

**METHOD OF DEVELOPMENT OF AN ANALGESIC PEPTIDE
PREPARATION, FOR THE TREATMENT OF ACUTE AND CHRONIC PAIN.**

The present invention relates to the fields of medicine and veterinary
5 treatments, particularly to drugs that are useful for the treating of acute and
chronic pain.

The present invention based on an analgesic peptide substance with a
structure H-Tyr-D-Arg-Phe-Gly-NH₂ and/or H-Tyr-D-Arg-Phe-Sar-OH. This
pharmaceutical composition can be recommended to treat chronic pain of
10 cancer patients at the late stages of the disease and is effective in treating
acute pain under extreme conditions.

The objective of the present invention was to develop and design a
compound, and a method of preparation, including methods of application of an
active formulation based on the tetrapeptide H-Tyr-D-Arg-Phe-Gly-NH₂ and/or
15 H-Tyr-D-Arg-Phe-Sar-OH, which is considered to be a highly effective pain
killer when it is used for subcutaneous, intravenous, intradermally, intranasal,
skin patches, rectal suppositories or transdermal therapeutic systems to treat
chronic pain in patients with cancer at the late stages.

In summary: Depending on a particular clinical scenario and the
20 physiological characteristics of a patient the 0.5-10 mg tetrapeptide is
recommended to be injected subcutaneously or intradermal. Before application,
it is recommended to dissolve the lyophilized dosage in 1-2 ml of water for
injection and administer parenterally, preferably subcutaneously or
intradermally. The current pharmaceutical composition has high antinociceptive
25 activity, long lasting effects, mild side effects and is non-toxic.

The standard approaches used to prevent acute pain are based on
screening of ligands to opioid receptors (OR) in humans. At present, three major
groups of OR are discussed: μ -, δ - and κ -receptors. It is believed that μ -
receptors are responsible for analgesic effects, while the δ - and κ - have

different physiological effects: analgesic, euphorogenic and dysphorogenic effects, including respiratory depression and so on.

For cases of mild or moderate symptoms of pain of a periodic and chronic nature, drugs of the first and second line are used. These drugs do not belong to opioid analgesics, non-steroidal anti-inflammatory drugs or weak opioids (codeine, dionin, Tramal) group of analgetics. Non-narcotic analgesics have no effect on the respiratory center, do not cause euphoria, physical or mental dependence. However, their analgesic activity appears to be a link with neurologic, muscle, joint pain, headache, and toothache relief. For severe pain associated with injuries, surgical interventions, malignant tumors, etc., they are de facto ineffective. Another undesirable side effect of these drugs is a negative effect on the gastrointestinal tract, hematopoietic system, and excretory system.

Patients with generalized forms of cancer usually suffer from a combination of several types of pain. Pain leads to disability, reduced activity, emotional disorders, regional and systemic disturbance of microcirculation, which is a cause of secondary immune depression and impaired activity of the visceral systems. However, the pain might be also a result of tumor progression or is the result of metastasis dissemination. In other cases the pain can occur due to complications linked with anticancer treatment. Pain and clinical symptoms vary, but have a common property, that they are temporary and progressive. Nearly 3.5 million patients suffer from daily pain of varying intensity. According to statistics, about 40% of patients with intermediate stages of cancer, and 60-87% with late stages of the disease, suffer from pain ranging from mild to strong.

Over time, cancer patients who utilized weak analgesic drugs stop responding to their therapeutic efficacy. In this regard, the use of opioid-based drugs is prescribed. This group of analgesics includes low-molecular narcotic analgesics (morphine and its derivatives) that have a strong analgesic effect, that enables their use in case of injuries and diseases accompanied by severe pain (cancer, heart attack, and others). This group has a big disadvantage - they have an impact on the central nervous system, which manifests itself in a

state of euphoria, and after repeated use, exhibit addictive syndromes, including psychological and physical dependence. It is beyond a question that precisely this property of the drug leads to narcotic dependence. This limits their widespread use, and requires strict regulations due to their potential use in drug trafficking. The other disadvantage is the fairly low therapeutic range of the doses. The risk for adverse effects such as respiratory depression and loss of consciousness, lead to severe consequences, including fatalities.

The search for new analgesics is one of the current challenges of modern pharmacology, since the analgesic drugs that are in use are lacking the required balance for efficacy and safety.

The development of peptide based analgesic drugs is an interesting and challenging problem of modern experimental pharmacology. Being closely related analogs of endogenous peptides, this group of drugs possess high efficiency and selectivity against the determination of analgesic and euphoric effects the features of the chemical structure (very few amino acids) prevents the use of a peptide drug as a «recreational» drug or to modify their chemical structure to induce euphoric effects.

