Research Results in Pharmacology

Research Results in Pharmacology 10(1): 23–43
UDC: 615.015.11
DOI 10.18413/rrpharmacology.10.443

Research Article

# Study of the anti-inflammatory, analgesic, ulcerogenic and anti-ulcerogenic activity of N-isopropenylimidazole zinc complex derivative

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**Academic editor:** Oleg Guderev ♦ **Received** 07 December 2023 ♦ **Accepted** 23 January 2024 ♦ **Published** 13 February 2024

**Citation:** Galenko-Yaroshevsky PA, Shelemekh OV, Popkov VL, Zadorozhniy AV, Nektarevskaya IB, Bunyatyan ND, Murashko RA, Lebedeva SA, Zelenskaya AV, Uvarov AV, Gulevskaya ON, Alukhanyan LO, Glechyan TR, Sergeeva AV, Kornetskaya AV, Korovaykin NE (2024) Study of the anti-inflammatory, analgesic, ulcerogenic and anti-ulcerogenic activity of N-isopropenylimidazole zinc complex derivative. Research Results in Pharmacology 10(1): 23–43. https://doi.org/10.18413/rrpharmacology.10.443

## Abstract

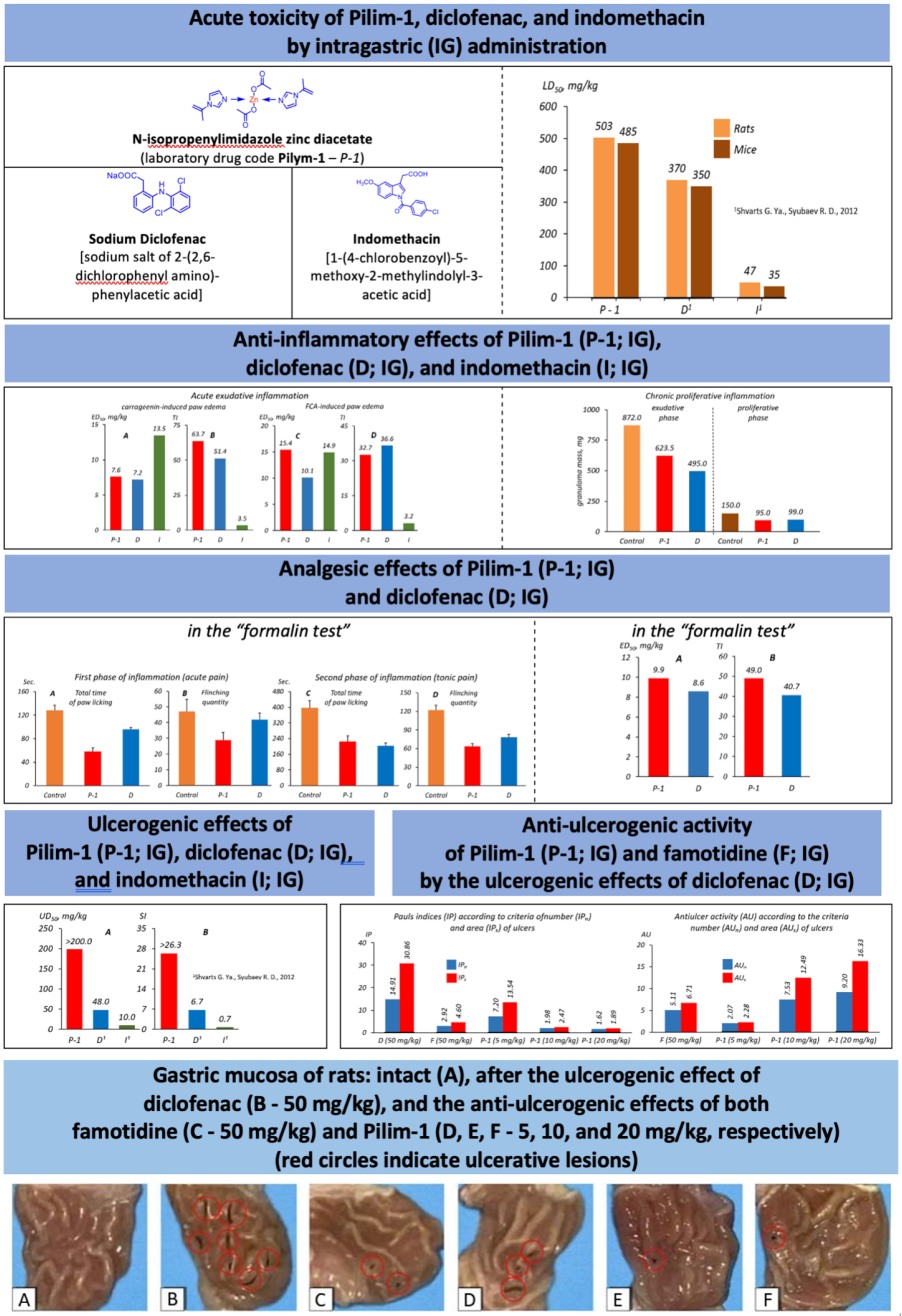
**Introduction**: Although nonsteroidal anti-inflammatory drugs (NSAIDs) have a clear therapeutic benefit, they can also have serious side effects. The most serious of these is the ulcerogenic effect, which can result in a decline in the quality of life or even death. This calls for the search for and creation of novel compounds with potent anti-inflammatory properties that do not have adverse effects on the digestive system. **The aim of the study was** to investigate the anti-inflammatory, analgesic, ulcerogenic and anti-ulcerogenic activities of N-isopropenylimidazole zinc complex derivative.

**Materials and methods:** Experiments in rats and mice were used to determine the median lethal dose $LD_{50}$ for N-isopropenylimidazole zinc complex derivative (laboratory name Pilim-1). Experimental models of acute exudative inflammation and chronic proliferative inflammation were used. In the first case, the inflammation was induced by sub-plantar injection of carrageenan and a complete Freund's adjuvant into the hind paw of rats. In the second case, the inflammation was induced by the implantation of a sterile cotton pellet ("cotton pellet–induced granuloma") under the skin of rats. Acute and tonic pain, visceral, and somatic deep pain were simulated using the formalin test in rats and the acetic acid-induced writhing test in mice, respectively. The ulcerogenic and anti-ulcerogenic effects of Pilim-1 were studied in rat experiments. Pilim-1 and the reference drugs diclofenac, indomethacin and famotidine were administered intragastrically.

**Results and Discussion:** Pilim-1 showed marked anti-inflammatory and analgesic effects, demonstrated less toxicity than diclofenac and indomethacin, while its activity is comparable to diclofenac and superior to indomethacin. Its therapeutic index is higher than that of indomethacin and diclofenac and, in contrast, it essentially has no ulcerogenic effect and exhibits stronger anti-ulcerogenic activity than famotidine. It appears that the anti-inflammatory, analgesic, and gastroprotective properties of Pilim-1 are largely due to its antioxidant and antihypoxic properties, its inhibitory effects on cyclooxygenase and 5-lipoxygenase, and the presence of imidazole and zinc in its structure, both of which have a wide range of biological activity.

**Conclusion:** Pilim-1 can be recommended for further preclinical studies due to its relatively low (practically absent) ulcerative activity, strong anti-inflammatory, analgesic, and anti-ulcerative effects, greater therapeutic index, and less acute toxicity in comparison with diclofenac and indomethacin.

# Graphical Abstract




## Keywords

analgesic activity, anti-ulcerogenic activity, diclofenac, indomethacin, N-isopropenylimidazole zinc complex derivative, acute toxicity, anti-inflammatory activity, ulcerogenic effect

# Introduction

Inflammation is a typical pathological process characterized by two cardinal signs: external (swelling, redness, fever, pain, and dysfunction) and internal (alteration, microcirculation disorder – exudation+migration, and proliferation).

Currently, inflammation is considered as a complex of dynamic reactions to tissue damage induced by exogenous factors (mechanical – wounds, fractures, bruises, bedsores; physical – exposure to high and low temperatures, ultraviolet radiation, electric current, radiation; chemical – organic and inorganic substances; biological – viruses, bacteria, fungi, insects, mites) and endogenous factors (blood clots, tissue necrosis, vascular calcification, stone formation, hemorrhage, formation of cytotoxic immune complexes) (Serebrennikova et al. 2023; Stone et al. 2022). The presence of an inflammatory process requires complex treatment, and pharmacotherapy plays a significant role in this.

Non-steroidal anti-inflammatory drugs (NSAIDs) are the drugs of choice in the treatment of inflammatory diseases. Although non-steroidal anti-inflammatory drugs (NSAIDs) have a clear therapeutic benefit, they can also have serious side effects. The most serious of these is the ulcerogenic effect, which can result in a decline in the quality of life or even death (Welts et al. 2018; Karateev et al. 2018; Khoroshun and Lazareva 2022; Kamchatnov et al. 2023; Minhas et al. 2023). This calls for the search for and development of novel compounds with potent anti-inflammatory properties that do not have adverse effects on the digestive system, in order to create new drugs with an appropriate mechanism of action. Numerous studies have demonstrated that the medications atmizole, hesperidin, hypoxene, petaprot, acizole and mexidol, combining antihypoxant and antioxidant properties, have anti-inflammatory effects and are also able to greatly increase the therapeutic efficacy of NSAIDs (Bobr et al. 2010; Novikov et al. 2010; Turgeneva et al. 2011; Babaniyazova et al. 2013; Novikov and Ilyukhin 2013; Pozhilova 2014; Maskurova et al. 2018; Ivanova et al. 2021).

In the above-mentioned aspect, the compound N-isopropenylimidazole zinc complex derivative (laboratory name Pilim-1) caught our attention because, in an experimental setting, it has antioxidant and antihypoxic effects (Shakhmardanova and Galenko-Yaroshevsky 2015; Lebedeva et al. 2023), can significantly speed up the healing of incised and planar wounds, has an antimicrobial effect (Lebedeva et al. 2021, 2022, 2022a; Lebedeva et al. 2023, 2023a), and exhibits anti-inflammatory and regenerative activity in gum tissues in endodontic-periodontal lesions (Galenko-Yaroshevsky et al. 2023).

# Materials and Methods

### Experimental compounds

The chemical substance N-isopropenylimidazole zinc complex derivative with the laboratory name Pilim-1 (A.E. Favorsky Irkutsk Institute of Chemistry, Siberian Branch of the Russian Academy of Sciences), diclofenac, the active ingredient of which is diclofenac sodium [gastro-resistant tablets, 50 mg and a solution for intramuscular injection, 25 mg/mL, 3 mL (Ozon Pharmaceutical Company, Russia)], indomethacin [gastro-resistant tablets, 25 mg (Ozon Pharmaceutical Company, Russia)], famotidine [lyophilizate for solution for intravenous injections, 20 mg (FARMAKLAB LLC, Russia)].

