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Research Article

Conjugation of a WOW Peptide with silver nanoparticles to face the increase of antimicrobial resistance during COVID 19 pandemic

Ali H. Salama[1]

1 *Faculty of Pharmacy, Middle East University, Amman, Jordan*

Corresponding author: Ali H. Salama (asalama@meu.edu.jo)

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Abstract

Bacterial resistance is a difficult limitation in the treatment of infections. The potential antibacterial activity of WOW peptide conjugation with silver nanoparticles against selected pathogens is investigated in this study. The peptide WOW was created by combining two tryptophan subunits and one ornithine amino acid, and its purity was determined using reverse phase high performance liquid chromatography. Mass spectrometry and electrospray ionization mass spectrometry were used to confirm the WOW peptide. Silver nanoparticles conjugated with WOW were created by adding WOW to a solution of silver nitrate in the presence of the reducing agent sodium borohydride. The yellow-brown color indicated the presence of WOW-AgNPs, which was confirmed by ultraviolet/visible spectrophotometry. The minimum inhibitory and bactericidal concentrations of WOW nanoparticles were determined using the micro dilution method against *Staphylococcus aureus*, *Escherichia coli*, Methicillin resistant *Staphylococcus aureus* (MRSA), and ESBL Escherichia coli. The Erythrocyte Hemolytic Assay was used to assess the toxicity of nanoparticles conjugated with WOW. WOW alone was effective (MICs between 120 and 215 μgml^{-1}) against both standard and resistant strains of bacteria. WOW –AgNPs, on the other hand, were more effective, with MICs ranging from 30 to 100 μgml^{-1} depending on the bacteria used. WOW -–after 30 minutes of incubation, silver nanoparticles at a concentration of 100 μgml^{-1} caused only 3% hemolysis in human erythrocytes.in conclusion, WOW –silver nanoparticles were found to have good antibacterial activity against pathogenic strains of gram positive and gram negative bacteria. Furthermore, the conjugate demonstrated low hemolytic activity and cytotoxicity. As a result, WOW conjugation with AgNPs is a promising treatment candidate for bacterial infection with low toxicity.

Keywords

silver nanoparticles, antibiotics, minimum inhibitory concentration, antimicrobial peptides

Introduction

Antibiotic resistance is one of the world's most serious problems, and it is on the rise around the world, posing a risk to the global population and raising concerns among health officials and governments (Matzov et al. 2017). The widespread use of conventional antibiotics to treat a wide range of ailments is thought to be a major contributor to the problem. Because of the rise of antibiotic-resistant bacteria, the number of antibiotics developed for

clinical use has decreased dramatically (Sifri et al. 2019). Antibiotics used to treat various types of organisms have decreased by 90% in the last 30 years, according to the US Food and Drug Administration. As a result, there is an urgent need to develop new antibiotic alternatives or strategies to improve the efficacy of existing ones. Antimicrobial peptides (AMPs) are a new and promising alternative to traditional antibiotics (Salama et al. 2021). These are small molecules (less than 10 kDa) that come in a variety of sizes and amino acid configurations. AMPs have positive charges ranging from +3 to +9, as well as more than 30% amphipathic hydrophobic residues. When AMPs come into contact with plasma membranes, their amphipathic properties allow them to create pores in the target membranes, resulting in intracellular cell leakage and cell death. AMPs also have an important role in DNA replication and transcription inhibition after crossing the bacterial cytoplasm without damaging the cell membrane (Moravej et al. 2020). As a result, these AMPs may serve as a substitute for antibiotics, or they may work in conjunction with antibiotics to combat various infections (Kumar et al. 2018). Despite the advantages of AMPs as a replacement for conventional antibiotics, a number of issues have hampered their clinical application. These problems arise as a result of the low blood stability caused by the interaction of lipoproteins and negative charge albumins (Kumar and Sanil 2017). More importantly, AMPs lack selectivity, resulting in unwanted interactions with host cells and erythrocyte toxicity. (Raheem and Straus 2019). As a result of these major issues, new research approaches focusing on the design of novel AMP subfamilies known as ultra-short antimicrobial peptides (USAMPs), which contain three to ten amino acids, have been developed. These USAMPs have numerous structural and economic advantages over traditional AMPs, and could thus play an important role in overcoming traditional AMP limitations. (Niu et al. 2021). Furthermore, due to their structural features and restricted number of amino acids, USAMPs have various advantages for development over AMPs, including inexpensive cost of production, reduced mammalian cell toxicity, and enhanced potency. This would eventually give the scientific community with a viable antibacterial chemical choice. Nanotechnology is a fast developing discipline with a wide range of applications in drug research and development. At the same time, silver has been used as an antibacterial and antiseptic agent with little negative effects. Silver nanoparticles (AgNPs) have been discovered to have broad-spectrum antibacterial, antifungal, and antiviral activity. AgNPs are thought to act by passing through bacterial cell walls, altering the structure of cell membranes, and potentially causing cell death by releasing Ag ions that interact with the thiol group (Saadh 2021) . As a result, we present the design and characterization of a tri conjugated ultra-short antimicrobial peptide (WOW) made up of alternating W: tryptophan subunits and ornithine : O and to further improve the hydrophobic character of the ultra-short peptide, the tri was conjugated to para hydroxy cinnamic acid. The resulting WOW peptide was also conjugated with AgNPs to increase its efficacy against resistant and common potentially pathogenic gram positive and gram negative bacteria.