A well known study of various peptide based substances confirms the ability of peptides to produce analgesic effects. Among endogenous analgesic peptides, there are β -endorphins, met-enkephalin, leu-enkephalin, and dynorphin A. However, these natural peptides are not convenient to use as drugs, because of their biochemical characteristics and large molecules.

In the 1980s the natural peptide dermorphin, having a high affinity to opioid receptors was discovered. Analogs of dermorphin peptide and their modifications were examined (Yemelyanov TG Nezavibatko VN, Usenko AB, Andreeva LA). Heptapeptide possessing analgesic effects, combined with thermoregulatory and / or vasomotor activity and / or influence on the behavioral response of the subject, has been approved in Patent No. 2134121, issued August 10, 1999, Russia.

Many of the peptides belonging to dermorphin group of analgesics possess analgesic effects. We have proposed to use the peptides H-Tyr-D-Arg-Phe-Gly-NH₂ and H-Tyr-D-Arg-Phe-Sar-OH to prevent acute and chronic pain in patients with cancer and traumatic pain. These peptides act as agonists of the μ -opioid receptor. Based on these observations the aim of this study was the search of antagonists of the μ -receptor with reduced or with the elimination of euphoric effects, and its influence on the respiratory system.

We used laboratory animals - rats and mice to examine the biological effects, and examined the effects of these compounds on the behavior of analgesic effects and toxicity.

Example 1

Peptides H-Tyr-D-Arg-Phe-Gly-NH₂ and H-Tyr-D-Arg-Phe-Sar-OH were synthesized by the automatic solid-phase method using amino acid activation. Peptides were cleaved from the resin and de-protected with a mixture of TFA:m-cresole. Purification of the peptides was performed by reverse-phase HPLC. Peptides were characterized by mass spectrometry (MALDI). The purity of the product is 98%.

The basis for the effective preparation, in addition to the active substance, is the formulation of the required dosage. Proper selection of the components of the dosage, maintains the biological effect of the active ingredients of the drug for a long period of time. For the dosage compound that provides long-term preservation of the biological properties of the active peptide, we developed the following composition 1ml solution: 0.4 mg of sodium acetate, sodium chloride 1.8mg, 10 mg mannitol, 40 mg polyvinylpyrrolidone 40000, glycine 20 mg, 1.5-3 mg of the tetrapeptide, pH = 4.5-5.5 before lyophilization. That provides long-term preservation of the biological properties of the active peptide. The preparation is poured into vials and lyophilized.

After lyophilization, and capping the vials, we tested residual moisture, which was in the range of 3 – 4.3 % under selected lyophilization conditions.

The composition of the peptide formulation retains its biological activity and stability, when stored at room temperature for a year or more. We suggest storing the drug in a dark place at a temperature between 6-10°C.

Example 2

5 Peripheral opioid activity of the peptide H-Tyr-D-Arg-Phe-Gly-NH₂ (4Xm) was evaluated based on its' ability to inhibit the contraction of isolated organs - guinea pig ileum (GPI) caused by electrical stimulation. The tests were carried out on a special apparatus for opioid activity testing, using guinea pig ileum (GPI) assay. GPI, taken from conventional animals were placed in a container
10 of 10 ml with electrodes inserted into a Krebs solution at 34°C in a thermostatic water bath (HOECHST Organbad, Germany), including a thermostat for maintaining a constant temperature in the working area. The lower end of GPI segment was rigidly attached to the vessel bottom. The upper end of the segment was attached to a K-30 isometric transducer ("Hugo Sachs Elektronik
15 KG", Germany) connected via dual-channel amplifiers to recorder MS 6601 ("Watanabe", Japan). The initial tension was 1g. The buffer solution with GPI segments was constantly aerated. GPI segments were equilibrated in Krebs solution for one hour.

The preparations were stimulated transmurally by a series of four
20 rectangular pulses, each lasting 0.5 ms with 1.5 ms interpulse interval and 7.5s intervals between series at 80V voltage using an electric impulse stimulator (type 215/I, "Hugo Sachs Elektronik KG", Germany). The pulse was registered under the conditions of isometric conditions via dual channel enhancers to an MC 6601 reader ("Watanabe", Japan).

25 At the beginning stimulation was applied for a minimum of 45 min, that allowed us to establish reproducible responses.

The test substance was dissolved in distilled water and was administered to the solution in containers with GPI segments in a volume of 5 to 15 µl, at final concentrations of 1×10^{-9} - 1×10^{-6} M. Each subsequent administration of the
30 substances was made 1 minute after the previous dose, without prior cleaning

of the chamber, on a cumulative basis. Based on the data obtained, the dose-response curve was determined for morphine and the test substance. The potency of the opioid receptor agonists in the absence and presence of the antagonist was assessed as the negative logarithm of the concentration required to cause 50% of the maximum response (pD2) using the logistic equation described by DeLean et al. (1978).

