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INFLUENCE OF NEW ANTIMICROBIAL PEPTIDES OF THE MEDICINAL LEECH *HIRUDO MEDICINALIS* ON THE FUNCTIONAL ACTIVITY OF NEUTROPHIL GRANULE PROTEINS

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BACKGROUND: Resistance of microorganisms caused dangerous to human health infections to traditional antibiotics is a serious problem for healthcare. In this regard, the development of new effective antimicrobial drugs and therapeutic approaches is an urgent task. Antimicrobial peptides (AMPs) are considered a promising alternative to traditional antibiotic in the fight against resistant microorganisms.

AIM: The aim of this work is to study the effect of new synthesized AMPs of the medicinal leech *Hirudo medicinalis* (including under conditions of development of oxidative/halogenative stress) on the functional activity of neutrophils granular proteins — the main effector cells of the immune system.

MATERIALS AND METHODS: Myeloperoxidase peroxidase activity was assessed by the rate of *o*-dianisidine oxidation. Neutrophil elastase activity was determined by the fluorescence method using a specific substrate MeOSuc-AAPV-AMC. Lactoferrin iron-binding activity was assessed spectrophotometrically by the change in absorption of protein solution after addition of Fe^{3+} salt. Lysozyme activity was determined by the rate of *M. lysodeikticus* bacterial cells lysis.

RESULTS: Native AMPs 536_1 and 19347_2 inhibited and 12530 increased myeloperoxidase peroxidase activity, this tendency persisted after these AMPs modification by hypochlorous acid (HOCl). In contrast to the native AMP halogenated AMP 3967_1 acquired the ability to enhance myeloperoxidase enzymatic activity. In the presence of AMP 3967_1 neutrophil elastase amidolytic activity increased insignificantly, while AMP 19347_2 inhibited neutrophil elastase activity. After HOCl modification these AMPs retained their ability to regulate neutrophil elastase activity. Synergistic effects (~20%) against gram-positive bacteria *M. lysodeikticus* were revealed for combination of lysozyme with AMPs 12530 and 3967_1. Inhibition lysozyme antimicrobial activity was observed in the presence of AMPs 19347_2 and 536_1, however the severity of this effect decreased after AMPs modification by HOCl. After HOCl modification AMP 3967_1 increased, while AMP 12530 on the contrary acquired the ability to inhibit lysozyme mucolytic activity.

CONCLUSIONS: The use of drugs based on studied AMPs of medicinal leech will have a beneficial effect on the body's fight against infectious agents due to the antimicrobial action of AMPs themselves. But in addition studied AMPs are capable to modulate the biological activity of own endogenous antimicrobial proteins and peptides: to enhance it, if it is necessary to eliminate pathogen and to inhibit — if it necessary to protect against damage to the body's own tissues.

Keywords: antimicrobial peptides; medicinal leech; myeloperoxidase; lysozyme; elastase; lactoferrin; neutrophils; hypochlorous acid; halogenative stress.

List of abbreviations

AMP: antimicrobial peptides; LF: lactoferrin; MIC_{max}: the maximum value among the minimum AMP concentrations required to achieve 100% inhibition of the growth of microorganisms; MPO: myeloperoxidase; NE: neutrophilic elastase; PA_{MPO}: peroxidase activity of myeloperoxidase; HOCl: hypochlorous acid.

ВЛИЯНИЕ НОВЫХ АНТИМИКРОБНЫХ ПЕПТИДОВ МЕДИЦИНСКОЙ ПИЯВКИ *HIRUDO MEDICINALIS* НА ФУНКЦИОНАЛЬНУЮ АКТИВНОСТЬ БЕЛКОВ ГРАНУЛ НЕЙТРОФИЛОВ

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Обоснование. Серьезной проблемой здравоохранения является резистентность к традиционным антибиотикам микроорганизмов, вызывающих опасные для здоровья человека инфекции. В связи с этим разработка новых эффективных антимикробных препаратов и терапевтических подходов представляется актуальной задачей. Антимикробные пептиды считают перспективной альтернативой традиционным антибиотикам в борьбе с резистентными микроорганизмами.

Цель — изучение влияния новых синтетических антимикробных пептидов медицинской пиявки *Hirudo medicinalis* на функциональную активность гранулярных белков нейтрофилов — основных эффекторных клеток иммунитета, в том числе в условиях развития окислительного/галогенирующего стресса.

Материалы и методы. Пероксидазную активность миелопероксидазы оценивали по скорости окисления *о*-дианизидина. Амидолитическую активность нейтрофильной эластазы определяли флуоресцентным методом с использованием специфического субстрата MeOSuc-AAPV-AMC. Железосвязывающую активность лактоферрина оценивали спектрофотометрическим методом по изменению поглощения раствора при добавлении соли Fe^{3+}. Активность лизоцима определяли по скорости лизиса бактериальных клеток *M. lysodeikticus*.

Результаты. Нативные антимикробные пептиды 536_1 и 19347_2 ингибировали, а антимикробный пептид 12530 — усиливал пероксидазную активность миелопероксидазы, данная тенденция сохранялась и после модификации указанных пептидов хлорноватистой кислотой (HOCl). В отличие от нативного галогенированный антимикробный пептид 3967_1 усиливал ферментативную активность миелопероксидазы. В присутствии антимикробного пептида 3967_1 амидолитическая активность нейтрофильной эластазы незначительно увеличивалась, в то время как антимикробный пептид 19347_2 ингибировал активность фермента. После модификации HOCl данные соединения сохраняли свою способность регулировать активность нейтрофильной эластазы. Синергетические эффекты (~20 %) против грамположительных бактерий *M. lysodeikticus* выявлены для комбинации лизоцима с антимикробными пептидами 12530 и 3967_1. Ингибирование антимикробной активности лизоцима наблюдалось в присутствии антимикробных пептидов 19347_2 и 536_1, но выраженность данного эффекта снижалась после их модификации под действием HOCl. После модификации HOCl антимикробный пептид 3967_1 усиливал, в то время как 12530, наоборот, приобретал способность ингибировать муколитическую активность лизоцима.

