Effect of the amphipatic α-helix structure and the cysteine redox state of GIBIM-P5 analogues on the antimicrobial activity against *Escherichia coli* O157:H7, methicillin resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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Trabajo de Grado para Optar el título de Química

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Dedicatoria

A Dios por ser mi fortaleza, por su infinito amor para conmigo y los míos y por permitirme cumplir mis sueños

A mis padres, Juan y Hermelinda por su amor, dedicación, esfuerzo e incondicionalidad

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Resumen

TITULO: EFECTO DE LA ESTRUCTURA A-HÉLICE ANFIPÁTICA Y DEL ESTADO REDOX DE LA CISTEÍNA DE LOS ANÁLOGOS DE GIBIM-P5 SOBRE LA ACTIVIDAD ANTIMICROBIANA CONTRA *ESCHERICHIA COLI* O157: H7, *STAPHYLOCOCCUS AUREUS* RESISTENTE A LA METICILINA Y *PSEUDOMONAS AERUGINOSA

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PALABRAS CLAVE: Peptidos antmicrobianos/ estructura α-hélice/ *P.aeruginosa*/ SARM/ *E.coli O157:H7*

DESCRIPCIÓN: El efecto de la estructura secundaria α-hélice y el estado redox de la cisteína sobre la actividad antimicrobiana de GIBIM-P5 se estudió mediante el diseño de siete péptidos análogos. La secuencia GIBIM-P5 se modificó para aumentar o disminuir la estabilidad de la α -hélice cambiando los residuos Gly por Ala o por residuos Pro, respectivamente. Además, los residuos de Phe y Cys fueron cambiados por Trp y Ser, respectivamente; en algunos péptidos análogos, se incluyó una Cys adicional cambiando uno de los residuos de la secuencia peptídica. Cuando los residuos de Gly fueron cambiados por Ala para generar GAM019, la MIC_{99} disminuyó de 10 a 1.48 μM para Pseudomonas aeruginosa (P. aeruginosa), de 100 a 4.32 μM para Staphylococcus aureus resistente a meticilina (SARM) y de 100 a 29.34 μM para Escherichia coli 0157: H7 (E. coli O157: H7); por el contrario, cuando los residuos de Gly fueron cambiados por Pro en GAM020, la MIC_{99} aumentó a 29 μM para P. aeruginosa, a 91 μM para SARM y 132 μM para E. coli O157: H7. El contenido helicoidal fue mayor para GAM019 (33,7%), seguido de GIBIM-P5 (22,5%) y menor para GAM020 (2,9%). La actividad antibacteriana de péptidos análogos con dos Cys fue mayor cuando ambos estaban en el mismo lado de la α -hélice, (GAM022 y GAM023) que cuando estos Cys estaban en el lado opuesto (GAM024 y GAM025). Curiosamente, estos péptidos presentaron baja citotoxicidad frente a las células de mamíferos, presentando un índice terapéutico entre 2 y 88, que eran más altos que el índice de GIBIM-P5, mostraron un alto potencial para ser utilizados contra P. aeruginosa y SARM.

*** Trabajo de grado para optar por el título de Química**

**** Facultad de Ciencias. Escuela de Química. Director: Mauricio Urquiza Martínez. Co-directora: Stelia Carolina Méndez Sánchez**

Abstract

TITLE: EFFECT OF THE AMPHIPATIC A-HELIX STRUCTURE AND THE CYSTEINE REDOX STATE OF GIBIM-P5 ANALOGUES ON THE ANTIMICROBIAL ACTIVITY AGAINST *ESCHERICHIA COLI* O157:H7, METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* AND *PSEUDOMONAS AERUGINOSA**

AUTHOR: YENNY PAOLA BAUTISTA******

Keywords: antimicrobial peptides / α-helix structure / *P.aeruginosa* / SARM / *E.coli O157:H7*

