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Research Article

Effective wound healing agents based on N-alkenylimidazole zinc complexes derivatives: future prospects and opportunities

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Abstract

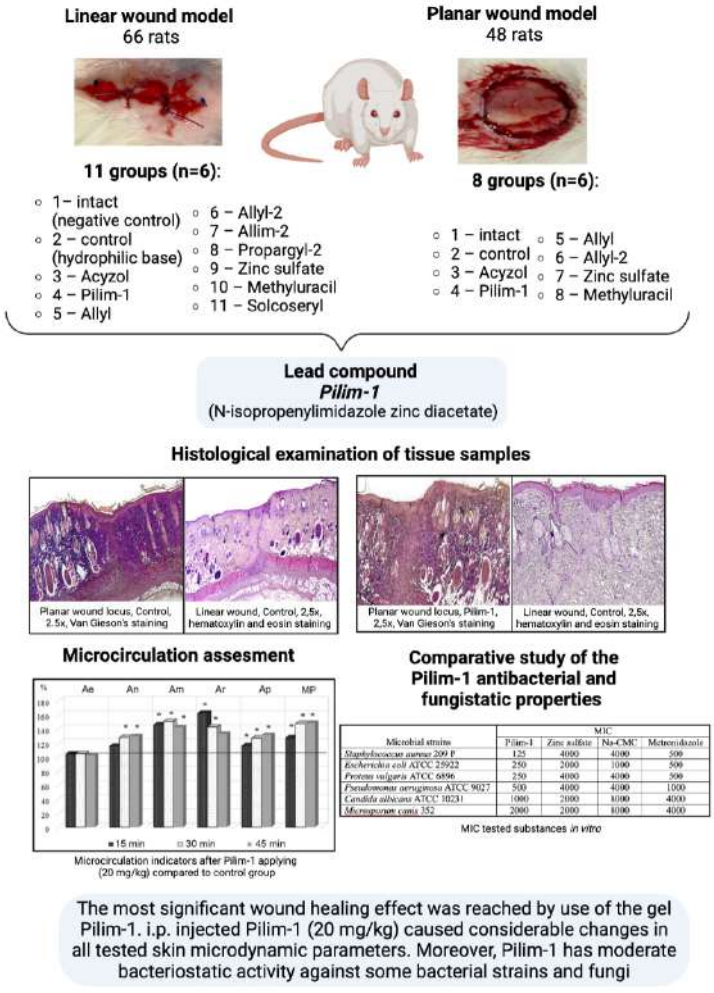
Introduction: The therapeutic effect of commercially available domestic and foreign drugs for the treatment of various skin injuries is far from optimal. These drugs have no universal effects, but cause pronounced side reactions. There is a clear demand for development of innovative wound-healing drugs with antimicrobial properties, which increase the natural protective function of the skin. Pharmaceutical compounds with zinc nanoparticles have been increasingly recognized as a promising therapeutic direction. These drugs can easily penetrate into damaged tissues and stimulate metabolic processes. Zinc complexes with imidazole derivatives are of a particular interest. Imidazole acts as a structural fragment of many natural physiologically active compounds, thus providing targeted delivery of this essential trace element into the wound for inclusion in the multicascade mechanism of wound healing. **The aim of the study:** to provide experimental evidence for effects of recently developed zinc complexes with N-alkenylimidazole as wound healing agents.

Materials and Methods: Wound-healing effects of six 1% gels containing distinct N-alkenylimidazole zinc complexe derivatives based on the Na-carboxymethylcellulose (Na-CMC) were comparatively studied in 128 outbred white rats of both genders. The Na-CMC-based Zinc Sulfate 1% gel, Methyluracil and Solcoseryl served as reference drugs. After performing the local tolerance study of zinc complexes, linear and planar sterile wounds of comparable size were inflicted in anesthetized animals. The degree of healing was evaluated on the day 8 and day 28 after the treatment start by wound sizes and histological examination of inflammatory response, epithelization, granulation tissue, angiogenesis, and necrosis. The skin microcirculation system was evaluated using the laser Doppler Flowmetry (LDF), whereby the blood flow indicators were recorded 30 and 60 minutes after intraperitoneal administration of the trial compound. The antimicrobial activity of the zinc compounds was determined *in vitro* by means of their minimum inhibitory concentration suppressing the bacteria and fungi growth using the double serial dilution method in liquid culture media. The statistical data processing was performed using the Statistica 12 software package.

Results and Discussion: In the linear wound model, all animals treated with either of six experimental zinc compounds showed almost complete reduction in wound size (92-100%, $p<0.05$) on the day 8, significantly exceeding the wound healing in the control animals (reduction by 67-88 %, $p<0.05$) and effects of the reference drugs (reduction by 83-86%, $p<0.05$). In the planar wound model, the most significant wound healing effect was reached by using the gel containing N-isopropenylimidazole zinc diacetate (encoded as Pilim-1). The respective histological examination showed signs of complete epithelialization, absence of destructive changes in the epidermis, restoration of skin appendages and presence of mature granulation tissue. Intraperitoneal Pilim-1 administration at a dose of 20 mg/kg improved microcirculation in the rat skin, as judged by significant effects on perfusion and the amplitudes of the isolated rhythms of the LDF-gram. In addition, Pilim-1 exerted a moderate bacteriostatic and fungistatic activity, which was 2 times greater than the antimicrobial activity of Metronidazole.

Conclusion: Topical application of gels containing 1% N-alkenylimidazole zinc complex derivatives accelerates the healing of uninfected linear and planar wounds in comparison with the established reference drugs. The Pilim-1 zinc compound exhibited the most pronounced therapeutic effect. The observed *in vitro* antimicrobial action of Pilim-1 is of further interest for potential implications in treatment of infected skin wounds. The regenerative effect of this substance opens prospects for development of new drugs with improved wound healing properties.

Graphical Abstract



Abstract legend: Out of the six tested N-alkenylimidazole zinc complex derivatives, Pilim-1 exhibited the strongest wound healing action, including improved microcirculation and moderate antimicrobial effects.

Keywords

linear wound, N-alkenylimidazole derivatives, reparative regeneration, skin, planar wound, topical therapy, wound healing, zinc

Introduction

Skin wound treatment remains to be a major challenge for modern medicine and cosmetology due to the high incidence of home, industrial and combat injuries. Development of new drugs that accelerate regenerative and reparative skin processes is of high clinical relevance despite a considerable progress in the translational wound healing research.

