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Research Article

Maximum tolerant dose and analgesic activity of PT1 peptide

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Abstract

Introduction: The article presents the results of the study of the maximum tolerant dose (MTD) and the analgesic activity of peptide PT1 isolated from *Alopecosa marikovskyi* spider venom. PT1 is the first compound of polypeptide nature, capable of exerting a selective modulating effect on purinergic P2X3 receptors.

Materials and methods: The study was conducted on 174 ICR mice. The analgesic activity of the peptide was evaluated in a thermal hypersensitivity test triggered by CFA and in a model of chemical irritation.

Results and discussion: The determined MTD for the peptide PT1 when administered intravenously provides evidence to attribute it to low-toxic compounds. The maximum analgesic activity of PT1 using the biomodel of hypersensitivity induced by CFA when tested 15 minutes after the administration was recorded at doses of 0.1 and 0.5 mg/kg. In the visceral pain test, the maximum analgesic activity 15 minutes after the administration of the chemical stimulus was observed at a dose of 0.01 mg/kg.

Conclusions: According to the results of testing peptide PT1, it is shown that it belongs to low-toxic compounds, has a pronounced analgesic activity in a wide range of doses of 0.0001–10 mg/kg.

Keywords

P2X3 receptors, analgesics, pain models.

Introduction

Nowadays pain is one of the main reasons for seeking medical help. The perception of pain is an important protective ability of the body, informing us of the harmful effects that damage tissues and organs or pose a potential danger to the body. Pain is not just a symptom of many acute and chronic inflammatory pathologies, but also a complex psychophysiological phenomenon involving the formation of emotions, motor, humoral and hemodynamic manifestations (Chiao and Boretsky 2017, Grant et al. 2018, Linton and Shaw 2011, Monaghan et al. 2018). Pain response is a systemic response of the body, and this indicates the extreme complexity of its mechanisms. It was established that the pain arising in case of tissue damage has a phase character: first, it is acute and well localized, and then after a few seconds, it is replaced by diffuse, less acute and more emotionally colored. Such dynamics of pain is associated with the participation of various afferent systems in conducting nociceptive impulses (Al Darwish Alopecosa marikovskyi et al. 2016, Dyachenko et al. 2018, Palikova et al. 2018a, Palikova et al. 2018b, Sharkey 2013, Sharma et al. 2015).

Existing painkillers can regain control of pain sensitivity in only ~ 30% of patients with injuries of the nervous system. A significant number of elderly people, patients with oncological diseases complain of daily pain. In addition, chronic pain leads to serious economic losses due to a decrease in the ability to work of a significant part of the population. Patients with chronic pain syndrome often become depressed, restless, and suffer from sleep disorders. The quality of life of such persons is drastically reduced (Avez-Couturier and Wood 2016, Mercadante 2017, Vickers et al. 2017). Based on the above, the search for and study of new drugs with an analgesic activity is a relevant area for scientific research.

It turned out that natural poisons can serve as a source of powerful new analgesics (Lewis and Garcia 2003). In 2012, in a targeted search for natural analgesic compounds in the laboratory of neuroreceptors and neuroregulators of the IBCh RAS from the Central Asian wolf spider, purotoxin PT1, a peptide that inhibits one of the most important subtypes of human pain receptors was isolated and comprehensively characterized. The amino acid sequence PT1 (protein sequence data in the UniProt knowledge base [http://www.uniprot.org] under accession number P86269) was determined using automated Edman degradation and verified by matrix-activated laser desorption mass spectrometry/ionization and specific proteolysis.

Recombinant PT1 was produced by *E. Coli* as a cleaved thioredoxin fusion, and the equivalent of the native peptide was proven by analytical chromatography, MC, and biological testing. It was shown that PT1 in nanomolar concentrations significantly reduces ATP receptor activation, stabilizing the receptor desensitization stage (Grishin et al. 2010).