The characteristics of the opioid peptide activity were evaluated in comparison with a standard solution of morphine. During the experiment the amplitude contractions were fixed. We used parallel monitoring in 4 chambers with GPI segments for each substance.

The inhibitory effect of morphine and substance 4Xm caused by electrical stimulation were prevented by addition of the opioid receptor antagonist naloxone washing solution at a concentration of 10^{-6} M.

The EC50 for morphine was 3.26×10^{-7} M, and pD2 – 6.48. For 4Xm substance EC50 was 2.37×10^{-7} M, a pD2 – 6.62. Thus, the activity of the 4Xm peptide was higher than morphine when the drug was applied to the opioid μ -receptors located on GPI.

Example 3

A comparative study of the analgesic activity of 4Xm peptide versus morphine solution in equal molar concentrations was performed using in-vivo methods "Tail Flick" and "Hot Plate" on a rat.

We have examined two doses of 4Xm - 0.1 and 5 mg/kg. As reference substances morphine was used at doses of 5 and 15 mg/kg.

The experimental and control groups of rats were taken from the same batch, nearly the same weight of about 160g. 6 animals were used per dose.

The 4Xm substance and morphine were injected intraperitoneally in equal volume to laboratory animals of experimental and control groups, respectively. Injection of the substances was performed in a solution of water

for injection of total amount less than 0.5 ml. Measurements were started 15 minutes after injection.

Both "Tail Flick" and "Hot Plate" tests were carried out in duplicates, one after the other. Each animal was tested prior to the substances injection, with a
5 delay of 15 min after administration. The substances were administered intraperitoneally in 0.5 mL saline separately on groups "morphine", "peptide".

The "Tail Flick" test is based on the spinal reflex withdrawal of the rats' tail during high temperature stimulation. This reflexive response is an indicator of pain sensitivity

10 The tail-flick latency was assessed by applying radiant heat on a selected spot on the tail using a tail-flick analgesymeter. The device has a lock with a groove for the animal's tail. Above the groove there is a bulb with a focusing lens. To perform a measurement, the rat had to remain calm, without unconditional unexpected movements of the tail. The lamp is switched on by
15 pressing a button and turns off when withdrawal of the tail takes place. The device measures the time when the button is pressed, and fixes it on the display and prints on a paper tape. The administration of substances with the analgesic effect increases the time of withdrawal of the tail. Since animals differ in individual pain sensitivity, the animals were each used in two measurements -
20 before and after injection. A maximum tail-flick latency of 10 sec. was used to minimize tissue damage to the tail. The experiment was conducted on male rats, weighing 160-180g- 6 animals for each dose.

"Hot Plate" test is based on the reflex of the animal licking its' feet after contact with a hot surface. The device is a metal surface with a diameter of 30
25 cm whose temperature is regulated by software. The site is surrounded by a clear fence, with a height of 40 cm. The temperature of the metal pad was set at exactly 52°C. The experiments were started when the rat was placed in the area and the licking of a hind paw was considered as the end point. At this moment the latency to respond was recorded and the rat was immediately

removed. Maximal latency accepted was 45 sec. The administration of substances with the analgesic effect increases the time before feet licking.

Our studies have shown the pronounced analgesic activity of the 4Xm substance compared with control animals which received a placebo or reference drug -, morphine. Effect of full analgesia was observed with a dose of an analgesic peptide in the amount of 5 mg/kg. The same effect was reached when we used 0.1 mg/kg 4Xm substance in comparison to 5 mg/kg of morphine. The data obtained confirm significantly higher activity of 4Xm nearly 10-20 times greater.

10 **Example 4**

The examination of the effects of substances on spontaneous locomotor activity and exploratory behavior by the open field test, and test on the ability to balance on a horizontal bar.

The aim of the experiment was to identify an effective dose of a substance that might affect the behavior of the animals. We have studied different high doses of substances.

Evaluation of doses and the methods of administration

1- Control animals, without injection

2 - Injection of 300 ml of a physiological solution (stress control)

20 3 - 4Xm 0.5 mg/kg (EC50 on the analgesic effect)

4 - 4Xm 1.5 mg/kg

5- 4Xm 5 mg/kg

6.- 4Xm 10 mg/kg (20 EC50 dose on the analgesic effect)

7.- 4Xm 20 mg/kg (40 EC50 dose on the analgesic effect)

25 8.- 4Xm 100 mg/kg (200 EC50 dose on the analgesic effect)

9.- Morphine 120 mg/kg (20 EC50 dose on the analgesic effect)

Experimental and control groups of male mice used were of the same weight and from the same batch (22-24 g).

