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THE PROGNOSIS AND INVESTIGATION OF α-DEFENSIN-1 (HNP-1) INFLUENCE ON MORPHOLOGICAL CHANGES OF *STAPHYLOCOCCUS AUREUS* CELLS BY THE ATOMIC-FORCE MICROSCOPY DATA

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ПРОГНОЗИРОВАНИЕ И ИССЛЕДОВАНИЕ ВЛИЯНИЯ α-ДЕФЕНЗИНА-1 (HNP-1) НА МОРФОЛОГИЧЕСКИЕ ИЗМЕНЕНИЯ КЛЕТОК *STAPHYLOCOCCUS AUREUS* ПО ДАННЫМ АТОМНО-СИЛОВОЙ МИКРОСКОПИИ

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Methods of computer chemistry were used to model the spatial structures of α-defensin-1 (human neutrophil peptide-1, HNP-1) and peptidoglycan molecules. Geometric optimization has been carried out, quantum-chemical characteristics and charge density distribution of the molecules have been studied, molecular docking has been carried out. Using high-resolution atomic-force microscopy, the influence of HNP-1 on the character of morphological changes in the cell wall of gram-positive microorganisms (*Staphylococcus aureus*) was studied. A pronounced difference in the morphological features of bacterial populations is shown by the nature of the response to the action of HNP-1. Positively charged sites of HNP-1, binding to the negatively charged lipopolysaccharides (LPS) of the outer bacterial membrane, either create cracks in the LPS layer, or bind to sites in LPS that are responsible for interaction with the Ca^{2+} and Mg^{2+}, cations necessary for stabilizing the cell surface.

Keywords: defensin, HNP-1, antimicrobial peptide, antimicrobial resistance, molecular docking, atomic-force microscopy, Staphylococcus aureus

Методами компьютерной химии выполнено моделирование пространственных структур α-дефензина-1 (human neutrophil peptide-1, HNP-1) и молекулы пептидогликана. Осуществлена геометрическая оптимизация, изучены квантово-химические характеристики и распределение плотности заряда исследуемых молекул, проведен молекулярный докинг (molecular docking). С помощью высокоразрешающей атомно-силовой микроскопии, изучено влияние HNP-1 на характер морфологических изменений клеточной стенки грамположительных микроорганизмов (*Staphylococcus aureus*). Показана выраженная разность морфологических особенностей бактериальных популяций по характеру реагирования на действие HNP-1. Положительно заряженные участки HNP-1, связываясь с отрицательно заряженными липополисахаридами (ЛПС) внешней мембраны бактерий, либо создают трещины в ЛПС-слое, либо связываются с участками в ЛПС, отвечающими за взаимодействие с катионами Ca^{2+} и Mg^{2+}, необходимыми для стабилизации поверхности клетки.

Ключевые слова: дефензин, HNP-1, антимикробные пептиды, антимикробная резистентность, молекулярный докинг, атомно-силовая микроскопия, Staphylococcus aureus

Nowadays one of the most significant medical problems is the ubiquitous growth of the antimicrobial resistance [2, 13, 14]. It can be concluded that in the nearest future medicine is to face great difficulties in the treatment of infections caused by resistant pathogens, which, in its turn,

will result increased lethality, longer treatment time. It is impossible to justify and standardize a choice of an antibacterial agent for infectious processes is possible without objective information on the structures of pathogens and their sensitivity to antimicrobial drugs [8].

Creating new antimicrobial agents and as improving those ones already used requires a great variety of methods allowing to comprehensively complex the mechanisms and consequences of their effects on bacterial cells [4].

At present of great interest is one of such methods, namely, atomic–force microscopy (AFM) [3], it is based on estimating the interaction between a resilient probe (cantilever) with a surface of the test sample under investigation. The surface side force effecting the probe [9] leads to cantilever bending, and the latter registers the surface relief while moving on it and reacting to the force interaction. Earlier we have studied cefotaxime influence on morphological changes of *Staphylococcus aureus* using this method [1].

