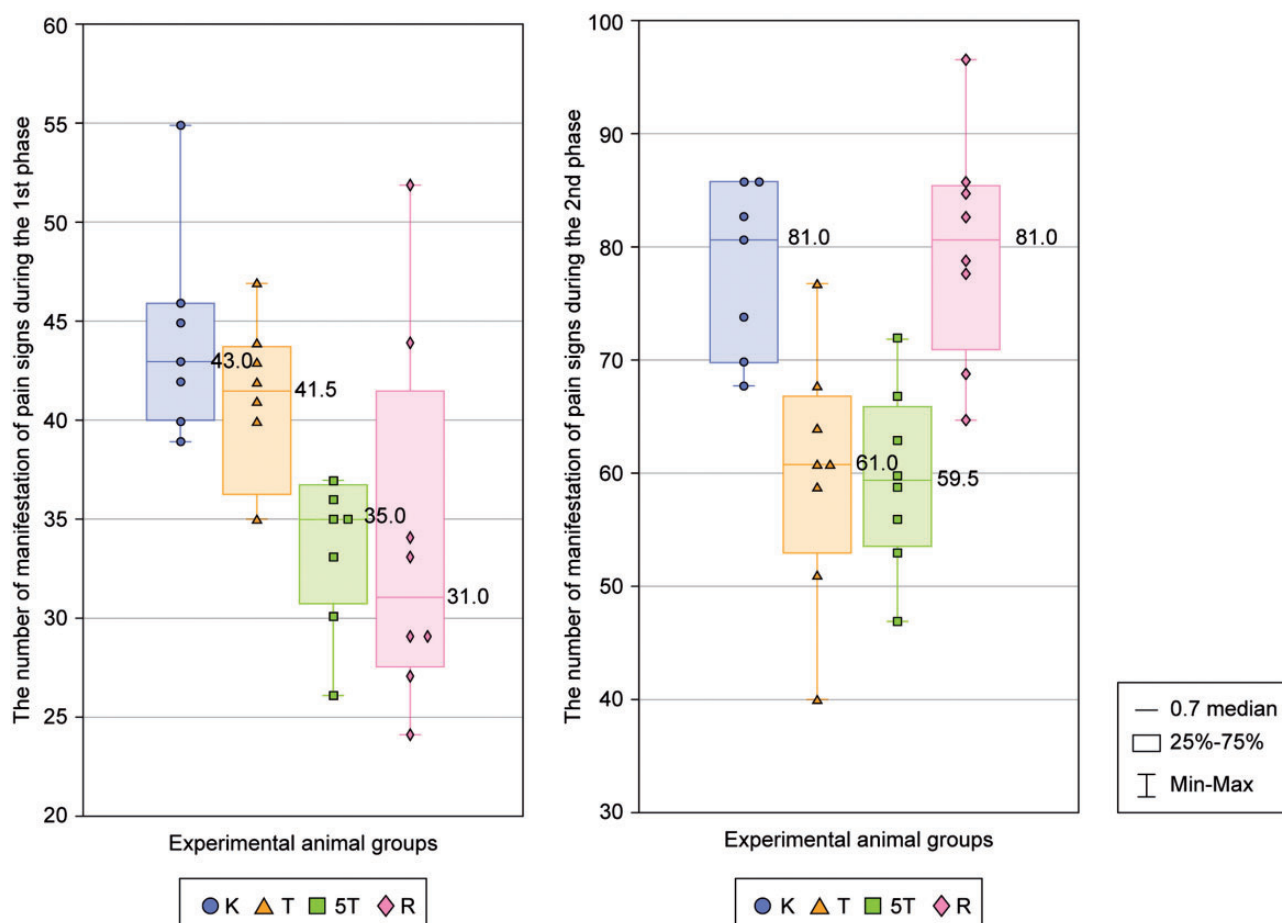
### Statistical analysis for the study of specific activity

To identify the differences in analgesic activity between the studied drugs, all indicators that can be quantified were statistically described. The experimental data distribution normality testing was performed by Shapiro–Wilk test and the variance homogeneity by Levene test. For the data distributed differently from the normal, the nonparametric Kruskal–Wallis criterion and the Mann–Whitney criterion were used to perform paired comparisons. All calculations were performed in the IBM SPSS statistics software package.

## Results

### Comparative study of analgesic activity of low-molecular synthetic peptides with non-narcotic type of analgesia and ketorolac tromethamine

**Results of the “formalin test”.** Experimental data testing showed that the distribution is different from the normal for most of the indicators, and therefore the differences can be estimated by non-parametric criteria only. As such, the Kruskal–Wallis test was carried out to screen for differences, and the Mann–Whitney test was applied to find differences between the specific groups. Using these criteria made it possible to reveal significant differences in the rest period duration and in the number and frequency of flinching in the second phase (Figure 3) between the experimental groups with rats which were administered a synthetic peptide (T, 5 T) and groups with rats which were administered a saline solution and ketorolac tromethamine (K, R).



