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EXPERIMENTAL EVALUATION OF
ANTIMICROBIAL EFFECTS OF THE
SYNTHETIC PEPTIDE ON PATHOGENIC
BACTERIA

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EKSPERIMENTALNA EVALUACIJA
ANTIMIKROBNOG EFEKTA SINTETIČKOG
PEPTIDA NA PATOGENE BAKTERIJE

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Key words

antimicrobial peptides, antibiotic resistance, de novo peptide design, computational modelling, bacterial growth

Ključne reči

antimikrobni peptidi, rezistencija na antibiotike, de novo dizajniranje peptida, kompjutersko modelovanje, rast bakterija

Abstract

Antibiotics are common anti-infection drugs used these days. However, their excessive applications have led to the rising antibiotic resistance, a serious phenomenon in modern medicine that is regarded as one of the pre-eminent public health concerns of the 21st century. As a result, there is a growing need to find alternative drugs to eradicate the microbial resistance. Antimicrobial peptides (AMPs) are natural defence molecules found in virtually all life forms. They are evolutionary conserved components of the innate immune defence. AMPs utilize different mechanisms of action for killing bacteria, which can vary depending on a type of particular bacterium. Generally, AMPs are broad-spectrum antibiotics that act not only against bacteria but also certain viruses and fungi. A number of studies suggested a possibility of using AMPs as an alternative therapy for treatment of microbial infections.

Azurocidin (Azu) is a natural antibiotic from human neutrophils. The Resonant Recognition Model (RRM) was employed to computationally design a bioactive peptide analogue that can mimic the bioactivity of natural antimicrobial peptide Azu. In this study the antimicrobial activity of a synthetic peptide analogue of Azurocidin (Azu-RRM) was experimentally evaluated on selected bacteria and compared with the antimicrobial effect of native Azu on these bacteria. The findings revealed that Azu-RRM affected the growth of both Gram-positive (*Staphylococcus aureus* ATCC 25923, and *Staphylococcus aureus* 344) and Gram-negative (*E. coli* 25922) bacteria. The results clearly indicate that synthetic Azu-RRM shows a potent antimicrobial activity and can potentially present a new alternative therapeutic compound to control bacterial infections.

INTRODUCTION

Antibiotics are common anti-infection drugs applied in clinics today. Infections caused by antibiotic resistance to common pathogenic bacteria are associated with increased morbidity, prolonged hospital stays, greater direct and indirect medical and health care costs. These extended periods of stay in hospital lead to a greater chance for infections to be spread out from one infectious individual to another. The shortcomings of currently used therapies to combat a variety of infections have prompted a search for alternatives and development of new antimicrobial drugs.

AMP is an evolutionarily conserved component of the innate immune response, the principal defence system for majority of living organisms. AMPs are found in virtually all life forms, where they work as a first line of defence against invading pathogens [1,2]. They work as a first line of defence against invading pathogens in a human body. Often AMPs target the cytoplasmic membranes but may also interact with DNA and protein synthesis, protein folding, and cell wall synthesis. It has recently been discovered that some AMPs are not directly bactericidal, but rather exert their effects by immunomodulation [1,2,4]. AMPs are relatively small (6 to

100 amino acids) amphipathic molecules of variable length, sequence and structure, with biological activity against a wide range of microorganisms including bacteria, protozoa, yeast, fungi, viruses and even cancer cells. They usually act through relatively non-specific mechanisms resulting in membranolytic activity but they can also stimulate the innate immune response. AMPs represent a new family of antibiotics that have stimulated research and clinical interest as new therapeutic options for infections caused by multidrug-resistant bacterial^[4,5]. In particular, there are many experiments on the effect of antimicrobial peptide Azurocidin. It is reported that this peptide shows great antimicrobial activity against the gram-negative bacteria and gram-positive bacteria^[6-9].