To overcome the increasing antimicrobial resistance, it is necessary to develop such antibacterial agents that microorganisms can't get resistant with time. These medicines should be very efficient and safe. In this respect, the most promising idea is developing new antimicrobial agents based on antimicrobial peptides (AMPs). AMPs are peptides having from 5 to ~100 amino acid residues with a wide range of killing activity against bacteria, fungi, protozoa, and even viruses [12].

We think that human α-defensin-1, or human neutrophil peptide-1 (HNP-1), produced by neutrophils is of special interest among AMPs [17].

HNP-1 (α-defensin-1) is a cationic peptide; it has a wide range of antimicrobial activity regarding many gram-positive and gram-negative bacteria including MRSA. The mechanism of action of this antimicrobial peptide (as well as all others) relates to its direct effect on microbial membranes, i.e. pore formation, cell leakage and successive bacteria lysis. It is considered that the specific destruction of a microbial wall is due to the electrostatic attraction of positively charged AMPs and negatively charged membrane [5]. Besides, Erik de Leeuw et al showed that HNP-1 could bind with the cell wall precursor lipid II thus inhibiting cell wall synthesis [6].

The aim of the work was to study the mechanism of intermolecular interaction of α-defensin-1 (HNP-1) and bacterial cell wall components (peptidoglycan molecule) as well as to investigate morphological changes and their nature in *S. aureus* microbe cells upon HNP-1 impact, which was achieved by atomic-force microscopy.

Material and Methods. The main object under study was *S. aureus* strains sensitive to beta-lactam antibiotics (MSSA). The bacteria were grown on Becton Dickinson mannitol-salt agar (18–24 h, 37 ºC) in the bacteriological laboratory of LLC «Center of Clinical Pharmacology and Pharmacotherapy» (Stavropol, Russia).

Recombinant HNP-1 produced by Cloud-Clone Corp., USA was used to affect *S. aureus.* The preliminary mechanism of HNP-1 action on a microbe wall was described above. HNP-1 was used in two concentrations: 2.5 μg/ml and 5 μg/ml. These concentrations were empirically chosen in accord with MIC (minimum inhibitory concentration) data for *S. aureus* (The Antimicrobial Peptide Database http://aps.unmc.edu/AP/database/query_output.php?ID=00176).

Applying computer chemistry methods, space structures of HNP-1 and peptidoglycan molecule were simulated. The geometrical optimization was made, quantum-chemical characteristics and charge density distribution of the molecules under investigation were studied.

The bacterial material was fixed on the surface of mica plates with the adhesive treatment (glutaraldehyde). The necessity of employing the given method was due to its capability to fix cell morphology changes happening at its interacting with antimicrobial peptide HNP-1. While the main HNP-1 action is to destroy a cell membrane, bacterial cells touching a substrate surface in a suspension drop are firmly fixed without any further changes and any significant influence on the microbe cell morphology [16].

Bacterial cells were washed from the culture medium surface with the physiological solution, then the extinction coefficient was led to its standard 10 ME of the turbidity standard. At first the samples of the first (control, without any HNP-1 action), second and third groups (HNP-1, its concentration of 2.5 μg/ml and 5 μg/ml, respectively) were kept at 37 ºC for 20 minutes. After that a control drop (about 5 μl) of newly prepared suspension with bacterial cells as well as a bacterial cell suspension with HNP-1 for experimental samples were put onto the surface of a fresh mica plate cleavage. The washout was performed with a small amount of injection water, and then the samples were immediately dried with a large flow of compressed air at room temperature (20–22 ºC). Their natural drying was made for 16–20 hours. The same sequence of samples, namely, the reference group (without HNP-1 action – the first group), second and third (HNP-1, its concentration of 2.5 μg/ml and 5 μg/ml, respectively) groups were put into the thermostat (37 ºC, 60 min), with their further fixation on the surface of a fresh mica plate cleavage [10]. There were some copies of all samples (there was a series of repetitions for each experimental and reference sample). The measurement of topological features was repeatedly performed both within one sample (in its different parts) and a series of samples.