Заключение. Использование препаратов на основе исследуемых антимикробных пептидов медицинской пиявки будет оказывать благоприятное влияние на борьбу организма с возбудителями инфекций не только за счет антимикробного действия самих антимикробных пептидов, но и за счет модуляции данными соединениями биологической активности собственных эндогенных антимикробных белков и пептидов: усиления — в случае необходимости элиминации патогена и ингибирования — для защиты от повреждения собственных тканей организма.

Ключевые слова: антимикробные пептиды; медицинская пиявка; миелопероксидаза; лизоцим; эластаза; лактоферрин; нейтрофилы; хлорноватистая кислота; галогенирующий стресс.

Background

Antibiotic resistance is a serious public health problem due to the increasing global availability of antibacterial drugs and inappropriate prescription, dosage, and course duration, as well as the use of such drugs in animal husbandry. Therefore, large-scale research is globally performed to develop new non-traditional anti-infectious treatment methods. Antimicrobial peptides (AMPs) quickly attracted the attention of researchers as promising candidates for creating drugs based on them, devoid of several disadvantages of traditional antibiotics [1]. AMPs represent a large and diverse group of short (10–50 amino acid residues), amphipathic, and as a rule, positively charged molecules that are produced by many

tissues and cell types in various species of invertebrates, plants, and animals.

The mechanisms of action of AMPs are diverse, they disrupt the integrity of the plasma membrane, inhibit the synthesis of cell wall components, modify the cytoplasmic membrane by inhibiting septum formation, trigger autolysis processes, bind to DNA, and suppress replication processes, transcription, and translation, as well as inhibit the activity of several enzymes, and exhibit a microbicidal effect due to the regulation of innate and acquired immunity [2]. AMPs appear to be a promising compound base for the development of new antibacterial, antifungal, antiviral, and anticancer therapeutic agents due to such a wide range of biological activities [3, 4].

AMP can be obtained from microorganisms, plants, and animals. Such AMPs can cause side effects with a low probability; therefore, natural AMPs are used as a base for creating new synthetic AMPs with various modifications aimed at improving their antimicrobial activity, stability, and efficiency, reducing toxicity related to the macroorganism's cells, as well as increasing their resistance to digestive enzymes [1, 5].

The salivary gland secretion of the medicinal leech *Hirudo medicinalic* is a humoral agent of hirudotherapy, which is a widely used method of treating many diseases using leeches. Hirudotherapy has a multifactorial effect, including the antimicrobial and immunostimulating effects induced, among other things, by antimicrobial proteins and peptides [6, 7]. Earlier bioinformatic analysis of the genome of the medicinal leech *Hirudo medicinalic* identified and synthesized cationic peptides with antimicrobial properties Phe-Arg-Ile-Met-Arg-Ile-Leu-Arg-Val-Leu-Lys (AMP 3967_1), Lys-Phe-Lys-Lys-Val-Ile-Trp-Lys-Ser-Phe-Leu (AMP 12530), Arg-Trp-Arg-Leu-Val-Cys-Phe-Leu-Cys-Arg-Arg-Lys-Lys-Val (AMP 536_1), and Arg-Pro-Ile-Leu-Ile-Arg-Val-Arg-Arg-Ile-Arg-Val-Ile (AMP 19347_2) [8]. However, it should be kept in mind that using AMPs as antibacterial therapeutic agents in foci of infection, these compounds will inevitably come into contact with neutrophils, which are cells that primarily migrate from the bloodstream to the site of pathogen localization. Neutrophilic granulocytes serve as the most significant effector cells of the innate immune system and provide the body's primary line of defense against infectious agents [9]. Neutrophilic granulocytes possess powerful oxygen-dependent and oxygen-independent mechanisms that ensure the performance of microbicidal, cytotoxic, and cytolytic functions, initiating a basic inflammatory response development. The cytolytic and cytotoxic potential of neutrophils is concentrated in the granular apparatus and secretory vesicles [10, 11]. The secretion of granular contents occurs not only during phagocytosis at the sites of inflammation. Granular proteins and peptides, the levels of which can vary both in physiological conditions and in certain diseases, always occur in normal blood plasma.

When AMPs interact with granular proteins of neutrophils, the biological functions of these compounds are modified. For example, [12] demonstrated that when defensin neutrophils own AMPs bind to proteins from the family of serine protease inhibitors (serpins), the inhibitory effect of the latter against proteases decreases, and the antimicrobial activity of defensins is also blocked. In [13], data were obtained that support the assumption of the possibility of binding of defensins and some other AMPs to the serpin family proteins that do not exhibit antiprotease activity (corticosteroid-binding globulin [transcortin] and thyroxine-binding globulin) and do not modulate the biological activity of transcortin. Therefore, studying the effect of new synthesized AMPs of medicinal leech on the biological activity of the main granular proteins of neutrophils, namely myeloperoxidase (MPO), neutrophil elastase (NE), lactoferrin (LF), and lysozyme is of interest.

Hypochlorous acid (HOCl) should be kept in mind to inevitably be produced in the inflammation foci due to the functioning of the enzyme of azurophil granules of MPO neutrophils, which is, on one hand, the main antimicrobial agent of neutrophils, on the other hand, retains the ability to damage own biologically important molecules, thereby changing their basic functions. Thus, NE modification with high concentrations of HOCl (when the molar ratio of HOCl/enzyme exceeded 10–40 times) was previously revealed to lead to enzyme inactivation [14]. Lysozyme modified by HOCl lost its ability to lyse bacterial cells of *Micrococcus lysodeikticus* [15]. Earlier, we established that after HOCl treatment in the MPO molecule, heme degrades, which leads to a sharp decrease in the catalytic activity of the enzyme [16]. After HOCl modification,

the iron-binding activity of LF decreases [17]. The ability of the studied cationic AMPs of the medicinal leech to regulate the biological activity of the main granular proteins of neutrophils will change after the HOCl modification is unknown.