Peptides and proteins are used in design of new drugs and other pharmaceutical products. The Resonant Recognition Model (RRM) ^[10,11] is a theoretical approach capable of analysing protein structure-function relationships, and is used to identify the selectivity of protein interactions within the amino acid sequence. The RRM concept is based on the finding that there is a significant correlation between spectra of the numerical presentation of amino acids and their biological activity. The RRM ^[10,11] is a physico-mathematical model that interprets protein sequence linear information using digital signal processing methods. In the RRM the protein primary structure is represented as a numerical series by assigning to each amino acid in the sequence the electron-ion interaction potential (EIIP) value to each amino acid ^[10]. These numerical series can then be analysed by appropriate digital signal processing methods (fast Fourier transform is generally used). To determine the common frequency components in the spectra for a group of proteins, the multiple cross-spectral function is used. Peaks in this function denote common frequency components for the sequences analyzed. Once the characteristic frequency for a particular protein function/interaction is identified, it is possible then to utilize the RRM approach to predict the amino acids in the protein sequence, which predominantly contribute to this frequency and thus, to the observed function, as well as to design de novo peptides having the desired periodicities. The predictive capabilities of the RRM approach have been tested previously on a vast number of different proteins ^[10-18]. In this study, the antimicrobial effects of computationally designed Azu-RRM peptide were evaluated on the selected bacterial cultures.

MATERIALS AND METHODS

Application of the Resonant Recognition Model to bioactive peptide design

In order to design biologically active peptides, it is of primary importance to determine which amino acids are responsible for the biological activity of a native protein. It is known that the biological function of a protein is determined by its primary structure, i.e. a linear sequence of amino acids. It has been found through an extensive research that proteins with the same biological function have a common frequency in their numerical spectra. This frequency was found then to be a characteristic feature for protein biological function or interaction ^[10-12,14, 16-18]. In the RRM, a

protein characteristic frequency can be identified from analysis of the power spectra of the selected protein sequences. In addition, from the analysis of their phase spectra we can identify the corresponding phase for a particular frequency.

On the basis of determined frequency and phase, a *de novo* short bioactive peptide having the desired biological activity of a given protein can be designed. In our previous study ^[3] we applied the RRM to computational analysis of native Azurocidin (Azu) proteins and design of its peptide analogue, Azu-RRM. The experimental evaluation of its efficacy and comparison of the anti-microbial activities of Azu and Azu-RRM are conducted here on the selected Gram-negative and Gram-positive bacteria.

MATERIALS

The Gram-positive bacterial strains were *Staphylococcus aureus* ATCC 25923, and *S. aureus*344 (ampicillin resistant strain). The Gram-negative bacterium used in this study was *E. coli* ATCC 25922. The bacterial isolates were obtained from the Microbiology Laboratory, School of Applied Sciences. The bacterial isolates were cultures in Mueller-Hinton Agar (MHA) and Mueller-Hinton Broth (MHB) (ThermoFisherScientific, Australia). The optical density (OD₆₀₀) of bacterial suspensions in broth cultures was measured by Eppendorf OD₆₀₀ reader (Biophotometer). Ascent software for Multiskan Ascent Reader for OD₆₀₀ was from Thermo Electron Co. The *de novo* designed peptide Azu-RRM was commercially synthesized to 95% purity by GL Biochem, China.

Antimicrobial Assay

To assess the antimicrobial activity of the synthetic peptide Azu-RRM, a well isolated colony of either *S. aureus* ATCC 25923; *S. aureus*344; or *E. coli* ATCC25922 from a fresh MHA plate was inoculated into 2ml of MHB (to ensure a proper viability), and incubated at 37°C overnight. The starting concentration for inoculation was setup similarly to Weigand ^[19], with a sample of bacterial suspension measured via optical density reading by Eppendorf BioPhotometer to determine an approximate concentration of bacteria. The sample then is appropriately diluted to create the desired concentration of 1x10⁷, which is used as a basis for the experiment starting inoculation.

To examine the antimicrobial activity of Azu-RRM peptide at different concentrations, the assay was conducted in a 96-well microlitre-plate as explained below:

1. Firstly, 100µl of MHB were added to each well from column 1 to column 11 in all rows of A, B, C, F, G, and H.

2. Then, 100µl of Azu-RRM working solution with an initial dose of 1000 µM was added to the first well in rows A, B and C. Serial dilutions of the peptides were then created in wells (2-10).

3. In the next steps, all wells of rows F, G, and H were used for halving the concentration of the peptide in each consecutive well. Then the extra 100µl solution in each well from the last column of rows F, G, and H were discarded. This was followed by adding 100µl of the bacterial suspension to all wells in column 1 to 10. Negative growth controls

of sterile MHB (200µl) were added in all wells in column 11. And 200µl of bacterial suspensions (without the peptide) were added to wells in column 12 as full growth controls.

