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Restoring antibiotic sensitivity to lincomycin in compositions with nanosilver and humic substances

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ABSTRACT

Aim. To study the effect of compositions with nanosilver and humic substances on restoration of sensitivity of methicillin-resistant *Staphylococcus aureus* to lincomycin.

Materials and methods. Compositions of humic substances with silver nanoparticles were synthesized from commercial sodium humate Powhumus and silver nitrate in the presence of NaOH (1 M) to modulate alkaline pH. To synthesize one of the two compositions, sodium humate was modified with hydroquinone. To describe the characteristics of the resulting compositions, surface plasmon resonance spectra of silver nanoparticles and their images obtained by transmission electron microscopy were recorded. Sensitivity of a clinical strain of methicillin-resistant *Staphylococcus aureus* was determined by measuring the minimum inhibitory concentration (MIC) with the addition of lincomycin and tetracycline to the compositions.

Results. 100% conversion of ionic silver into metallic silver with a characteristic nanoparticle size of 6 nm was shown. The effects of tetracycline and lincomycin on the studied strain of *Staphylococcus aureus* were compared, and high sensitivity to tetracycline (MIC < 10 μg / ml) and resistance to lincomycin (MIC > 200 μg / ml) were shown. Studying the effect of the composition containing sodium humates with nanosilver with the introduction of lincomycin into it showed that this approach can significantly reduce MIC of lincomycin to 0.1 μg/ml in the presence of compositions with hydroquinone-modified sodium humate at a concentration of 40 μg / ml and compositions with unmodified sodium humate at a concentration of 60 μg / ml.

Conclusion. The study demonstrated that the use of compositions with humic substances and nanosilver completely restores sensitivity of methicillin-resistant *Staphylococcus aureus* to lincomycin.

Keywords: silver nanoparticles, humic substances, lincomycin, synergistic effect, antibiotic resistance, *Staphylococcus aureus*

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Восстановление антибиотикочувствительности к линкомицину в составе композиций с наносеребром и гуминовыми веществами

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РЕЗЮМЕ

Цель – исследование восстановления чувствительности метициллин-резистентного штамма *Staphylococcus aureus* к линкомицину при добавлении гуминовых веществ с наночастицами серебра.

Материалы и методы. Композиции гуминовых веществ с наночастицами серебра синтезировали из коммерческого гумата натрия Powhumus и нитрата серебра в присутствии NaOH (1 моль) для создания щелочной среды. Для синтеза одной из двух композиций гумат натрия модифицировали гидрохиноном. Для описания характеристик полученных составов снимали спектры поверхностного плазмонного резонанса наночастиц серебра и их изображения, полученные методом просвечивающей электронной микроскопии. Чувствительность клинического штамма *Staphylococcus aureus*, устойчивого к метициллину, определяли, измеряя минимальную ингибирующую концентрацию (МИК), с введением в состав композиций линкомицина и тетрациклина.

Результаты. Показана 100%-я конверсия ионного серебра в металлическое с характерным размером наночастиц 6 нм. Проведено сравнение действия тетрациклина и линкомицина на исследуемый штамм *S. aureus*, показаны высокая чувствительность к тетрациклину (МИК < 10 мкг/мл) и отсутствие чувствительности к линкомицину (МИК > 200 мкг/мл). Исследование действия композиции гуматов натрия с наносеребром при введении в них линкомицина показало, что подобный подход позволяет существенно снизить МИК линкомицина до 0,1 мкг/мл в присутствии композиций с модифицированным гидрохиноном гумата натрия в концентрации 40 мкг/мл и композиций с немодифицированным гуматом натрия в концентрации 60 мкг/мл.

Заключение. В проведенном исследовании продемонстрировано, что при использовании композиций гуминовых веществ с наносеребром происходит полное восстановление чувствительности к линкомицину устойчивого к метициллину штамма *Staphylococcus aureus*.