### Animals

The experiments were performed in 100 white outbred male mice and 448 male Wistar rats weighing 18-32 and 210-320 g, respectively, which were kept in standard vivarium conditions, got a standard food ration according to the rules of good laboratory practice (GOST 33216-2014). The research was conducted in accordance with the rules of *The European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes* (ed. Strasbourg, 2006), the Law of the Russian Federation "On the Animal Protection from Animal Cruelty" dated 06/24/1998, Good laboratory practice in preclinical studies in the Russian Federation (GOST 3 51000.3-96 and GOST R 53434-2009), the provisions of the World Medical Association Declaration of Helsinki (Report of the AVMA Panel on Euthanasia JAVMA, 2001), Guidelines for Maintenance and Care of Laboratory Animals (interstate standard GOST 33216-2014 dated July 1, 2016), Guidelines for Preclinical Trials of Medicines (Mironov 2012), and Guidelines for handling laboratory (experimental) animals when conducting preclinical (non-clinical) studies (recommendations of the The Eurasian Economic Commission's Board No.33 dated Novermber 14, 2023, Moscow). The experiments were approved by the Ethics Committee of Rostov State Medical University of the Ministry of Health of the Russian Federation (Minutes No. 16/19 of September 17, 2019).

### The experimental design

The following animal groups were established to conduct targeted studies of Pilim-1 and the reference medications indomethacin and diclofenac:

- acute toxicity (determination of the median lethal dose $LD_{50}$). Pilim-1 was administered intragastrically (IG) in a wide range of doses in rats (300, 400, 500, 600 and 700 mg/kg) and in mice (300, 400, 500 and 600 mg/kg), 5 and 4groups respectively of 5 animals each;
- acute exudative inflammation: the model of carrageenan-induced paw edema included 16 groups of 10 rats each. The first group was control-1 (sub-plantar injection (SP) of physiological solution), the 2nd group was control-2 (carrageenan SP), the 3rd–5th groups were administered with Pilim-1 (5, 7.5 and 10 mg/kg intragastrically (IG), respectively), the 6th–8th groups were administered with diclofenac (5, 7.5 and 10 mg/kg IG, respectively), the 9thand 10th groups were control–3 (physiological solution SP) and control-4 (carrageenan SP), the 11th–14th groups were administered with indomethacin (5, 10, 15 and 20 mg/kg IG, respectively), the 15th and 16th groups were administered with Pilim-1+diclofenac (5 mg/kg IG) and Pilim-1+indomethacin (5 mg/kg IG), respectively. The model of CFA-induced paw edema included 12 groups of 10 rats each. The first group was control–1 (physiological solution SP), the 2nd group was control-2 (complete Freund's adjuvant (CFA) SP), the 3rd–6th groups were administered with Pilim-1 (5, 10, 15 and 20 mg/kg IG, respectively), the 7th–9th groups were administered

diclofenac (5, 10 and 15 mg/kg IG, respectively), the 10th–12th groups were administered with indomethacin (5, 10 and 20 mg/kg IG, respectively);

- chronic proliferative inflammation in the cotton pellet–induced granuloma. This model included 3 groups of 10 rats each. The first group is the control (subcutaneous implantation of a cotton pellet), the 2ndand 3rd groups were administered with Pilim-1 and diclofenac (10 mg/kg IG), respectively;

- analgesic activity. The "formalin test" included 3 groups of 10 rats each. First group was the control (formalin SP), the 2nd and 3rd groups were administered with Pilim-1 (8 mg/kg IG) and diclofenac (7 mg/kg IG), respectively. The acetic acid-induced writhing test included 8 groups of 10 mice each. The first group was the control (intraperitoneal injection (IP) of acetic acid), the 2nd-4th groups were administered with Pilim-1 (5, 10 and 15 mg/kg IG, respectively), the 5th–8th groups were administered with diclofenac (2.5, 5, 10 and 15 mg/kg IG, respectively);

- ulcerogenic effect was studied in rats, which were divided into 2 groups of 5 and 10 rats, respectively. The first group was the control (distilled water IG), the 2nd group was administered with Pilim-1 (200 mg/kg IG);

- anti-ulcerogenic effect was studied in rats, which were divided into 6 groups of 6, 22, 10, 10, 10 and 10 rats, respectively. The first group included intact animals, the 2nd group was the control (diclofenac 50 mg/kg IG), the 3rd group was administered with famotidine (50 mg/kg IG), the 4th–6th groups were administered with Pilim–1 (5, 10 and 20 mg/ kg IG, respectively) (Fig. 1).

**Experimental models of the research**

***Determination of the median lethal dose LD50 of Pilim-1*** was carried out in experiments on mice and rats with intragastric administration. Pilim-1 was dissolved in distilled water and given via a metal stomach tube according to the method of Litchfield and Wilcoxon (1949). The animals were kept watch over for 14 days.

***The effect of Pilim-1 on acute exudative inflammation*** was studied in experiments on rats using the phlogogens carrageenan (i-carrageenan, TYPE.V, Sigma, USA) and the Complete Freund's Adjuvant - CFA (InvivoGen, France).

Carrageenan-induced paw edema was simulated by the method described by Schwartz and Syubaev (2012), by sub-plantar injection of 0.1 mL of 1% carrageenan solution into the right paw of animals. The volume measurement of the right (experiment) and left (control-1 and control-2) paws was carried out 3 hours after phlogogen injection using a plethysmometer IITC Life Science (USA). An increase in paw volume and inflammation inhibition were the criteria used for the evaluation of the anti-inflammatory effect of Pilim-1 in comparison to the reference medications diclofenac and indomethacin. An increase in the paw volume was calculated using the formula (1):

$$E = \frac{V_1 - V_0}{V_0} \times 100\,\% \qquad (1),$$

where $E$ is increase in edema; $V_1$ is a paw volume after phlogogen injection; $V_0$ is a paw volume before phlogogen injection.

Inflammation inhibition was calculated using the formula (2):

$$100\% - [(V_1 - V_0 / V_0(e) \ : \ V_1 - V_0 / V_0(c)] \times 100\,\% \qquad (2),$$

where e is treated animals (experimental); c is non-treated animals (control-2).

Twelve hours after food deprivation, a single dose of Pilim-1 (5, 7.5, and 10 mg/kg), diclofenac (5, 7.5, and 10 mg/kg), and indomethacin (5, 10, 15, and 20 mg/kg) in tablets were given to the animals via a metal stomach tube one hour prior to the induction of edema with carrageenan. The tablets were crushed to powder and dissolved in 1% potato starch gel (Kirichenko 2020). Control animals were administered with the same volume of 1% potato starch gel.

The median effective dose ($ED_{50}$) that causes 50% inhibition of inflammation and the therapeutic index (TI), or $LD_{50}/ED_{50}$, were calculated for each drug in order to compare the efficacy of Pilim-1 with the reference drugs.

To identify the possible enhancement of the anti-inflammatory effect of diclofenac by Pilim-1, both substances were used in minimal effective doses (5 mg/kg) and given concurrently in 1% potato starch gel via a metal stomach tube 1 hour before carrageenan-induced inflammation.

CFA-induced paw edema was simulated according to the method described by Schwartz and Syubaev (2012). CFA in a volume of 0.1 mL was injected into the right hind paw. The volume measurement of the right (experiment) and left (control-1 and control-2) paws was performed 3 days after CFA injection using a plethysmometer IITC Life Science (USA), which allows us to estimate the primary reaction at the site of CFA injection (Gromyko and Gritsuk 2012; Schwartz and Syubaev 2012). The evaluation criteria of the anti-inflammatory effect were an increase in paw volume and inflammation inhibition, which were calculated using the above formulas (1 and 2).

A single dose of Pilim-1 (5, 7.5, and 10 mg/kg), tablets of diclofenac (5, 7.5, and 10 mg/kg), and indomethacin (5, 10, 15, and 20 mg/kg) were given via a metal stomach tube one hour prior to the CFA administration. The tablets were crushed to powder and dissolved in 1% potato starch gel. Control animals were administered with the same volume of 1% potato starch gel. The median effective dose ($ED_{50}$) and the therapeutic index (TI) were calculated for each drug in order to compare the efficacy of Pilim-1 with the reference drugs.

***Chronic proliferative inflammation*** was simulated according to the method described by Schwartz and Syubaev (2012), Khnychenko and Okunevich (2015). A sterilized cotton pellet weighing 40 mg was aseptically implanted subcutaneously into the back of rats (under light ether anesthesia).

Pilim-1 and diclofenac tablets, crushed to powder, were given intragastrically in 1% potato starch gel at a dose of 10 mg/kg one hour prior to the implantation of a cotton pellet and for the following eleven days. The implanted cotton pellet with the granuloma that had developed around it was removed on the 12th day of the experiment, and it was then promptly weighed. Then the cotton pellet was dried at 60ºC in a dry-air thermostat TS-1/80 SPU model 1001 (Smolensk Special Design and Technology Bureau of Programmed Control Systems, Russia), at the end of the day, it was also weighed. The proliferative process was evaluated by comparing the dried cotton pellet to its initial mass, and the exudative process was evaluated by comparing the mass of the raw and dried cotton pellet.

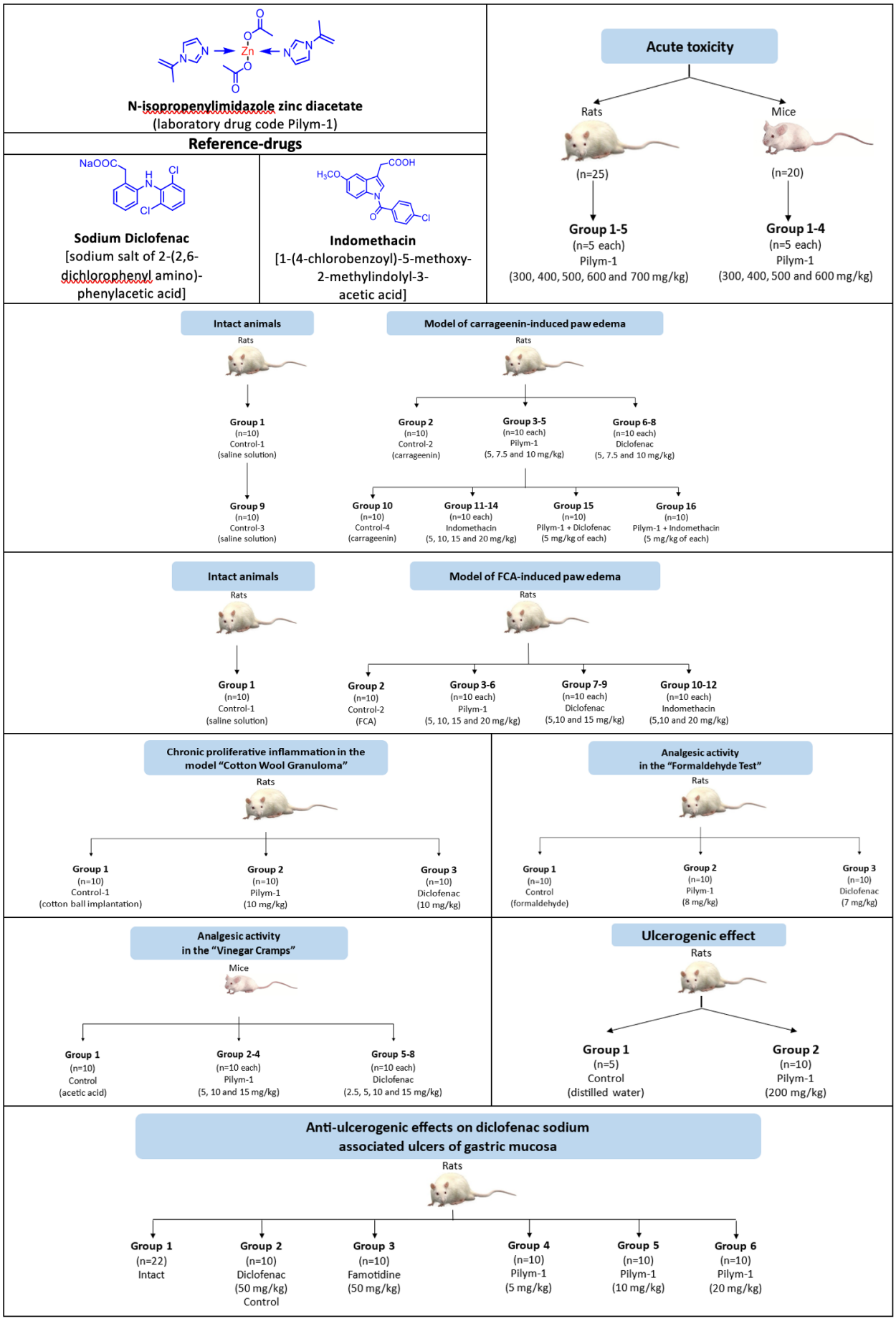



**Figure 1.** Block diagram of the research design.

***The analgesic effect of Pilim-1 in the formalin test and the acetic acid-induced writhing test***

***The formalin test*** was simulated according to the method described by Bondarenko et al. (2011), Voronina and Guzevatykh (2012). The rats were injected with 50 µL of 2% formalin solution subplantarly into the right hind paw (GOST 1625-86 FM, Russia). The formalin test is divided into two phases: acute pain, which lasts for five minutes, and tonic pain, which lasts for an hour or longer.