Drugs based on coordination compounds of vital trace elements typically show substantial activity as ligand systems, simplifying the delivery and incorporation of the ttrace elements at the molecular level. This makes the development of complex substances and their implementation in practical medicine a promising scientific direction.

The complex cascade mechanism of wound healing is known to be controlled by multidomain zinc metalloproteinases (Krishnaswamy et al. 2017). Thus, the epidermal metalloproteinase plays an important role in the supply of zinc, which then accumulates in proliferating cells (Krarup et al. 2017). Previous studies on zinc metabolism during wound healing in the skin of mice showed that zinc accumulates in the wound surface after the induction of metallothionein expression (Lin et al. 2017).

Zinc is required for the processes of migration, proliferation and differentiation of keratinocytes (Lebedeva et al. 2023) and exerts anti-inflammatory, antimicrobial and membrane-stabilizing activity as well. These properties imply wound-healing effects of zinc compounds, such as zinc-containing N-alkenylimidazole derivatives.

Currently used zinc-containing drugs (zinc pyrithione, zinc oxide, and zinc sulfate) have considerable toxic effect, while their efficacy is rather low due to poor absorption. This fact emphasizes the urgent demand for new, effective and safe zinc drugs with wound-healing properties.

The aim of the study: to provide experimental evidence for efficiency of recently developed N-alkenylimidazole-zinc complexes as wound healing agents.

Materials and Methods

Experimental animals

All experiments were performed in healthy outbred white rats of both genders (64 males and 64 females), weighing 220-320 g. Animals were housed in individual cages under stable environmental conditions, humidity (50-70 %), and temperature (20±2°C) in a room with a 12/12-h reversed light/dark cycle. Rats received food and drinking water ad libitum.

The study protocol was approved by the Local Bioethics Committee of I.M. Sechenov First Moscow State Medical University (Sechenov University) of the Ministry of Health of the Russian Federation (8-2 Trubetskaya St., Moscow, Russia), Minutes No. 03-23 of February 16, 2023. The conditions for animal housing and handling complied with the principles of the Declaration of Helsinki on the humane handling to animals, GOST 33044–2014 "Principles of Good Laboratory Practice" approved by order of the Federal Agency for Technical Regulation and Metrology No. 1700-ST of November 20, 2014, and Guidelines for Conducting Preclinical Drug Trials (Mironov 2012) applicable on the territory of the Russian Federation.

The study protocol was designed to minimize animal suffering and decrease the experimental animal number according to the 3R (Reduction, Replacement, Refinement) principle, described in GLP (Good Laboratory Practice) guidelines. Surgical procedures were performed under general anesthesia using intramuscular injection of Zoletil® (Virbac, France) at a dose of 5 mg/100 g of body weight. Animals were humanely euthanized by low flow rates of 100% carbon dioxide in a CO_2-box.

Pharmaceutical substances

Six complexes of zinc with N-alkenylimidazole were synthesized at A.E. Favorsky Irkutsk Institute of Chemistry, Siberian Branch of the Russian Academy of Sciences (Russia). The structural and chemical formulas of these compounds are presented in Table 1.

Acyzol represents the best studied national zinc-containing compound developed in the Russian Federation. Its crystal and molecular structures are presented in Figure 1.

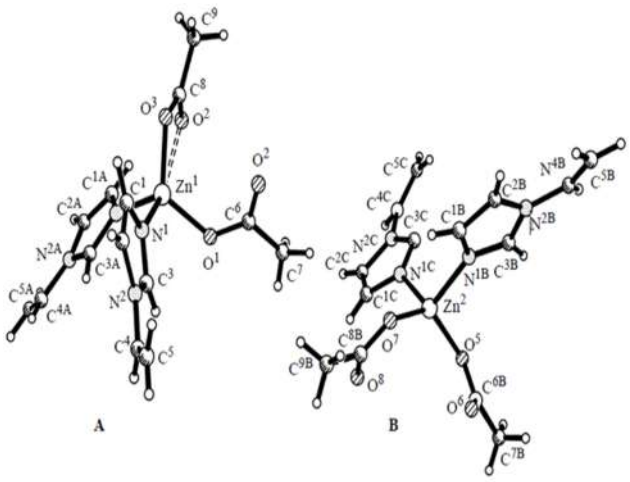
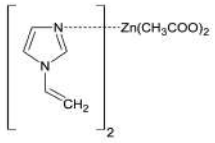
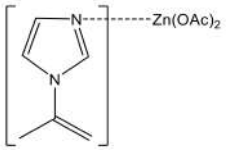
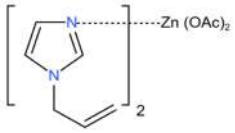
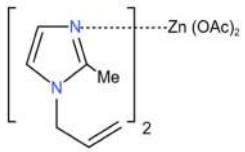
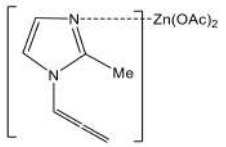
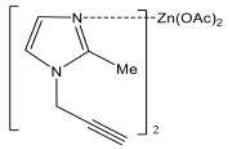


Figure 1. Acyzol crystal and molecular structure (Baikalova et al. 2005).

Initially, Acyzol was implemented in clinical medicine as an antidote for carbon monoxide and other combustion products poisoning (Barinov and Nechiporenko 2006). Further studis have demonstrated its remarkable anti-inflammatory, reparative, detoxifying, immunomodulatory and bacteriostatic effects. Due to the antihypoxic and antioxidant properties, Acyzol exerts membrane-protective, hepatoprotective, adaptogenic and cardioprotective actions (Shakhmardanova et al. 2017; Aliev et al. 2019). Acyzol may further be instrumental for treatment of neurodegenerative diseases requiring correction of zinc homeostasis (Yakimoskii et al. 2017). Acyzolic zinc has been shown to facilitate numerous metabolic cascades associated with zinc-dependent enzyme systems. Hence, this drug can be used in zinc-deficient conditions such as Prasad's syndrome, immunosuppressive disorders, allergic dermatitis, prostate dysfunction or psoriasis. (Aliev et al. 2019).