DESCRIPTION: The effect of the secondary α-helix structure and the cysteine redox state on the antimicrobial activity of GIBIM-P5 was studied by design seven analogue peptides. GIBIM-P5 sequence was modified to increase or decrease the α-helix stability by changing Gly residues by Ala or by Pro residues, respectively. Moreover, the Phe and Cys residues were changed by Trp and Ser, respectively; in some analogue peptides an additional Cys was included by changing one of the peptide sequence residue. When the Gly residues were changed by Ala to generate GAM019 the MIC_{99} decreased from 10 to 1.48 μM for *Pseudomonas aeruginosa* (*P. aeruginosa),* from 100 to 4.32 μM for Methicillin-resistant *Staphylococcus aureus* (SARM) and from 100 to 29.34 μM for *Escherichia coli* 0157:H7 (*E.coli* O157:H7*)*; on the contrary, when Gly residues were changed by Pro in GAM020 the MIC_{99} increased to 29 μM for *P. aeruginosa,* to 91 μM for SARM and to 132 μM for *E. coli O157:H7*. The helical content was higher for GAM019 (33.7%), followed by GIBIM-P5 (22.5%) and lower for GAM020 (2.9%). The antibacterial activity of analogue peptides having two Cys was higher when both of them were on the same side of the α-helix, (GAM022 and GAM023) than when these Cys were on the opposite side, (GAM024 and GAM025). Interestingly, these peptides presented low cytotoxity against mammalian cells, displaying and therapeutic index between 2 and 88, which were higher than the index of GIBIM-P5, having showed high potential to be used against *P. aeruginosa* and SARM.

***Research project to achieve Bachelor Degree in chemistry.**

**** Science Faculty. Chemistry Departament. Director: Mauricio Urquiza Martínez.**

Co-director: Stelia Carolina Méndez Sánchez

Introduction

The growing resistance of bacteria to conventional antibiotics, mostly associated with the indiscriminate and incorrect use of antibiotics(Ventola, 2015) , is a threat for the worldwide public health that requires an effort of the scientific community to design or discovery new potential antibiotics (Health World Organization, 2017)(Wernli & Jorgensen, 2016). The antibiotic treatment of these resistant bacteria is deficient, promoting the persistence of infectious diseases and increasing the probability of pathogen transmission. The increased multiresistant infection leads to a high mortality rate (Hover et al., 2018), disability and increase in treatment cost. In fact, 700,000 people die per year worldwide because of these pathogens and the annual deaths in 2050 could reach 10'000,000 (Willyard, 2017).

In February 2017, the World Health Organization (WHO) published the list of twelve pathogen families that urgently requires new efficient drugs to control the infections caused by them (Organización Mundial de la salud, 2017). in this high priority list are *Pseudomonas aeruginosa* and Methicillin-resistant *Staphylococcus aureus* (MRSA) because both of them caused lethal infections and they are resistant to most of the conventional antibiotics; *P. aeruginosa* causes a mortality rate of 50-70% of infected patients and present resistance to cephalosporins, macrolides and tetracyclines (Gómez, Leal, Pérez, & Navarrete, 2005). and MRSA causes skin-level infections, pneumonias, septic arthritis, among others (Togneri, Podestá, Pérez, & Santiso, 2017)(Tacconelli & Magrini, 2017). Peptides with antimicrobial activity (AMPs) could be used to control the infections generated by these multiresistant

microorganisms considering their broad spectrum of antimicrobial activity and that pathogens rarely generate resistance against them (Chingaté, Guzman, & L, 2016)(J Cruz, Ortiz, Guzmán, Fernández-Lafuente, & Torres, 2014). AMPs frequently interact with lipopolysaccharide and lipoteicnoic acid on the membrane of Gram-negative and Gram-positive bacteria, respectively(Migliolo et al., 2016)(Beisswenger & Bals, 2005); but at a lesser extent with the eukaryotic cell membranes probably because it is composed of zwitterionic lipids such as phosphatidyl choline and phosphatidyl ethanolamine (Y. Huang, Huang, & Chen, 2010)(Epand & Vogel, 1999).

The AMP antimicrobial activity is associated with the physicochemical peptide characteristics such as to the positive net charge (cationic peptides), the amphipathicity, the size and the secondary α-Helix structure. These characteristics facilitate the peptide interaction with the bacterial lipid membrane (J. Huang et al., 2011)(Sun, Xia, Li, Du, & Liang, 2014). The experimental evidence suggests that an AMP folded into an amphipatic α-Helix structure having a positive side opposed to hydrophobic side interacts with microbial membrane disrupting its integrity (Y. Huang et al., 2010)(J. Huang et al., 2011). The proposed mechanism is an electrostatic initial interaction between the AMP positive charge and the microbe negative charge of the bacterial membrane phospholipids (Rudi, Müller, Siano, Simonetta, & Tonarelli, 2010)(Migliolo et al., 2016). Moreover, the peptide hydrophobic side interacts with the lipids of the membrane and the hydrophilic amino acids interact with the phospholipid polar heads resulting in the peptide insertion into the membrane (J Cruz et al., 2014)(Y. Huang et al., 2010). This peptide insertion can generate membrane damage resulting the exit of the cytoplasmic material or followed by targeting the cytoplasmic microbial proteins (Zhao, 2003). According to