Rats were given Pilim-1 and the reference medication, diclofenac tablets (crushed into powder), in 1% potato starch gel via a metal stomach tube at doses of 8 and 7 mg/kg, respectively 60 minutes prior to phlogogen injection. For these animals, these doses are close to $ED_{50}$ in exudative inflammation induced by carrageenan. The analgesic effect was assessed by the total time of an animal licking the paw with the injected formalin and the total number of flinches for 60 minutes. In addition, the analgesia index was calculated according to the formula (3) described by Kuzmin and Zvartau (1998):

$$A = \frac{B_c - B_e}{B_c} \times 100\,\% \qquad (3),$$

where *A* is an analgesia index; *Bc* is the number of behavioral reactions in the control group; *Be* is the number of behavioral reactions in the experimental group.

***The acetic acid-induced writing test*** was simulated according to the method described by Schwartz and Syubaev (2012) by intraperitoneal injection of a 0.75% solution of acetic acid at the rate of 0.1 mL per 100 g of animal body weight. For fifteen minutes, a painful reaction was registered in the form of ”writhes”, which were characterized by the stretching of hind paws, contraction and relaxation of the abdominal wall muscles and bowing of the back. Sixty minutes prior to the acetic acid-induced writing test, the animals were given Pilim-1 (5, 10 and 15 mg/kg) and powder-crushed diclofenac tablets (2.5, 5, 10 and 15 mg/kg), which were taken as a reference medication, in 1% potato starch gel via a metal stomach tube. Control animals were given 1% potato starch gel in the equivalent to the studied substances volume. The analgesic effect of Pilim-1 was estimated by reducing the number of writhes compared to those in the control group. As an extra criterion for assessing the potency of the analgesic effect, the latent time of the writhe onset was noted (in seconds).

The analgesic effect, or inhibition of pain reaction (IPR, %) was estimated by reducing the number of writhes compared with the control and calculated using the formula (4):

$$IPR = \frac{W_c - W_e}{W_c} \times 100\,\% \qquad (4),$$

where *Wc* is the number of writhes in the control group, *We* is the number of writhes in the experimental group.

The median effective dose ($ED_{50}$) that causes 50% inhibition of the pain response and the therapeutic index (TI) were calculated to compare the analgesic activity of Pilami-1 and diclofenac.

***The possible ulcerogenic effect*** of Pilim-1 was studied in rats using the method described by Schwartz and Syubaev (2012). Single dose of Pilim-1 was administered intragastrically to the animals deprived of food 24 hours before the study. Three hours after the rats were given a 200 mg/kg aqueous solution of Pilim-1, they were sacrificed with an overdose of chloroform anesthesia. The stomach was dissected, cut along a lesser curvature and rinsed with saline solution to remove the contents.

The ulcerogenic effect was evaluated using a four-grade scale: 0 – absence of damage; 0.5 – hyperemia; 1 – single insignificant lesion (1 or 2 petechial hemorrhages); 2 – multiple lesions (erosion, petechial hemorrhages); 3 – significant and multiple lesions (erosion, hemorrhages); 4 – gross lesions covering the entire surface of the mucous membrane (massive hemorrhages, erosions, perforations), followed by calculation of the median ulcerogenic dose ($UD_{50}$) of the test substance causing grade 2 damage to the gastric mucosa, and the safety margin (SM) – $UD_{50}/ED_{50}$.

***The anti-ulcerogenic activity*** was studied in rats according to the method described by Buzlama et al. (2017). A single intragastric administration of diclofenac solution in a “ulcerogenic” dose of 50 mg/kg at a rate of 0.2 mL per 100 g of animal body weight was used to simulate ulcerative lesions of the stomach mucosa 24 hours after food deprivation in animals with free access to water.

Pilim-1 (5, 10 and 20 mg/kg, IG) and reference medication famotidine (50 mg/kg, IG), which blocks H2-histamine receptors, were given once, fifty minutes prior to the administration of diclofenac. The intact group of rats was not exposed to any effects. Three hours after the rats were given diclofenac, animals of all 6 groups were sacrificed with an overdose of chloroform. The stomach was dissected, cut along a lesser curvature and rinsed to examine the mucous membrane and measure ulcerative lesions.

The effectiveness of famotidine and Pilim-1 was compared using two criteria: alternative and graded. The total area of ulcerative lesions (in $mm^2$), calculated planimetrically, and the average number of ulcers in the group per animal were compared using the first criterion. The area of ulcers was assessed by calculating the area index according to the formula (5):

$$AI = \frac{S}{N} \qquad (5),$$

where *AI* is the area index, *S* is the total area of ulcers on average per animal in $mm^2$, *N* is the number of ulcers per animal

The Pauls’ index was calculated according to two criteria: the number of ulcers and their area, using the formulas (6 и 7):

$$IP_n = \frac{n \times F\,\%}{100\,\%} \qquad (6),$$

where *IPn* is the Pauls’ index by the number of ulcers, *n* is the average number of ulcers per animal, pcs/animal, *F* is the number of animals with ulcers in the group, %.

$$IP_s = \frac{S \times F\,\%}{100\,\%} \qquad (7),$$

where *IPs* is the Pauls’ index by the area of ulcers, *S* is the total area of ulcers on average per animal, $mm^2$, *F* is percentage of animals with ulcers in the group, %.

The anti-ulcerogenic activity was calculated according to the formula (8):

$$AU = \frac{IP_k}{IP_e} \quad (8),$$

where $AU$ is the anti-ulcerogenic activity of the test substance, $IPk$ is the Pauls' index in the control group, $IPe$ is the Pauls' index in the experimental group.

If the AU index was less than 2.0, the substance was thought to have an anti-ulcerogenic activity.

If the ulcer area was 50% smaller than the mean value in the control group (diclofenac), these animals were considered to have a small ulcer area.

### Statistical data processing

The statistical processing of the obtained results (mean values - M, standard error of the mean – m, $LD_{50}$, $ED_{50}$, and $UD_{50}$) was carried out using the Statistic Version 6.0 software (StatSoft Inc.) and unique IBM PC computer programs created at the Department of Pharmacology of Kuban State Medical University. Using the Student's t-test, hypotheses regarding the mean values were investigated. The differences were considered significant at $p<0.05$.

# Results

### Study of the acute toxicity of Pilim-1

The results showed that the $LD_{50}$ of Pilim-1 in intragastrical administration to rats and mice is 503 (424÷582) and 485 (438÷532) mg/kg, respectively. On the other hand, the literature data in Schwartz and Syubaev (2012) indicates that the $LD_{50}$ of diclofenac and indomethacin (administered in the same way to the rats and mice) is 370 and 350 mg/kg, 47 and 35 mg/kg, respectively (Fig. 2).

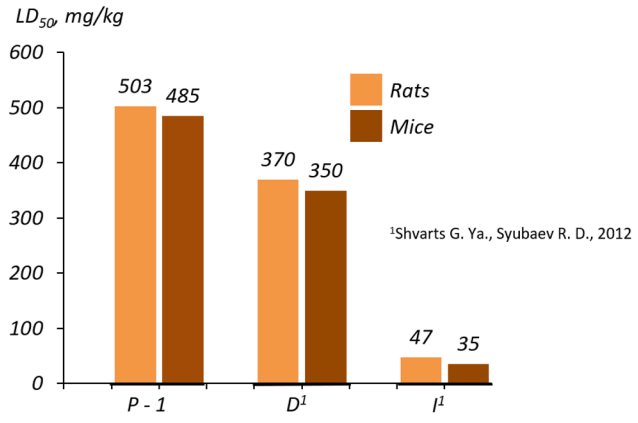



**Figure 2.** Comparative acute toxicity (according to the median lethal dose – $LD_{50}$) of Pilim-1 (P-1), diclofenac (D) and indomethacin (I) in intragastrical administration to rats and mice.

Therefore, $LD_{50}$ indicates that Pilim-1 is 1.4 and 10.7 times less toxic when administered intragastrically to rats than diclofenac and indomethacin, respectively. Based on the GOST 12.1.007 "Harmful substances. Classification and general safety requirements" (Interstate Standard. Moscow, Standartinform, 2007), Pilim-1 can be categorized as a moderately toxic substance (hazard class 3) when given to rats at a single intragastrical dose.

### The effect of Pilim-1 on acute exudative inflammation

#### *The effect on carrageenan-induced edema*

Sub-plantar injection of a saline solution to the right paw of the intact rats (control-1) did not lead to any significant changes in the volume of the paw after 3 hours later compared with the volume of the left paw (1.39 mL versus 1.38 mL, respectively), whereas carrageenan injection (control-2) at the peak of the inflammatory response (3 h) led to an increase in the volume of the right paw to 2.48 mL versus 1.42 mL of the left paw, while the volume of the right paw increased by 74.6% (Table 1).

The administration of Pilim-1 at the doses of 5, 7.5 and 10 mg/kg caused dose-dependent statistically significant inhibition of edema of the right paw. The volume of the paw was 2.29, 1.97 and 1.76 mL, respectively, versus 2.48 mL in the control-2. The paw volume gain decreased by 55.8, 35.9 and 22.2%, and inflammation inhibition increased by 25.2, 51.9 and 70.2%, respectively (Table 1).