Ключевые слова: наночастицы серебра, гуминовые вещества, линкомицин, синергетический эффект, антибиотикорезистентность, *Staphylococcus aureus*

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

After the discovery of the first antibiotic penicillin, already in 1942, drug-resistant strains of *Staphylococcus aureus* emerged [1]. An increase in the number of antibiotic-resistant bacterial strains over time led to the need to develop new antibiotics, which, in turn, brought about new multidrug-resistant microorganisms [2]. As a result, searching for ways to enhance the effects of existing antibiotics became a pressing issue [3]. Silver nanoparticles, which are known for their antibacterial properties, attracted particular attention [4]. This is because resistance to metal particles develops much more slowly than to antibiotics [5].

Numerous studies on antibacterial activity of compositions with silver nanoparticles and antibiotics showed that nanosilver is capable of not only enhancing the effect of antibiotics against bacteria [6], but also of restoring sensitivity of resistant bacterial strains to antibiotics [7, 8]. Previous studies showed that the use of silver nanoparticles together with a wide range of antibiotics provided a synergistic antibacterial effect of both components against various gram-positive bacteria. It was shown that sensitivity of *Bacillus cereus* to lincomycin increased four-fold in the presence of silver nanoparticles [9]; sensitivity of *Entorococcus faecium* to ampicillin rose by 16 times, and to amikacin – by 32 times [10]. The shape and size of synthesized nanoparticles had a significant impact on antibacterial activity [11].

The main problem with the use of silver nanoparticles (NPs) in clinical practice is a potential increase in cytotoxicity of this kind of compositions. The problem is complicated by a poor understanding of the mechanism of action of such compositions [12, 13]. It is assumed that they might increase the concentration of Ag+ near the bacterial cell wall, which exerts a higher cytotoxic effect [14], induces hydroxyl radical formation [15], and affects DNA transcription [16].

Studies on the biological activity of natural and modified humic substances (HS) show that they have a great potential for incorporation into nanoparticle-based compositions due to plentiful properties and functional groups in their molecular ensemble [17–19]. The use of HS as both reducing and stabilizing agents for the synthesis of silver NPs without application of additional reducers is of particular interest [20–23]. In our previous studies, we described a method for synthesizing nanosilver-based drugs with a strong safety profile, which resulted from high antioxidant activity and low cytotoxicity of HS [24]. Addition of antibiotics to compositions with silver NPs and HS might be of particular interest in terms of developing novel combination drugs to overcome antibiotic resistance. Various mechanisms regulating interaction of HS with a wide range of antibiotics were discussed in the review article [25]. We hypothesized that antibiotic binding to HS on the surface of silver NPs could facilitate its penetration into the cell together with silver NPs.

The choice of antibiotics for the experimental studies was guided by the aim of the study – to develop effective bionanomaterial for treatment of infected wounds with a possibility of application in semisolid dosage forms (ointments, gels, etc.). Hence, an antibiotic was to penetrate both into soft tissues and bones to treat chronic and sluggish purulent inflammations, such as pressure ulcers and diabetic foot. These requirements are met by penicillins, lincosamides, fluoroquinolones, aminoglycosides, tetracyclines, etc. An antibiotic should mix well with lipophilic ointments for external application in semisolid dosage forms. This requirement is met only by ointments with lincomycin and tetracycline, since penicillins are not used externally, levofloxacin (fluoroquinolones) is used in the form of an aqueous spray, and neomycin (aminoglycosides) is applied in the form of an aerosol. As a result, two antibiotics were chosen for the study – lincomycin and tetracycline, resistance to which evolved in the methicillin-resistant *Staphylococcus aureus* (MRSA).

The MRSA strain F-182 was chosen as one of the most abundant and dangerous forms of *Staphylococcus aureus* causing a broad spectrum of diseases, including bloodstream infections, pneumonia, and skin and bone infections. Moreover, MRSA strains are highly resistant to the majority of existing antibiotics, which makes it difficult to treat and control. The strain F-182 is very well studied and described in the literature, which allowed for more in-depth research. At the next stages of our research, we plan on looking into other strains in search of promising combinations.

The aim of the study was to investigate the effect of compositions with nanosilver and humic substances on restoration of sensitivity of MRSA to lincomycin and tetracycline.