Therefore, **this study aimed** to study the ability of native and HOCl-modified new artificially synthesized AMPs of the medicinal leech *Hirudo medicinalic* to modulate the biological activity of the main granular proteins of neutrophils (MPO, NE, LF, and lysozyme).

Materials and methods

Reagents

The reagents lysozyme, sodium hypochlorite (NaOCl), *M. lysodeikticus*, and *o*-dianisidine produced by Sigma-Aldrich (USA) and MeOSuc-AAPV-AMC produced by Santa Crus Biotechnology (USA) were used in this study, as well as the rest of the reagents produced by Reakhim (Russia) and Belmedpreparaty (Belarus).

This study also used MPO and NE that was isolated from frozen leukocytes of healthy donors using affinity chromatography using heparin-sepharose and aprotinin-agarose, hydrophobic chromatography (phenyl-sepharose), and gel filtration (Sephacryl S-200 HR), as described earlier [18–20]. The ratio of the absorption values of the MPO preparation at a wavelength of 430 and 280 nm (A_{430}/A_{280}) served as a characteristic of the purity and homogeneity of the isolated MPO and was usually no <0.85. The NE preparation was homogeneous according to electrophoresis and mass spectrometry data and did not contain any impurities of cathepsin G and proteinase-3.

This study also used recombinant human LF that was isolated from milk of transgenic goat producers bred within the scientific and technical program of the Union State "Development of technologies and organization of pilot production of highly effective and biologically safe new generation drugs and food products based on human LF obtained from the milk of animal producers" ("BelRosTransgen-2") [21]. The study [22] demonstrated that recombinant LF is similar in physical, biochemical, and biological characteristics to natural human LF isolated from human milk.

This study used four synthesized AMPs based on bioinformatic analysis of the medicinal leech *Hirudo medicinalis* genome. The characteristics of these AMPs (amino acid sequence, molecular weight, charge, MIC_{max} as the maximum value among the minimum required AMP concentrations to achieve 100% growth inhibition of microorganisms *Escherichia coli, Chlamydia trachomatis*, and *Bacillus subtilis* in a standard test) are presented in Table.

Modification of AMPs by HOCl

A NaOCl solution was immediately prepared before the experiment by diluting the stock commercial solution in phosphate-buffered saline (PBS: 137 mM of NaCl, 2.7 mM of KCl, 8.7 mM of Na_2HPO_4, 1.5 mM of KH_2PO_4, and pH of 7.4). The molecular form of the acid HOCl and its anion OCl^- is approximately in equal amounts, HOCl implies the HOCl/OCl^- mixture since the pKa of HOCl is 7.5 and at physiological pH values in the medium under study.

The studied AMPs contain a different number of amino acid residues that are sensitive to HOCl (Table), thus the molar ratio of AMP to HOCl was individually selected for each AMP so that all HOCl molecules would interact with AMP. The absence of unreacted HOCl was assessed by the fluorescence method using scopoletin, which is instantly oxidized in the presence

Table / Таблица

Characteristics of AMP used in the work
Характеристики используемых в работе антимикробных пептидов

AMP code	AMP amino acid sequence	Length	Molecular weight, Da	Charge	MIC_{max}, μM
3967_1	Phe-Arg-Ile-**Met**-Arg-Ile-Leu-Arg-Val-Leu-**Lys**	11	1444.89	+4	10
12530	**Lys**-Phe-**Lys**-**Lys**-Val-Ile-**Trp**-**Lys**-Ser-Phe-Leu	11	1423.81	+4	90
536_1	Arg-**Trp**-Arg-Leu-Val-**Cys**-Phe-Leu-**Cys**-Arg-Arg-**Lys**-**Lys**-Val	14	1863.36	+6	17
19347_2	Arg-Pro-Ile-Leu-Ile-Arg-Val-Arg-Arg-Ile-Arg-Val-Ile	13	1660.13	+5	77

N o t e. Amino acids that are most sensitive to the HOCl actions are marked in bold.

of HOCl. AMP modification was performed at room temperature (23°C) for 1.5–2 h with moderate stirring (once at the beginning and once at the end of the modification). The molar ratio of AMP:HOCl for AMP 536_1 was 1:20; 12530 was 1:10; 3967_1 was 1:10; and 19347_2 was 1:1.

Amidolytic activity of NE

NE activity was assessed by the fluorescence method using a specific substrate MeOSuc-AAPV-AMC [18]. NE (50 nM) in PBS containing 1 mM of $CaCl_2$ and 0.5 mM of $MgCl_2$ was incubated for 3 min at 37°C with various concentrations of native or HOCl-modified AMP, after which 20 μM MeOSuc-AAPV-AMC substrate was added, and the kinetics of the NE substrate hydrolysis was recorded, accompanied by the release of a fluorophore, aminomethylcoumarin, by increased fluorescence intensity at a wavelength of 460 nm (excitation 380 nm). The area under the kinetic curve of the fluorescence intensity change at 10 min after the NE addition was calculated, including both the maximum fluorescence intensity value due to the formation of a fluorescent reagent and the rate of its formation to quantitatively characterize the NE activity index.

Peroxidase activity of MPO

The MPO peroxidase activity (PA_{MPO}) was assessed by the rate of oxidation by the enzyme *o*-dianisidine [23]. MPO preparation (0.5 nM) was incubated with various native or HOCl-modified AMP concentrations for 5 min at 23°C and was added to 0.1 M of Na-phosphate buffer (pH 6.2) containing *o*-dianisidine (380 μM), after which H_2O_2 (50 μM) was added to the mixture, and the optical density changes of the solution were recorded at a wavelength of 460 nm at 23°C. The tangent of the initial slope of the recorded curves was calculated, which characterizes the rate of *o*-dianisidine oxidation in the presence of MPO, for a quantitative assessment.

The mucolytic activity of lysozyme

Lysozyme activity was determined by the rate of lysis of *M. lysodeikticus* bacterial cells [24]. Lysozyme (50 μg/ml) was incubated with various native or HOCl-modified AMP concentrations for 5 min at 23°C and added to a suspension of *M. lysodeikticus* bacterial cells in 0.1 M of Na_2HPO_4/KH_2PO_4 buffer (pH 6.2) at 37°C, and the change in the light transmission of the resulting suspension was recorded at a wavelength of 540 nm. To quantitatively characterize the activity of lysozyme, the change in light transmission was calculated by the sample compared to the initial level of light transmission at 3 min after the addition of lysozyme to the bacterial cells.