Table 1. Tested N-alkenylimidazole metal complexes derivatives

№	Chemical substance	Chemical name of substances	Structural formula	Chemical formula
1	Acyzol	bis(N-vinylimidazole) zinc diacetate		$C_{14}H_{18}N_4O_4Zn$
2	Pilim-1	(N-isopropenylimidazole) zinc diacetate		$C_{10}H_{14}N_2O_4Zn$
3	Allyl	bis(N-allylimidazole) zinc diacetate		$C_{16}H_{22}N_4O_4Zn$
4	Allyl-2	bis(N-allylmethylimidazole) zinc diacetate		$C_{18}H_{26}N_4O_4Zn$
5	Allim-2	(N-allenyl-2-methylimidazole) zinc diacetate		$C_{11}H_{14}N_2O_2Zn$
6	Propargyl-2	bis(N-propargyl-2-methylimidazole) zinc diacetate		$C_{18}H_{22}N_4O_4Zn$

The molecular structure of Allyl confirmed by NMR, mass and IR spectroscopy is presented in Figure 2 (Parchina et al. 2019).

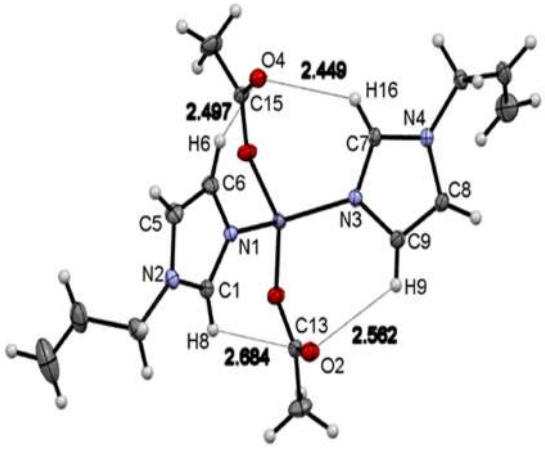


Figure 2. Molecular structure of Allyl with ellipsoids at 50% probability. Selected bond lengths (Å) and angles (°): Zn1–O1 1.977(9), Zn1–O3 1.968(9), Zn1–N1 2.008(1), Zn1–N3 2.002(1); O1–Zn1–O3 99.2(4), O1–Zn1–N1 113.3(4), O1–Zn1–N3 111.4(4), O3–Zn1–N1 107.0(4), O3–Zn1–N3 109.9(4), N1–Zn1–N3 114.8(4) (Parchina et al. 2019).

The hydrophilic base was provided by sodium carboxymethyl cellulose (Na-CMC) – the sodium salt of cellulose ether and glycolic acid, which is a uniform powder of white or gray color; the viscosity of the 1% solution at 25°C is 2200 mPa.s. Solutions of this substance show high binding, dispersing, wetting and adhesive properties and are resistant to microorganisms, non-toxic, physiologically inert, stable in a wide pH range and well absorbed. Na-CMC interacts easily with a number of inorganic substances and can be used in the complex drug development (Ogai et al. 2010). These properties make Na-CMC a suitable gel carrier.

The reference agents included Zinc sulfate 0.5% Na-CMC-based gel, Methyluracil (2,4-dioxo-6-methyl-1,2,3,4-tetrahydropyrimidine) 10% ointment for local use (CJSC "Nizhpharm", STADA, Russia), Solcoseryl, 5% ointment for local use (Legacy Pharmaceuticals, Switzerland), and Metronidazole chemical substance.

Previous research showed that Zinc sulfate transiently dampened inflammation and reduced bacterial growth (Larsen et al. 2017). Study of the healing process showed that the use of $ZnSO_4$ at 1/10 of the LC_{50} caused a significant improvement in tissue reepithelization, angiogenesis and tissue remodeling through overexpression of COLI alpha 1, TGF-beta 1, VEGF-A and FGF-7 (El-Adl et al. 2018).

Methyluracil wound healing properties are based on the stimulation of epidermal proliferation in the injury area (Nozdrin et al. 2002). According to the literature data, Solcoseryl is able to accelerate wound healing by 30% compared with the traditionally used drugs (Andrianova et al. 2016).

Metronidazole (2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanol) is a synthetic chemotherapeutic drug with a broad spectrum of action against anaerobic microorganisms and protozoa. It can be used in combination with Miramistin for the treatment of experimental septic wounds (Grigoryan et al. 2017; Tiganov et al. 2018).

Experimental design

The design of the experiment is schematically shown in Figure 3.