Diclofenac and indomethacin also showed dose-dependent and statistically significant suppression of carrageenan-induced right paw edema. At the same time, the first two doses of diclofenac (5 and 7.5 mg/kg) were comparable to the same doses of Pilim-1 in its anti-inflammatory effect. So, in diclofenac groups, paw volume, paw volume gain and inflammation inhibition were 2.27 and 1.95 mL, 56.6 and 36.4%, 24.1 and 51.3%, respectively for the doses of 5 and 7.5 mg/kg. In Pilim-1 groups, paw volume, paw volume gain and inflammation inhibition were 2.29 and 1.97 mL, 55.8 and 35.9%, 25.2 and 51.9%, respectively. At a dose of 10 mg/kg, diclofenac significantly exceeded Pilim-1 in anti-inflammatory effect. For the reference drug, paw volume, paw volume gain and inflammation inhibition were 1.61 ml, 10.3 and 86.2%, and for the experimental substance these indicators were 1.76 ml, 22.2 and 70.2%, respectively (Table 1).

In the approved experimental conditions, the anti-inflammatory effect of indomethacin was less than that of diclofenac and Pilim-1. This led to its administration at higher doses, which produced similar anti-inflammatory effects to those of diclofenac and Pilim-1. Thus, indomethacin at doses of 5, 10, 15 and 20 mg/kg induced a statistically significant suppression of right paw edema to 2.36, 2.18, 2.00 and 1.78 mL, respectively, versus 2.45 mL in the control-4, while the paw volume gain decreased by 63.7, 43.4, 35.1 and 21.9%, respectively, and the inflammation inhibition increased by 17.8, 44.0, 52.9 and 71.7%, respectively (Table 1).

Based on the inflammation inhibition, the $ED_{50}$ values for Pilim-1 and diclofenac were 7.6 and 7.2 mg/kg, respectively, and 13.5 mg/kg for indomethacin (Table 1, Fig. 3A). This suggests that Pilim-1 has an anti-inflammatory activity in carrageenan paw edema, which that is 1.8 times greater than indomethacin and almost equal to diclofenac.

**Table 1.** The effect of Pilim-1, diclofenac and indomethacin on carrageenan-induced paw edema in rats (M±m, n=10)

| Animal groups | Dose, mg/kg | Paw volume, mL | | Paw volume gain, % | Inflammation inhibition, % | $ED_{50}$, mg/kg | TI |
|---|---|---|---|---|---|---|---|
| | | right | left | | | | |
| Control-1–saline solution SP [1] | | 1.38±0.03 (1.31÷1.45) | 1.39±0.03 (1.32÷1.46) | | | | |
| Control-2– potato starch gel IG + carrageenan SP [2] | | 1.42±0.03 (1.35÷1.49) $p_{1-2}>0.05$ | 2.48±0.02 (2.44÷2.52) $p_{1-2}<0.001$ | 74.6 | | | |
| **Pilim-1 IG + carrageenan SP** | | | | | | | |
| Pilim-1 [3] | 5 | 1.47±0.05 (1.35÷1.59) $p_{1-3}>0.05$ $p_{2-3}>0.05$ | 2.29±0.03 (2.22÷2.36) $p_{1-3}<0.001$ $p_{2-3}<0.001$ | 55.8 | 25.2 | 7.6 | 63.7 |
| Pilim-1 [4] | 7.5 | 1.45±0.03 (1.38÷1.52) $p_{1-4}>0.05$ $p_{2-4}>0.05$ | 1.97±0.02 (1.93÷2.01) $p_{1-4}<0.001$ $p_{2-4}<0.001$ | 35.9 | 51.9 | | |
| Pilim-1 [5] | 10 | 1.44±0.05 (1.32÷1.56) $p_{1-5}>0.05$ $p_{2-5}>0.05$ | 1.76±0.03 (1.69÷1.83) $p_{1-5}<0.001$ $p_{2-5}<0.001$ | 22.2 | 70.2 | | |
| **Diclofenac IG + carrageenan SP** | | | | | | | |
| Diclofenac [6] | 5 | 1.45±0.04 (1.35÷1.55) $p_{1-6}>0.05$ $p_{2-6}>0.05$ | 2.27±0.03 (2.20÷2.34) $p_{1-6}<0.001$ $p_{2-6}<0.001$ | 56.6 | 24.1 | 7.2 | 51.4 |
| Diclofenac [7] | 7.5 | 1.43±0.03 (1.36÷1.50) $p_{1-7}>0.05$ $p_{2-7}>0.05$ | 1.95±0.02 (1.91÷1.99) $p_{1-7}<0.001$ $p_{2-7}<0,001$ | 36.4 | 51.3 | | |
| Diclofenac [8] | 10 | 1.46±0.05 (1.32÷1.58) $p_{1-8}>0.05$ $p_{2-8}>0.05$ | 1.61±0.02 (1.57÷1.65) $p_{1-8}<0.001$ $p_{2-8}<0.001$ | 10.3 | 86.2 | | |
| Control-3 – saline solution SP [9] | | 1.40±0.06 (1.25÷1.55) | 1.41±0.04 (1.31÷1.51) | | | | |
| Control-4 – potato starch gel IG + carrageenan SP [10] | | 1.38±0,05 (1.26÷1.50) $p_{9-10}>0.05$ | 2.45±0.,03 (2.38÷2.52) $p_{9-10}<0.001$ | 77.5 | | | |
| **Indomethacin IG + carrageenan SP** | | | | | | | |
| Indomethacin [11] | 5 | 1.50±0.06 (1.35÷1.65) $p_{9-11}>0.05$ $p_{10-11}>0.05$ | 2.36±0.02 (2.32÷2.40) $p_{9-11}<0.001$ $p_{10-11}<0.05$ | 63.7 | 17.8 | 13.5 | 3.5 |
| Indomethacin [12] | 10 | 1.52±0.06 (1.37÷1.67) $p_{9-12}>0.05$ $p_{10-12}>0.05$ | 2.18±0.03 (2.11÷2.25) $p_{9-12}<0.001$ $p_{10-12}<0.001$ | 43.4 | 44.0 | | |
| Indomethacin [13] | 15 | 1.48±0.04 (1.38÷1.58) $p_{9-13}>0.05$ $p_{10-13}>0.05$ | 2.00±0.02 (1.96÷2.04) $p_{9-13}<0.001$ $p_{10-13}<0.001$ | 35.1 | 52.9 | | |
| Indomethacin [14] | 20 | 1.46±0.03 (1.39÷1.53) $p_{9-14}>0.05$ $p_{10-14}>0.05$ | 1.78±0.03 (1.71÷1.85) $p_{9-14}<0.001$ $p_{10-14}<0.001$ | 21.9 | 71.7 | | |
| Pilim-1 + Diclofenac [15] | 5 5 | 1.42±0.02 (1.38÷1.46) $p_{9-15}>0.05$ $p_{10-15}>0.05$ | 1.52±0.02 (1.48÷1.56) $p_{9-15}<0.001$ $p_{10-15}<0.001$ | 7.0 | 91.0 | | |
| Pilim-1 + Indomethacin [16] | 5 5 | 1.36±0.06 (1.21÷1.51) $p_{9-16}>0.05$ $p_{10-16}>0.05$ | 1.55±0.02 (1.51÷1.59) $p_{9-16}<0.01$ $p_{10-16}<0.001$ | 13.9 | 82.1 | | |

***Note:*** 1. $ED_{50}$ – the median effective dose that causes 50% inhibition of inflammation, TI – therapeutic index, SP – subplantarly, IG – intragastrically. 2. Confidence interval at $p<0.05$ is in the round parenthesis; the number of the animal group is in the square brackets.

The TIs for Pilim-1, diclofenac, and indomethacin were calculated based on their $LD_{50}$ and $ED_{50}$ values. It was discovered that this indicator was 63.7 for Pilim-1, 51.4 for diclofenac, and 3.5 for indomethacin (Table 1, Fig. 3B). Consequently, TI of Pilim-1 is 1.2 times higher than that of diclofenac, and 1.8 times higher than that of indomethacin.

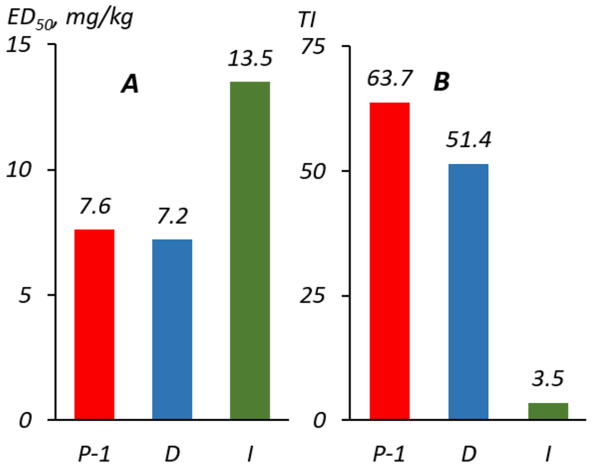



**Figure 3.** Comparative activity (according to the median effective dose $ED_{50}$, diagram **A**) and therapeutic indices, diagram **B**) of Pilim-1 (P-1), diclofenac (D) and indomethacin (I) in carrageenan-induced acute exudative inflammation in rats.

It was of interest to investigate the anti-inflammatory effect of the combined use of Pilim–1, diclofenac and indomethacin administered intragastrically at minimally effective doses of 5 mg/kg, in carrageenan-induced paw edema in rats.

Pilim-1 was discovered to significantly enhance the anti-inflammatory effect of diclofenac and, to a lesser degree, indomethacin. Concurrently, the first series of experiments showed an increase in paw volume and the inflammation inhibition of 7.0 and 91.0%, respectively, while the second series showed an increase in paw volume and the inflammation inhibition of 13.9 and 82.1%, respectively (Table 1).

### *The effect on CFA-induced paw edema*

In the intact rats (control-1), as in the earlier series of the experiments with carrageenan-induced edema, the volume of the right paw practically did not change 3 days after sub-plantar injection of saline solution compared to the left paw (1.36 mL versus 1.37 mL, respectively). But CFA injection (control-2) dramatically increased the volume of the right paw over the observed time period, which reached 2.36 mL compared to 1.34 mL of the left paw, and the volume of the right paw increased by 76.1% (Table 2).

Administration of Pilim-1 to the animals at doses of 5, 10, 15 and 20 mg/kg caused dose-dependent statistically significant suppression of right paw edema to 2.31, 2.28, 1.87 and 1.68 mL, respectively, compared to 2.36 ml in the control-2. The paw volume gain decreased by 68.6, 60.5, 35.5 and 24.4%, and the inhibition inflammation increased by 9.9, 20.5, 53.4 and 67.9%, respectively (Table 2).

Diclofenac and indomethacin also demonstrated a dose-dependent and statistically significant suppression of CFA-induced edema of the right paw. At the same time, diclofenac at doses of 5, 10 and 15 mg/kg caused a statistically significant inhibition of right paw edema to 2.16, 1.94 and 1.68 mL compared to 2.36 ml in the control-2, the decrease in paw volume gain by 60.0, 38.6 and 16.7% and the increase in inflammation inhibition by 21.2, 49.3 and 78.1%, respectively (Table 2).

Indomethacin at doses of 5, 10 and 20 mg/kg induced statistically significant suppression of right paw edema to 2.32, 2.10 and 1.70 mL versus 2.36 mL in the control-2, while the decrease in paw volume gain was 62.2, 54.4 and 23.2%, and the increase in inflammation inhibition was 18.3, 28.5 and 69.5% respectively (Table 2).

Based on the inflammation inhibition, the $ED_{50}$ values for Pilim-1 and indomethacin were 15.4 and 14.9 mg/kg, respectively, and 10.1 mg/kg for diclofenac (Table 2, Fig. 4A).