The iron-binding activity of LF

The ability of 10 mg/ml of the LF preparation to bind iron ions in the absence and presence of native or HOCl-modified AMPs at various concentrations was spectrophotometrically recorded by the change in the absorption of the test solution at a wavelength of 465 nm with the successive addition of the Fe^{3+} salt $[NH_4Fe(SO_4)_2 \cdot 12\,H_2O]$ at 23°C and constant stirring [17]. LF in the iron-saturated form is characterized by the appearance of an additional maximum (in comparison with the apo-form) in the region of 400–500 nm. The area under the kinetic curve of the optical density changes of the LF solution after 20 successive additions of the Fe^{3+} salt $[NH_4Fe(SO_4)_2 \cdot 12\,H_2O]$ was determined to quantitatively characterize the iron-binding activity of LF.

A computerized spectrofluorimeter CM2203 (SOLAR, Belarus) was used to implement all fluorescence methods, as well as a computerized spectrophotometer PB2201 (SOLAR, Belarus) – for spectrophotometric methods.

Statistical data analysis

Statistical analysis of results was performed using the Origin 7.0 software package. Data were presented as mean ± standard error of the mean. The student's *t*-test was used to analyze the differences between the mean values. Differences were considered statistically significant at a significance level of $p < 0.05$.

Results and discussion

MPO (donor: H_2O_2-oxidoreductase, K.F. 1.11.2.2) belongs to the family of heme-containing mammalian peroxidases. The main substrate of MPO is H_2O_2 produced *in vivo* during the respiratory burst of neutrophils. In the presence of H_2O_2, MPO is converted into a highly reactive compound I, which has two oxidative equivalents and implements sequential one-electron oxidation of various substrates (nitrite,

ascorbate, tyrosine, etc.) through the formation of compound II with the return of the enzyme to its original form and closing the so-called peroxidase cycle. Unlike most peroxidases, MPO compound I is also capable of simultaneously performing two-electron oxidation of halides with hypohalous acid formation [25, 26].

Figure 1 presents the evaluation results of the peroxidase activity of MPO in the presence of native and HOCl-modified AMPs of the medicinal leech *Hirudo medicinalis*. AMP 536_1 can be seen to have the greatest modulating effect. Thus, in the presence of AMP 536_1, PA_{MPO} changed in a biphasic manner, it increased (by ~1.2 times) in the concentration range of 0.5–2.5 μM and significantly decreased at concentrations of 20–40 μM (for example, PA_{MPO} decreased by a factor of approximately 2 in the presence of AMP 536_1 at a concentration corresponding MIC_{max}) (Fig. 1, *a*). A less pronounced inhibitory effect on PA_{MPO} was exerted by AMP 19347_2, namely at concentrations corresponding to MIC_{max} and higher. This AMP inhibited PA_{MPO} in a concentration-dependent manner (Fig. 1, *b*). A study of AMP 536_1 and 19347_2 effects on the kinetic parameters of PA_{MPO} showed that these compounds are non-competitive inhibitors of MPO since they did not affect the Michaelis constant for H_2O_2 (data not presented). The presence of AMP 12530 at concentrations corresponding to MIC_{max} and higher increases PA_{MPO} by approximately 1.2–1.3 times (Fig. 1, *c*), which may be associated with enzyme native structure stabilization. AMP 3967_1 in the entire studied concentration range did not affect PA_{MPO} (Fig. 1, *d*).

After HOCl modification, the ability of AMP 536_1 to modulate the enzymatic activity of MPO decreased (at low concentrations, modified AMP 536_1 lost its ability to enhance PA_{MPO}, whereas its inhibitory effect decreased by approximately 2 times at high concentrations) (Fig. 1, *a*). After treatment with HOCl, AMP 19347_2 also lost its ability to inhibit the enzymatic activity of MPO by a factor of approximately 2 (Fig. 1, *b*). In the presence of AMP 12530 modified with HOCl, PA_{MPO} increased higher (by ~1.4–1.6 times) than in the presence of native AMP (Fig. 1, *c*). PA_{MPO} in the presence of HOCl-modified AMP 3967_1 most significantly increased (by ~30%–40%) in the range of low (0.5–5 μM) AMP concentrations (Fig. 1, *d*).

The data presented concluded that AMP 536_1 and 19347_2 can be considered promising compounds for the synthesis of more effective pharmacological inhibitors of MPO on their basis. Indeed, for effective inhibition of MPO activity in some cases, a small amino acid sequence consisting, for example, of 10 amino acid residues, is sufficient, as was demonstrated for the ceruloplasmin fragment in [27]. AMP 12530 enhances the catalytic activity of MPO, thereby increasing the production of bactericidal that are reactive halogen species that destroy the pathogen. After HOCl modification, the ability of AMP to regulate the enzymatic activity of MPO changes. The data obtained when developing anti-infectious therapeutic agents based on AMP should be taken into account.

Additionally, the effect of AMP was investigated on another marker enzyme of azurophilic granules of neutrophils, NE. NE belongs to the group of serine proteases, which active center comprises the classical conservative triad of Ser, His, and Asp residues. These residues form an ensemble that implements a nucleophilic attack on the peptide bonds of proteins, which results in their hydrolytic cleavage. Proteases of this class have different substrate specificities. NE is specific for peptide bonds formed by Gly, Ala, Val, Leu, and Ile, which comprise hydrophobic radicals [28]. Depending on the physicochemical factors in pathological processes, NE can function both as a pro-inflammatory and anti-inflammatory agent [29]; therefore, the search for ways to regulate its activity seems to be an urgent task.