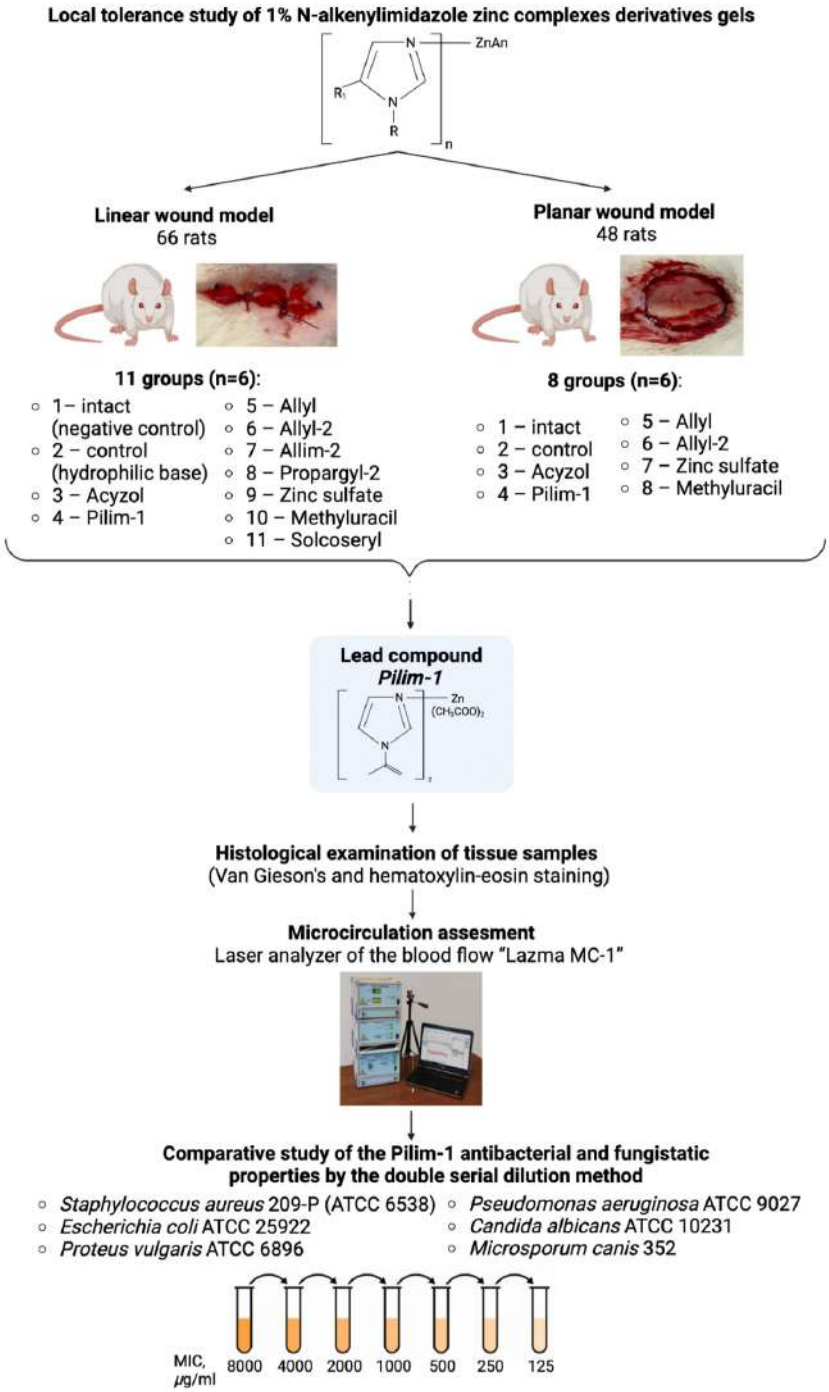


Figure 3. Experimental design. Six experimental zinc-containing compounds were involved in local tolerance study (groups 3-8) controlled by the intact group (group 1), the group receiving gel with the hydrophilic base only (group 2), as well as positive control groups receiving previously established drugs (groups 9-11). The ensuing evaluation of the experimental compounds in linear and planar wounds revealed Pilim-1 as the first-choice candidate. Further histological, microcirculation, and antiseptic studies were performed with Pilim-1 only.

Animal procedures

To perform the local tolerance study of 1% gel containing zinc complexes and Na-CMC, we applied the substances on a 4x4 cm area of rat skin for 4 hours. The day before the experiment, the skin was clean-shaved on both sides of the spine, leaving a 2 cm wide wool coat between the areas. One side was used as a control and the other – for the tested applications. Skin was observed at 1 and 16 h after the substance removal for appearance of erythema as a sign of functional microcirculation disorders or inflammation (Lepeshko and Bondarenko 2017).

Linear and planar wound models in rats were reproduced in compliance with aseptic rules under anesthesia.

Linear wounds. Animals were randomly divided into 11 groups (n = 6 each) including the operated untreated (intact) rats (I) for negative control, C – control (hydrophilic base), or experimental animals receiving Acyzol (D1), Pilim-1 (D2), Allyl (D3), Allyl-2 (D4), Allim-2 (D5), Propargyl-2 (D6), Zinc sulfate (D7), Methyluracil (D8), Solcoseryl (D9). The wool was removed on a 20 mm wide and 50 mm long area of the animal back, and a 30 mm long linear wound was inflicted with a scalpel (wound dimensions were measured to an accuracy of 1 mm). Then, two seams were applied with silk thread at an equal distance. To adequately monitor the wound epithelialization, the applied sutures were not completely tight so that the epithelial layers on the wound side edges had no direct contact to each other.

Immediately after the surgical operation, 1% gel of N-alkenylimidazole zinc complexes or reference drugs were applied to the wound area in the respective experimental groups of animals. The animals of the control group received gel with the same amount of the hydrophilic base (Na-CMC). In intact animals (negative control), the wound healed "naturally". Wounds were left uncovered and air-exposed for the entire time to monitor the native healing process. The location of the wound prevented the rats from accessing the wounds and reopening them. Animals were placed in individual cages.

To assess the wound healing dynamics, the wound size was measured and the skin state was recorded. The general condition of the rats was evaluated by the body weight dynamics. The placebo gel and tested drugs were applied once a day for 7 days.

Re-epithelialization was calculated using the following formula (1):

$$\frac{L\,(mm)}{Ln\,(mm)} \times 100\%, \qquad (1)$$

where L is initial wound length, mm; L_n is length of the wound per day of measurement, mm.

At the end of the study (day 8), the animals were euthanized to obtain skin samples for histological analysis.

Planar wounds. We randomly divided 48 rats into 8 groups of 6 animals in each (I – intact, C – control, D1 – Acyzol, D2 – Pilim-1, D3 – Allyl, D4 – Allyl-2, D5 – Zinc sulfate, D6 – Methyluracil). The wound-healing effect of the zinc metal complexes (Acyzol, Pilim-1, Allyl, Allyl-2) was compared to the control group receiving the Na-CMC-containing gel. Wound healing in the Methyluracil and Zinc sulfate groups compared with intact group with naturally healing wounds. We excluded Allim-2 and Propargyl-2 from the further study as they showed less pronounced effects than the other zinc compounds in the linear wound model.

The planar wound was obtained by cutting a skin section of 706.86 mm^2 with the subcutaneous fat removal. Skin wounds were open throughout the observation period. Wound healing in rats was evaluated by periodical wound patterns on tracing paper and calculating wound area. Local conservative wound treatment was performed in an open manner (without bandage) by applying the test drugs to the wound surface once a day until complete healing for 28 days. Wounds were measured two times a week. The wound healing progress was evaluated by weight change and calculating the wound area. The inflammatory process in each animal group was assessed visually.

The wound healing was calculated by the following formula (2):

$$x = \frac{(S - S_n)}{S} \times 100\%, \qquad (2)$$

where S is initial wound area, mm^2; S_n is wound area on the measurement day, mm^2.