MATERIALS AND METHODS

Chemicals and materials. Sodium hydroxide (Kemphasol), silver nitrate (Molychem), Powhumus sodium humate (Humintech, Germany), and *Staphylococcus aureus* Rosenbach 1884 (strain

F-182) were used for bioassays. The value of pH was measured using the pH meter 713 (Metrohm, Switzerland) equipped with a glass electrode.

Synthesis of the compositions was carried out according to the procedure described in [24]. In brief, an aqueous solution of sodium humate and hydroquinone (HQ) derivatives at a concentration of 11.8 g / l was prepared. The pH value of the solution was brought to 12 by adding 1 M NaOH. Then, 50 ml of the solution was placed in a 100 ml three-neck round-bottom flask equipped with a reflux condenser and heated to 80 °C with constant stirring. When the temperature reached 80 °C, 2 ml of $AgNO_3$ solution at a concentration of 110 g / l was added dropwise to the hot humate solution, while maintaining pH at 12 with 1 M NaOH. Then, the solution was maintained at 80 °C with constant stirring for 4 hours. The total concentration of silver in the resulting solution was 41 mM / l.

Analysis of the synthesized compositions containing nanosilver and HS. The synthesized Ag NPs stabilized with HS were analyzed by transmission electron microscopy (TEM). The experiment was carried out using the JEOL JEM-2100F analytical electron microscope (JEOL, Akishima, Japan). The data were processed using the ImageJ program. Morphology and size of the particles were analyzed for both compositions.

The total silver concentration was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using the 720-ES ICP-OES spectrometer (Agilent Technologies, USA). Ultraviolet-visible absorption spectra were registered to confirm the presence of silver NPs, which were detected by a surface plasmon resonance (SPR) peak at 400–430 nm for silver NPs. Absorption spectra were measured using the UV / Vis spectrometer (Cary 50 Probe, USA). The results of UV-visible spectroscopy and ICP-AES were compared to determine the degree of conversion of ionic silver to metallic silver.

The minimum inhibitory concentration (MIC) of the synthesized compositions to MRSA was determined using 96-well plates with Mueller Hinton broth (MHB). To determine MIC, the required volume of 0.9% NaCl and 0.142 ml of MHB containing the appropriate concentrations of lincomycin as part of compositions of HS with silver NPs were added to each well. The range of resulting concentrations was 0.1–20 μg / ml for lincomycin and 0–150 μg / ml for compositions of silver NPs and HS. MRSA colonies were pre-incubated at 35 °C in MHB for 12 hours. 0.025 ml of a bacterial suspension adjusted to McFarland 2 standard was added to each well and analyzed on the ImmunoChem-2100 microplate reader (High Technology Inc., USA) at a wavelength of 630 nm for 24 hours. MHB without the addition of test substances, to which a bacterial suspension was also added, served as a control.

Statistical processing of the obtained results was carried out by measures of variability using the SPSS statistical package (version 15.0, SPSS Inc., Chicago, IL, USA) with determination of the mean and the error of the mean and the probability of differences p for small samples with the Bonferroni correction (significance of differences at $p < 0.05$).