Materials and methods

The PT1 peptide under study consists of 35 amino acid residues, and can be isolated by chromatographic methods from venom of the spider *Alopecosa marikovskyi*, or obtained by peptide synthesis, as well as by genetic engineering methods. PT1 has the following amino acid sequence:

Gly1-Tyr2-Cys3-Ala4-Glu5-Lys6-Gly7-Ile8-Arg9-Cys10-Asp11-Asp12-Ile13-His14-Cys15-Cys16-Thr17-Gly18-Leu19-Lys20-Cys21-Lys22-Cys23-Asn24-Ala25-Ser26-Gly27-Tyr28-Asn29-Cys30-Val31-Cys32-Arg33-Lys34-Lys35

The recombinant peptide PT1 modulates the activity of the purinergic receptors P2X3 and exhibits an analgesic activity in animal models (Grishin et al. 2010, Kabanova et al. 2012, RF Patent No. 2422459 2011). The recombinant peptide PT1 was provided by the Laboratory of Neuroreceptors and Neuroregulators of the IBCh RAS for *in vivo* preclinical studies.

Maximum tolerant dose study

The study of the maximum tolerant dose (MTD) and the anesthetic activity of the PT1 polypeptide were carried out in compliance with the requirements of the current Guidelines for the preclinical study of new pharmacological substances and according to the Rules of Laboratory Practice in the Russian Federation (National Standard of the Russian Federation, GOST 33647-2015). The procedures with animals were reviewed and approved by the Bioethical Commission of the Institute of Bioorganic Chemistry of RAS (Minutes No. 330/12).

The toxicity class of the test drug is determined in accordance with the GHS (Globally Harmonized System of Classification and Labeling of Chemicals).

To determine the MTD of the PT1 peptide after its single administration to mice, an intravenous route of administration was chosen. The study used female ICR outbred mice. Observation of the animals to detect abnormalities in health and mortality was performed daily in the morning. A detailed clinical examination of each animal was carried out 10–60 minutes after the administration of the substance, then daily in the first 48 hours and weekly thereafter.

Hypersensitivity induced by complete Freund's adjuvant

Thermal hypersensitivity was modeled by intraplantar administration of a mixture of CFA (Sigma-Aldrich, USA) and 0.9% sodium chloride solution (1:1) 24 hours before the injection of the peptide and the comparator was administered, followed by placing the animal on a thermostatted surface (53 °C) until a characteristic response in the form of shaking the hind paw, in which local inflammation was induced. In the control group, animals received a 0.9% sodium chloride solution. Testing on the thermostatted surface was carried out at different time points after the injection of substances (15 min, 1 h, 4 h, 24 h). For the recombinant peptide PT1, an intravenous route of administration was chosen. The selected bio-model of thermal hypersensitivity provoked by CFA is the basic model for evaluating the analgesic activity of substances.

Method to evaluate visceral pain – writhing test

To simulate visceral pain, male mice were intraperitoneally (i.p.) injected with a 0.6% solution of acetic acid after prior administration of the test substances (1 h, 4 h, 24 h beforehand). In the control group, animals received a 0.9% sodium chloride solution. The testing took into account the time of the first specific nociceptive cramp-like response, as well as their number for 15 minutes.

The results of the obtained studies were processed statistically and presented in the form of tables and figures. When performing statistical calculations, the normality of the distribution was preliminarily estimated using Shapiro-Wilks tests. In the case of a normal distribution of quantitative variables, a Duncan's test (univariate analysis of variance: a posteriori multiple comparisons) was used for independent samples to compare the two groups. When comparing several groups with an abnormal distribution of the studied traits, the nonparametric Kruskal-Wallis criterion was used, which is a rank analysis of variations. The difference in the dynamics of treatment was assessed using Student's t-test for related samples. The difference between the experimental groups was considered significant at p≤0.05. All calculations were done on a personal computer using the statistical program STATISTICA 7.0 (StatSoft, USA).