Histological analysis

Wound histological samples were taken on the 8th (linear wound) or 29th (planar wound) day from the beginning of treatment after the animal euthanasia procedure by placing them in a CO_2 chamber. The material was taken by excision of skin with subcutaneous fiber from the wound area to the fascia. After fixing the samples in a 10% neutral formalin solution, paraffin embedding and sectioning (3μm sections) were performed for ensuing hematoxylin-eosin or Van Gieson's staining. Samples were morphologically examined for inflammatory response, epithelization, severity and maturity of granulation tissue, angiogenesis, and necrosis.

Antimicrobial activity tests

We used the double serial dilution method in liquid culture media to study the bacteriostatic and fungistatic activity *in vitro*. As test microorganisms, we used the pathogenic Gram-positive bacteria *Staphylococcus aureus* 209-P (ATCC 6538), Gram-negative bacteria *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 6896 and *Pseudomonas aeruginosa* ATCC 9027, yeast-like fungi *Candida albicans* ATCC 10231 and mycelial fungi *Microsporum canis* 352.

The bacteriostatic and fungistatic drug properties were evaluated using the meat-peptone broth (MPB) or Fluid Sabouraud medium, respectively. For fungistatic tests, cultures were incubated at 30-32°C for 48 hours in the presence of yeast-like fungi or for 10-14 days in the presence of mycelial fungi. At least three independent experiments were performed for each setting. Bacteriostatic and fungistatic effects were defined by means of the minimum inhibitory drug concentration (MIC) suppressing the bacterial growth at which no microorganisms growth was visually observed.

Skin microcirculation

The skin microcirculation was evaluated 15, 30 and 45 min after intraperitoneal (i.p.) injection of the test substance at a dose of 20 mg/kg. To this end, 14 animals with the average motor activity and low affectivity (according to the "open field" test) were divided into the control group (n=7; Group 1) receiving single i.p. injection of the physiological saline (0,2 mL) and the experimental group receiving the test substance at the dose of 20 mg/kg i.p. (n=7; Group 2).

Changes in the skin microcirculation were detected using the laser analyzer of the blood flow "Lazma MC-1" (R&D Company "Lazma Ltd.", Russia). LDF-metry was used to record the following non-oscillometric indicators of the basal flow: microcirculation perfusion (MP, perfusion units, PU), mean square deviation ("flux", RMS, PU), and coefficient of variation (CV, %) (Krupatkin and Sidorov 2005; Krupatkin 2018).

We used the wavelet analysis of the LDF signal to evaluate the amplitudes of blood flow oscillations of different frequency ranges. Endothelial oscillations have the lowest frequency (0.0095-0.02 Hz) due to periodic contractions of the endothelial cells cytoskeleton. Endothelial oscillations reflect the humoral and metabolic factors effects on the microvascular bed and characterize the blood flow condition. Myogenic oscillations have the frequencies of 0.07-0.15 Hz. They are caused by periodic activity of smooth muscle fibers of arterioles, leading to changes in the lumen diameter. Myogenic range is overlapped with neurogenic oscillations (0.02-0.046 Hz) reflecting sympathetic regulatory activity. High-frequency oscillations include respiratory (0.15-0.4 Hz) and pulse (0.8-0.16 Hz) waves. Respiratory waves are represented by periodic pressure changes in the venous section of the vascular channel caused by respiratory excursions of the chest. Pulse blood flow oscillations are caused by intravascular pressure variations, which are to a greater or lesser extent synchronized with cardiorhythm (Hoffman et al. 1990).

Due to the range of oscillations in the amplitude of rates (A), their normalized characteristics were analyzed

$$A_{norm} = \frac{A}{3\sigma}, \qquad (3)$$

where A is the amplitude of oscillations in any range from 0.02–2 Hz.

Such normalization allows excluding the non-standard research condition affects (Krupatkin and Sidorov 2005).

Statistical analysis

The normality of the samples was checked using the Shapiro-Wilk test. As the samples distribution was near to the Gaussian distribution model, the significance of differences between the experimental groups was evaluated with unidimensional analysis of variance with further processing by Student's method of multiple comparisons with Bonferroni adjustment. The statistical processing of the data obtained in the study was carried out using a Statistica 12 software package (StatSoft, USA). The differences were significant at $p \leq 0.05$.

For the statistical processing of the microcirculation parameters, we used nonparametric statistical methods, as the distribution of variable values differed from the Gaussian distribution model. The calculations, statistical processing and graphic design of the data obtained in the study were carried out using a Microsoft Excel program, StatSoft/ STATISTICA 8 software package. Statistical significance of differences between control (i.p. injection of physiological saline) and experimental groups with different measurement interval (15, 30, and 45 min) was determined using the Mann-Whitney U test at $p \leq 0.05$.

Results and Discussion

Evaluation of skin condition 1 and 16 h after application of 1% gels of Na-CMC-based zinc complexes revealed no appearance of skin erythema in rats.

Linear wound model

The dynamics of regenerative processes reflected by daily wound size decreases showed significantly faster reductions in would size in D1-6 animal groups compared to control rats (Fig. 4).

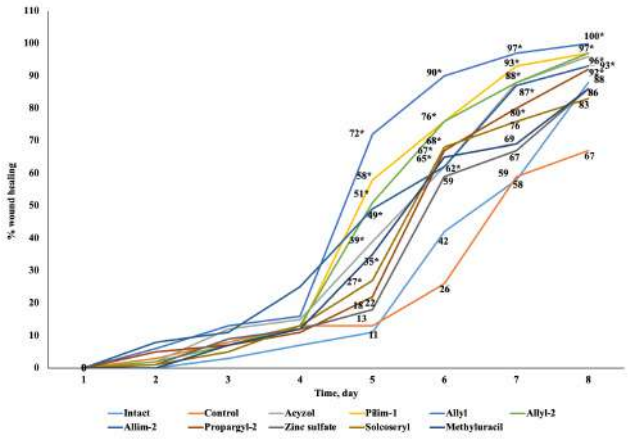


Figure 4. Comparative analysis of linear wound length dynamics. *Note:* * – $p \leq 0.05$ compared to the intact and control groups. The percentage values of wound healing refer to the initial wound length.

By the end of the observation period (8 days), all animals in the D1-D6 groups reached 92-100% wound healing (Fig. 5).