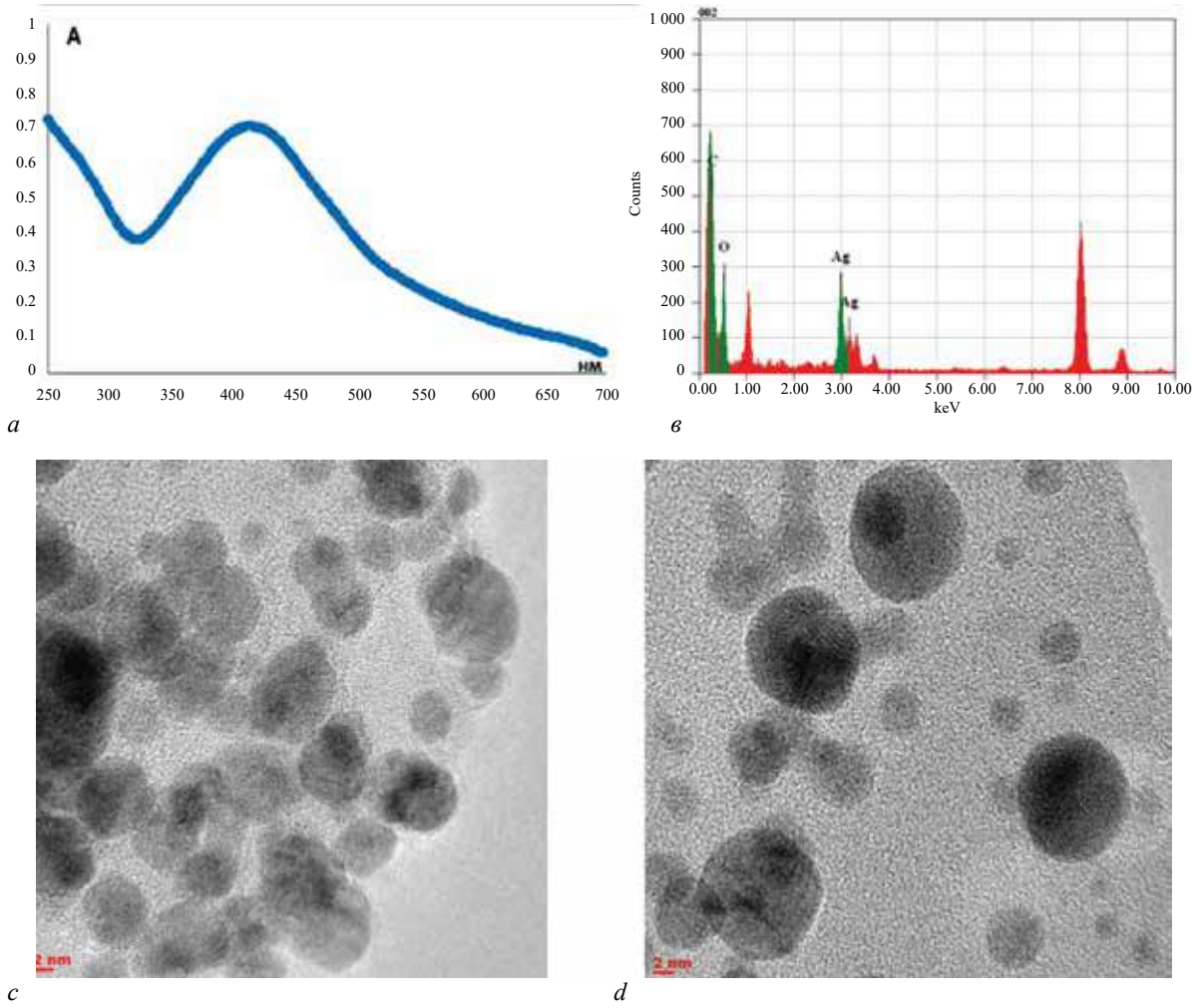
RESULTS

Synthesis of compositions of silver NPs with HS was accompanied by 100% conversion of ionic silver into metallic particles. The particles were spheric and around 6 nm in size. The resulting compositions contained 11.8 g / l of HS and 4.6 g / l (43 mM) of silver NPs. Figure 1 shows the surface plasmon resonance (SPR) spectra of silver NPs and their TEM images.

Figure 1*a* demonstrates a characteristic SPR peak of nanosilver at 414 nm, which indicates small size of synthesized nanoparticles. TEM images of AgNP – CHP (Fig. 1*b*) confirm that the average size of silver NPs is 6–7 nm. The size of nanoparticles is of particular importance for their biological activity. AgNPs with sizes less than 10 nm were reported to have maximum antimicrobial activity [13]. The size of the AgNPs used in this study satisfied this condition.