Experiments were partially carried out using the equipment provided by the IBCh core facility, supported by Russian Ministry of Education and Science, grant RFMEFI62117X0018).

Results and discussion

Initially, the study of MTD of the PT1 peptide after intravenous administration was started with a dose of 50 mg/kg, and further the dose was increased, since no death of the experimental animals was observed. Increasing the dose to 300 mg/kg did not cause any manifestation of toxic effects. At a dose of 900 mg/kg in mice, an intense licking of the site of injection was observed, indicating an irritant effect. Subsequently, the dose was increased to 2000 mg/kg, at which the death of all animals was recorded. Reducing the dose to 1800 mg/kg also caused the death of experimental animals. When the dose was reduced to 1400 mg/kg, PT1 caused pronounced toxic effects, while death of one animal (12.5%) out of eight was observed. Increasing the dose to 1600 mg/kg caused the death of two (40%) animals out of five, and pronounced toxic effects were observed after administration. The range of clinical signs of intoxication in determining MTD in experimental animals is given in Table 1.

Table 1. The Range of Clinical Signs of Intoxication in Determining the Maximum Tolerant Dose of Peptide PT1.

Clinical signs	Observation Day	I/v injection of peptid PT1:			
		900 mg/kg (n=3)	1400 mg/kg (n=8)	1600 mg/kg (n=5)	1800 mg/kg (n=3)
Animal death	day 1	0	1	1	0
	day 2	0	0	1	3
	Total:	0	1	2	3
Hunched pose, piloerection	day 1	2	5	3	3
	day 2	1	3	2	0
	Total:	3	8	5	3
Lethargy, decreased motor activity	day 1	1	5	3	3
	day 2	1	3	2	0
	Total:	2	8	5	3
Impaired hearing and pain sensitivity	day 1	2	3	4	3
	day 2	1	0	1	0
	Total:	3	3	5	3
Convulsions, muscle stiffness	day 1	0	5	3	3
	day 2	0	1	2	0
	Total:	0	6	5	3
Heavy intermittent breathing, rapid breathing	day 1	1	1	3	3
	day 2	0	0	2	0
	Total:	1	1	5	3

Note: here and below *n* – the number of animals in the group.

Table 2. Body Weight and Weight Gain in Mice CD-1 after a Single Injection of Peptide PT1.

Body weight of experimental animals (g) and weight gain (%)	i/v injection of peptide PT1:	
	1400 mg/kg	1600 mg/kg
Body weight on the 1st day of administration, g	23.4±0.9 (n=8)	22.3±0.4 (n=5)
Body weight on the 7th day of administration, g	24.3±1.1 (n=7)	23.2±0.2 (n=3)
Weight gain on the 7th day of administration,%	3.9±2.3 (n=7)	14.8±4.0 (n=3)
Body weight on the 14th day of administration, g	25.8±1.1 (n=7)	24.1±0.5 (n=3)
Weight gain on the 14th day of administration,%	12.0±2.8 (n=7)	24.9±4.7 (n=3)

The average body weight and weight gain in experimental animals with intravenous administration of the peptide PT1 at doses of 1400 and 1600 mg/kg are presented in Table 2. With intravenous administration of the peptide PT1, the body weight of animals treated with doses of 1400 and 1600 mg/kg was 23.4±0.9 (n=8) and 22.3±0.4 (n=5), respectively. The dynamics of body weight gain on the 7th and 14th days after administration of the test peptide showed that the animals completely recovered from the toxic effects of large doses of the drug over the study period. No significant differences between the groups of mice that received different doses were identified.

In surviving animals, 14 days after the administration of the peptide PT1, planned necropsy was performed. Necropsy revealed no abnormalities in the internal organs, which indicated low toxicity of the substance. Thus, as a result of the study, MTD of the PT1 peptide was determined for female CD-1 mice after intravenous administration, which was 1400 mg/kg. In accordance with GOST 12.1.007.-76 and the OECD classification of chemical substances, the results of the determination of the MTD of the PT1 peptide upon its intravenous administration give grounds for attributing it to low-toxic compounds.