**Figure 4.** Total number of manifestations of pain signs during the first and second phases in the “Formalin Test” for the various experimental animal groups (K—control, T—animals that received the synthetic peptide 2 µg, 5 T—animals that received the synthetic peptide 10 µg, R—animals that received ketorolac tromethamine 2.14 mg).

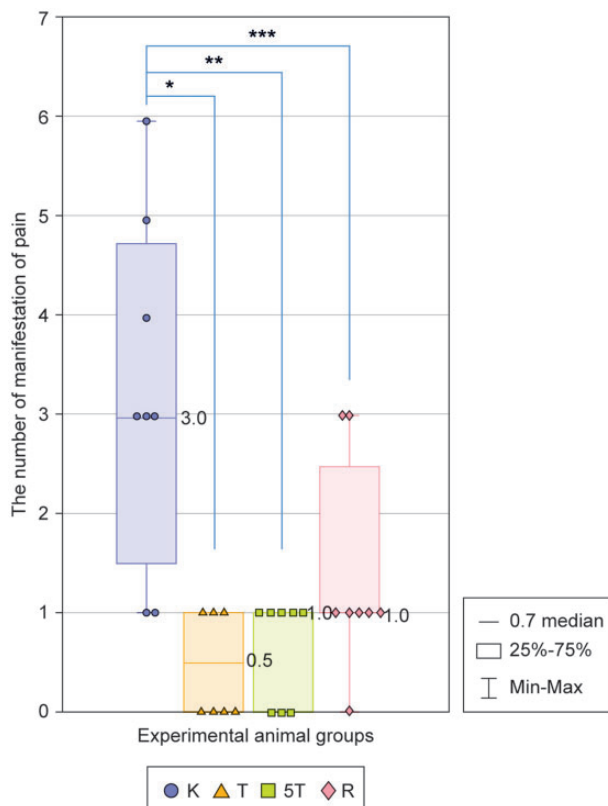


According to other criteria (licking, drawn up paw, duration of the first phase), no significant differences were found. Also, the formalin test did not reveal any significant effects of ketorolac tromethamine (Figure 4).

### Results of the “Haffner Tailset Pinch Test”

The testing for distribution of the sum of points of pain manifestations showed that the main data set is distributed differently from the normal and should be processed using non-parametric methods. The evaluation of differences between the results in various experimental groups by Kruskal–Wallis revealed a significant difference between them. The pairwise comparison of different groups’ results by the Mann–Whitney test revealed a significant difference between the control group and all the others.

Wherein statistical criteria of the differences significance for Mann–Whitney paired (with the control group) comparisons for the results of the “Haffner Tailset Pinch Test” for animals that received the



**Figure 5.** The number of manifestations of pain signs in the “Haffner Tailset Pinch Test” for various experimental animal groups (K—control, T—animals that received the synthetic peptide 2 µg, 5T—animals that received the synthetic peptide 10 µg, R—animals that received ketorolac tromethamine 2.14 mg). \* $P < 0.006$  significantly different from the control. \*\* $P < 0.003$  significantly different from the control. \*\*\* $P < 0.04$  significantly different from the control.

synthetic peptide 2 µg ( $P < 0.006$ ) and animals that received the synthetic peptide 10 µg ( $P < 0.003$ ) was significantly different from the criterion for animals, which were administered a ketorolac tromethamine ( $P < 0.04$ ).

The “Haffner Tailset Pinch Test” results (Figure 5) lead to the conclusion that the synthetic peptide, at doses 1000 times lower than those of the comparison drug, reduces more effectively the pain symptoms of in experimental animals.

### Results of the study of the possible mechanism of analgesic action of the peptides

No peaks were identified on the chromatograms obtained during elution of rat brain preparations, using the empty matrix and the matrix with the cross-linked reverse sequence peptide taken as a control. The protein fraction elution results for the peptide under study are shown in Figure 2. After reaching the maximum sodium chloride concentration, the presence of two peaks which coincides with the change in the nature of the conductivity curve, was detected in the range from 32nd to 34th min.

The samples corresponding to the above two peaks were selected and concentrated. The electrophoresis results for the fraction obtained are shown in Figure 1. The upper major spot corresponding to a mass of ~190 kDa was identified as a clathrin heavy chain by 13 peptide sequences. The second major spot corresponding to a mass of ~55 kDa was identified as different tubulin chains by 22 peptide sequences.