The atomic-force microscopy was made in the «Nanobiotechnology and biophysics» laboratory of the biotechnological engineering center (Stavropol, Russia) by AFM NTegra Life (NT-MDT, Moscow) in a semi-contact mode. HA_NC *Etalon*, *Resonant frequency* 151 kHz, Force constant 3,5±20 N/m, Curvature radius <10 nm cantilevers were employed in the scanning process. The optimal values of the main parameters at scanning were: the cantilever oscillation amplitude – *Resonance* 11, its initial oscillation – *Phase* 180˚, scanning – *Frequency* 0.47–0.51 Hz, feedback loop gain – *FB Gain* 0.15–0.17 and *Set Point* 5.6. The images obtained were analyzed by applied programs Nova Px 3.4. (NT-MDT, Russia), which allow editing AFM images and presenting them in 2D and 3D formats.

Results and Discussion. At the first stage, the principal quantum-chemical characteristics have been studied to predict and analyze the interaction of peptidoglycan molecules (the basic component of a cell membrane) and HNP-1 by computer chemistry methods as well as the molecular docking has been made, which allows determining the molecule orientation and forming a stable complex compound.

The peculiarity of *S. aureus* cell wall as well as most gram-positive bacteria is that it is presented by 5–6 peptidoglycan chains comprising up to 90 % of the cellular dry shell mass. To estimate the effect of HNP-1 and peptidoglycan interaction the protein database was used, the geometric optimization of molecules was done by molecular mechanics method. This method takes atoms as Newton's particles interacting with one another at the expense of empirically given potential fields. The optimization of molecule geometry implies searching for stable molecule structure conditions where the system energy is minimal. Molecular properties of peptidoglycan and HNP-1 are summarized in Table 1.

Table 1

Molecular properties of peptidoglycan and HNP-1

Indicators	Peptido-glycan	HNP-1 (α-defensin-1)
Potential energy, kcal/mol	–7838.5	–2873.2
Dipole moments, Debye	212.3	44.5
RMS gradient kcal/(Å×mol)	0.088	0.10

The data obtained support the accuracy of the geometric optimization of molecular models. The potential energy values of peptidoglycan and HNP-1 are small (–7838.5 and –2873.2 kcal/mol), which justifies the stability of both molecules. RMS gradient approaches zero (0.088 and 0.10 kcal/(Å×mol), which justifies the efficiency of minimizing potential energy and energy balance of system characteristics. Dipole moments characterize the charge distribution irregularity on molecule surfaces (212.3 for peptidoglycan and 44.5 Debye for HNP-1).

The efficiency of destroying *S. aureus* cell shell can be analyzed by the molecular docking method, it allows determining the orientation among molecules to form a stable complex compound. Peptidoglycan (receptor) and HNP-1 (ligand) complexes are the factor of transmitting a chemical signal between two molecules (inhibiting or catalytic). The results of molecular docking between peptidoglycan and HNP-1 are presented in Figure 1 (A-D), they testify that both molecules have hydrophobic zones Figure 1 (A), 1 (B); Figure 1 (C) the complex is formed through one of these zones (Fig. 1 (D)). Figure 1 (C) clearly presents that α-defensin-1 molecule penetrates into peptidoglycan fragment. To estimate the space location of the complex compound the structural model formed during docking was analyzed Figure1 (E-F).