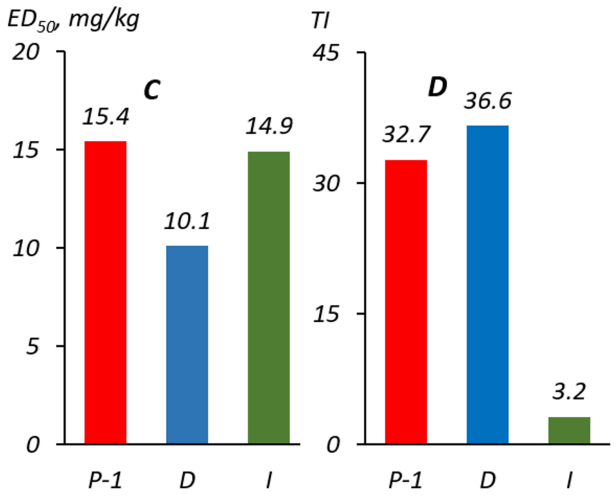



**Figure 4.** Comparative activity (according to the median effective dose $ED_{50}$, diagram **C)** and therapeutic indices, diagram **D)** of Pilim-1 (P-1), diclofenac (D) and indomethacin (I) in CFA-induced acute exudative inflammation in rats.

Based on these data, the anti-inflammatory activity of Pilim-1 is 1.5 times less than that of diclofenac and comparable to that of indomethacin in the model of CFA-induced paw edema in rats.

The TIs for Pilim-1, diclofenac and indomethacin indomethacin were 32.7, 36.6 and 3.2, respectively (Table 2, Fig. 4D). Consequently, the TI of Pilim-1 is 10.2 times greater than that of indomethacin and 1.1 times lower than that of diclofenac.

Therefore, Pilim-1 has the ability to prevent exudative inflammation caused by carrageenan and CFA in rat experiments.

Thus, Pilim-1 in experiments on rats has the ability to inhibit exudative inflammation induced by carrageenan and CFA. At the same time, in the carrageenan-induced paw edema model, its anti-inflammatory activity is comparable to that of diclofenac and exceeds that of indomethacin, and its TI is more significant than that of diclofenac and, especially, indomethacin. A noticeable potentiation of the anti-inflammatory effect was observed with combined administration of Pilim-1+diclofenac and Pilim-1+indomethacin at minimal anti-inflammatory dosages. In the CFA-induced paw edema model, Pilim-1 is less effective than diclofenac, but comparable to indomethacin, and its TI is less than that of diclofenac, and significantly higher than that of indomethacin.

**Table 2.** The effect of Pilim-1, diclofenac and indomethacin on CFA-induced paw edema in rats (M±m, n=10)

| Animal groups | Dose, mg/kg | Paw volume, ml | | Paw volume gain, % | Inflammation inhibition, % | $ED_{50}$, mg/kg | TI |
|---|---|---|---|---|---|---|---|
| | | right | left | | | | |
| Control-1–saline solution SP [1] | | 1.36±0.03 (1.29÷1.43) | 1.37±0.04 (1.27÷1.47) | | | | |
| Control -2– potato starch gel IG + CFASP [2] | | 1.34±0.02 (1.30÷1.38) $p_{1-2}$>0.05 | 2.36±0.03 (2.29÷2.43) $p_{1-2}$<0.001 | 76.1 | | | |
| **Pilim-1 IG + CFASP** | | | | | | | |
| Pilim-1 [3] | 5 | 1.37±0.04 (1.27÷1.47) $p_{1-3}$>0.05 $p_{2-3}$>0.05 | 2.31±0.03 (2.24÷2.38) $p_{1-3}$<0.001 $p_{2-3}$>0.05 | 68.6 | 9.9 | 15.4 | 32.7 |
| Pilim-1 [4] | 10 | 1.42±0.03 (1.35÷1.49) $p_{1-4}$>0.05 $p_{2-4}$>0.05 | 2.28±0.04 (2.18÷2.38) $p_{1-4}$<0.001 $p_{2-4}$>0.05 | 60.5 | 20.5 | | |
| Pilim-1 [5] | 15 | 1.38±0.02 (1.34÷1.42) $p_{1-5}$>0.05 $p_{2-5}$>0.05 | 1.87±0.03 (1.80÷1.94) $p_{1-5}$<0.001 $p_{2-5}$<0.001 | 35.5 | 53.4 | | |
| Pilim-1 [6] | 20 | 1.35±0.03 (1.28÷1.42) $p_{1-6}$>0.05 $p_{2-6}$>0.05 | 1.68±0.02 (1.64÷1.72) $p_{1-6}$<0.001 $p_{2-6}$<0.001 | 24.4 | 67.9 | | |
| **Diclofenac IG + CFA SP** | | | | | | | |
| Diclofenac [7] | 5 | 1.35±0.02 (1.31÷1.39) $p_{1-7}$>0.05 $p_{2-7}$>0.05 | 2.16±0.04 (2.06÷2.26) $p_{1-7}$<0.001 $p_{2-7}$<0.001 | 60.0 | 21.2 | 10.1 | 36.6 |
| Diclofenac [8] | 10 | 1.40±0.03 (1.33÷1.47) $p_{1-8}$>0.05 $p_{2-8}$>0.05 | 1.94±0.03 (1.87÷2.01) $p_{1-8}$<0.001 $p_{2-8}$<0.001 | 38.6 | 49.3 | | |
| Diclofenac [9] | 15 | 1.44 ±0.02 (1.40÷1.48) $p_{1-9}$>0.05 $p_{2-9}$>0.05 | 1.68±0.04 (1.58÷1.79) $p_{1-9}$<0.001 $p_{2-9}$<0.001 | 16.7 | 78.1 | | |
| **Indomethacin IG + CFA SP** | | | | | | | |
| Indomethacin [10] | 5 | 1.43±0.04 (1.33÷1.53) $p_{1-10}$>0.05 $p_{2-10}$>0.05 | 2.32±0.02 (2.28÷2.36) $p_{1-10}$<0,001 $p_{2-10}$>0,05 | 62.2 | 18.3 | 14.9 | 3.2 |
| Indomethacin [11] | 10 | 1.36 ±0.02 (1.32÷1.40) $p_{1-11}$>0.05 $p_{2-11}$>0.05 | 2.10±0.03 (2.03÷2.17) $p_{1-11}$<0.001 $p_{2-11}$<0.001 | 54.4 | 28.5 | | |
| Indomethacin [12] | 20 | 1.38±0.02 (1.34÷1.42) $p_{1-12}$>0.05 $p_{2-12}$>0.05 | 1.70±0.02 (1.66÷1.74) $p_{1-12}$<0.001 $p_{2-12}$<0.001 | 23.2 | 69.5 | | |

***Note:*** 1. CFA – the Complete Freund's Adjuvant; $ED_{50}$ – the median effective dose that causes 50% inhibition of inflammation, TI – therapeutic index, SP – subplantarly, IG – intragastrically. 2. Confidence intervals at $p$<0.05 are in the round parenthesis; the number of the animal group is in the square brackets.

**Table 3.** The effect of Pilim-1 and diclofenac on chronic proliferative inflammation in rats (M±m, n=10)

| Animal group, the dose of substances | Granuloma mass, mg | | Granuloma mass, characterizing the inflammation phases, mg | |
|---|---|---|---|---|
| | raw | dried | exudative | proliferative |
| Control – potato starch gel IG + subcutaneous implantation of a cotton pellet [1] | 1062.0±17.1 (1023.3÷1100.7) | 190.0±9.4 (168.8÷211.2) | 872.0 | 150.0 |
| Pilim-1 IG, 10 mg/kg + subcutaneous implantation of a cotton pellet [2] | 758.5±13.7 (727.7÷789.3) $p_{1-2}$<0.001 | 135.0±8.2 (116.4÷153.6) $p_{1-2}$<0.001 | 623.5 / 28.5 | 95.0 / 36.7 |
| Diclofenac IG, 10 mg/kg + subcutaneous implantation of a cotton pellet [3] | 634.0±12.8 (606.1÷661.9) $p_{1-3}$<0.001 $p_{2-3}$<0.001 | 139.0±8.4 (119.9÷158.1) $p_{1-3}$<0.001 $p_{2-3}$>0.05 | 495.0 / 43.4 | 99.0 / 34.0 |

***Note:*** 1. IG – intragastrically. 2. Confidence intervals at $p$<0.05 are in the round parenthesis; the number of the animal group is in the square brackets. 3. The numerator of the exudative phase is the difference in the masses of raw and dried granuloma. The numerator of the exudative phase is the difference in the masses of the dried granuloma and the cotton pellet. The denominator is the indicators of the inflammation phases relative to the control (%).

### *The effect of Pilim-1 on chronic proliferative inflammation*

Research using a chronic proliferative inflammation model in rats revealed that by the 12th day of the experiment, a granuloma had formed around a cotton pellet implanted subcutaneously into the back. The raw mass of the cotton pellet was 1062 mg, and the dried mass was 190 mg. The masses of the granulomas, which represent the exudative proliferative phase of inflammation, were 872 mg and 150 mg, respectively (Table 3, Fig. 5).

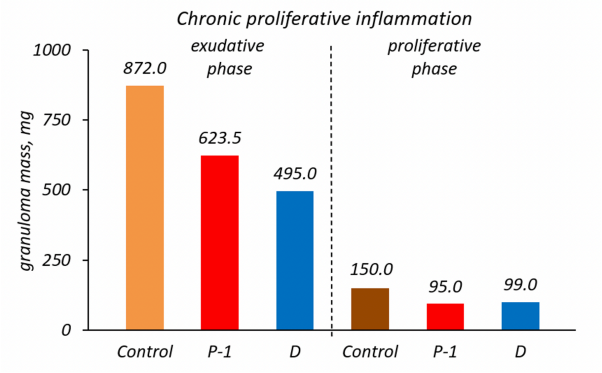



**Figure 5.** The effect of Pilam-1 (P-1) and diclofenac (D) on chronic proliferative inflammation (exudative and proliferative phases) in rats.

In the animals that were intragastrically administered with Pilim-1 and diclofenac at a dose of 10 mg/kg for 11 days the masses of raw and dried granulomas, as well as masses indicating the exudative and proliferative phases of inflammation, were 758.5, 135.0, 623.5, and 95.0 mg respectively under the influence of Pilim-1, and 634.0, 139.0, 495.0, and 99.0 mg respectively under the influence of diclofenac (Table 3, Fig. 5). Simultaneously, Pilim-1 and diclofenac reduced the exudation phase by 28.5% and 43.4%, respectively, in comparison to the control. Treatment with Pilim-1 resulted in slightly higher proliferative process intensity compared to that resulted from treatment with diclofenac one (36.7 versus 34%).

Therefore, in the rat model of chronic proliferative inflammation, Pilim-1 slightly exceeds diclofenac in suppressing the proliferative phase but falls short in inhibiting the exudative phase.

### Study of the analgesic effect of Pilim-1

### *The formalin test*

Rats receiving a subplantar injection of formalin only (control) exhibited marked pain responses during both the first and second phases of inflammation.

The administration of Pilim-1 and diclofenac significantly reduced the pain response in the form an animal licking paws in the first phase of inflammation (with acute pain)by 54.4 and 25.4%, and in the second one (with tonic pain) by 43.1 and 48.8%, respectively (Table 4, Fig. 6A-6G).