Materials and methods

Bacterial cultures

S. aureus (ATCC 29213), *E. coli* (ATCC 25922), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC BAA-41) and ESBL- *E. coli* (ATCC BAA-3054) were obtained and used in the study from the American Type Tissue Culture Collection (ATCC, Manassas, VA, USA).

Design and synthesis of WOW

WOW is a tri-USAMP that was rationally designed to include three alternating subunits of both tryptophan and ornithine in conjugation with a hydrophobic moiety of para hydroxy cinnamic acid. The designed peptides used in the present study were synthesized by (GL Biochem Ltd., Shanghai, China) using solid-phase method and Fmoc chemistry finally obtained as a lyophilized state. Reverse phase high-performance liquid chromatography (RP-HPLC) was used for purification of the peptide using a C18 internsil ODS-SP column, the column was eluted with acetonitrile / H2O-TFA gradient at flow rate of 1.0ml/minute. The purification and identification of the synthesized peptides were confirmed by ESI-MS mass spectrometry (Almaaytah et al. 2017). Fig. 1 show the structure of the peptide

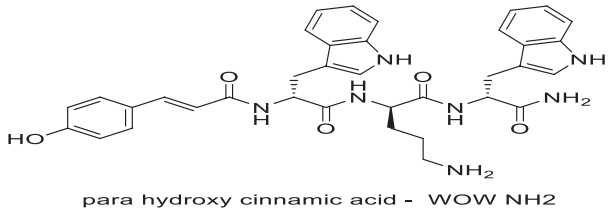

Figure 1. The overall structure of WOW.

Determination of the Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) for WOW

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of both WOW and the eight individual antibiotics (levofloxacin, chloramphenicol, rifampicin, amoxicillin, clarithromycin, doxycycline, vancomycin, and cefixime) representing a variety of modes of antimicrobial activity were determined using the microbroth dilution method as outlined by the Clinical and Laboratory Standards In-

stitute and as described previously (CLSI Guideline Addresses Identification of Bacteria and Fungi Using DNA Target Sequencing 2010). Briefly, bacterial strains were grown in Muller Hinton broth (MHB) medium and diluted to 10^6 CFU/mL in the same medium before use. WOW and the different antibiotics were prepared in different concentrations and aliquot with the bacteria in 96-well plates and incubated for 18–24 h at 37 °C. The cell growth and the MICs were determined by reading the plates on an ELISA reader at OD λ = 570. The MBC was determined by transferring 10 µL from the negative well onto agar plates and incubated for 24 h at 37 °C. The MBC was determined as the concentration that caused the eradication of 99.9% of viable cells. All experiments were performed in triplicate.

Synthesis of AgNPs conjugate with WOW (WOW -AgNPs)

A 5 mL (0.1 mM) aliquot of WOW solution was mixed with a 5 mL (0.1 mM) silver nitrate solution and agitated for 10 minutes. A 20 ml solution of 5 mM sodium borohydride (NaBH4) was added to the mixture. A reducing agent (sodium borohydride solution (NaBH4)) was added to the solution to cause silver ion reduction and then the formation of WOW -AgNPs, as evidenced by the color changing from clear to yellow-brown. The nano particles (NPs) were centrifuged for 1 hour at 12,000 x g, after which the supernatant layer was collected and freeze-dried. The yield was calculated as a percentage of the amount of active component in 100 mg of dry nanoparticles (Masri et al. 2018).

Minimum Inhibitory Concentrations (MICs), and Minimum Bactericidal Concentrations (MBCs) Determination of the WOW *nanoparticles NPs*

Bacterial cells were grown overnight in MHB before being diluted in the same medium to a concentration of 10^6 CFU ml^{-1}. WOW –AgNPs were diluted with sterile distilled water to achieve final concentrations ranging from 0.5 to 100 (gml^{-1}).