4. Then the seeded plates were incubated at 37°C for 4hours, 8hours and overnight.

Antibiotics susceptibility testing

In order to measure the activity of Azu-RRM against the studied bacterium, Multiskan Ascent Reader for OD₆₀₀ reading was carried out with Ascent software. The antimicrobial activity was assessed by measuring the optical density of the seeded plates, as previously indicated, were incubated at 37°C for 4hours, 8hours and overnight. The experiments were performed in triplicates with three repeats for each experiment. The average of the three times OD₆₀₀ reading was taken for the data analysis.

Viable cell counts

The colony-forming unit (CFU) count is an estimate of viable bacterial numbers. Unlike direct optical density counts, where all bacterial cells, dead and living, are counted, CFU estimates viable cells only. This method is employed here to assess the effects induced by the synthetic peptide treatment on the selected Gram-negative and Gram-positive bacteria.

Firstly, 10ul samples of bacterial suspensions were collected from wells with 8h incubation, treated with Azu-RRM at the concentration of 2uM and 4uM, and positive control after OD₆₀₀ reading. Secondly, the collected samples were diluted to final concentrations of 10⁻², 10⁻⁴, 10⁻⁵, 10⁻⁶, and 10⁻⁷.

Then, 100ul from the last 3 dilutions (10⁻⁵, 10⁻⁶, and 10⁻⁷) were spread as lawn culture on MHA plates and incubated overnight at 37°C. The next day, the data of CFU was conducted according to the standard methods of surface plate dilution and cell counting in a similar way to Roszak&Cowell[20].

Data analysis

The statistical analysis was performed using the Microsoft Office 2003 Excel and MATLAB software. The statistical significance of the differences between the Azu-RRM treatments of bacteria and the positive control group were analysed by one-way ANOVA.

RESULTS

A. Computational analysis and design

The RRM approach was applied to analysis of eight Azu peptides. The Azu protein primary sequences were collected from the NCBI protein database.

A multiple cross-spectral analysis was performed resulting in two prominent RRM characteristic frequencies identified at $f_1=0.1133$ (prominent peak) and $f_2=0.0293$ (less significant peak) shown in Figure 1. These frequencies are related to the biological activity of the analysed native Azu peptides. According to the RRM concepts, the prominent peak(s) characterizes the common biological activity of the analyzed Azu peptides.

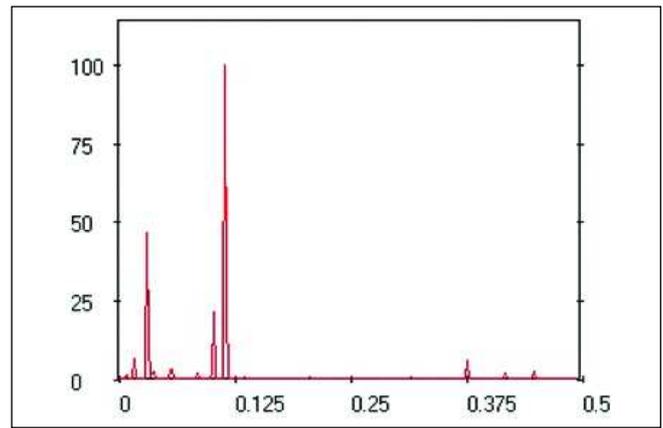


Figure 1. Multiple cross-spectral function of eight Azurocidin protein sequences. The abscissa represents RRM frequencies, and the ordinate - relative signal intensities [3].

Less prominent peaks observed in Figure 1 indicate that these selected Azu peptides can be involved in different biological processes (interact with other proteins or small molecules). We then calculated the phase at the more prominent frequency $f_1=0.1133$. On the basis of the determined characteristic frequency $f_1=0.1133$ and the phase at this frequency $\phi_1=-1.880$ we designed the short peptide analogue, Azu-RRM. ProtParam

(<http://au.expasy.org/tools/protparam.html>)

was used as a tool to compute physical and chemical parameters of the synthetic Azu-RRM peptide analogue. This computationally designed peptide (18 mer long) is 4.4358 kDa; theoretical pI: 6.00; estimated half-life in mammalian reticulocytes: 5.5 h; and instability index: 16.84 which classifies the protein as stable [3].