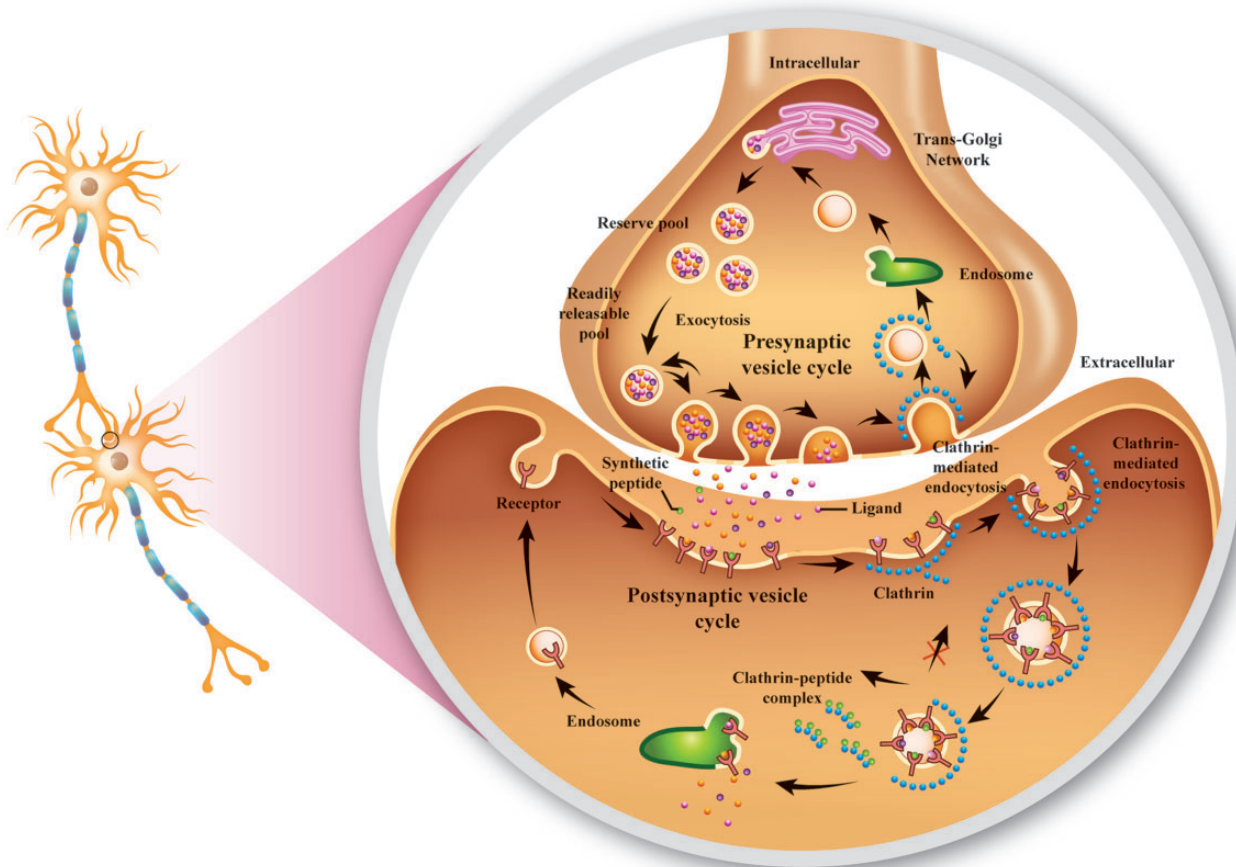
MALDI protein identification data (Mascot score  $> 1700$ ) are given in Table 2.

## Discussion

It is obvious that different mechanisms underlie the anti-inflammatory and analgesic activity of the synthetic peptide and ketorolac tromethamine.

In the experiment, there was a significant decrease in the number and frequency of flinches in the second phase and an increase in the duration of rest between the first and second phases in the formalin test in rats which were administered a synthetic peptide. This analgesic effect is typical for non-opioid analgesics with central activity, including for calcitonin salmon.<sup>39</sup>

The activity mechanism of ketorolac tromethamine is associated with non-selective inhibition of the COX-1 and COX-2 activity which catalyzes the formation of prostaglandins from arachidonic acid, which play an important role in the pathogenesis of pain, inflammation and fever. Ketorolac tromethamine should lower the number of behavioral reactions in the second phase of formalin test,<sup>40</sup> but we did not observe any significant effects compared to the control. Herewith, in the



**Figure 6.** The special role of clathrin-dependent endocytosis (CME) in the presynaptic and postsynaptic vesicular cycles during the process of pain synaptic transmission. Specific binding (inactivation) of the heavy clathrin chain by analgesic peptide entails the loss of activity of the entire clathrin complex. Inhibition of CME by the clathrin blockage leads to irregular recycling of pain receptors in the postsynaptic vesicular cycle, which results in the analgesic effect.

