The Effects of Synthetic Azurocidin Peptide Analogue on Staphylococcus Aureus Bacterium

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***Abstract*— Antibiotics are commonly used as anti-infection drugs. However, the rising of microbial resistance to antibiotics imposes a major challenge to their widespread applications. Hence, there is a growing need to find alternative drugs to eradicate the microbial resistance arising from the excessive use of antibiotics. Antimicrobial peptides (AMPs) are natural defence molecules found in human body. These AMP are present virtually in all life forms where they act as the first line defence agents against invading pathogens. Published studies [1] [2] suggest the possible use of AMPs as alternative anti-infective drugs. In this study we evaluated the anti-microbial activity of a synthetic Azurocidin peptide analogue and compared its efficacy with the native natural antimicrobial peptide Azurocidin. The Resonant Recognition Model (RRM) was employed here to computationally design a short Azurocidin peptide analogue, Azu-RRM. According to the RRM, this *de novo* designed peptide analogue will mimic and exhibit the activity of the natural Azurocidin (Azu) protein. Within this study the antimicrobial activity of Azu-RRM was investigated on *Staphylococcus aureus* (ATCC 25923) bacterium. The results obtained reveal that the synthetic peptide analogue affected the growth of this gram positive bacterium. The findings also showed that the Azu-RRM is exhibiting the anti-microbial effects on the growth of the studied bacteria comparable with the suppressing effects induced by the natural Azu protein.**

I. Introduction

NOWADAYS antibiotics are common anti-infection drugs applied in clinical environment. Infections caused by antibiotic resistant pathogenic bacteria are associated with increased morbidity, prolonged hospital stays, greater direct and indirect health care costs. These infections also contribute to extended disease periods when infected individuals can spread infections to healthy people. The decline in the efficiency of currently used antibiotics for treatment of various infections has prompted research into new antimicrobial drugs.

Antimicrobial peptides (AMPs) are evolutionarily conserved components of the innate immune response, the principal defence system for majority of living organisms, where AMPs play a key role as defence agents against invading pathogens [1] [2]. AMPs are relatively small (6 to 100 amino acids) amphipathic molecules of variable length, sequence, and structure with bioactivity against a wide range of microorganisms including bacteria, protozoa, yeast, fungi, viruses and even tumor cells [2]. 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 treatment of infections caused by multidrug-resistant bacteria [3] [4]. There are a number of published studies on the effects of APM Azurocidin that show its strong antimicrobial activity against different gram-negative and gram-positive bacteria [5] [6] [7] [8], which is of particular interest to this study.

Proteins are linear macromolecules built up of sequentially linked amino acids. The information contained in the amino acid sequence determines a protein's chemical properties, chain conformation and protein's function. The Resonant Recognition Model (RRM) [9] [10] is a physico-mathematical approach that interprets protein sequence linear information using digital signal processing methods, Fourier and Wavelet Transforms. The application of the RRM involves two stages of calculation. The first is the transformation of the amino acid sequence into a numerical sequence. Each amino acid is represented by the value of the Electron-Ion Interaction Potential (EIIP) describing the average energy states of all valence electrons in a given amino acid. The EIIP values for each amino acid were calculated using the following general model of pseudopotentials [9] [10]:

$$\langle k+q|w|k\rangle = 0.25\frac{Z\sin(1.04*\pi Z)}{2\pi}$$

where q is the change of momentum of the delocalised electron in the interaction with potential w, while

$$Z = \frac{\sum_i Z}{N}$$

where Zi is the number of valence electrons of the i-th component of each amino acid and N is the total number of atoms in the amino acid. A unique number can thus represent each amino acid or nucleotide, irrespective of its position in a sequence. Numerical series obtained in this way are then analysed by digital signal analysis methods. As the average distance between amino acid residues in a polypeptide chain is about 3.8 Å, it can be assumed that the points in the numerical sequence derived are equidistant. For further