B. Experimental Evaluation of anti-microbial effects of Azu-RRM peptide analogue by OD595 reading

The anti-microbial effects of Azu-RRM peptide analogue on the growth of Gram-positive *S. Aureus* ATCC 25923, *S. aureus* 344 and Gram-negative *E. coli* bacteria are shown in Figures 2-7. Different concentrations of Azu-RRMat8h and overnight incubations were used in order to find the optimal treatment concentrations and time that can induce the maximum suppressing effects on bacterial growth.

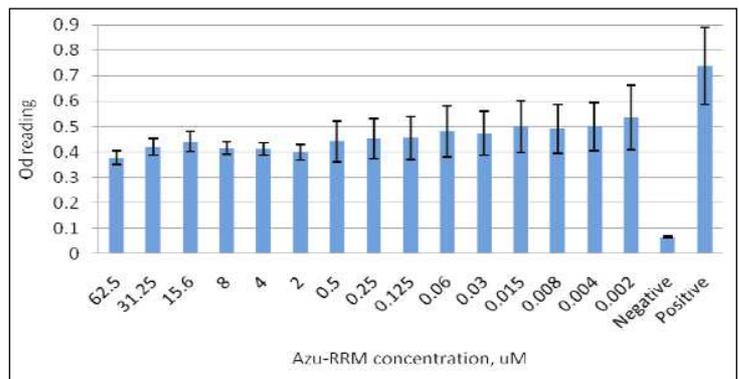


Figure 2. The effects of Azu-RRM on the growth of *S. aureus* ATCC25923 at 8hours incubation

Figure 2 demonstrates the effects of different concentrations of Azu-RRM peptide analogue on Gram-positive *S. aureus* ATCC25923 bacteria at 8 hour incubation. Its anti-microbial activity is noticeably strong at the concentration of 2µM, when compared to the positive control. A relative change of 48% in absorbance (OD reading) can be seen at this concentration of the synthetic peptide treatment.

Figure 3 demonstrates the effects of different concentrations of Azu-RRM peptide on Gram-positive *S. aureus* ATCC25923 bacteria at overnight incubation. Its anti-micro-

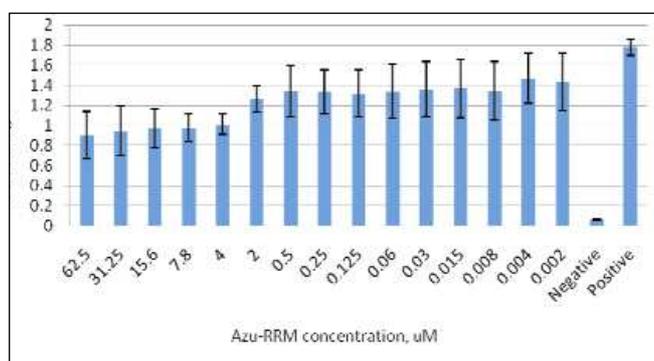


Figure 3. The effects of Azu-RRM on the growth of *S. aureus* ATCC 25923 at overnight incubation

0.5µM, when compared to the positive control. A change of 40% in absorbance (OD reading) can be seen at this concentration of the synthetic peptide treatment.

Figure 5 demonstrates the effects of different concentrations of Azu-RRM peptide on Gram-positive *S. aureus* 344 (ampicillin resistant strain) at overnight incubation. Its anti-microbial activity is noticeably strong in the concentration of 4µM when compared to the positive control. A change of 44% in absorbance (OD reading) can be seen at this concentration of the synthetic peptide treatment.