Subsequently, the PT1 polypeptide was tested in a thermal hypersensitivity test triggered by CFA. PT1 was administered intravenously at doses of 0.001, 0.01, 0.05, 0.1, 0.5, 1, 2 mg/kg to ICR mice 21–24 hours after simulating local inflammation induced by CFA. Testing was carried out 15 minutes after the administration of PT1. The control group of animals received saline (solvent). The results of the study are presented in Figure 1.

At a dose of 0.001 mg/kg (7.4±0.3 s), no significant differences from the control group were observed. In-

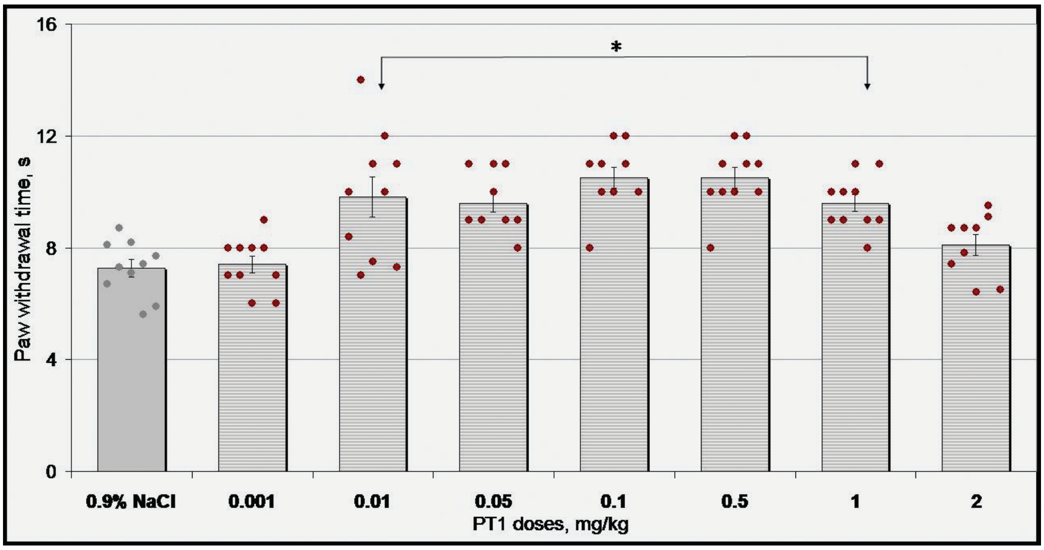

Figure 1. The study of the analgesic activity of the peptide PT1 on the biomodel of thermal hypersensitivity provoked by CFA The data are presented as arithmetic mean ± error of the arithmetic mean, the number of animals in each group is not less than 9, p≤0.05 when compared with the "0.9% NaCl" group; statistical analysis was performed using the ANOVA-2 test (Post-Hoc Duncan-test).