**Table 2.** Proteomic parameters of the MALDI identified proteins (Mascot score > 1700).

No	Protein name	GN, designation	Mascot score	Theoretical MW (kDa)	Matched peptides	Sequence coverage, %
1	Tubulin alpha-1A chain	Tuba1a, TBA1A_RAT	2.043,3	50,800	22	48,1
2	Tubulin alpha-1B chain	Tuba1b, TBA1B_RAT	2.022,8	50,816	22	48,1
3	Tubulin beta-2A chain	Tubb2a, TBB2A_RAT	1.926,3	50,281	33	53,3
4	Tubulin beta-4B chain	Tubb4b, TBB4B_RAT	1.919,3	50,233	35	56,0
5	Clathrin heavy chain	Cltc, CLH1_RAT	1.904,2	193,216	35	24,2
6	Tubulin beta-2B chain	Tubb2b, TBB2B_RAT	1.795,9	50,385	33	53,3
7	Tubulin beta-5 chain	Tubb5, TBB5_RAT	1.794,4	50,100	34	55,2

MALDI: matrix-assisted laser desorption/ionization.

“Haffner Tailset Pinch Test,” the comparison drug reduced effectively the manifestations of pain signs in experimental animals in case of intranasal administration. A significant decrease in the manifestations of pain symptoms in the “Haffner Tailset Pinch Test” in experimental animals which were administered the

synthetic peptide is also an evidence that it is highly effective as an analgesic at doses 1000 times lower than those of the ketorolac tromethamine.

The analgesic effect of the peptide as an analogue of the calcitonin fragment of salmon due to its interaction with the calcitonin receptors localized on the cell surface

membrane<sup>14</sup> and the subsequent endocytosis of the receptor and its ligand into the cell.<sup>41</sup> By a similar mechanism, a fragment of human calcitonin 9–32 enters the cells through the nasal mucosa.<sup>42</sup> The experimental results presented in this article allow to determine intracellular targets with which the low molecular weight synthetic peptide interacts.

The proteins, which were identified after desorption from the sepharose matrix with immobilized peptide and electrophoresis, were the clathrin heavy chain and various tubulin chains. Under the same experimental conditions, these proteins were not adsorbed on free unmodified sepharose matrix and sepharose matrix modified with peptide with the reverse amino acid sequence. This suggests that the sorption of these proteins on analgesic peptides is specific. The presence of tubulin is common when the chosen technique is used.<sup>43</sup>

Therefore, we may uphold with a high degree of probability that the analgesic effect of the peptide under study is associated with a specific binding of a clathrin heavy chain. It is fundamentally important that inactivation of the clathrin heavy chain entails the loss of activity of the entire clathrin complex.<sup>44</sup>

Inhibition of CME by clathrin blockage leads to irregular recycling of pain receptors in the postsynaptic vesicular cycle, which results in the analgesic effect. This process and the special role of CME during the pain synaptic transmission are schematically shown in Figure 6.

Facts about analogous analgesic mechanisms are known. For example one of the mutations in the clathrin heavy chain leads to a rare disease in humans—loss of pain and tactile sensitivity,<sup>45</sup> and the intensity of inflammatory and, as a consequence, pain reactions depends on the clathrin heavy chain.<sup>46,47</sup>

Also important is the fact that CME is inhibited by a full-sized calcitonin molecule in osteoclasts.<sup>48</sup>

Study findings presented in this article indicate that the analgesic effect of the developed synthetic peptides to be associated with the specific binding of the clathrin heavy chain. The inhibition of clathrin-mediated endocytosis of pain receptors in the postsynaptic vesicular cycle may be a more effective means of analgesia than simply the inhibition of activity of these receptors on the plasma membrane and the use of NSAIDs, what in the future will allow to replace opioid and NSAIDs analgesics with a much less toxic low-molecular synthetic peptides with non-narcotic type of analgesia.

#### Author contributions

All the authors are responsible for the data reported and all of them participated in the discussion on the manuscript.

#### Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: The authors of the work are employees of research centers: Science-Research Center «BIOPHARMOS», Russia; PHARMBIOTECH OY, Finland.


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#### Research data

Supplemental material files. Baseline data on experiments and statistical measurements: (1) Proteomic parameters of the MALDI identified proteins. (2) The results of statistical processing of the experiment.

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