this model the stability of the α-Helix secondary structure seems to be very important for the AMP antimicrobial activity; this structure is stabilized not only by hydrogen bonds but also for the presence of amino acids that in the process of peptide α-Helix folding the change in peptide configurational entropy is low (J. Huang et al., 2011)(David & Michael, 2005).

We have reported GIBIM-P5, an AMP having the MIC_{99} of 10 and 100 μM against *P.aeruginosa*, SARM and *E.coliO157:H7*, respectively. GIBIM-P5 displays an amphipatic α-Helix in 30% TFE with the helical content of 23% (Jenniffer Cruz et al., 2018). Having into account that GIBIM-P5 is suitable to study the effect of the stability of the α-Helix in the antimicrobial activity, its sequence was modified to determine the role of Gly, Phe, Cys residues and the peptide secondary structure in the antibacterial activity. Seven peptides were designed and tested against *P.aeruginosa*, SARM and *E.coli* O157:H7.

1. Objetivos

1.1 Objetivo General

Determinar el efecto de la estructura α- hélice y el estado de oxidación de las cisteínas del péptido antimicrobiano GAM019 y sus análogos en la actividad antibacterial contra Escherichia coli, Staphylococcus aureus resistente a meticilina y Pseudomonas aeruginosa.

1.2 Objetivos Específicos

Sintetizar y caracterizar péptidos con posible actividad antimicrobiana.

Determinar la estructura secundaria y la posible formación de puentes disulfuro de los péptidos.

Comparar la actividad *in-vitro* de los péptidos en diferentes estados de oxidación contra cepas como *E. coli* O157:H7, *P. aeruginosa* y *S. aureus* resistente a Meticilina.

2. Materials and methods

2.1 Materials

Rink-amide resin AM (100-200 mesh) substitution of 0.58 mmol/g) from novabiochem, TBTU (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate), HBTU (N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate) of Merck, DMF (Dimethylformamide), DCM (dichloromethane), Acetonitrile for HPLC, gradient grade ≥99.9% from Merck, DIEA (N,N-diisopropylethylamine) Sigma Aldrich, DCC (dicyclohexylcarbodiimide) from Alfa Aesar, F-moc-L-amino acids from Sigma Aldrich ,TFA (Trifluoroacetic acid), oxyme pure, TIS (triisopropylsilane), CHCA matrix (a-cyano-4-hydroxycinnamicacid) marca sigma aldrich, , Luria Bertani Broth y Mueller Hinton Broth from EMD millipore, column HPLC (ZORBAX Eclipse XDB-C18 (4.6 × 150 mm, 5 µm)) from Agilent.

2.2 Peptide Design

Based on GIBIM-P5, which is an AMP previously reported as having antibacterial activity against *P. aeruginosa*, SARM and *E. coli O157:H7* (Jenniffer Cruz et al., 2018), a new set of analog peptides were designed changing the potential helix stability, the aromatic character and the redox activity of this peptide. Initially GAM019 were designed with a more stable a-helix by changing the Glycines by Alanines and changing Phe by Trp. This a-helix was destabilized in

GAM020 by changing the Alanines by Prolines. In GAM021 the peptide redox activity was modified by changing Cys by Ser. GAM022 to GAM025 contain an additional Cys in different part of the sequence. The theoretical antimicrobial activity of these sequences was determined by using the CAMP software ("CAMP R3 (Collection of Anti-microbial peptides," n.d.).