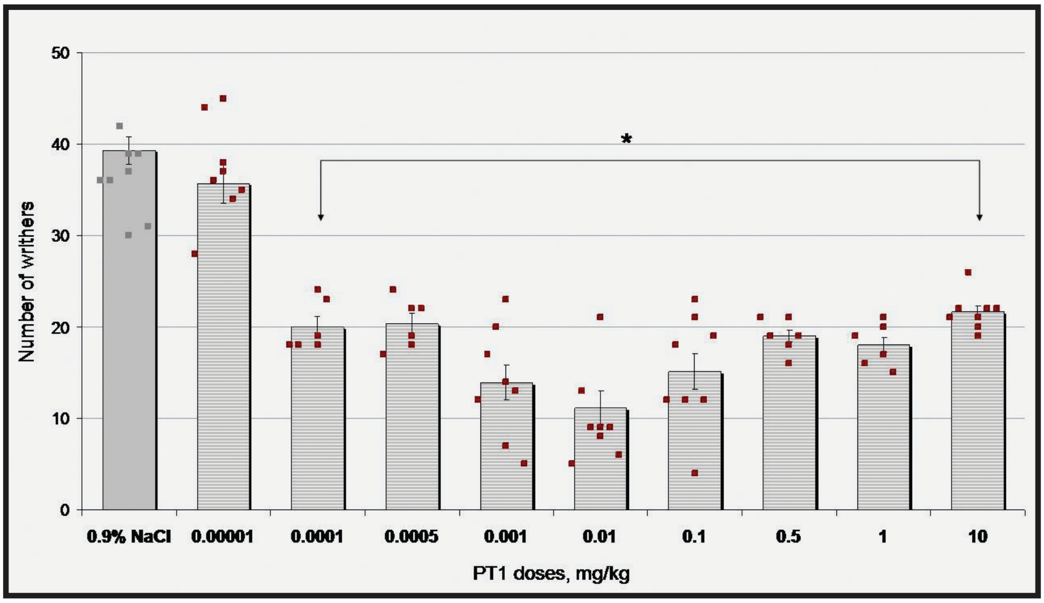

Figure 2. The study of the analgesic activity of the peptide PT1 in the test of visceral pain. The data are presented as the arithmetic mean ± error of the arithmetic mean, the number of animals in the group is not less than 6, p≤0.05 when compared with the "0.9% NaCl" group; statistical analysis was performed using the ANOVA-2 test (Post-Hoc Duncan-test).

travenous administration of PT1 in the dose range from 0.01 mg/kg (9.5±0.8 s) to 1 mg/kg (9.6±0.3 s) showed significant differences in the time that animals spent on a thermostatically controlled plate relative to the control group treated with saline (7.3±0.3 s). The maximum analgesic activity of PT1 using the biomodel of hypersensitivity triggered by CFA in tests 15 minutes after the administration was recorded at doses of 0.1 and 0.5 mg/kg.

In addition, the PT1 peptide was examined in a visceral pain test. The results of the study are presented in Figure 2. The test peptide was administered intravenously at doses of 0.00001, 0.0001, 0.0005, 0.001, 0.01, 0.1, 0.5, 1 and 10 mg/kg to ICR mice; the study was performed 15 minutes after peptide administration. The control group of animals received saline (solvent).

In the visceral pain test, the number of specific nociceptive "writhing" type responses and the time of the first "writhe" after the administration of the stimulus (0.6% acetic acid) were recorded. In the study of the analgesic activity of the PT1 peptide 15 minutes after the administration, no significant differences between the groups in terms of the "time of the first writhe" indicator after the injection of the chemical stimulus were detected relative to the control group treated with saline. Statistical analysis of the parameter "total number of writhes over 15 minutes" revealed a significant difference in all studied doses relative to the control group (39.3±1.5), except for the minimum dose of 0.00001 mg/kg (35.7±2.1). A significant difference in the dose of 0.00001 mg/kg relative to the other studied doses was observed.

The results of testing the peptide PT1 by intravenous administration to ICR mice showed that the maximum analgesic activity 15 minutes after the injection of the chemical stimulus occurs at a dose of 0.01 mg/kg.

Conclusions

The results of the study showed that the PT1 peptide showed pronounced activity in vivo in a wide range of doses (0.0001–10 mg/kg). The peptide significantly reduced the pain response in both the thermal hypersensitivity test triggered by CFA at doses of 0.01–1 mg/kg and in the visceral pain model at doses of 0.0001–10 mg/kg. Thus, according to the results of testing of the peptide PT1, it is shown that it belongs to low-toxic compounds and has a pronounced analgesic activity, which has practical value when creating further effective analgesics of a new generation.

Conflict of interest

The authors declare neither competing financial interests, nor conflict of interests.

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