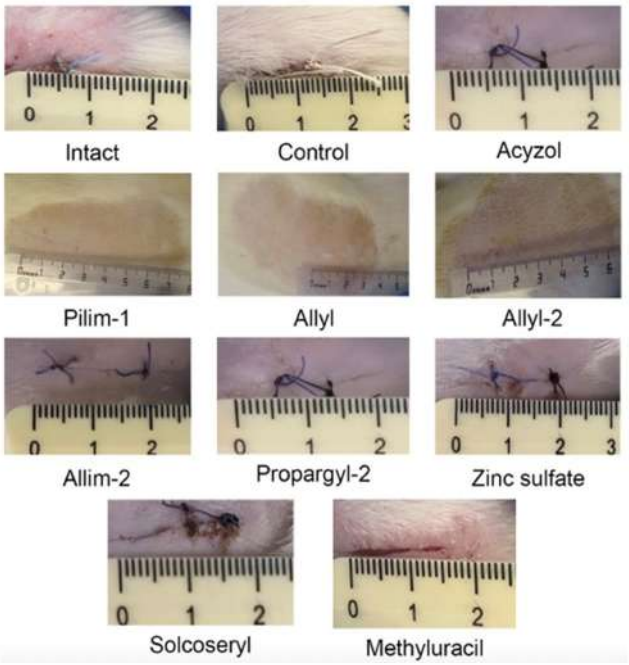


Figure 5. Linear wounds appearance on the 8th day of the experiment. Note nearly complete wound closure in the D1-6 animal groups.

Compared to the experimental compound groups (D1-D6), the wound-healing in the intact and control (compound-free gel) groups showed significantly slower

progress reaching 67% and 88% by the end of observation period, respectively.

In positive control animal groups treated with Methyluracil and Solcoseryl, significant differences with the untreated rats were observed on the 5th and 6th days. Wound healing in these groups amounted to 35% and 65% (Methyluracil) and 27% and 68% (Solcoseryl) on the 5th and 6th days, whereas in the untreated group the wound healing reached 11% and 42%, respectively.

Planar wound model

We observed the positive dynamics in the regeneration process in all studied animals by the 10th day of the observation (Table 2).

We observed increases of wound areas in Methyluracil, Zinc sulfate and Allyl groups on the 3rd observation day, as well as in the Acyzol group on the 3rd and 7th days. These increases may reflect a hyper-reaction at the stage of acute inflammation due to severe swelling in the lesion area affecting the surrounding intact tissue.

As shown in Table 2, significant reductions in wound sizes compared to control animals were observed in the Pilim-1 and Allyl groups starting from the 21st day of the treatment. In addition, significantly stronger healing compared with the control was detected in the Pilim-1 group on the 10th day of treatment.

The photographical images below demonstrate the wound appearance on the 29th day after surgery (Fig. 6).

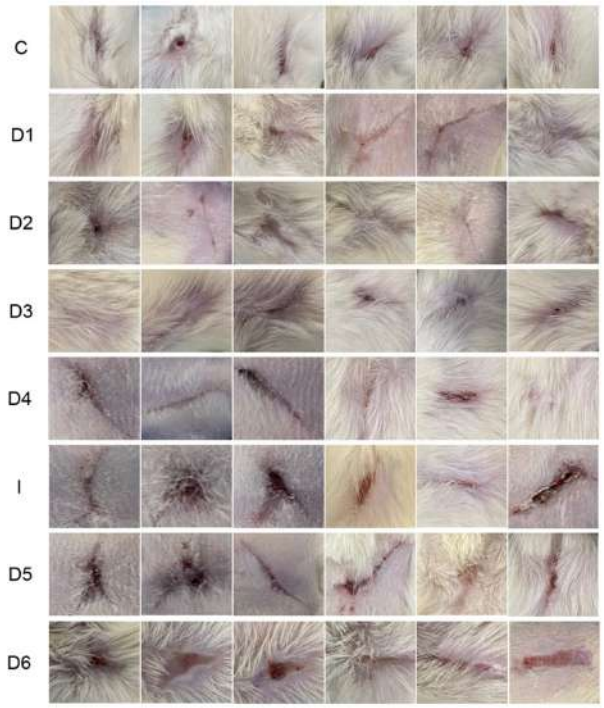


Figure 6. Photographs of planar wounds macroscopic appearance on 29th day after surgery (n=6). *Note:* **C** – control, **D1** – Acyzol, **D2** – Pilim-1, **D3** – Allyl, **D4** – Allyl-2, **I** – intact, **D5** – Zinc sulfate, **D6** – Methyluracil

Table 2. Decrease dynamics of the planar wound area (S, mm²)

Indicators	Experiment time, day							
	3	**7**	**10**	**14**	**17**	**21**	**24**	**29**
Control (Na-CMC)								
S, mm²	659.6±104.90	461.9±90.95	279.2±25.22	92.4±13.50	75.1±6.54	52.6±6.54	28.2±7.07	12.6±0.53
%	7	35	60	87	89	93	96	98
Acyzol								
S, mm²	780.1±121.20	799.3±136.70	338.4±35.30	140.6±18.04	96.0±7.07	56.6±2.65	17.1±6.01	0.5±0.20
%	-10	-21*	52	80	86	92	98	100*
Pilim-1								
S, mm²	625.9±97.04	455.7±87.45	176.5±68.6	70.1±13.08	63.8±17.5	21.3±7.60	2.1±0.53	0
%	11	36	75*	90	91	97*	100*	100*
Allyl								
S, mm²	806.0±208.0	466.6±89.03	244.6±67.0	103.3±17.5	70.6±8.66	5.5±2.47	2.08±0.53	1.04±0.53
%	-14	34	65	85	90	99*	100*	100*
Allyl-2								
S, mm²	632.0±46.79	375.2±63.84	153.7±9.68	125.5±10.59	60.1±11.17	27.0±6.17	15.9±3.62	3.5±1.29
%	11	47	78	82	91	96	98	99
Intact								
S, mm²	743.4±103.1	353.4±48.59	190.9±16.7	114.3±6.56	77.8±17.79	40.6±4.71	32.4±5.89	22.9±5.14
%	-5	50	73	83	89	94	95	97
Zinc sulfate								
S, mm²	841.2±196.4	652.1±206.6	358.7±79.43	130.8±23.44	87.8±18.15	43.0±6.48	21.8±4.51	13.5±5.19
%	-19	8	49	81	88	93	96	98
Methyluracil								
S, mm²	855.8±245.8	612.9±244.7	274.0±99.5	126.1±13.08	123.5±9.5	80.4±13.08	34.3±12.0	6.7±0.5
%	-21	13	61	82	83*	89*	95	99

Note: Results are presented as mean±standard error of the mean (M±m). * – statistically significant differences ($p \leq 0.05$) in comparison with the control or intact groups. The percentage of wound-healing refers to the initial wound area on day 0 (706.86 mm²).