The studied AMPs can modulate the MPO activity at concentrations comparable to the MIC_{max}, thus to study the effect of AMPs on the biological activity of the remaining granular proteins of neutrophils, these compounds were used at MIC_{max} concentrations and those 2 times higher and 2 times lower than the MIC_{max}. As seen from the data presented in Fig. 2, the enzymatic activity of NE increased by approximately 30% in the presence of AMP 3967_1 in the studied concentration range (Fig. 2, *a* and *c*). AMP 536_1 and 12530 at the concentrations used did not significantly affect the enzymatic activity of NE (Fig. 2, *c*). AMP 19347_2 in a concentration-dependent manner inhibited the ability of NE to cleave the fluorescent substrate (Fig. 2, *b* and *c*). The inhibitory effect of

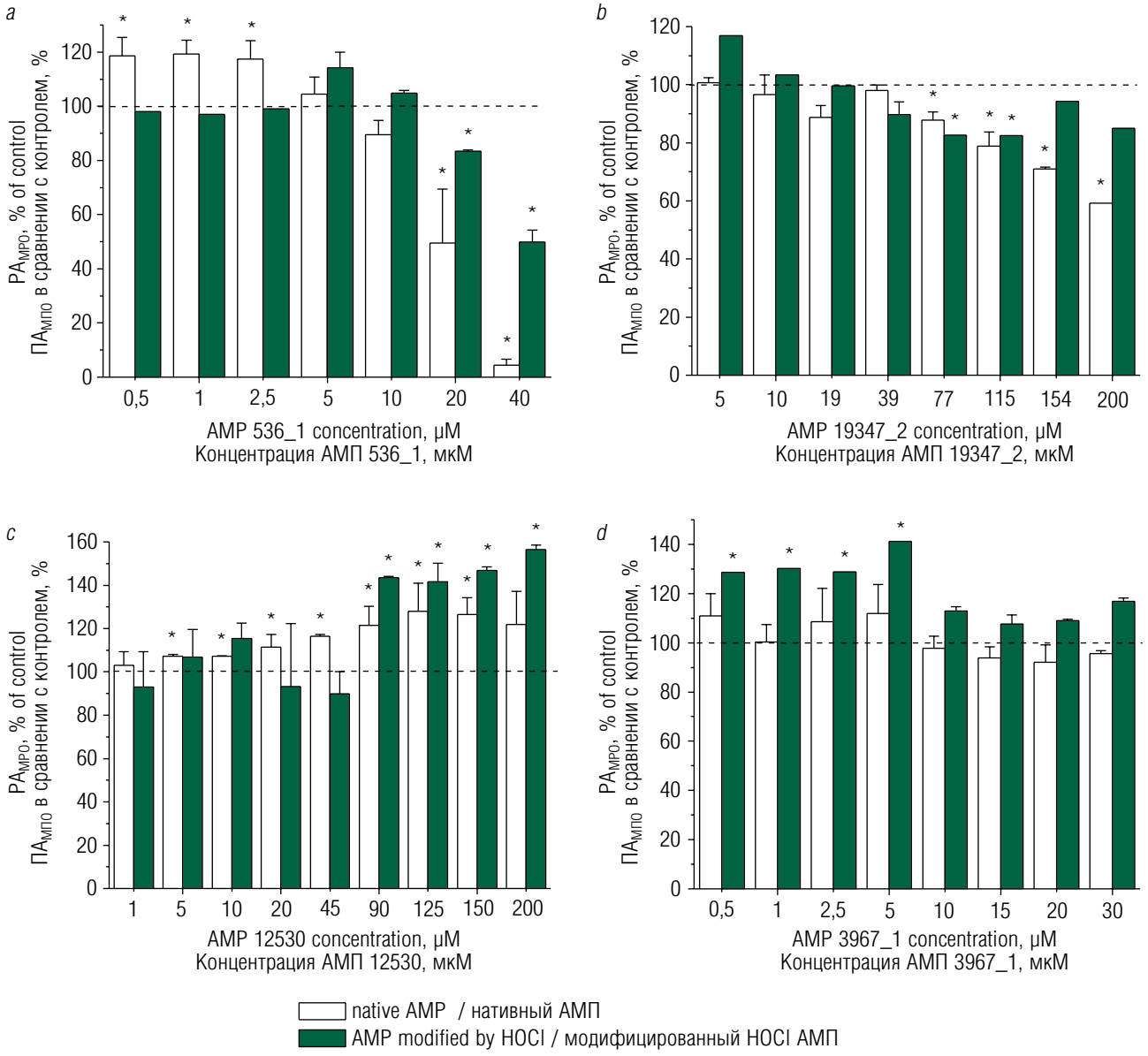
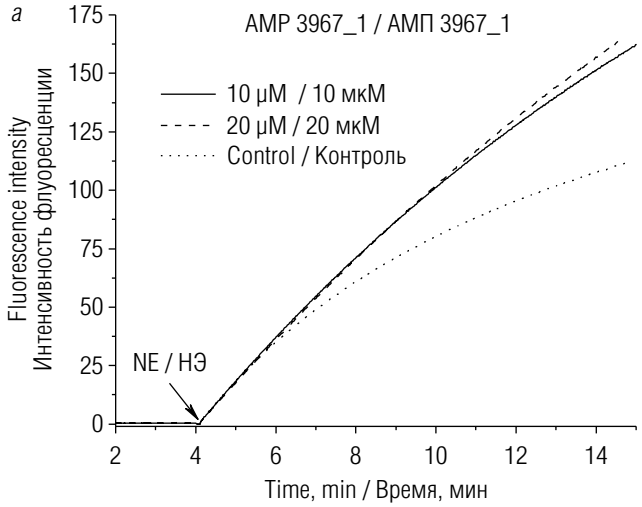