At the first stage of the experiment, we studied the effect of HS on the antibacterial activity of tetracycline and lincomycin. The studied strain was found to lack resistance to tetracycline: the antibiotic inhibited bacterial growth over the entire range of the studied concentrations. Lincomycin, on the contrary, did not suppress bacterial growth over the entire range of HS (0–1, 500 μg / ml) and lincomycin (10–200 μg / ml) concentrations.

Both combined compositions of AgNPs – CHP and AgNPs – CHP – HQ did not cause any changes in sensitivity of MRSA to tetracycline, but resulted in complete recovery of sensitivity to lincomycin (HS > 0 μg / ml for AgNPs – CHP, and HS 200 μg / ml for AgNPs – CHP – HQ). As a result, we decided to study the properties of the lincomycin-containing compositions where sensitivity to the antibiotic was restored.



a *в* *c* *d*

Fig. 1. Characteristics of silver nanoparticles (AgNPs) synthesized in the presence of coal humate (CHP): *a* – UV-visible absorption spectrum of the AgNP – CHP with a SPR peak at 414 nm; *b* – TEM elemental map; *c* and *d* – TEM images of AgNPs stabilized with HS

The antibacterial effect of AgNP synthesized in the presence of HS toward MRSA growth was studied in the presence or absence of lincomycin. We studied suppression of bacterial growth by AgNPs stabilized with CHP and CHP – HQ in the concentration range of 20–150 μg / ml and lincomycin in the concentration range of 0.1–20 μg / ml (Table 1, Fig. 2).

Table 1

Changes in the optical density of MRSA cells over 24 hours registered for MIC in the compositions with AgNPs, lincomycin, and modified sodium humate (CHP – HQ), mg / l

Lincomycin concentration	Concentration of AgNPs in the presence of CHP – HQ							Concentration of AgNPs in the presence of CHP						
	0	20	40	60	80	100	150	0	20	40	60	80	100	150
0.1	0.5	0.4	**0**	**0**	**0**	**0**	**0**	0.4	0.4	0.4	**0**	**0**	**0**	**0**
0.2	0.4	**0.2**	**0**	**0**	**0**	**0**	**0**	0.3	0.3	0.4	**0**	**0**	**0**	**0**
0.5	0.5	**0.2**	**0**	**0**	**0**	**0**	**0**	0.3	0.4	0.4	**0**	**0**	**0**	**0**
1	0.4	0.3	**0**	**0**	**0**	**0**	**0**	0.3	0.4	0.4	**0**	**0**	**0**	**0**
2	0.5	0.3	**0**	**0**	**0**	**0**	**0**	0.3	0.4	0.4	**0**	**0**	**0**	**0**
5	0.5	0.3	**0**	**0**	**0**	**0**	**0**	0.4	0.4	0.4	**0**	**0**	**0**	**0**
10	0.5	0.3	**0**	**0**	**0**	**0**	**0**	0.4	0.4	0.4	**0**	**0**	**0**	**0**
20	0.5	0.4	**0**	**0**	**0**	**0**	**0**	0.4	0.3	0.4	**0**	**0**	**0**	0

Note. Cases of statistically significant suppression of MRSA growth compared to the controls are presented in bold ($p < 0.05$).