The number of flinches during the first and second phases of inflammation decreased by 38.3 and 47.5%, respectively, under the influence of Pilim-1, and by 10.6 and 36.1%, respectively, under the influence of diclofenac. This is an alternative indicator of pain response to formalin (Chayka et al. 2015) and, according to Wheeler-Aceto and Cowan (1991), is more reliable than licking paws (Table 4, Fig. 6). It should be noted that Pilim-1 caused a more pronounced decrease in this pattern compared to diclofenac.

Thus, based on the duration of licking paws and the number of flinches, the ”formalin” test in rats revealed that Pilim-1 has a more pronounced analgesic effect in the first phase of inflammation (reflecting acute pain), and it is less effective in the second phase of inflammation (reflecting tonic pain) in comparison to diclofenac.

**Table 4.** The effect of Pilim-1 and diclofenac on pain sensitivity in the "formalin test" in rats (M±m, n=10)

| Animal group, the dose of substances | The total duration of the pain reaction, sec. | First phase of inflammation | | Second phase of inflammation | |
|---|---|---|---|---|---|
| | | The total time of licking paws, sec. | The total number of flinches | The total time of licking paws, sec. | The total number of flinches |
| Control – potato starch gel IG + Formalin SP [1] | 524.6 | 128.4±3.8 (119.8÷137.0) | 47.0±3.4 (39.2÷54.8) | 396.2±16.5 (359.0÷433.4) | 122.0±3.4 (114.2÷129.8) |
| Pilim-1 IG (8 mg/kg) + Formalin SP [2] | 284.2 | 58.6±2.7 (52.5÷64.7) $p_{1-2}$<0.001 | 29.0±2.1 (24.3÷33.7) $p_{1-2}$<0.001 | 225.6±12.7 (197.0÷254.2) $p_{1-2}$<0.001 | 64.0±1.8 (59.8÷68.2) $p_{1-2}$<0.001 |
| Diclofenac IG (7 mg/kg) + Formalin SP [3] | 298.6 | 95.8±1.6 (92.1÷99.5) $p_{1-3}$<0.001 $p_{2-3}$<0.001 | 42.0±1.8 (37.8÷46.2) $p_{1-3}$>0.05 $p_{2-3}$<0.001 | 202.8±6.3 (188.6÷217.0) $p_{1-3}$<0.001 $p_{2-3}$>0.05 | 78.0±2.2 (73.1÷82.9) $p_{1-3}$<0.001 $p_{2-3}$<0.001 |

***Note:*** 1. SP – subplantarly, IG – intragastrically. 2. Confidence intervals at $p$<0.05 are in the round parenthesis; the number of the animal group is in the square brackets.

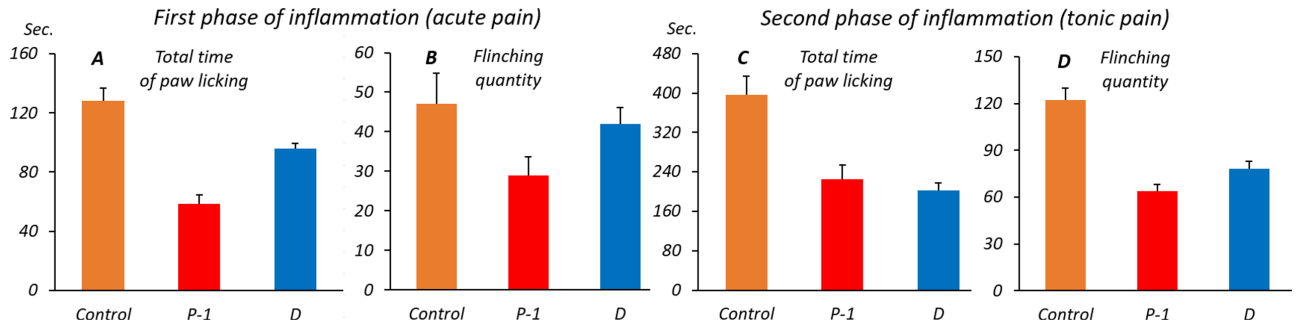



**Figure 6.** Comparative analgesic activity of Pilim-1 (P-1) and diclofenac (D) in the first and second phases (nonspecific inflammation) of the "formalin test" in rats for acute and tonic pain, respectively.

### *The acetic acid-induced writing test*

The administration of acetic acid to mice caused a pronounced pain reaction, manifested in the form of writhes, the average number of which was 29.6 during a 15-minute observation. The latent time of their onset was 268 seconds (Table 5).

Pilim-1 at doses of 5, 10, and 15 mg/kg caused a statistically significant suppression of visceral pain response: the average number of writhes was 20.2, 12.4, and 9.8 versus 29.6 in the control, meaning the pain response decreased by 31.8, 58.1, and 66.9%, respectively, the latent time of the onset of writhes was 316, 364, and 396 seconds, meaning it increased by 17.9, 35.8, and 47.8%, respectively (Table 5).

Diclofenac at doses of 2.5, 5, 10 and 15 mg/kg caused statistically significant suppression of the pain response: the average number of writhes was 22.6, 15.7, 13.2 and 9.4 compared to 29.6 in the control, meaning the pain response decreased by 23.6, 47.0, 55.4 and 68.2%, respectively. The latent time of the onset of writhes was 305 (statistically insignificant), 370, 398 and 414 seconds; it increased by 13.8, 38.1, 48.5 and 54.5%, respectively (Table 5).

The $ED_{50}$ and TI of Pilim-1 were 9.9 mg/kg and 49.0, and for diclofenac they were 8.6 mg/kg and 47.0, respectively. Thus, the $ED_{50}$ of Pilim-1 is 1.2 times lower, and its TI is 1.2 times higher compared to these indicators for diclofenac (Table 5, Fig. 7A and 7B).

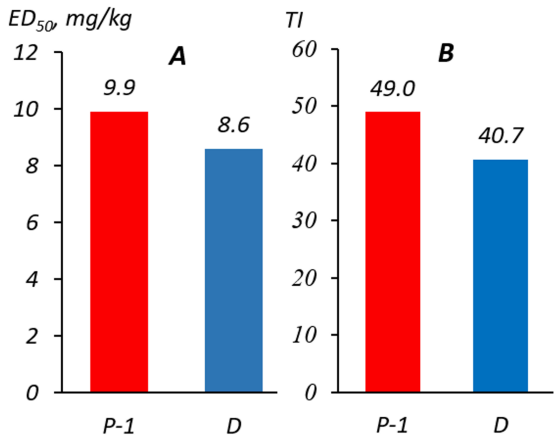



**Figure 7.** Comparative analgesic activity (according to the median effective dose $ED_{50}$, diagram **A**) and therapeutic indices, diagram **B**) of Pilim-1 (P-1) and diclofenac (D) in the acetic acid-induced writing test in mice.

**Table 5.** The effect of Pilim-1 and diclofenac on the pain sensitivity of mice in the acetic acid-induced writing test (M±m, n=10)

| Animal group | Dose, mg/kg | The number of writhes for 15 min | Latent time of onset of writhes, sec. | Inhibition of pain response, % | $ED_{50}$, mg/kg | TI |
|---|---|---|---|---|---|---|
| Control – potato starch gel IG + acetic acid IP [1] | | 29.6±1.9 (25.2÷34.0) | 268±12 (240÷296) | | | |
| **Pilim-1 IG+acetic acid IP** | | | | | | |
| Pilim-1 [2] | 5 | 20.2±2.2 (15.3÷25.1) $p_{1-2}$<0.01 | 316±14 (284÷348) $p_{1-2}$ <0.02 | 31.8 | 9.9 | 49.0 |
| Pilim-1 [3] | 10 | 12.4±1.5 (9.0÷15.8) $p_{1-3}$<0.001 | 364±13 (335÷393) $p_{1-3}$<0.001 | 58.1 | | |
| Pilim-1 [4] | 15 | 9.8±1.6 (6.7÷13.5) $p_{1-4}$<0.001 | 396±11 (372÷420) $p_{1-4}$<0.001 | 66.9 | | |
| **Diclofenac IG+acetic acid IP** | | | | | | |
| Diclofenac [5] | 2.5 | 22.6±1.8 (18.4÷26.8) $p_{1-5}$<0.02 | 305±15 (372÷ 338) $p_{1-5}$>0.05 | 23.6 | 8.6 | 40.7 |
| Diclofenac [6] | 5 | 15.7±1.7 (11.8÷19.6) $p_{1-6}$<0.001 | 370±13 (341÷399) $p_{1-6}$<0.001 | 47.0 | | |
| Diclofenac [7] | 10 | 13.2±1.8 (9.0÷17.4) $p_{1-7}$<0.001 | 398±11 (374÷422) $p_{1-7}$<0.001 | 55.4 | | |
| Diclofenac [8] | 15 | 9.4±1.4 (6.2÷12.6) $p_{1-8}$<0.001 | 414±10 (391÷ 437) $p_{1-8}$<0.001 | 68.2 | | |

***Note:*** 1. $ED_{50}$ – the median effective dose that causes 50% inhibition of pain response, TI – therapeutic index, IP – intraperitoneally, IG – intragastrically. 2. Confidence intervals at p<0.05 are in the round parenthesis; the number of the animal group is in the square brackets.

Therefore, the analgesic effect of Pilim-1 is close to or comparable to that of diclofenac in the acetic acid-induced writing test in mice. Pilim-1 slightly exceeds diclofenac with regard of TI.

### *The study of the possible ulcerogenic effect of Pilim-1*

In the rat experiments, the ulcerogenic effect Pilim-1 (200 mg/kg) had an average value of 0.3 points. We can conclude that it was practically absent. Nevertheless, it can be assumed that the $UD_{50}$ for Pilim-1 is more than 200 mg/kg, while for diclofenac and indomethacin, these indicators, according to the literature data (Schwartz and Syubaev, 2012), are 48 and 10 mg/kg, respectively (Table 6, Fig. 8A).

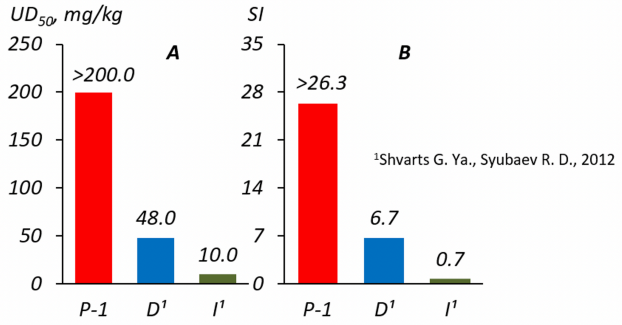



**Figure 8.** Comparative ulcerogenic activity (according to the ulcerogenic dose $UD_{50}$, diagram **A**) and safety margin, diagram **B**) of Pilim-1 (P-1, diclofenac (D) and Indomethacin (I) in the rat experiments.