In 96-well plates, an aliquot of 50 µL of each concentration and 50 µL of diluted bacterial solution were placed in each well. Each experiment was repeated three times. Plates were incubated at 37 °C for 24 hours. Bacterial growth was measured using an enzyme-linked immunosorbent assay (ELISA) for plate stability, and the MIC was determined as the lowest concentration that inhibited growth (turbidity). Each plate contained a positive control (50 50 µL of bacterial suspension plus 50 50 µL of MHB without antibacterial drugs) and a negative control 200 50 µL MHB). Each experiment was repeated three times. MBCs were determined by taking 10 µL from clear negative wells and from turbid positive control wells after which the aliquots were streaked on sterile labeled nutrient media (How et al. 1985).

Erythrocyte hemolytic assay

A conventional hemolytic experiment was used to test the capacity of WOW conjugation with silver nanoparticles to destroy mammalian erythrocytes. An aliquot of 2 ml of human blood in a 50-ml centrifuge tube at 3000 xg for 5 minutes. The supernatant was then collected, and the cell pellet was suspended in 48 ml of Phosphate-buffered saline and centrifuged at 3000 g for 5 minutes three times. The cells were suspended in sterile tubes containing 50 ml of PBS to obtain a final concentration of 4 percent erythrocytes. An amount of 1 ml of each peptide concentration was added to 1 ml of erythrocyte suspension (4%). Positive controls were made by diluting 1 ml of the erythrocyte solution with 5 µl of triton X100. Negative controls were prepared by combining 1 ml of erythrocyte suspension with 1 mL of PBS. The suspension was incubated at 37 °C, for one hour. The mixture was gently mixed and 1 mL was aspirated of each sample into sterile Eppendorf tubes, then centrifuged at 3000 xg for 5 minutes. An amount of 100 µl of the mixture were taken from each Eppendorf and placed in a 96-well plate (Maturana et al. 2017). At a wavelength of 570 nm, the absorbance was measured. The following formula was used to compute the percentage of hemolysis:

$$\%\ \text{Hemolysis} = \frac{(A - A0)}{(AX - A0)} \times 100$$

Where A: is Optical density 450 with the peptide solution;
A0: is Optical density 450 of the blank;
And A_X: is Optical density 450 of control (0.1% triton X-100).

Results and discussion

Infections caused by drug-resistant bacteria are a serious and growing global health issue. As a result, significant efforts are being made to develop new products (Prestinaci et al. 2015).

Despite these efforts, a growing number of multidrug-resistant bacteria, such as methicillin-resistant *S. aureus* (MRSA) and extended-spectrum beta-lactamase, are being reported on a regular basis (ESBL) (Alabi et al. 2013).

In this study, a novel peptide WOW and its conjugate with silver nanoparticles (WOW -AgNPs) were tested against important pathogenic bacteria such as *S. aureus*, MRSA, *E. coli*, and ESBL *E. coli*. WOW was synthesized and conjugated with AgNPs. Fig. 2 shows the confirmation of WOW-AgNPs through the UV-vis spectrum at 404 nm.

WOW is effective against both standard and resistant gram positive and gram negative bacterial strains, as shown in Table 1, and thus has the potential to be used as a therapeutic alternative to conventional antibiotics. When compared to the other AMPs (Niu et al. 2021), however, the MIC value was slightly higher, which can be explained by WOW net charge. The ideal net charge is +3, but our peptide had a net charge of +2. This would explain the lower antibacterial activity (Xu et al. 2021).

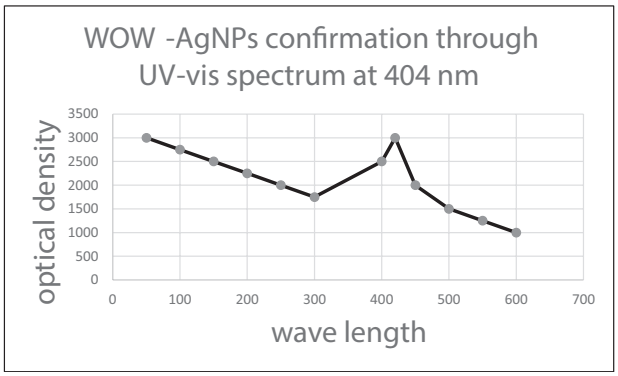

Figure 2. Absorption had shown of silver nanoparticles surface plasmon resonance at 420 nm.

Table 1. MIC and MBC of WOW and WOW -AgNPs conjugate on standard and resistant bacterial strains.