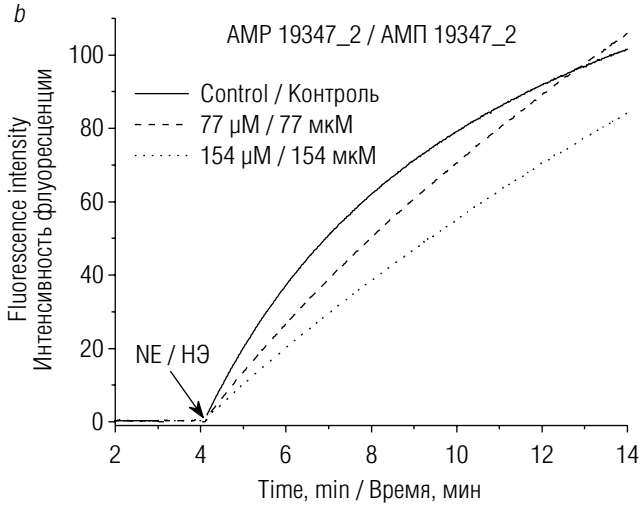
Fig. 1. Effect of native and modified by HOCl AMP 536_1 (*a*), 19347_2 (*b*), 12530 (*c*), and 3967_1 (*d*) in various concentrations on MPO (0.5 nM) peroxidase activity (PA_{MPO}) which was recorded by the oxidation of chromogenic substrate *o*-dianisidine (380 μM) in the presence of H_2O_2 (50 μM). PA_{MPO} in the absence of AMP is accepted as 100%. *$p < 0.05$ compared with the PA_{MPO} in the control (in the absence of AMP)

Рис. 1. Влияние нативных и модифицированных хлорноватистой кислотой (HOCl) антимикробных пептидов (АМП) 536_1 (*a*), 19347_2 (*b*) 12530 (*c*) и 3967_1 (*d*) в различных концентрациях на пероксидазную активность очищенной миелопероксидазы ($ПА_{МПО}$) (0,5 нМ), регистрируемую по окислению ферментом хромогенного субстрата *o*-дианизидина (380 мкМ) в присутствии H_2O_2 (50 мкМ). За 100 % принята $ПА_{МПО}$ в отсутствие АМП. *$p < 0,05$ по сравнению с $ПА_{МПО}$ в контроле (в отсутствие АМП)

AMP 19347_2 on the enzymatic activity of NE may be due AMP 19347_2 contains the most (7) aliphatic amino acid residues among all the used AMPs (4 Ile, 1 Leu, and 2 Val). Peptide bonds formed by these amino acids, which compete with the used substrate to record the enzyme activity, can be targets for NE. AMPs modified by HOCl did not have any different effect from their native analogs on the amidolytic activity of NE (data not presented).

LF is a cationic iron-binding glycoprotein of the transferrin family. LF is mainly contained in the secretions of exocrine glands, respiratory and reproductive systems, breast milk, tears, synovial fluid, and saliva, which indicates the important role of LF in nonspecific protection against pathogenic microorganism invasion. The source of LF in blood plasma are neutrophils, in which this protein is synthesized during their differentiation and maturation, stored in





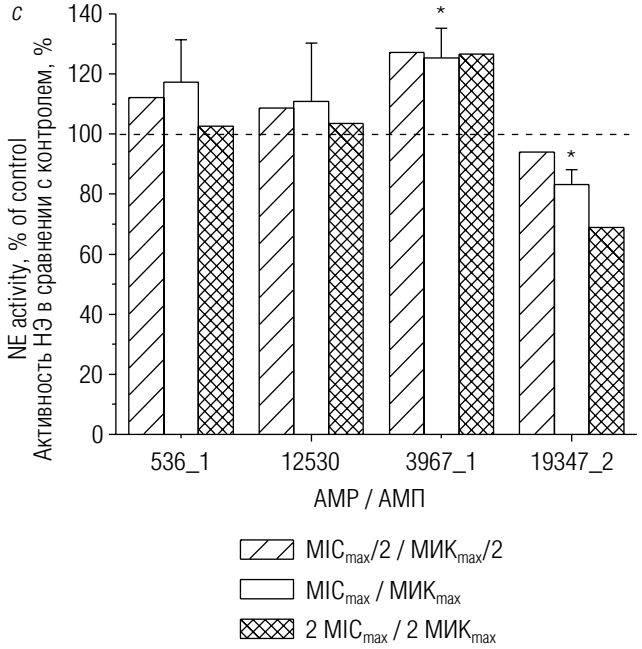


Fig. 2. Effect of AMP on NE enzymatic activity. Typical kinetic curves (*a*, *b*) of an increase in the fluorescence intensity of aminomethylcoumarin formed during the cleavage of a specific substrate MeOSuc-AAPV-AMC (20 μM) by purified NE (50 nM) in the absence and in the presence of AMP 3967_1 (*a*) and 19347_2 (*b*) at various concentrations. AMP concentrations are indicated on the legend to the figure. The arrow indicates the moment of NE addition. Fluorescence intensity was measured at 460 nm, excitation wavelength was 380 nm. Dependence of NE enzymatic activity on AMP concentration (*c*). NE activity in the absence of AMP is taken as 100%. MIC_{max} — minimal inhibitory concentrations against three bacterial species (*Escherichia coli*, *Chlamydia thrachomatis* and *Bacillus subtilis*). $*p < 0.05$ compared to NE activity in the control (in the absence of AMP)

Рис. 2. Влияние антимикробных пептидов (АМП) на ферментативную активность нейтрофильной эластазы (НЭ). Типичные кинетические кривые (*a*, *b*) увеличения интенсивности флуоресценции аминометилкумарина, образующегося при расщеплении специфического субстрата MeOSuc-AAPV-AMC (20 мкМ) очищенной НЭ (50 нМ) в отсутствие и в присутствии АМП 3967_1 (*a*) и 19347_2 (*b*) в различных концентрациях (указаны на легенде). Момент добавления НЭ отмечен стрелкой. Длина волны возбуждения флуоресценции — 380 нм, регистрации — 460 нм. Зависимость ферментативной активности очищенной НЭ от концентрации АМП (*c*). За 100 % принята активность НЭ в отсутствие АМП. $МИК_{max}$ — максимальное значение среди минимальных концентраций АМП, необходимых для достижения 100 % ингибирования роста микроорганизмов *Escherichia coli, Chlamydia trachomatis* и *Bacillus subtilis* в стандартном тесте. $*p < 0,05$ по сравнению с активностью НЭ в контроле (в отсутствие АМП)

specific granules, and successfully degranulated into the extracellular space in case of inflammation. LF has antibacterial, antiviral, antifungal, immunomodulatory, antioxidant, and other beneficial properties [30]. One of the aspects of the antimicrobial action of LF is the high affinity of this molecule for iron ions. Structurally, the LF molecule is divided into two parts, called N- and C-lobes, each of which contains an iron-binding site consisting of 1 Asp, 1 His, and 2 Tyr [31]. The process of binding iron ions is cooperative, as binding of iron in the C-lobe significantly stabilizes this process in the N-lobe.