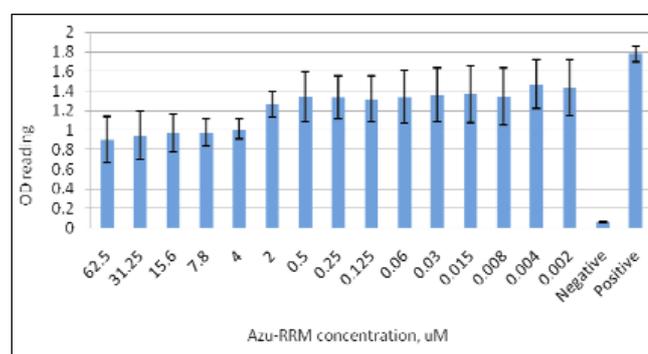


Figure 4. The effects of Azu-RRM on the growth of *S. aureus* 344 at 8h incubation

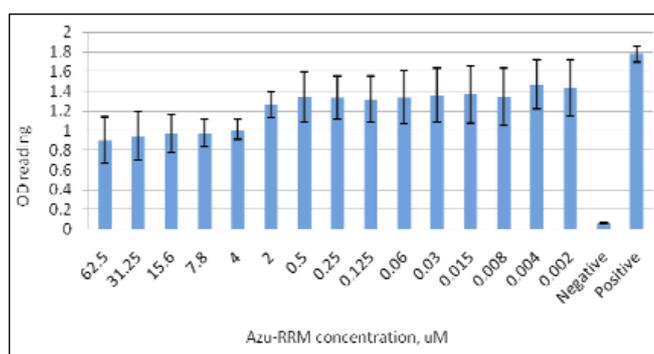


Figure 5. The effects of Azu-RRM on the growth of *S. aureus* 344 at overnight incubation

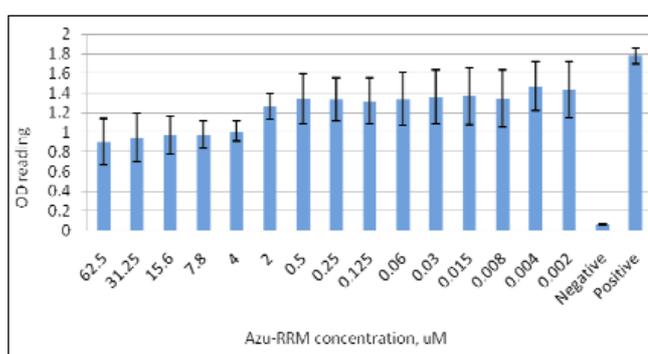


Figure 6. The effects of Azu-RRM on the growth of *E. coli* 25922 at 8 hours incubation

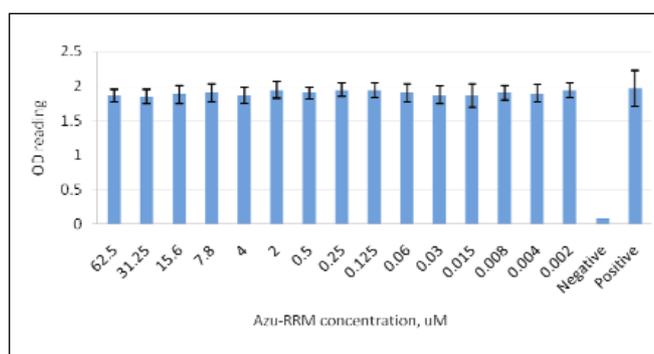


Figure 7. The effect of Azu-RRM on the growth of *E. coli* 25922 at overnight incubation

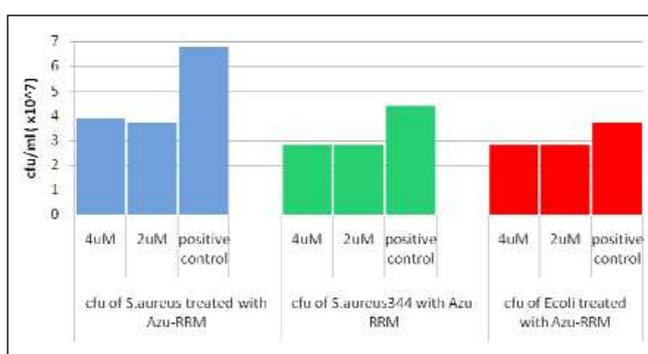


Figure 8. Viable cell counts (CFU) of *S. aureus* ATCC 25923, *S. aureus* 344 and *E. coli* ATCC 25922 cultures following treatment with Azu-RRM at 8h incubation

bial activity is noticeably strong at the concentration of 4µM, when compared to the positive control. A change of 44% in absorbance (OD reading) can be seen at 4µM concentration of the synthetic peptide treatment.