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numerical analysis the distance between points in these numerical sequences is set at an arbitrary value d = 1. Then the maximum frequency in the spectrum is fmax = 1/2d = 0.5. The total number of points in the sequence influences the resolution of the spectrum only. Thus, for an N-point sequence the resolution in the spectrum is equal to 1/N. The n-th point in the spectral function corresponds to the frequency f = n/N. In order to extract common spectral characteristics of sequences having the same or similar biological function, the following cross-spectral function was used:

$$Sn = XnYn^* \quad n = 1,2,..., N/2$$

where Xn are the Discrete Fourier Transform (DFT) coefficients of the series x(n) and Yn* are complex conjugate discrete Fourier transform coefficients of the series y(n). Peak frequencies in the amplitude cross-spectral function define common frequency components of the two sequences analysed. To determine the common frequency components for a group of protein sequences, the absolute values of multiple cross-spectral function coefficients M have been calculated as follows:

$$|Mn| = |X1n|.|X2n|...|XMn| \quad n = 1,2,..., N/2$$

Peak frequencies in such a multiple cross-spectral function denote common frequency components for all sequences analysed. Signal-to-noise ratio (S/N) for each peak is defined as a measure of similarity between sequences analysed and is calculated as the ratio between signal intensity at the particular peak frequency and the mean value over the whole spectrum. The presence of a peak frequency with significant signal-to-noise ratio (at least 20) in a cross-spectral function implies that all of the analysed sequences within the group have one frequency component in common. This frequency is related to the biological function provided the following criteria are met:

- Only one peak exists for a group of protein sequences sharing the same biological function.
- No significant peak exists for biologically unrelated protein sequences.
- Peak frequencies are different for different biological functions [9] [10] [11] [12].

Once the characteristic frequency for a particular biological function or interaction is determined, it becomes possible to identify the individual "hot spot" amino acids that contributed most to this specific characteristic frequency and thus, to the observed biological behavior of the protein [9] [10]. It is also possible to design *de novo* short bioactive peptides having the desired biological activity of a given protein. In this study we applied the RRM to computational analysis of native Azurocidin (Azu) proteins and design of its peptide analogue, Azu-RRM. The experimental evaluation and comparison of the anti-microbial activities of Azu and Azu-RRM was conducted on the selected gram-positive *S. aureus* bacteria.

II. MATERIAL AND METHODS

A. Resonant Recognition Model (RRM) – bioactive peptide design

In this study, the bioactive Azu-RRM peptide analogue was designed using the following strategy for the defined peptide design:

1) The RRM characteristic frequency is determined from the multiple cross-spectral functions for a group of protein sequences that share a common biological function.

2) The phases are calculated for the characteristic frequency or frequencies of a particular protein, which is selected as a parent for an agonist/antagonist.

3) The minimal length of the designed peptide is defined by the appropriate frequency resolution. An Inverse Fourier Transformation (IFT) is used to calculate a numerical sequence of different lengths, which exhibits the same prominent characteristic frequency as a parent protein.

4) To determine the amino acids that correspond to each element of the new numerical sequence defined above, the tabulated Electron Ion Interaction Potential (EIIP) parameter values are used. The resulting new amino acid sequence represents the anticipated designed peptide. The *de novo* designed peptide has no homology to the original protein sequence but is expected to exhibit the same biological function as an original/parent protein

B. Experiment Setup

Mueller-Hinton Agar (MHA) and Mueller-Hinton Broth (MHB) were obtained from ThermoFisher Scientific, Australia. The *de novo* designed peptide Azu-RRM was commercially synthesized to 95% purity by GL Biochem (China). The Human Neutrophilpeptide (Azu) was purchased from Sigma-Aldrich Pty Ltd, Australia. The gram-positive bacterium *S. aureus* ATCC 25923 was used here to assess the antimicrobial effects of the studied Azu and Azu-RRM peptide analogue.

To assess the antimicrobial activity of Azu-RRM and the native Azu peptide, a well isolated *S. aureus* colony from a fresh MHA plate was inoculated into 2ml of MHB and incubated at 37^0C overnight. Then the optical density (OD_{600}) of the bacterial cell density in broth was adjusted to 0.5 McFarland.

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

1. First, 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 or Azu-RRM working solution with an initial dose of 0.625 µM for Azu or an initial dose of 1.25µM for Azu-RRM were 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 step, all wells of rows F, G, and H were used for halving the concentration of the peptide in each consecutive well.
4. The extra 100µl solution in the last column well of rows F, G, and H was discarded.
5. 100µl of bacterial suspension was added to all wells in column 1 to 10.
6. Negative growth controls of sterile MHB (200µl) were added in all wells in column 11. And 200µl of bacterial

suspensions (without peptide) were added to wells in column 12 as full growth controls.

7. The seeded plate was then incubated at 37°C overnight.

The antimicrobial experiments were repeated three times. The average of the three times OD_{595} reading was taken for the data analysis.