The study on the effect of AMP on the iron-binding activity of LF revealed that none of the new synthetic AMPs of the medicinal leech in the study in the used concentration range ($MIC_{max}/2$, MIC_{max}, and 2 MIC_{max}) had a significant effect on the ability of LF to bind iron ions (Fig. 3). AMPs modified by HOCl also did

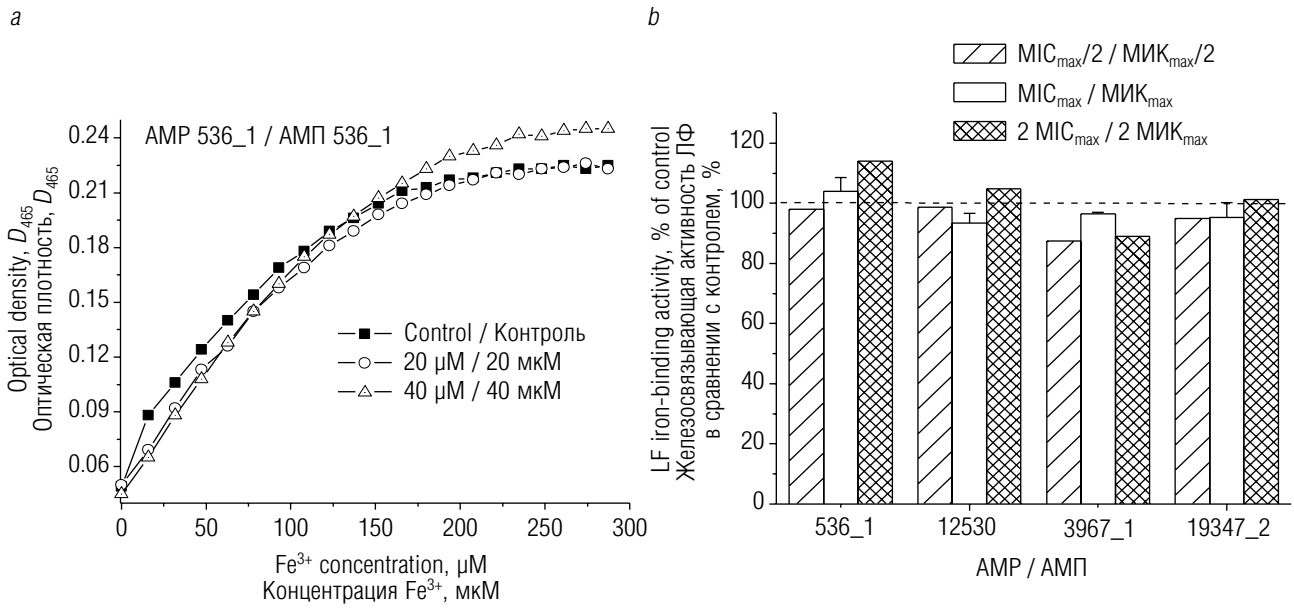


Fig. 3. Effect of AMP on LF iron-binding activity: *a* — typical kinetic curves of LF solution (10 mg/ml) optical density changes at a wavelength of 465 nm, corresponding to the absorption peak of LF iron-saturated form (holo-LF), in the absence and in the presence of AMP 536_1 at various concentrations after 20 successive additions of iron salt [$NH_4Fe(SO_4)_2 \cdot 12\ H_2O$] (16 μM each); *b* — effect of AMP 536_1, 12530, 3967_1 and 19347_2 at various concentrations on LF iron-binding activity. MIC_{max} — minimal inhibitory concentrations against three bacterial species (*Escherichia coli*, *Chlamydia thrachomatis* and *Bacillus subtilis*). LF iron-binding activity in the control (in the absence of AMP) was taken as 100%

Рис. 3. Влияние антимикробных пептидов (АМП) на железосвязывающую активность лактоферрина (ЛФ): *a* — типичные кинетические кривые изменения оптической плотности раствора ЛФ (10 мг/мл) на длине волны 465 нм, соответствующей пику поглощения насыщенной железом формы ЛФ (холо-ЛФ), в отсутствие и в присутствии АМП 536_1 в различных концентрациях после 20 последовательных добавок соли железа [$NH_4Fe(SO_4)_2 \cdot 12\ H_2O$] (по 16 мкМ); *b* — влияние АМП 536_1, 12530, 3967_1 и 19347_2 в различных концентрациях на железосвязывающую активность ЛФ. $МИК_{max}$ — максимальное значение среди минимальных концентраций АМП, необходимых для достижения 100 % ингибирования роста микроорганизмов *Escherichia coli, Chlamydia trachomatis* и *Bacillus subtilis* в стандартном тесте. За 100 % принята железосвязывающая активность ЛФ в контроле (в отсутствие АМП)

not affect the iron-binding activity of LF (data not presented).

Moreover, the effect of AMP on the biological activity of lysozyme contained in azurophilic and specific and gelatinase granules of neutrophils was investigated. Lysozyme (muramidase) is a low molecular weight cationic protein, a hydrolase class enzyme that destroys bacterial cell walls by hydrolysis of 1,4-beta-glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine of peptidoglycans that are part of the plasma membrane of bacteria, thereby protecting macroorganism from exogenous and endogenous microflora. In this case, peptidoglycan binds to the active center of lysozyme (in the form of a pocket), which contains two amino acid residues that are critical for enzyme functioning (Glu at position 35 and Asp at 52) [32].