Substances were injected intraperitoneally in equal volume into laboratory animals of experimental and control groups, respectively. The
5 substance was administrated in a water solution, so that the total volume of solution injected was less than 0.5 ml. Measurement was started 15 minutes after an injection.

Placebo group was tested 15 minutes after an injection into the abdominal wall without the introduction of substances, the administration of
10 physiological saline, morphine or investigated peptide, without any impact on following procedure:

Open field Test

The mice were placed individually in the center of an open field arena, with a floor divided into 16 equal squares with sides of 10 cm. The motor activity
15 of the mice was recorded by a video camera. Locomotion (number of floor units entered and time of locomotion) and number of times the animals stood on their hind legs were measured manually. The movement of the mouse in the center of the arena and in the periphery were recorded separately. Then the number of times the animal stood on its hind legs, "stand" and looking into a hole in the
20 floor "burrow" were recorded. Monitoring is conducted visually while entering data into a computer via a keyboard (semi-automatic registration). The effects of drugs were examined separately in three equal (100-sec.) periods for a total 5-min. observation interval. The effect of each drug was examined by comparison with saline control by calculating percentage inhibition value for
25 each activity measured in the open field test.

Balancing on a horizontal rod test

Animals were placed on a wooden rod with a diameter of 2 cm and 40 cm long, at a height of not less than 60 cm. Then we recorded the times of equilibrium maintenance on the rod.

Our studies have shown that the large doses of 4Xm does not lead to a significant loss of orientation of the animals or reduce their behavioral activity. Even at a dose of 100 mg/kg of 4Xm peptide, the mouse continued to actively move around the field, although with slightly reduced activity (by 20-25% at a dose of 20-100mg/kg). There was observed a 20-30% reduction in exploratory activity (a measure of "stand-burrow").

Our experiment did not attain a dosage level that caused toxic effects. Even at a dose of 100 mg/kg 4Xm, animals continued to move actively, breathing was not inhibited, and heart beats were stable.

It should be noted that the administration of the control substance morphine (20 EC50 doses), physical activity becomes significantly reduced by 50-70%.

We did not observe significant differences between the experimental and control groups in the test on maintaining balance on a horizontal rod. The experimental animals were well balanced on the shaft. Some difference in the experimental group from the control animals should be noted. Reduced activity in horizontal movement on the shaft (10-20%) was observed under conditions of high doses 20-100mg/kg of the 4Xm peptide.

We concluded that the substance 4Xm at the proposed dosage does not significantly affect behavioral characteristics of the animal. The use of the substance at doses of up to 200 EC50 does not result in toxic effects.

Our observations demonstrate that the tetrapeptide 4Xm possess significantly superior analgesic effects, compared to morphine. At the same time, unlike morphine 4Xm peptide does not cause toxic effects or psychological and behavioral disorders.

The result of our experiments is that we have produced a stable peptide (4Xm) that has an analgesic effect that is 10-times higher than that of morphine. An important consequence of our findings is that the 4Xm peptide could be used in a wider therapeutic range or "safety margin" than other opioids, and

which exceeds 200 therapeutic doses. This means that the drug does not represent a severe danger of an overdose, and can be used in a wide variety of applications, depending on the severity of pain.

5 Using the method of extrapolation, it was determined that the recommended dose for humans will range 0.5-10 mg/dose, depending on the nature of pain.

CLAIMS

1. A biologically active pharmaceutical formulation with analgesic peptide which can be used for treating acute pain in emergency medicine and the treatment of chronic pain, including the late stages of cancer.
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2. The formulation as in claim 1 wherein the analgesic peptide has the amino acid sequence H-Tyr-D-Arg-Phe-Gly-NH₂.
- 10 3. The formulation as in claim 1 wherein the analgesic peptide has the amino acid sequence H-Tyr-D-Arg-Phe-Sar-OH.
- 15 4. The formulation as in claim 1 wherein the injectable dosage form has the following composition: 0.2-0.8 mg sodium acetate, 1.8mg sodium chloride, 5-15 mg mannitol, 20-60 mg polyvinylpyrrolidone 40000 or equivalent, 10-40 mg Glycine, 0.5-10 mg tetrapeptide, pH 4.5-5.5 and is poured into vials.
- 20 5. The formulation as in claim 4 wherein the composition is poured into vials and lyophilized.
- 25 6. The formulation as in claim 1 wherein the dosage may have any form suitable for enteral administration: nasal spray, nasal drops, sublingual or buccal tablets, rectal suppositories and transdermal transport systems.