III. Result and Discussion

A. Computational analysis and design

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

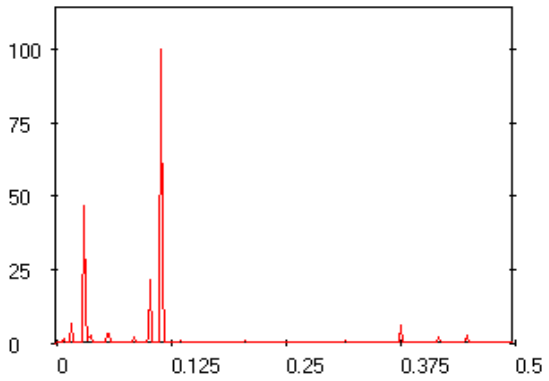

Figure1. Multiple cross-spectral function of eight Azurocidin protein sequences. The abscissa represents RRM frequencies, and the ordinate - relative signal intensities.

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. Less prominent peaks observed in Fig. 1 indicate that these selected Azu peptides can be involved in a 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 phase φ=-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.

B. Experimental Evaluation of anti-microbial effects of Azu-RRM peptide analogue and Azurocidin protein by OD_{595} reading

The anti-microbial effects of Azu-RRM peptide analogue and Azu against *S. aureus* bacterial growth are shown in Figures 2, 3. Different concentrations of native and synthetic peptides have been used in order to find the optimal concentrations that induce the maximum suppressing effects on bacterial growth. For Azu-RRM, we started from a concentration of $6.25x10^{-1}$(μM) then reduced in half until it reached $1.25x10^{-7}$(μM). For native Azu, the concentration started from $3.1x10^{-1}$(μM) to $5x10^{-6}$(μM). Figures 2, 3 demonstrate the window of the effect with the most visible change. In addition, the full growth control which is *S. aureus* without any treatment is shown with red line, while the green line shows the no growth negative control.

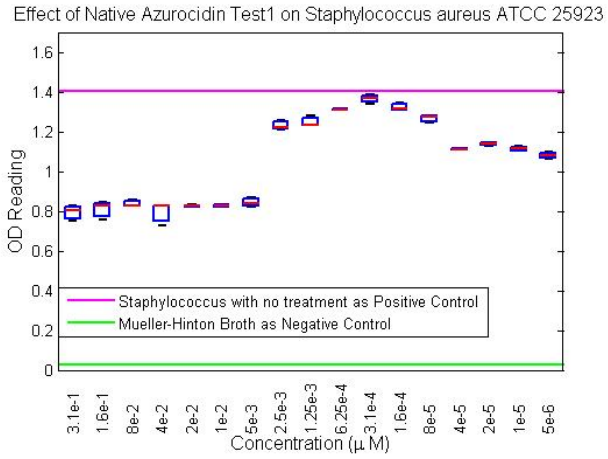

Figure 2. The effect of the native Azurocidin on the growth of gram-positive bacteria *S. aureus.*

Figure 2 demonstrates the significant effects of several concentrations of the native Azu peptide on *S. aureus.* Its anti-microbial activity is noticeably strong in the concentration range from $3.1x10^{-1}$(μM) to $5x10^{-3}$(μM). Apparently, the anti-microbial effects of the native Azu are reduced at lower concentrations: from $3.1x10^{-4}$(μM) to $6.25x10^{-7}$ (μM), at which no significant effects can be observed when compared to the positive control.

Figure 3 represents the antimicrobial activity of the synthetic Azu-RRM, computationally designed peptide analogue. As it can be observed from Figure 3, the concentration range of $6.25x10^{-1}$(μM) to $5x10^{-3}$ (μM) demonstrated a significant antimicrobial activity of Azu-RRM on *S. aureus*.

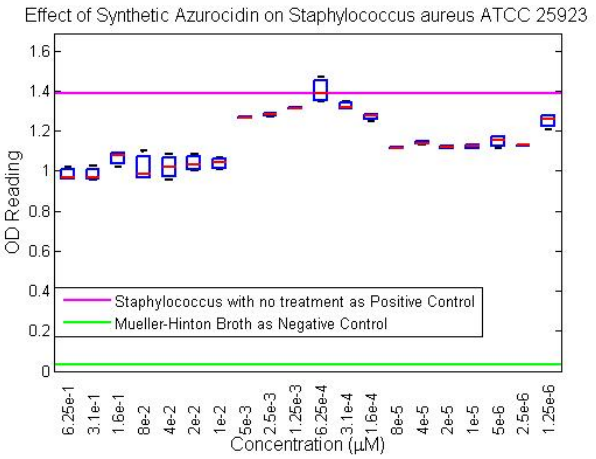

Figure 3. The effect of RRM-Azu on the growth of *S. aureus*.