Lysozyme lyses cells of various bacilli, micrococci, staphylococci, *Escherichia coli, Salmonella, Shigella*, and actinomycetes, as well as some types of yeast and fungi. The standard test to determine the mucolytic activity of lysozyme is the registration of lysis of bacteria *M. lysodeikticus* [33]. However, given the ability of the studied AMPs of the medicinal leech to integrate into the bacterial cell wall and disrupt its permeability, the effect of AMPs themselves on *M. lysodeikticus* bacteria was first studied, and then the combined effect of synthesized AMPs and lysozyme on these bacteria.

The individual antimicrobial activity of the studied AMPs of the medicinal leech against *M. lysodeikticus* was revealed to be rather low, and the addition of all AMPs to *M. lysodeikticus* led only to an insignificantly decreased light transmission of cell suspensions (data not presented), which may indicate the incorporation of AMPs into the bacterial membrane and im-

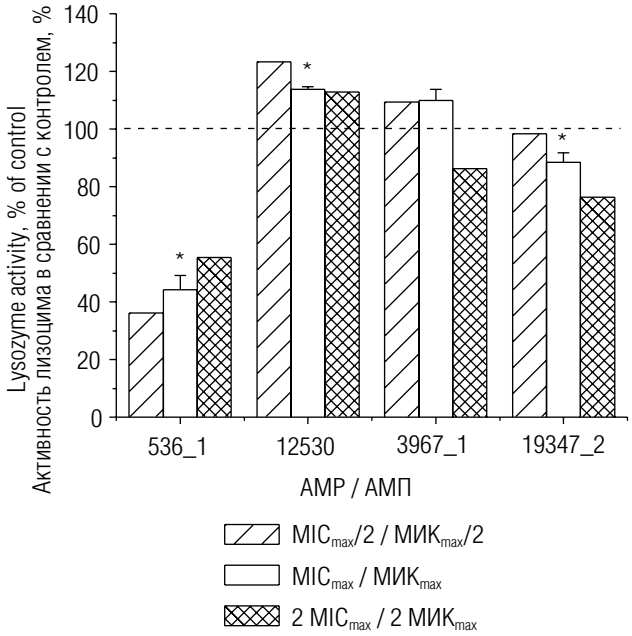


Fig. 4. Effect of AMP 536_1, 12530, 3967_1, and 19347_2 at various concentrations on lysozyme ability to lyse *M. lysodeikticus* bacterial cells. Lysozyme mucolytic activity in the control (in the absence of AMP) was taken as 100%. MIC_{max} — minimal inhibitory concentrations against three bacterial species (*Escherichia coli*, *Chlamydia thrachomatis* and *Bacillus subtilis*). $*p < 0.05$ compared to lysozyme activity in the control (in the absence of AMP)

Рис. 4. Влияние антимикробных пептидов (АМП) 536_1, 12530, 3967_1 и 19347_2 в различных концентрациях на способность лизоцима лизировать бактериальные клетки *M. lysodeikticus*. За 100 % принята муколитическая активность лизоцима в контроле (в отсутствие АМП). $МИК_{max}$ — максимальное значение среди минимальных концентраций АМП, необходимых для достижения 100 % ингибирования роста микроорганизмов *Escherichia coli, Chlamydia trachomatis* и *Bacillus subtilis* в стандартном тесте. $*p < 0,05$ по сравнению с активностью лизоцима в контроле (в отсутствие АМП)

pairment of its permeability to ions and, thereby swelling of bacterial cells.

The study of the combined AMP and lysozyme action revealed that the synergistic effects (~20%) against the gram-positive bacteria *M. lysodeikticus* for the combination of lysozyme with AMP 12530 (in the entire studied concentration range of AMP), as well as AMP 3967_1 was at a concentration of 10 μM or less. Antimicrobial activity was inhibited with the combined addition of lysozyme and AMP 3967_1 at a concentration of 15 μM and higher, as well as AMP 536_1 in the entire concentration range under study (Fig. 4). Strong mucolytic activity inhibition of lysozyme after preliminary incubation with AMP 536_1 can be associated with the interaction of cationic amino acid residues of AMP 536_1 (this AMP has the highest positive charge [+6] among all studied AMPs) with negatively charged amino acid residues of the active center of lysozyme, which play a key role in enzymatic cleavage of bacterial wall peptidoglycans. In the case of an additive combination of lysozyme with AMP 19347_2 at concentrations lower than the MIC_{max}, the overall effect was equal to the individual lysozyme action; with an increased concentration of AMP 19347_2, the antibacterial activity of the mixture under study decreased (Fig. 4), which again may be associated with the electrostatic interaction of negatively charged Asp and Glu in the active center of the enzyme with positively charged (+5) AMP 19347_2.

After the modification of HOCl, the ability of AMP 536_1 and 19347_2 to inhibit the mucolytic activity of lysozyme was retained, but the effect was less than for native AMPs (data not presented), which may be a consequence of a decreased cationic charge of AMP after HOCl treatment and a decreased interaction with anionic amino acid residues that are included in the composition of the active enzymatic center. The ability of AMP 3967_1 to enhance the lytic activity of lysozyme increased after HOCl modification, whereas AMP 12530 inhibited the mucolytic activity of lysozyme after halogenative modification (data not presented).

Conclusion

Therefore, based on the obtained data, the use of drugs based on the new investigated AMPs of medicinal leech provides a potential opportunity to exert a positive effect in the body's combat against infectious agents, not only due to the antimicrobial action of AMPs themselves but also due to the modulation by these compounds of the biological activity of own endogenous antimicrobial proteins and peptides, namely enchancement in the case of infection elimination and inhibition in the case of protect against damage to the body's tissues.

Additional information

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Author contributions. *D.V. Grigorieva* conducted the experiments, described them, and processed the results; *E.N. Grafskaia, I.A. Latsis,* and *V.N. Lazarev* performed the synthesis of AMP of medicinal leech; *A.V. Sokolov* isolated proteins from neutrophil extract; *I.V. Gorudko, O.M. Panasenko* selected the literature, discussed the results.

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