Bacterial strains	MIC µgml^{-1}			MBC µgml^{-1}	
	AgNPs alone	WOW alone	WOW -AgNPs	WOW alone	WOW -AgNPs
S. aureus (ATCC 29215)	120	100	32	100	32
Methicillin Resistant *S. aureus* (MRSA) (ATCC BAA-41)	230	210	95	210	95
E. coli (ATCC 25922)	140	95	29	95	29
ESBL- *E. coli* (ATCC BAA-3054)	220	195	81	195	81

Table 2. Minimum inhibitory concentrations in µgml^{-1} of the eight antibiotics against the tested bacterial strains.

Antibiotics	*S. aureus* (ATCC 29215)	MRSA (ATCC BAA-41)	*E. coli* (ATCC 25922)	ESBL *E. coli* (BAA-3054)
Levofloxacin	0.5	10	2	12
Chloramphenicol	20	25	80	150
Rifampicin	0.025	0.005	15	50
Amoxicillin	5	40	25	200
Clarithromycin	0.5	125	125	125
Doxycycline	2	10	1.5	16
Vancomycin	0.5	2	200	250
cefixime	4	30	6	80

To compare the efficacy of our conjugate, we studied the effect of conventional antibiotics against the same type of bacteria. Additionally, the results of MIC and MBC values were shown in Tables 3, 4, indicating that our peptide has good efficacy compared with traditional antibiotics.

The peptide was conjugated with silver nanoparticles in an attempt to improve it . Nanotechnology has enormous potential, particularly in diagnostics and drug delivery. Nanomaterial-based medication delivery methods have the potential to improve drug pharmacokinetics and pharmacodynamics (Ruden et al. 2009). Several drug-binding nanoparticles have been developed to eradicate drug-resistant bacterial infections because the smaller nanoparticle size provides a larger surface area for maximum drug delivery and availability. (Mussa Farkhani et al. 2016).

In this study, the conjugation of WOW with silver nanoparticles resulted in a nearly 70% reduction in the peptide's MIC value (Table 1). The decrease in the MIC could be due to WOW penetrating the outer membrane

Table 3. Minimum bactericidal concentrations in µgml-1 of the antibiotics against the tested bacterial strains.

Antibiotics	*S. aureus* (ATCC 29215)	MRSA (*ATCC* BAA-41)	*E. coli* (ATCC 25922)	ESBL *E. coli* (BAA-3054)
Levofloxacin	0.5	10	2	12
Chloramphenicol	30	40	100	200
Rifampicin	0.025	0.005	15	50
Amoxicillin	5	40	25	250
Clarithromycin	1.5	150	150	200
Doxycycline	10	20	15	25
Vancomycin	0.5	2	150	200
cefixime	4	30	6	80

Table 4. The *in vitro* hemolysis activity of WOW-AgNPs in human erythrocytes.

Concentration µg mL^{-1}	Hemolysis of AgNPs %	Hemolysis of WOW %	Hemolysis of WOW-AgNPs %
5	80	0	0
10	85	0	0
20	87	0	0
40	95	0	0
60	100	3	0
80	100	5	0
100	100	6	3

of the bacteria cell wall, increasing its permeability and, as a result, the antibiotic impact of the silver nanoparticles (Ramesh et al. 2016)

WOW –AgNPs were found to be effective, and the ability of this conjugate to damage mammalian erythrocytes, in particular, was investigated using the standard erythrocytes hemolysis assay. The erythrocytes were challenged with conjugate concentrations ranging from 5 to 100 g/mL. The results showed that after 60 minutes of incubation with human erythrocytes at a concentration of 100g/mL, the conjugate caused only 3% hemolysis (Table 4). The hemolytic assay confirmed the conjugate's lack of hemolytic activity.

Antimicrobial peptides have been shown to be promising medication candidates (Salama et al. 2021), but concerns about cell toxicity, metabolic stability, and high manufacturing costs have stymied their development. As reported in this study, our peptide has good antibacterial efficacy against a variety of typical pathogenic and resistant pathogens, including MRSA and ESBL E. coli, with a bactericidal mechanism of action. WOW AgNPs conjugate also demonstrated strong activity against human erythrocytes while displaying low hemolytic activity and cytotoxicity. As a result, the conjugation method with AgNPs demonstrated significant benefits in terms of peptide antibacterial activity and toxicity.

Conclusion

In this study, we describe the creation and antimicrobial evaluation of a new WOW-silver nanoparticle combination that displayed promising antibacterial properties

against clinically significant resistant Gram-positive and Gram-negative bacteria while exhibiting very weak hemolytic activities. Consequently, the combination of WOW with silver nanoparticles may offer a low-toxicity therapy option for bacterial illness. When compared to the nanoparticle or peptide alone, the peptide-silver nanoparticle combination has been found to have much greater stability and activity. Due to its distinct qualities in comparison to conventional antibiotics, this compound may one day be the remedy for the rising antimicrobial resistance.

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