Histological examination

Re-epithelialization is critical to wound healing. It involves inflammation, proliferation, migration and differentiation of keratinocytes. Skin defect size reduction is the main criterion of the wound healing process. Histological examination of wound marks allows assessing the nature of wound healing including the repair vs. purulent processes.

Histological analysis of skin samples obtained in the Pilim-1 group showed a complete wound closure at epidermal and dermal levels in the control and experimental groups on the 7th and 28th days after the application of linear or planar wounds, respectively. The thickness of collagen fiber bundles in the dermal reticular level directly in the damage zone both in the experiment and control groups was smaller compared to more intact zones at a distance from the damage zone. However, the damage zone appeared visually wider in control animals than in the Pilim-1 group, especially in the deep layer of the dermis.

The epidermis of control animals exhibited edema with marked vacuolar hydropic degeneration and hyperkeratosis, hydropic degeneration of the basal layer and partial rejection of the epithelium from the papillary layer of the dermis, indicating destructive processes in the basal membrane. In contrast, skin samples from the Pilim-1 group showed no obvious destructive alterations (Fig. 7).

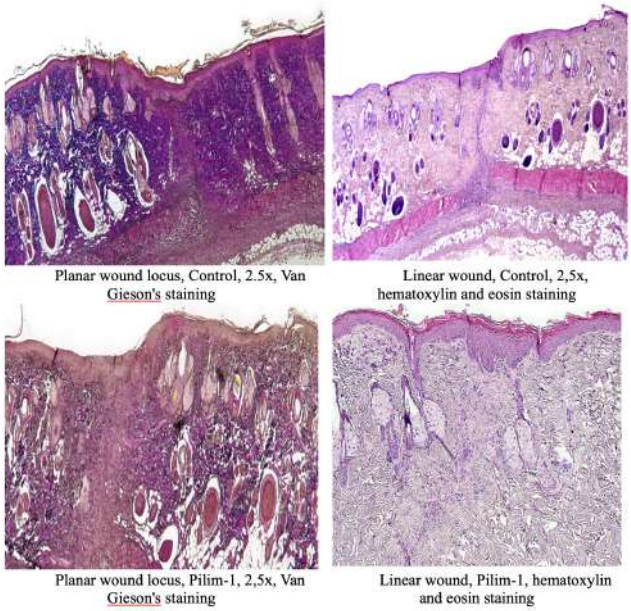


Figure 7. Comparative histological analysis of skin samples of linear and planar wound in rats treated with Pilim-1.

Effect of Pilim-1 on microcirculation

Assessment of microcirculation may help to understand the wound healing mechanism of the tested drugs. Microcirculatory disorders cause systemic and regional hemodynamic disturbances and impair tissue viability and functioning. We have used a previously established technique to evaluate microcirculation changes in skin after systemic application of Pilim-1 with the idea that detected effects may be extrapolated to its local action on the microvascular wound environment.

We found out that Pilim-1 (20 mg/kg) caused considerable changes in all tested skin microdynamic parameters compared to the control group 15 minutes after the i.p. administration of the drug (Fig. 3.).

The stability of the amplitudes of perfusion oscillations in the endothelial range (Ae) parameter throughout the whole time of the experiment indicates that the drug has no effect on the endothelial functions such as synthesis and release of molecules involved in the regulation of vascular tone.

The perfusion indicator (microcirculation perfusion, MP) parameter shows the value of integral erythrocyte velocity in the probed volume of tissue up to 1 mm^3 (PU), increased after 15 min by 27.51% ($p \leq 0.05$) and most pronouncedly after 30-45 min by an average of 48.41% ($p \leq 0.05$) in relation to the values of this index in the control group.

By the 30th min of the study, we observed an increase of amplitudes of perfusion oscillations in the neurogenic range (An) by 27.90% ($p \leq 0.05$) in comparison with the control group values. Maximum changes of An by 29.77 % ($p \leq 0.05$) were observed 45 min after administration of Pilim-1 at a dose of 20 mg/kg compared to hose in the control (Fig. 8).

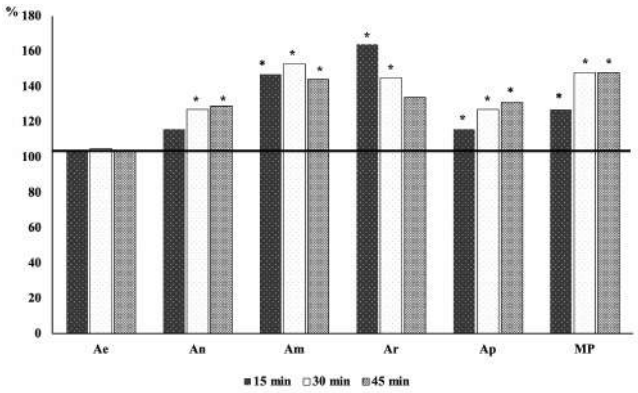


Figure 8. Microcirculation indicators after Pilim-1 applying (20 mg/kg) compared to control group. *Note:* The values obtained in the control group are set at 100%. Ae – amplitudes of perfusion oscillations in the endothelial range; An – amplitudes of perfusion oscillations in the neurogenic range; Am – amplitudes of perfusion oscillations in the myogenic range; Ap – amplitudes of perfusion oscillations in the pulse range; Ar – amplitudes of perfusion oscillations in the respiratory range. MP is a perfusion indicator. * – data labeling is the confidence level of the differences in the experimental group according to the Mann-Whitney U test, with $p \leq 0.05$.

The LDF-gram oscillation amplitudes in the neurogenic range represent the sympathetic adrenergic influences on smooth muscles of arterioles and arteriolar areas of arterio-venular anastomoses. Their increase suggests a decrease in peripheral resistance in circulatory system. The consequence of this process is an improvement of the peripheral blood flow.