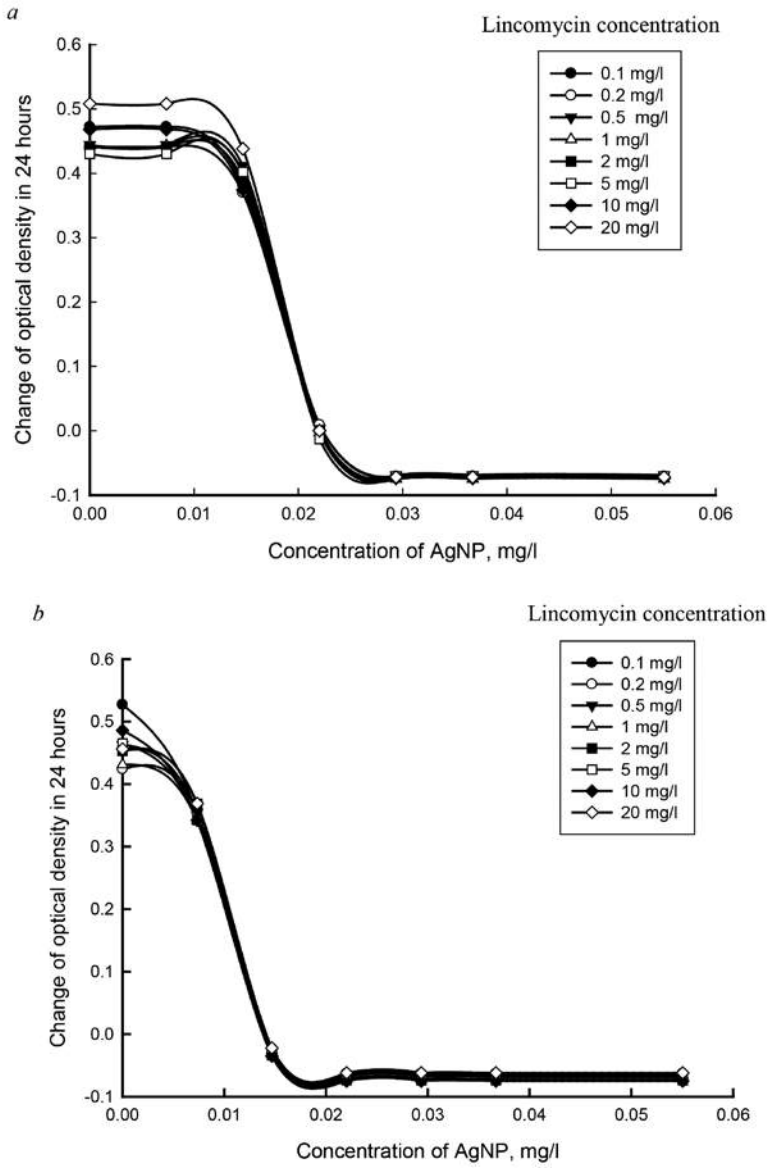


Fig. 2. A dependence of changes in the optical density of MRSA cells over 24 hours on the concentration of AgNPs at lincomycin concentrations in the range of 0.1–20 μg / ml in compositions with unmodified humic substances (*a*) and HQ-modified humic substances (*b*)

For lincomycin compositions in the absence of silver NPs, the MIC was > 200 μg / ml, whereas the presence of AgNPs in AgNP – CHP (40 μg / ml) and AgNP – CHP – HQ (20 μg / ml) compositions resulted in a drop in the MIC value down to 0.1 μg / ml (Fig. 2). The obtained data indicate significant suppression of bacterial growth compared to the use of the AgNP – HS – lincomycin composition.

DISCUSSION

The mechanism of lincomycin action is associated with the suppression of protein biosynthesis by binding to A-site in the 23S rRNA of the 50S subunit and, consequently, preventing the binding of tRNA and disrupting the translation process [26]. The mechanism of antibiotic resistance to lincomycin was described in detail in [27] and can be associated with inactivation of the antibiotic by enzymes [28], mutations in the MFS efflux pump [29], modification of the 23S rRNA binding site by methyltransferases [30] or mutation in the 50S ribosomal subunit. Although the mechanism of action of antibiotic compositions with AgNPs is not precisely known, it was shown that the use of

AgNPs could be of particular interest in the fight against such antibiotic-resistant microorganisms as MRSA [31, 32].

MRSA F-182 is a classical gram-positive bacterium used in numerous studies on a variety of antibacterial compositions [33]. The genome of this strain contains the *NorB* and *ErmY* genes, which are responsible for modification of the MFS efflux pump and methylation of the lincomycin binding site (23S rRNA), respectively. The above considerations allowed us to suggest that the combination of lincomycin with AgNP and HS made it possible to overcome the MLSB resistance to lincosamides, which is typical of this strain. The found MIC value for lincomycin among MRSA ATCC 43300 isolates was less than 0.1 μg / ml in the presence of 40 μg / ml of CHP and 20 μg / ml of CHP – HQ, respectively, while in the absence of AgNP – HS, the MIC for lincomycin exceeded 200 μg / ml.