**Table 6.** The ratio of ulcerogenic and anti-inflammatory (in carrageenan-induced paw edema) activity of Pilim-1, diclofenac and indomethacin in rat experiments

| Substance | $UD_{50}$, mg/kg | $ED_{50}$, mg/kg | SM | In comparison to | |
|---|---|---|---|---|---|
| | | | | diclofenac sodium | indomethacin |
| Pilim-1 | >200 | 7.6 | >26.3 | 3.9 | 37.6 |
| Diclofenac | 48[1] | 7.2 | 6.7 | 1.0 | 9.6 |
| Indomethacin | 10[1] | 13.5 | 0.7 | 0.1 | 1.0 |

***Note*:** $UD_{50}$ – the median ulcerogenic dose causing damage to 50% of the gastric mucosa. $ED_{50}$ – the median effective dose causing 50% inhibition of inflammation in the carrageenan-induced paw edema

The SM for Pilim-1 is above 26.3, and for diclofenac and indomethacin, it is 6.7 and 0.7, respectively. Thus, the SM of Pilim-1 is 3.9 and 37.6 times higher than that of diclofenac and indomethacin, respectively (Table 6, Fig. 8B).

Thus, in the rat experiments, Pilim-1 has an extremely low (practically absent) ulcerogenic effect, significantly surpassing both diclofenac and indomethacin in this indicator.

### Study of the anti- ulcerogenic effect of Pilim-1

There were no alterations (hyperemia, hemorrhages, erosions, or ulcers) in the stomach mucosa of the rats in the intact group (Fig. 9A).

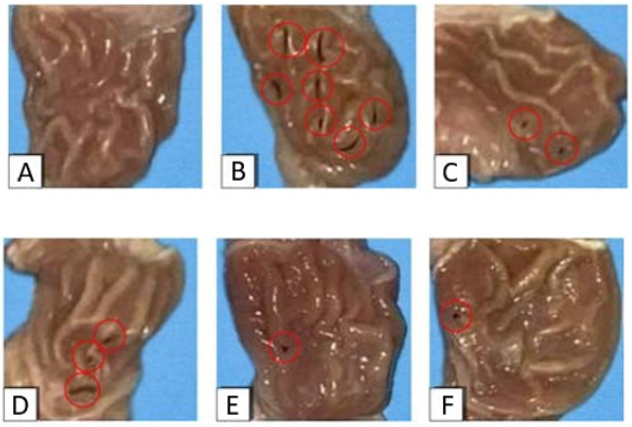



**Figure 9.** General appearance of the gastric mucosa of rats: intact, after the ulcerogenic effect of diclofenac and the gastroprotective effects of famotidine and Pilim-1. **Note:** ***A*** – the gastric mucosa of the intact animal, ***B*** – the influence of diclofenac (50 mg/kg), ***C*** – the anti–ulcerogenic effect of famotidine (50 mg/kg), ***D, E*** *and* ***F*** – the anti-ulcerogenic effect of Pilim-1 (5, 10 and 20 mg/kg, respectively). Ulcerative lesions are highlighted by red circles.

Diclofenac (50 mg/kg IG) produced ulcerative lesions of the gastric mucosa in 100% of the animals in the control group. The great majority of ulcers appeared deep and oblong, occupying a significant area (on average 30.86 mm$^2$, the area index 2.14). Only 36.36% of animals had ulcers in a small area. The Pauls' indices were 14.91 and 30.86 for the criteria "number" and "area" of ulcers, respectively (Table 7, Figs 9B and 10).

A reference drug famotidine (50 mg/kg, IG) caused a statistically significant decrease in the number and area of ulcers by 80.4 and 80.2%, respectively, while the area index showed a small (trend) decline to 43.9%. In comparison to the control, there were 43.64% more animals with a small area of ulcerative lesions. Based on the parameters of "number" and "area" of ulcers, the anti-ulcerogenic activity of famotidine was 5.11 and 6.71, respectively (Table 7, Figs 9B and 10).

The administration of Pilim-1 in a wide range of doses 5, 10 and 20 mg/kg (IG) decreased the number and area of ulcers by 51.7 and 49.6, 85.2 and 88.4, 87.9 and 92.4%, and the area index by – 21.5, 29.0 and 46.7%, respectively. The number of animals with a small area of ulcerative lesions increased by 36.34, 53.64 and 63.64%, respectively. Based on the parameters of "number" and " area" of ulcers, the anti-ulcerogenic activity of Pilim-1 at selected doses increased gradually, reaching 2.07 and 2.28, 7.53 and 12.49, 9.20 and 16.33 (Tables 7, Figs. 9D-F and 10).

Therefore, Pilim-1 exhibits dose-dependent anti-ulcerogenic activity at all three chosen doses in the model of NSAID-induced gastropathy in rats caused by diclofenac. Based on the parameters "number" and "area" of gastric mucosa ulcers, Pilim-1 at the lowest dose is 2.5 and 2.9 times less effective than famotidine, while at the second and third doses (10 and 20 mg/kg), it is 1.5 and 1.9, 1.8, and 2.4 times more effective, respectively.

## Discussion

Analyzing the results of the conducted research, it is important to first highlight that, in compliance with GOST 12.1.007 "Harmful Substances. Classification And General Safety Requirements" (Interstate Standard. Moscow, Standartinform, 2007), Pilim-1 can be categorized as a moderately toxic substance (hazard class 3) when given to rats at a single intragastrical dose.

The strong anti-inflammatory effect of Pilim-1, both in conditions of acute exudative (with carrageenan-induced and CFA-induced edema) and chronic proliferative inflammation, as well as potentiation of the NSAIDs diclofenac and indomethacin, can be attributed to a variety of metabolotropic properties (antihypoxant, antioxidant, membrane stabilizing, reparative, and bacteriostatic) resulting from the combined action of imidazole and zinc contained in Pilim-1, not just from its inhibitory effect on cyclooxygenase and 5-lipoxygenase (Shakhmardanova and Galenko-Yaroshevsky 2015, 2016).

The literature indicates that imidazole is present in a wide range of biologically important compounds, including numerous pharmaceuticals (e.g., acizole, omeprazole, metronidazole, naphthysine, clofelin, phentolamine, etymizole, ketocanazole, mebendazole, mercazolil, mercaptopurine, etc.).

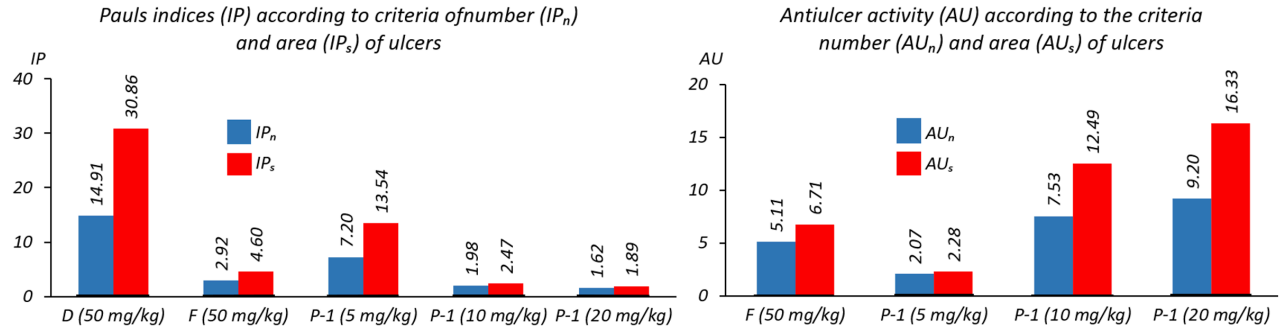



**Figure 10.** Comparative anti-ulcerogenic activity of Pilim-1 (P-1) and famotidine (F) under condition of the ulcerogenic action of diclofenac (D). ***Note***: *IP* – the Pauls' index, *IPn* – the Pauls' index by the number of ulcers; *IPs* – the Pauls' index by the area of ulcers; *AU* – anti-ulcerogenic activity; $AU_n$ – anti-ulcerogenic activity by the number of ulcers; $AU_s$ – anti-ulcerogenic activityby the area of ulcers.

**Table 7.** Comparative anti-ulcerogenic activity of Pilim-1 and famotidine on the ulcerogenic effect of diclofenac in rat experiments

| Criteria | Diclofenac, 50 mg/kg; control | Famotidine, 50mg/kg | Pilim-1, mg/kg | | |
|---|---|---|---|---|---|
| | | | 5 | 10 | 20 |
| Number of ulcers/ number of animals | 14.91±2.17 (10.40÷19.42) | 2.92±1.08 (0.47÷5.37) *** | 7.20±1.73 (3.28÷11.29) **+ | 2.20±0.65 (0.73÷3.67) *** | 1.80±0.43 (0.82÷2.78) *** |
| The difference with the control, % | - | -80.4 | -51.7 | -85.2 | -87.9 |
| The area of ulcers, mm² | 30.86 ±6.74 (16.84÷44.88) | 4.60±1.19 (1.64÷7.56) *** | 13.54±1.52 (10.11÷16.97) *++ | 2.74±0.76 (1.03÷4.45) *** | 2.10±0.53 (1.37÷2.83) *** |
| The difference with the control, % | - | -80.2 | -49.6 | -88.4 | -92.4 |
| The area index | 2.14±0.46 (1.19÷3.09) | 1.20±0.32 (0.71÷1.69) | 1.68±0.54 (0.95÷2.41) | 1.52±0.43 (0.54÷2.50) | 1.15±0.32 (0.43÷1.87) |
| The difference with the control, % | - | -43.9 | -21.5 | -29.0 | -46.7 |
| The number of the animals in the group | 22 | 10 | 10 | 10 | 10 |
| The number of the animals with a small area of ulcers≤15.4 mm² | 8 | 8 | 7 | 9 | 10 |
| The number of the animals with a small area of ulcers, % | 36.36 | 80.00 | 70.00 | 90.00 | 100.00 |
| The difference with the control, % | - | + 43.64 | + 36.34 | + 53.64 | + 63.64 |
| The number of the animals with ulcers, % | 100.00 | 100.00 | 100.00 | 90.00 | 90.00 |
| The Pauls' index according to the "number of ulcers" criterion | 14.91 | 2.92 | 7.20 | 1.98 | 1.62 |
| Anti- ulcerogenic activity according to the "number of ulcers" criterion | - | 5.11 | 2.07 | 7.53 | 9.20 |
| The Pauls' index according to the "area of ulcers" criterion | 30.86 | 4.60 | 13.54 | 2.47 | 1.89 |
| Anti- ulcerogenic activity according to the "area of ulcers" criterion | - | 6.71 | 2.28 | 12.49 | 16.33 |

**Note:** * – $p<0.02$, ** – $p<0.01$ and *** – $p<0.001$ in comparison to the control (diclofenac); + –$p<0.05$ and ++ – $p<0.001$ in comparison to the famotidine. Confidence intervals at $p = 0.05$ are in the parenthesis.

The ionizing and aromatic imidazole ring promotes the pharmacokinetic characteristics of pharmaceuticals. Many newly synthesized imidazole derivatives have demonstrated high levels of anti-inflammatory, analgesic, antimicrobial, antiviral, antifungal, antituberculosis, antitumor, anticonvulsant, anticoagulant, and other types of activity in recent years, typically surpassing those of well-established medications with the appropriate mechanism of action (Kaldybayeva et al. 2023; Fadhil and Qhanim 2020; Gas et al. 2020; Rajam et al. 2020; Wells et al. 2020; Vidhya and Malar 2020; Gurevich et al. 2021; Pham et al. 2021; Patel et al. 2022).