Moreover, at the concentration ranges of $1.25x10^{-3}$(μM) to $5x10^{-5}$ (μM), some anti-microbial activity can be seen. In the range of $6.25x10^{-1}$(μM) to $5x10^{-3}$ (μM), we can observe no significant anti-microbial activity of Azu-RRM. Comparison of Figure 2 and Figure 3 shows that the *in vitro* inhibitory effect of the synthetic Azu-RRM peptide analogue on *S. aureus* was comparable to the inhibitory effect of the native Azu against this pathogenic bacterium. The dose dependent activity of Azu-RRM was similar to that of the native Azu. Our findings showed that the peptides exhibited potent activity against the bacteria – peptides' treatment at the particular concentrations lead to reduced bacterial growth. The results showed that the survival level of bacterial cells treated by Azu-RRM and native Azu at concentrations range from 0.3μM to 0.005μM was significantly lower ($p<0.05$) when compared to positive control. The statistical analysis was performed using the Microsoft Office 2003 Excel and MATLAB software. The statistical significance of the differences between the Azu-RRM and native Azu treatments of bacteria and the positive control group were analysed by one-way ANOVA. These findings reveal that computationally designed Azu-RRM exhibits the inhibitory activity (similar to the native antimicrobial protein Azu) against pathogenic bacteria.

IV. Conclusion

In this study, the efficacy of the synthetic Azu-RRM peptide analogue was experimentally evaluated *in vitro* on the gram positive bacterium *S. aureus*. The experimental evaluation of its anti-microbial activity was compared to the activity of the native Azu peptide. The antimicrobial activity of Azu-RRM was comparable to the activity of the native Azu in the concentrations range of 0.3μM to 0.005μM when tested against on *S. aureus*. It was previously reported by Watorek[8] that Azu induced significant anti-microbial effects (the most effective dosage) at the concentrations range of 10^{-5}-10^{-6}(M) that corroborates our current experimental findings. The results obtained reveal that both native and synthetic peptides induce suppressing effects on *Staphylococcus 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.

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References

[1] Robert E.W. Hancock and Gill Diamond, "The role of cationic antimicrobial peptides in innate host defences", TRENDS IN MICROBIOLOGY VOL 8(9), 402-410, 2000

[2] Andreas Peschel, "How do bacteria resist human antimicrobial peptides", Trends in Microbiology Vol10(4), 179-186, 2002

[3] M. Wu, E. Maier, R. Benz and R.E.W. Hancock: "Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of Escherichia coli". Biochemistry, Vol. 38, 7235–7242, 1999

[4] Mannis MJ. "The use of antimicrobial peptides in ophthalmology: an experimental study in corneal preservation and the management of bacterial keratitis". Trans Am Ophthalmol Soc. 100:243–271, 2001

[5] David Campanelli, Patricia A Detmers and Joelle E Gaby,"Azurocidin and homologus serine protease from neutrophils". J Clin. Invest Vol.85, 904-915, 1990

[6] Roque P Almeida, Ann Vanet, Joelle E Gabay, "Azurocidin, a natural antibiotic from human neutrophils: expression, antimicrobial activity, and secretion". Protein Expression and Purification, Vol.7, 355-366, 1996

[7] Denise McCabe, Tali Cukierman and Joelle E Gabay, "Basic residues in Azurocidin/HBP contribute to both heparin binding and antimicrobial activity". The J. of biological Chemistry, Vol.227(30),27477-27488,2002

[8] Wieslaw Watorek, "Azuorcidin-inactive serine proteinase homolog acting as a multifunctional inflammatory mediator". Acta Biochimica Polonica, Vol.50(3),743-752, 2003

[9] Pirogova E, Istivan T, Gan E, Cosic I: Advances in methods for therapeutic peptide discovery, design and development. Curr Pharm Biotechnol,12:1117–1127, 2011

[10] Cosic I: Macromolecular bioactivity: Is it Resonant Interaction between Molecules? – Theory and Applications. IEEE Trans. On BME 1994, 41:1101-1114.

[11] Cosic I: The Resonant Recognition Model of Macromolecular Bioactivity Birkhauser Verlag; 1997.

[12] Taghrid S. Istivan1, Elena Pirogova, Emily Gan1, Nahlah M. Almansour1, Peter J. Coloe1, Irena Cosic : "Biological Effects of a De Novo Designed Myxoma Virus Peptide Analogue: Evaluation of Cytotoxicity on Tumor Cells". Vol. 6(9), 1-10, 2011