The obtained MIC values for AgNPs obtained in this study in the presence of lincomycin and HS were shown in Table 2 and were 0.022 and 0.015 μg / ml for AgNP – CHP and AgNP – CHP – HQ compositions, respectively. Comparison of these values with those reported in the literature indicated higher activity of the obtained compositions compared to values for both engineered AgNPs and bio-AgNPs synthesized by various microorganisms (Table 2).

Table 2

Comparison of the MIC values for lincomycin and AgNPs with respect to MRSA found in this study and in the literature

Antibacterial agent	NP size, nm	Microorganism	MIC			Reference
			Lincomycin, μg / ml	AgNP, μg / ml	HS, μg / ml	
Lincomycin	–	MRSA ATCC 43300	>200	–	–	This study
Humate + lincomycin	–	MRSA ATCC 43300	>200	–	>1,500	This study
Humate + AgNP + lincomycin	6.0	MRSA ATCC 43300	<0.1	0.022	40	This study
Humate – HQ + AgNP + lincomycin	6.0	MRSA ATCC 43300	<0.1	0.015	60	This study
Lignin + AgNP	20.0	Multidrug resistant S. aureus	–	10	–	[34]
Dendrimer-encapsulated AgNP	3.3	S. aureus USA 300	–	128	–	[34]
Polystyrene sulfonate particles, with incorporated AgNP	5.0	S. aureus ATCC 29213	–	1.14	–	[34]
AgNP synthesized using the extract of Dracocephalum kotschyi	19.0	MRSA ATCC 43300	–	15.6	–	[34]
AgNP synthesized using the extract of Artemisia haussk-nechtii	10.7	MRSA ATCC 43300	–	10.0	–	[34]
AgNP synthesized using marine Streptomyces sp.	13.5	MRSA ATCC 43300	–	0.039	–	[34]
AgNP immobilized on copolymer	25.0	MRSA ATCC 43300	–	0.54	–	[34]

The discovered synergistic effects of AgNPs with regard to lincomycin support viability of the idea of applying silver NPs [35] and their compositions [36] on clinical isolates that pose a significant danger to human life. Moreover, they show a significant advantage of the proposed approach to overcoming antibiotic resistance of gram-positive bacteria through the use of ternary compositions of humic substances with nanoparticles and antibiotics. Although similar results were achieved for antibiotic compositions with silver NPs [37] and nanoparticles of other metals [38], the results presented in this study are new and of particular importance for improving safety profile of compositions based on nanosilver due to biogenic properties of HS [24]. Moreover, successful restoration of susceptibility to antibiotics using a novel composition of lincomycin with AgNPs and HS is a significant breakthrough in the fight against antibiotic resistance.

CONCLUSION

The study for the first time demonstrated the synergistic effect of restoring MRSA susceptibility to lincomycin in the presence of silver NPs stabilized with humic substances. Modification of coal humate with hydroquinone slightly enhanced the observed

synergistic effect. We suggested that the synergistic effect of lincomycin and nanosilver stabilized with humic substances could be explained by formation of weak surface complexes between humic substances and lincomycin, which dissociate and release the antibiotic after silver nanoparticles enter the cell. This opens new avenues in the fight against antibiotic resistance. The synthesized compositions of lincomycin with AgNPs stabilized with coal humates could be considered as candidates for further preclinical and clinical trials with the aim of their rapid implementation into clinical practice.

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Authors' contribution

Zykova M.V., Belousov M.V. – final approval of the manuscript for publication. Zhang Yun –development of the method for synthesizing silver nanoparticles in the presence of humic substances, determination of the characteristics of the synthesized compositions. Lysenko I.V., Mikhalev D.A. – carrying out of the experiment. Arutyunyan D.A. – processing and analysis of the obtained data, drafting of the article. Azarkina L.A. – drafting of the original manuscript. Perminova I.V. – supervision of the project, setting tasks, discussion of the results, drafting of the article.

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