Animals receiving Pilim-1 at a dose of 20 mg/kg, showed significant Am level increases by 46.51% within 15 min, by 51.27% within 30 min and by 42.44% within 45 minutes after injection in comparison with the control group. Increased amplitudes of myogenic rhythms may reflect the decreased tone of precapillary sphincters and precapillary metarterioles. The myogenic range rhythms are caused by the Ca^{2+} concentration fluctuations through the muscle cells membranes; therefore, the increase in Am rhythms may be related with the reduced precapillary tone due to Ca^{2+}-dependent muscle relaxation in response to Pilim-1.

Pilim-1 administration at a dose of 20 mg/kg resulted in a significant change in amplitudes of perfusion

oscillations in the respiratory range (Ar) parameter, with a maximum increase of 62.86% ($p \leq 0.05$) observed 15 min after the administration of the drug. After 30 and 45 min of the study, increases of Ar reached 43.17% and 33.34%, respectively, as compared to the Ar parameter in the control group.

Respiratory range was represented by periodic pressure changes in the venous section of the vascular bed caused by diaphragmatic excursions and reflects venous outflow.

After the Pilim-1 administration at a dose of 20 mg/kg to rats, we observed a significant increase in Ap after 15, 30, and 45 minutes by 16.43%, 27.08%, and 31.65%, respectively, compared to the control group values.

Pulse blood flow fluctuations were caused by intravascular pressure variations, which are to a greater or lesser extent synchronized with cardiorhythm.

Thus, Pilim-1 i.p. administration at a dose of 20 mg/kg has significant effects both on perfusion and the isolated rhythms amplitudes of the LDF-gram. This effect is likely independent of endothelial metabolic processes such as nitric oxide release.

Study of the Pilim-1 antibacterial and fungistatic properties

Antibacterial properties of metal-containing compounds are of great interest because bacteria have not developed resistance to them yet. Antibacterial properties of zinc cations have been demonstrated on clinical isolates of the *Str. pyogenes* (Checknov et al. 2017), *S. aureus* and *P. aeruginosa* (Checknov et al. 2015). The protective effect of zinc cations applied to an antibiotic disk has now been shown against *S. aureus* bacteria (Checknov et al. 2019).

Recent study revealed the ability of zinc to block the SOS-system reactions, leading to the bacterial antibiotic resistance through the induction of hypermutagenesis (Bunnell et al. 2017). This should increase the efficacy of antibiotics against chronic infections and help to achieve the complete pathogen elimination.

ZnO nanoparticles (ZnONPs) are known to have antimicrobial activity against Gram-positive and Gram-negative bacteria, as well as against bacterial spores (Raghunath and Perumal 2017). The mechanisms of antibacterial activity of ZnO particles are not fully understood. Some studies have proposed that the main factor of antibacterial activity may be the formation of hydrogen peroxide (Xie et al., 2011) or binding of ZnO particles to the bacterial surface due to electrostatic forces. Nanoparticles also shown to produce the ROS amount that increase the membrane lipid peroxidation level, resulting in the leakage of membrane reducing sugars, proteins, DNA, and reduce the viability of bacterial cells (Tiwari et al. 2018).

We found out that Pilim-1 has moderate bacteriostatic activity against Gram-positive bacteria *Staphylococcus aureus* 209-P, Gram-negative bacteria *Esherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 6896, *Pseudomonas aeruginosa* ATCC 9027 at concentrations of 125, 250, 250 and 500 µg/mL, respectively. The study of fungistatic action of the test substances showed a weak activity against yeast-like fungus *Candida albicans* ATCC 10231 and mycelial fungus *Microsporum canis* 352 at a concentration of 1000 µg/mL The metronidazole activity against all studied microorganisms strains was two times lower (Table 3).

Table 3. MIC tested substances *in vitro*, µg/ml

Microbial strains	MIC			
	Pilim-1	Zinc sulfate	Na-CMC	Metronidazole
Staphylococcus aureus 209 P	125	4000	4000	500
Escherichia coli ATCC 25922	250	2000	1000	500
Proteus vulgaris ATCC 6896	250	4000	4000	500
Pseudomonas aeruginosa ATCC 9027	500	4000	4000	1000
Candida albicans ATCC 10231	1000	2000	8000	4000
Microsporum canis 352	2000	2000	8000	4000

The results of Pilim-1 antimicrobial action *in vitro* suggest therapeutic benefits of this compound for treatment of infected wounds.

Conclusion

Zinc-containing drugs, such as zinc oxide, zinc sulfate, zinc hyaluronate, and zinc pyrithione, are widely used in skin injury treatment. However, these low-molecular inorganic zinc compounds cannot provide sufficient zinc accumulation in the skin, due to poor bioavailability, which results in their low efficacy and pronounced side effects.

The obtained results on wound-healing effects of the studied zinc complexes with N-alkenylimidazoles point to a considerable potential for implementation in medical practice. Studied metal complexes represent structural fragments of many natural physiologically active compounds that play important roles in enzymatic processes in living organisms. Zinc is involved in the processes of keratinization, migration, differentiation and proliferation of keratinocytes, skin regeneration and has anti-inflammatory and antibacterial properties. Zinc is also necessary for the immune system, which plays an important role in wound healing processes.

Phases of wound healing depend on reactive species-mediated signaling, redox level, and wound oxygenation (Roy et al. 2006). The deteriorated subcellular redox signaling is considered to be the main factor for the chronic wound development. The redox modulating pharmacologically active substances may open a new avenue in wound healing management (Atayik and Çakatay 2023).

Our earlier study showed that the metal complex compounds derivatives of N-alkenylimidazole may act as redox regulators restoring the shift of the redox potential caused by hypoxic conditions (Shakhmardanova et al. 2016).

Thus, dermatotropic action of zinc complexes may be provided by the organic ligand N-alkenylimidazole enabling high bioavailability of zinc and allowing its efficient incorporation in metabolism by reproducing the functions of cell metalloenzymes. We assume that wound-healing effects of the tested compounds are mediated by enhanced bioavailability resulting in improved

therapeutic action by resolving hypoxia, microcirculation improvement, reduction of free-radical generation, and stimulation of cell proliferation.

Conflict of interest

The authors have no conflict of interests to declare.

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