Figure 4 demonstrates the effects of different concentrations of Azu-RRM peptide on Gram-positive *S. aureus* 344 (ampicillin resistant strain) at 8 hour incubation. Its anti-microbial activity is noticeably strong at the concentration of

Figure 6 demonstrates the effects of different concentrations of Azu-RRM peptide on Gram-negative *E. coli* 25922 bacteria at 8 hours incubation. Its anti-microbial activity is noticeably strong at the concentration of 0.5µM, when compared to the positive control. A change of 26% in absorbance (OD reading) can be seen at 0.5 µM concentration of the synthetic peptide treatment.

As can be observed from Figure 7, there are no noticeable effects of the synthetic Azu-RRM peptide treatment on Gram-negative *E. coli* 25922 at overnight incubation. In essence, the results shown in Figures 2-6 indicate that the synthetic, computationally designed Azu-RRM peptide analogue has a broad range of antimicrobial activity against the studied Gram-positive and Gram-negative bacteria at the particular concentrations and incubation times. It should be noted, that the studied Gram-positive bacteria are well susceptible to the Azu-RRM treatment at the concentrations of 62.5 μM to 0.5 μM at 8 hours incubation. It can be also noted that Gram-negative bacteria *E. coli* 25922 are susceptible (the effects vary depending on Azu-RRM concentration) to Azu-RRM treatment at the concentrations of 62.5 μM to 2 μM at 8 hours incubation; with the maximum suppressing effect achieved at 0.5 μM (Figure 6). However, there are no noticeable effects of Azu-RRM treatment on *E. coli* 25922 at overnight incubation (Figure 7).

C. Results of viable cell counts on Azu-RRM 8hr incubation

The viable cell count (CFU) results from plates inoculated with the 10^{-6} bacterial suspension dilutions are shown in Figure 8. The viable count of bacterial cultures (Figure 8) show that both Gram-positive bacteria (*S. aureus* ATCC 25923 and penicillin resistant *S. aureus* 344) are more susceptible to Azu-RRM treatment than the Gram-negative bacterium *E. coli* 25922 at 8 hours incubation.

It also can be seen that:

(i) there is a significant reduction of 44% in viable cell counts of *S. aureus* induced by Azu-RRM treatment at the concentrations of 2 μM and 4 μM , when compared to CFU of positive control;

(ii) a reduction of 36% in viable cell counts of *S. aureus* 344 induced by Azu-RRM treatment at the concentrations of 2 μM and 4 μM , when compared to CFU of positive control; and

(iii) a reduction of 25% in viable cell counts of *E. coli* induced by Azu-RRM treatment at the concentrations of 2 μM and 4 μM , when compared to CFU of positive control.

The findings reveal that the growth rates of these three selected bacterial species were significantly affected by Azu-RRM treatment at the concentrations in a range of 62.5 μM to 0.5 μM at 8h incubation, when compared to the non-treated positive control. The statistical analysis of the experimental data shows that the survival rates of bacterial cultures of *S. aureus* ATCC 25923, *S. aureus* 344 and *E. coli* 25922 treated with Azu-RRM (62.5 μM -0.5 μM) at 37°C, 8 hour incubation were significantly lower ($p < 0.01$), when compared to the non-treated cultures (positive control). Hence, these findings allow summarizing that the computationally designed Azu-RRM peptide analogue exhibits an inhibitory activity against the studied pathogenic bacteria.

DISCUSSION AND CONCLUSION

In this study, the efficacy of the synthetic Azu-RRM peptide analogue, as a candidate for anti-infection therapy, was experimentally evaluated *in vitro* on Gram-positive bacteria (*S. aureus* ATCC 25923, and *S. aureus* 344) and the Gram-

negative bacterium (*E. coli* 25922). In our previous study it was shown that antimicrobial effects of the native Azu peptide can be emulated by the synthetic computationally designed Azu-RRM peptide analogue [3]. In particular, the reported findings showed that both native Azu and synthetic Azu-RRM peptides induce suppressing effects on *S. aureus*, their treatments at the particular concentrations affect the bacterial growth. Moreover, the results showed that their activities against *S. aureus* were rather bacteriostatic than bactericidal [3].