Zinc is an important part in the structure of imidazole, assisting in the stabilization of cell membranes and the development of antioxidant status (Panasenko et al. 2018). It also has anti-inflammatory and antimicrobial properties, which are mainly related to its impact on the production of pro-inflammatory cytokines (TNF-D, IL-1B, IL-6, IL-8, and IL-12) by decreasing the activation of nuclear factor kappa B, or NF-kB, which regulates the expression of numerous genes involved in immune system function, inflammatory response, and other processes. Furthermore, NF-kB plays an essential role in regulating the proliferation and development of inflammatory T cells and cells involved in congenital immunity. Numerous external and internal causes, including pro-inflammatory cytokines, bacterial and viral infections, oxidative stress, and others, can activate NF-kB (Liu et al. 2017; Voelkl et al. 2018; Lebedeva et al. 2023). It is believed that the primary mechanism of the anti-inflammatory effect of zinc is the upregulation of the zinc-finger protein A20 (known as tumor necrosis factor, alpha–induced protein 3 or A20 - TNFAIP3). This protein functions as a central negative regulator of the NF-kB pathways triggered by the tumor necrosis factor receptor, or TNFR, toll-like receptor, or TLR, and peroxisome proliferator-activated receptors, or PPARs. These receptors are involved in the transcriptional regulation of metabolism, inflammation, angiogenesis, and fibrous response (Nakano et al. 2020; Tobita et al. 2020; Ho et al. 2020). It should be noted that the inflammatory process activates the PPAR subtypes (α, β, and γ) through inhibition of NF-kB (Korbecki et al. 2019; Tobita et al. 2021).

It has been shown that zinc metabolism and its participation in the immune system function have been directly linked to its ZIP8 transporter (Zrt/Irt-like protein 8), which is one of 14 transporters in the Solute Carrier family SLC39A1-A14 and serves as an essential negative feedback regulator. Through the nuclear factor NF-kB, the inflammation stimulates ZIP8 expression. Next, zinc is captured by ZIP8, which is present on the plasma membrane, and is transferred to the cytosol, where it suppresses the activity of IKK kinase, so diminishing the inflammatory process (Notova et al. 2022; Liu et al. 2013; Gammoh and Rink 2017). Figure 11 provides more detailed information on the zinc anti-inflammatory mechanism of action.

The fact that Pilim-1 exhibits analgesic action in the formalin test suggests that this substance can effectively reduce acute pain reaction (caused by the injection of phlogogen) as well as tonic pain reaction (caused by the development of an inflammatory process in peripheral tissues and the functional impairment of posterior horn neurons in the gray matter of the spinal cord).

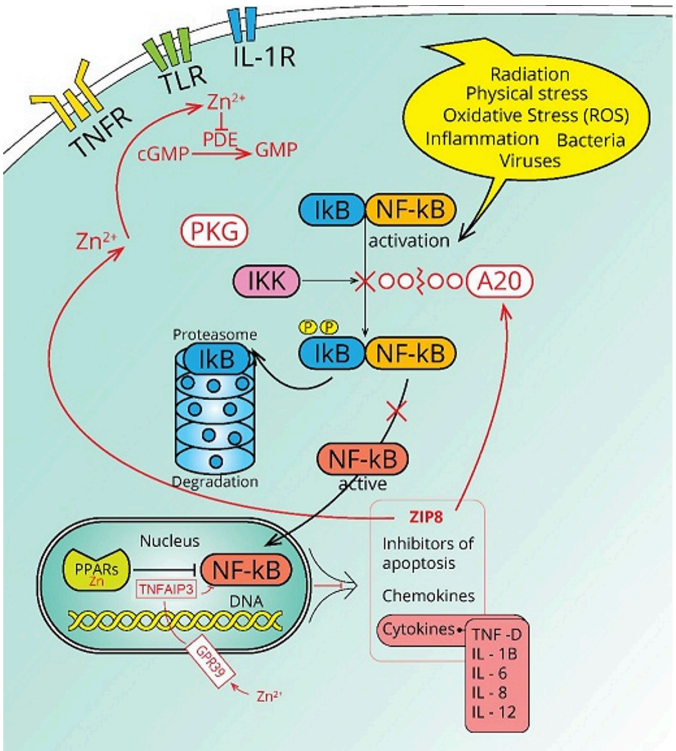



**Figure 11.** The mechanism of the anti-inflammatory effect of zinc mediated through the NF-kB signaling cascade. **Note:** TNFR – tumor necrosis factor receptor; TLR – toll-like receptor; IL-1R – interleukin 1 receptor; cGMP – cyclic guanosine monophosphate; PDE – phosphodiesterase; GMP – guanosine monophosphate; PKG – protein kinase G; IkB – nuclear factor B inhibitor; IKK (or IkB) – kinase complex inhibitor; A20 – zinc finger protein (contains about 20 amino acids); TNFAIP3 – tumor necrosis factor, alpha-induced protein 3 or A20; ZIP8 – Zrt/Irtlike proteins 8; PPARs – peroxisome proliferator-activated receptors; DNA – deoxyribonuclear acid; GPR39 – G protein-coupled receptor 39; TNF-α – tumor necrosis factor alpha; IL – interleukin.

These reactions represent the first and second phases of the formalin's action and can be triggered by a variety of damaging factors (injuries, burns, chemical agents, etc.). It should be highlighted that the ”formalin test” pain model most closely mimics pain syndrome, which is frequently observed in clinical practice (Chaika et al. 2015). (Chaika et al. 2015).

It is possible that Pilim-1 blocks non-selective $TRPA_1$ cation channels permeable to $Ca^{2+}$, $Na^{+}$, and $K^{+}$ and presented in nociceptive neurons of A-δ- and C-fibers channels because one of the algesic actions of formalin is the activation of these channels (Heber et al.2019; de Araujo et al. de Araujo et al. 2020; Beskhmelnitsyna et al. 2018). This is confirmed by our experiments, as the high efficiency of Pilim-1 in the first phase of the formalin test exceeds that of diclofenac.

The mechanism of the analgesic effect of Pilim-1 may involve an inhibitory effect on cyclooxygenase and 5-lipoxygenase, which are the primary enzymes in the synthesis of prostaglandins and leukotrienes, respectively, and potent mediators of both inflammation and pain. This hypothesis is supported by the inhibitory effect of this substance on carrageenan and CFA-induced edema (Karateev and Aleynikova 2016). Zinc, which is contained in Pilami-1 and tends to have an analgesic impact by inhibiting the inflammatory process, is likely to play a significant role in the analgesic effect of Pilim-1 (Koba et al. 2019). Furthermore, Pilim-1, like diclofenac (Björkman et al. 1992), tends to have a central analgesic effect linked to the impact on opioid receptors, as evidenced by the inhibition of both phases of inflammation induced by formalin (Chaika et al. 2015).

The inhibitory effect of Pilim-1 on prostaglandin synthesis and blockade of opioid receptors, the production and activity of which increase under the influence of chemogenous irritants, including acetic acid, may be the cause of a decrease in the pain response in the acetic acid-induced writing test, which simulates acute visceral and somatic deep pain in laboratory animals (Bondarenko et al. 2011; Ghorbanzadeh et al. 2016).

It is well-known that inflammation and pain are frequently linked. Histamine, which also serves as a physiological immune system regulator, and serotonin, which also acts as a pain mediator, are the two main mediators of inflammation. Furthermore, Bystrova et al. (2021) suggest that histamine and its receptors may function as parts of signaling pathways connected to the development of inflammatory and pain responses and that through a histamine-dependent mechanism, toll-like receptors (TLRs) may control the expression pattern of $TRPV_1$ receptors on sensory neurons, which are important for nociception and inflammation. It has recently been shown that diclofenac decreases the blood plasma histamine concentration, but has no effect on the serotonin level or the histamine and serotonin content of peritoneal exudates in rats with experimental acetic peritonitis, which is accompanied by a significant increase in the concentrations of histamine and serotonin in blood plasma (Ivanova and Voronina, 2018; Beskhmelnitsyna et al. 2019). Based on the above data, it is possible that the anti-inflammatory and analgesic effect of Pilim-1 is also associated with impaired histamine production.

The extremely weak or absence ulcerogenic action of Pilim-1 might be attributed to both its antioxidant qualities and the zinc that is included into its structure. Since impaired lipid peroxidation and respiratory chain disintegration are among the pathogenetic mechanisms of the gastrotoxic effect of NSAIDs, it has been demonstrated that medications with antioxidant effects (mexidol, quercetin, metaprot, hypoxen, etc.) exhibit a significant cytoprotective effect in NSAID gastropathy (Gladkikh 2017). Conjugation of indomethacin and complexation of ibuprofen and naproxen with zinc, in addition to potentiating the anti-inflammatory effect of these medications, caused a significant decrease in their damaging effect on the gastric mucosa of rats (Gladkikh 2017; Dillon et al. 2003; Jarosz et al. 2017).

The anti-ulcerogenic effect of Pilim-1 may be related to the biological properties of zinc and imidazole included in its structure. Thus, a number of studies have shown that zinc has a gastroprotective effect due to stimulation of tissue regeneration and effects on the neuroendocrine and immune systems (Podobed 2015; Ohkawara et al. 2006; Skrovanek et al. 2014). Zinc also has the anti-ulcerogenic effect because it can restore prostaglandin E2 biosynthesis, which is disrupted by the use of indomethacin and diclofenac (Podobed 2015). It can also inhibit the process of gastric mucosa cell apoptosis caused by indomethacin (Podobed 2015), suppress hydrochloric acid synthesis (Barbarino et al. 1992), stimulate the formation of mucus in the stomach (Mahmood et al. 2007), and increase energy metabolism in gastrointestinal tract cells (Skrovanek et al. 2014).

Remarkably, ulcerative lesions of the gastric mucosa have been linked to NSAID-gastropathies as well as an imbalance of the oxidant-antioxidant system, which disturbs free radical processes and causes peroxidation of polyunsaturated fatty acids in the lesion.

It is known that zinc, as well as many multifunctional compounds with imidazole in their structure, have antioxidant properties (Palamar et al. 2020). The available evidence suggests that the antioxidant activity of Pilim-1 may be a contributing factor to its anti-ulcerogenic impact, since it may be a result of the combination (potentially additive or mutually potentiating) activities of imidazole and zinc in the specified direction.

In summary, Pilim-1 has strong analgesic and anti-inflammatory properties; its effectiveness is comparable or somewhat less effective than that of diclofenac and above that of indomethacin. The TI of Pilim-1 is higher than that of diclofenac and, to a greater degree, indomethacin.

While diclofenac and indomethacin cause ulcers, Pilim-1 exhibits almost minimal gastrotoxicity.

The suggestions made about the possible mechanisms of anti-inflammatory, analgesic and anti-ulcerative effects of Pilim-1, as well as its nearly nonexistent ulcerogenic impact are theoretical and call for a more thorough investigation.

# Conclusion

The lower acute toxicity of Pilim-1 (in comparison to diclofenac and indomethacin), strong anti-inflammatory, analgesic, and anti-ulcerative effects, higher therapeutic index, and extremely low (almost nonexistent) ulcerogenic effect enable us to suggest it further preclinical research.available in the main text.

**Conflict of interest**

The authors have declared that no competing interests exist.

**Funding**

The authors have no funding to report.

**Data availability**

All of the data that support the findings of this study are available in the main text.

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