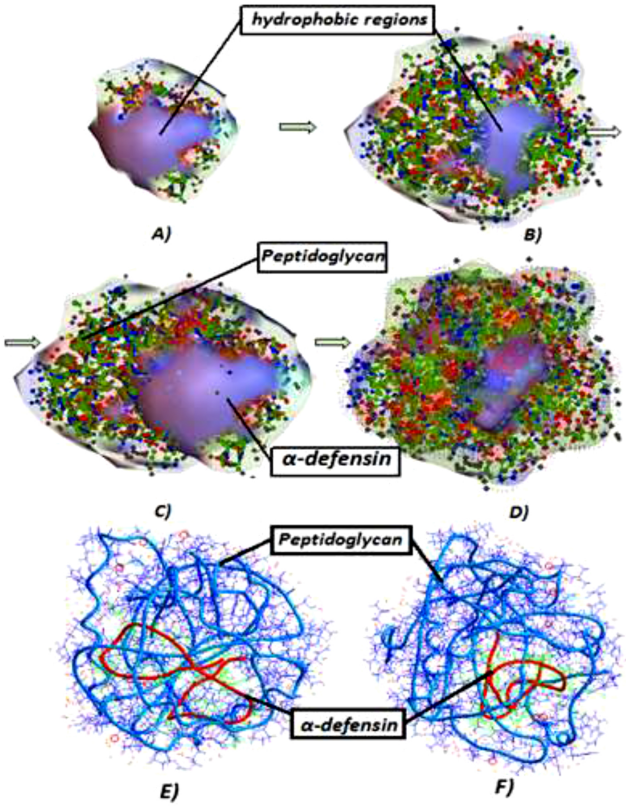

Fig. 1. Stages of molecular docking: A) molecule of α-defensin-1 (HNP-1); B) a fragment of the peptidoglycan molecule; C) intermediate stage of docking; D) complex compound; E) structural model of complex of peptidoglycan fragment with HNP-1 (frontal projection); F) it is the same, projection from the side

The complex compound model shows that HNP-1 deeply penetrates into the structure of peptidoglycan molecule fragment. It is evident that there is a potential energy decrease of intermolecular compound. However, to state the final HNP-1 effect on *S. aureus* cell walls its necessary to investigate the molecular properties of the compound formed at docking (Table 2).

Table 2

Molecular properties of the complex compound

Indicators	Research results
Potential energy (E_{pot}), kcal/mol	549.3
Dipole moment, Debye	67.6
RMS gradient kcal/(Å×mol)	0.093

Studying molecular properties of docked molecules states that the sum E_{pot} of two molecules will be –10711.7 kcal/mol (–7838.5 + (–2873.2). The compound E_{pot} should be lower than the energy sum of two initial components to form a stable complex. In our case, the potential energy of the complex is a sufficiently high value of 549.3 kcal/mol. It means that the compound is unstable, and the interaction of HNP-1 and peptidoglycan can lead to the damage of cell walls or serious changes of functional properties of the microorganism under study.

The next step was to evaluate the nature of α-defensin-1 action on *S. aureus* cells by AFM.

As the aim has been to investigate morphological changes and their nature in *S. aureus* microbe cells under the action of antimicrobial agent HNP-1, the results will be presented in images obtained without any numeric values of morphometric characteristics.

S. aureus morphology without HNP-1 action (control samples) are given in Figure 2 (A). The data obtained by AFM show *S. aureus* to be coccoid objects of a spherical shape adsorbed mainly like cell groups, sometimes like small aggregates or they form classical «staphyline» aggregates on the substrate surface.