The results presented in this study reinforce our previous research work on the antimicrobial activity of synthetic Azu-RRM. According to our results, the activity of Azu-RRM peptide against Gram-positive bacterium (*S. aureus* ATCC 25923, *S. aureus* 344) and Gram-negative bacterium (*E. coli* ATCC 25922) is dose-dependent. It is important to note, that at 8 hours incubation the most significant antimicrobial, suppressing effects of Azu-RRM against *S. aureus* ATCC 25922 was recorded at the concentration of 2 μM . While for the penicillin resistant strain *S. aureus* 344, the killing effect is most noticeable at the concentration of 0.5 μM . For *E. coli* ATCC 25922 bacterium, the most significant effect of Azu-RRM treatment is observed at the concentration of 0.5 μM .

Furthermore, the results also showed that the effects of Azu-RRM peptide against Gram-positive and Gram-negative bacteria are time-dependent, when compared to the same concentration treatment of Azu-RRM (0.5 μM , 2 μM and 4 μM) of these bacteria at 8 hours and overnight incubations. Interestingly, the test results of Gram-negative bacterium *E. coli* ATCC 25922 treatment by Azu-RRM at 8 hours incubation clearly demonstrate the antimicrobial effects. In contrary, the Azu-RRM treatment at overnight incubation induces no effects on the bacterial growth of *E. coli* ATCC 25922.

Our results also showed that the activity of Azu-RRM against the Gram-positive bacterial strains is stronger than against Gram-negative bacterium. The antimicrobial activity data from the live cells count revealed that Azu-RRM activity against the studied bacteria was rather bacteria-static than bacteria-cidal as the viable cell count (CFU) results showed that there were still live bacterial cells that survived the peptide treatment.

As was shown through the experimental evaluation, the RRM is capable of identifying the key amino acids in the native Azurocine peptide sequence which predominantly contributes to its characteristic frequency and thus, to the desired function, and design of the *de novo* peptide analogue. Since this *de novo* peptide carries the common characteristic of Azurocidin, specifically in terms of its antimicrobial activity, the peptide analogue will exhibit the same biological activity as the native Azurocidin. The experimental findings corroborate our previous results of using the RRM approach to *de novo* design of different peptide analogues [10-12, 14, 16-18].

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Sažetak

Antibiotici su uobičajeni anti-infektivni lekovi koji se danas primenjuju. Ipak, njihova široka primena dovela je do porasta rezistencije na antibiotike, ozbiljnog fenomena moderne medicine koji se smatra jednim od najozbiljnijih problema javnog zdravlja 21. veka. Kao posledica, javlja se potreba za alternativnim lekovima kojima bi se eradicala rezistencija mikroorganizama. Antimikrobni peptidi (AMP) su prirodni odbrambeni molekuli nađeni u skoro svim oblicima života. Oni predstavljaju evoluciono konzervirane komponente urođene imunske odbrane. AMP koriste različite mehanizme za ubijanje bakterija, koji variraju zavisno od tipa bakterije. Generalno, AMP su antibiotici širokog spektra, koji deluju ne samo protiv bakterija nego i protiv nekih virusa i gljivica. Brojni radovi ukazuju na mogućnost primene AMP kao alternativne terapije u tretmanu mikrobnih infekcija.

Azurocidin (Azu) je prirodni antibiotik izolovan iz humanih neutrofila. Model koji se naziva „Resonant Recognition Model” (RRM) je upotrebljen za kompjutersko dizajniranje analoga bioaktivnog peptida, koji može oponašati bioaktivnost prirodnog antimikrobnog peptida Azu. U ovom radu je antimikrobna aktivnost dobijenog sintetičkog peptida, analoga Azurocidina (Azu-RRM), eksperimentalno evaluirana na selektiranim bakterijama i upoređena s antimikrobnim efektom prirodnog Azu na tim bakterijama. Pokazano je da Azu-RRM utiče na rast i Gram-pozitivnih (*Staphylococcus aureus* ATCC 25923 i *Staphylococcus aureus* 344) i Gram-negativnih (*E. coli* 25922) bakterija. Rezultati jasno ukazuju da sintetički Azu-RRM pokazuje jaku antimikrobnu aktivnost i može potencijalno predstavljati novo alternativno terapijsko jedinjenje za kontrolu bakterijskih infekcija.

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