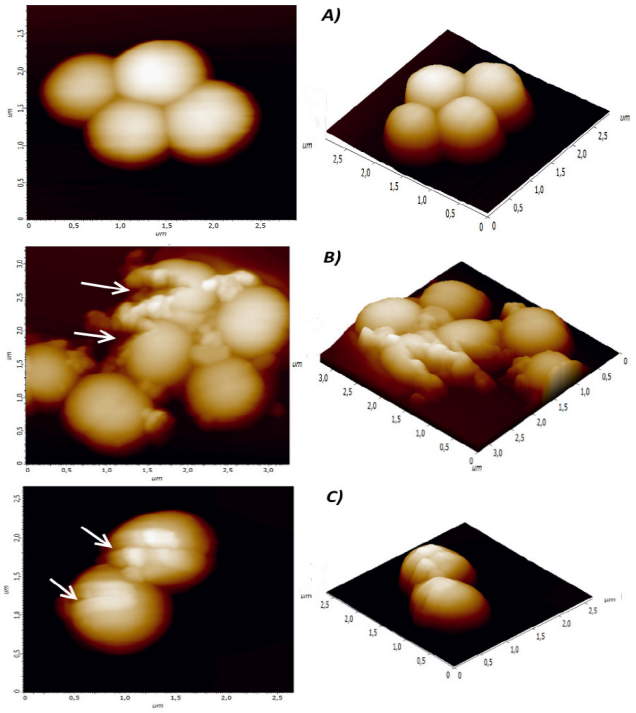

Fig. 2. AFM image of *S. aureus* (A) without affecting HNP-1 (control, after 20 min.); (B) an example of bacterial damage after exposure to HNP-1 (conc. 2.5 μg/ml, 20 min); (C) after exposure to HNP-1 (conc. 5 μg/ml, 20 min). The arrows indicate areas of the most pronounced damage

Atomic-force microscopy of the experimental samples Figure 2 (B–C) after HNP-1 (2,5 μg/ml) action showed that bacterial cells were determined as spherical objects with morphologically expressed damages on the surface.

The presence of positively charged zones in HNP-1 is an important feature of antimicrobial peptides. It is these zones where α-defensin-1 binds with negatively charged lipopolysaccharides (LPS) of the outer bacteria membrane. The structural changes appearing in the LPS-layer at this moment form the so called «vertical self-regulating channel» facilitating α-defensin-1 penetration into the plasmatic membrane of the pathogen. HNP-1 is supposed to create cracks in the LPS-layer or bind with LPS zones (Fig. 2 (B-C)) responsible for interacting with divalent cations Ca^{2+} and Mg^{2+} necessary to stabilize the cell surface and to perform cross-linking LPS negative charges [11].

AFM studies of control and experimental samples after HNP-1 action (5 mg/ml, 20 min) and incubation (60 min, 37 ºC) are given in Figure 3 (A-C). The cell wall damages are more expressed (than at 20 min incubation). The control samples in Figure 3 (A) were determined as regular spherical objects without visible damages of their cell walls. But experimental samples in Figure 3 (B-C) hardly ever had any whole cells in the scanning field compared with the samples after 20 min incubation.

Data from several studies indicate that many of the AMPs are aggregated after penetration into the pathogen membrane. HNP-1 molecule insertion in the lipid bilayer surface cases tangential stress between inner and outer lipid bilayer surface. Stress is compensated when a certain concentration of HNP-1 molecules is achieved on the outer lipid bilayer surface, which leads to different consequences: by redistribution of peptide molecules between the outer and inner surfaces of the membrane, the formation of pores of various structures or the destruction of the lipid membrane [15, 7].

Conclusions. This study shows the process of interaction of antimicrobial peptide α-defensin-1 (HNP-1) and peptidoglycan, the results are obtained through the computer-generated simulation. Complex compound model reveals that HNP-1 deeply penetrates the structure of peptidoglycan molecule. The study of docking molecules shows that potential energy of this aggregate equals to 549.3 kcal/mol, it means that interaction of HNP-1 and peptidoglycan is unstable and may lead to cell walls destruction.

High-resolution atomic-force microscopy suits perfectly well for bacterial cells practical diagnostic morphometry. AFM has an incredible opportunity to use morphologic and mechanic prokaryote and eukaryote cells to study, to visualize the consequences of the influence of different samples on model microorganisms.

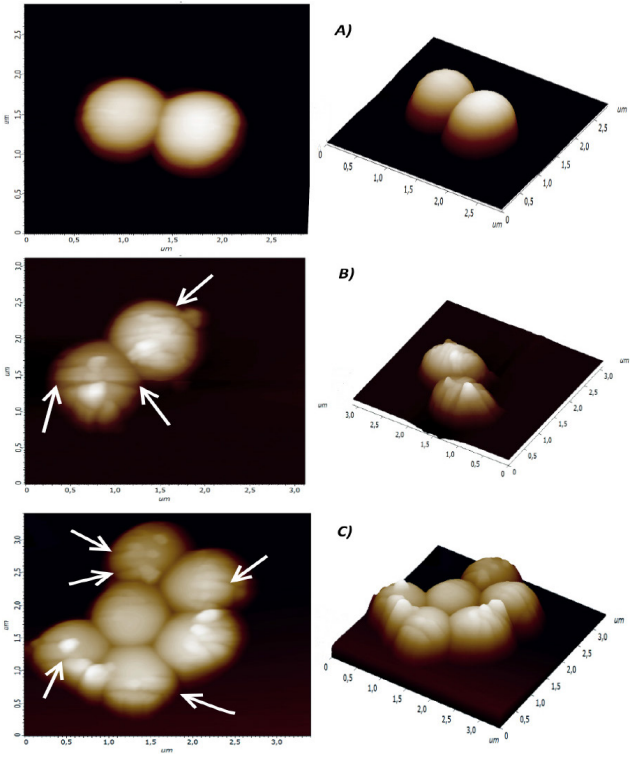

Fig. 3. AFM image of *S. aureus* (A) without affecting HNP-1 (control, after 60 min.); (B) an example of bacterial damage after exposure to HNP-1 (conc. 2.5 μg/ml, 60 min); (C) after exposure to HNP-1 (conc. 5 μg/ml, 60 min). The arrows indicate areas of the most pronounced damage

Using AFM for the first time, the morphofunctional reaction of *S. aureus* to the effect of the antimicrobial peptide HNP-1 was evaluated. Formation of the expressed heterogeneity of morphological and mechanical properties of the microorganism population is recorded. The data confirms that when the certain threshold HNP-1 molecule concentration is reached on the outer lipid membrane surface the tangential stress compensation happens, which at the end leads to pores with different structure development.

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METHIDICAL APPROACHES TO BIOASSAY OF PHENOLIC HYDROXYLENES CONTAIN SUBSTANCES

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МЕТОДИЧЕСКИЙ ПОДХОД К АНАЛИЗУ ВЕЩЕСТВ, СОДЕРЖАЩИХ В СТРУКТУРЕ ФЕНОЛЬНЫЕ ГИДРОКСИЛЫ, ПРИ БИОАНАЛИТИЧЕСКИХ ИССЛЕДОВАНИЯХ

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This article describes method of development approaches for bioassay of substances containing stable and unstable phenolic hydroxyls using methyldopa and mycophenolic acid for example

Keywords: phenolic hydroxyls, bioassay, mycophenolic acid, methyldopa, stabilization

Описаны подходы к разработке биоаналитических методик для определения веществ, содержащих стабильные и нестабильные фенольные гидроксилы, на примере метилдопы и микофеноловой кислоты.

Ключевые слова: фенольные гидроксилы, биоаналитика, микофеноловая кислота, метилдопа, стабилизация

The main step of bioequivalence and pharmacokinetic studies is a determination drug substance of concentration in biological fluids, such as plasma, serum and whole blood. Some substances are able to significantly decompose during the storage of samples. Are drugs, containing phenolic hydroxyls. Examples of the substances. The oxidation ability of phenols directly depends from the amount of phenolic hydroxyls in one benzene ring [2]. Mycophenolic acid (MPA) (Fig. 1 A) and methyldopa (MD) (Fig. 1 B) which contains one and two phenolic hydroxyls, respectively, were selected to work out approaches development of bioanalytical methods of the quantitative determination of drugs, containing phenolic hydroxyls.