2.3 Peptide synthesis and Purification

Peptides were synthesised using the F-MOC strategy utilizing the multiple peptide synthesis reported by Houghten (Houghten, 1985), L-amino acids with N-terminus protected by F-MOC group obtained from Merck Novabiochem and the Rink-amide resin AM (100-200 mesh) substitution of 0.58 mmol/g). The removal of the Fmoc group was carried out with a 20% piperidine in N, N-dimethylformamide (DMF). Carboxyl group activation was carried out with equimolar amounts of 2- (1H-benzotriazol-1-yl) -1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in DMF with N, N-diisopropylethylamine (DIPEA). The cleavage of peptide from the resin was performed by treatment with trifluroacetic acid, TIS (triisopropylsilane), ethanedithiol and water in a ratio of 92.5: 2.5: 2.5: 2.5 during one hour. The peptides were precipitated from this solution using cold ether, and extensively washed with cold ether to remove contaminants from the peptides (Jenniffer Cruz et al., 2014). These precipitated peptides were dissolved in water and lyophilized during several days. Lyophilized peptides were dissolved in water passed through syringes packed with stationary phase C-18 (Sep pak) with different solutions of acetonitrile of 0, 10, 25, 30, 35, 60, 100%, each fraction was liophilized and the fraction containing peptide was analyzed by HPLC. The peptide N-terminus was acetylated to remove the amino charge and also to protect the peptide from degradation by

exopeptidases, proteolytic enzymes acting on peptide bonds at the ends of the chain, as well as other factors (Tein, n.d.).

2.4 Peptide Characterization

To determine peptide purity and composition peptides GAM019, GAM020, GAM022 and GAM023 were analyzed using high resolution liquid chromatography in an Agilent model 1100 chromatograph, the column used was ZORBAX Eclipse XDB-C18 (4.6 × 150 mm, 5 μm) and the mobile phases were acetonitrile grade HPLC and water type I both with 0.1% TFA. The main peaks in HPLC were analyzed by MALDI-TOF (matrix-assisted laser desorption/ionization with a time of flight), on a Bruker Daltonics equipment with UltrafleXtreme using the matrix CHCA (a-cyano-4-hydroxycinnamicacid) from Sigma Aldrich, at 1mg/ml in 50:50 acetonitrile/water having 0.01% TFA (Daltonics, 2012).

The peptide secondary structure was analyzed by circular dichroism spectra, using peptide at 1 mg/ml, in 30% trifluoroethanol (TFE); the spectra were recorded from 190 to 250 nm, with a bandwidth of 0.5 nm and a speed of 50 nm per minute on a CD spectrometer (J-815 Jasco Corporation, Japan) at 25C. The data were plotted using GraphPad Prism 6 software and calculated the percentage of helicity using the K2D3 software (Louis-Jeune & Andrade-Navarro, Miguel A. Perez-Iratxeta, 2011) and also using the equation:

$$\% \alpha\text{-helix} = (-\Theta_{222\ nm}+3000)/39000$$

Where, $\Theta_{222\ nm}$ is molar ellipticity(Urquiza, Melo, Guevara, & Patarroyo, 2010).

2.5. Peptide antibacterial activity

Pseudomonas aeruginosa, methicillin-resistant *Staphylococcus aureus* and *Escherichia coli* O157: H7 were cultured in solid agar at 37 ° C for 12 hours; one colony of this plate was added in liquid medium (LB (Luria Bertani) for *E.coli O157: H7* and *Pseudomonas aeruginosa* and MH (Mueller Hinton) for MRSA) and incubated for 12 hours at 37 ° C at constant agitation (200 rpm). A 1:10 dilution of the microorganisms was performed in logarithmic phase and the optical density (OD) was measured at 595 nm to follow the bacterial growth. The kinetics of bacterial growth were carried out using the broth microdilution technique following the modification made by the research group of standards established by the Clinical Laboratory Standards Institute (CLSI) (J Cruz et al., 2017)(CLSI, 2015). The peptide activity on the growth of these bacteria were determined at different peptide concentrations in a 96 well plate in 200 ųL of bacterial culture; the starting OD of the culture was 0.1 and the volume of peptide added was lower than 10% of the final volume. The growth of these bacteria was followed by changes in OD during 8 hours, measuring it every hour. The percentage of inhibition was calculated

$$\% \text{ de inhibition} = 100 - \left(\frac{(Af - Ai)p*}{(Af - Ai)c*}\right) * 100$$

Where:

A_f : final absorbance at the 8 hour of culture

Ai: initial absorbance moment of adding the peptide

P*: bacterial peptide incubated with peptide

C*: bacterial culture without peptide

2.6 Peptide hemolytic activity

Erythrocytes obtained from human blood were washed 4 times with isotonic PBS buffer and used at 1% v/v in PBS buffer; 100 µL of this suspension containing four different peptide concentrations (16, 32, 64 and 128 µM) were incubated in a 96-well microplate during 4 hours at 37 ° C, once the incubation time finished these suspensions were centrifuged at 5000 rpm for 5 min, the released hemoglobin in the supernatants was measured at 550 nm. Erythrocytes in PBS and erythrocytes in 0.5% triton were used as a negative and positive control of hemolysis, respectively. The percentage of hemolysis was calculated as:

$$\%_{hemolysis} = \frac{(A_M - A_0)}{(A_{100} - A_0)} x100$$

A_M ei is the absorbance of the erythrocytes incubated with the peptide, A_{100} is the absorbance of the positive control and Ao is the absorbance of the negative control.

2.7 Eukaryotic cell toxicity of peptides

The Cos7 cell line was cultured in Eagle's minimal essential medium (EMEM Sigma-Aldrich) supplemented with 7% Fetal Bovine Serum (SFB), 2 mM L-glutamine, 2.2 g / L sodium bicarbonate and 100 μg/mL Streptomycin (Sigma-Aldrich). The cells were incubated in 5% CO2 atmosphere at 37° C. An average of 10000 cells was deposited per well in a 96-well microplate, incubated for a period of 24 hours at 37 ° C to 5% CO2 to ensure adherence; then the peptide was added at concentrations ranging from 16 to 128 μM and the cells were incubated for another 24 hours; then, 10 μL of neutral red was added per well and incubated in the same conditions during two hours; finally, cells were three time washes with isotonic PBS buffer, and lysed with 200 μL of a solution containing 50 % ethanol, 1% acetic acid, 49% water type I and the neutral red accumulated by the cells were measured at 540 nm (Repetto, del Peso, & Zurita, 2008). Cells no treated with peptides were used as a negative control of cytotoxicity.

2.8 Cysteine-reduction and -oxidation of peptides

Peptides at 600 μM were treated for 10 minutes at 80 °C with BME (2-mercaptoethanol) at 100 mM to reduce the cysteine of the peptides. To avoid the formation of Disulfide bridge these peptides were treated with 2 mM Iodoacetamide. The cysteine of peptides was oxidized with 5% v/v DMSO (dimethylsulfoxide) added to the peptide solutions and air was constantly bubbled for two hours at room temperature using a pump (Calce, Vitale, Scaloni, Amodeo, & De Luca, 2015). These peptide solutions were stored at -70 ° C until their use and were diluted in the appropriated buffer or medio before using them. Diluted reduction or oxidation buffers were used as a control in the experiments of antibacterial activity and they have no significant effect on the growth of the bacteria used in the research.

2.9 Statistical analysis

All the experiments were performed in triplicate, averages and standard deviations were determined, when the standard deviations were higher than 10% the experiments were repeated. The data were graphed using the GraphPad Prism 6 software; the analysis of variance was carried out by means of the t-student test when there were two groups of data or by ANOVA when the number of groups to be analyzed was greater than 2.

3. Results

The previously reported antimicrobial peptide GIBIM-P5 (1ATKKCGLFSILKGVGKI17) has antibacterial activity against *Escherichia coli* O157: H7 (*E. coli* O157: H7), methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* (P. aeruginosa); inhibiting 99% of the growth of these bacterial at 100, 100 and 10 μM, respectively (Jenniffer Cruz et al., 2018). This peptide contains the first 12 out of 37 residues of the antimicrobial CAMPSQ2827 peptide that against E. coli and S. aureus has the MIC of 1μM and 10μM, respectively (Waghu, Barai, Gurung, & Idicula-Thomas, 2015). The GIBIM-P5 in 30% TFE displays CD spectrum characteristic of α-helix, that according to Helical Wheel Projections (Armstrong & Zidovetzk, 2009) of its amino acid sequence could be an amphipathic helix presenting a hydrophobic side formed from the amino acids L7, I10, L11, V14 and I17 opposed to hydrophilic side formed by amino acids T2, C5, S9 K12 and K16; F8 and G15 residues are in helix side flanked by residues

K4 and K12; C5 is between residues K12 and K16. According to the proposed model of interaction these residues will be in the initial membrane contact area of this peptide (Figure 1).

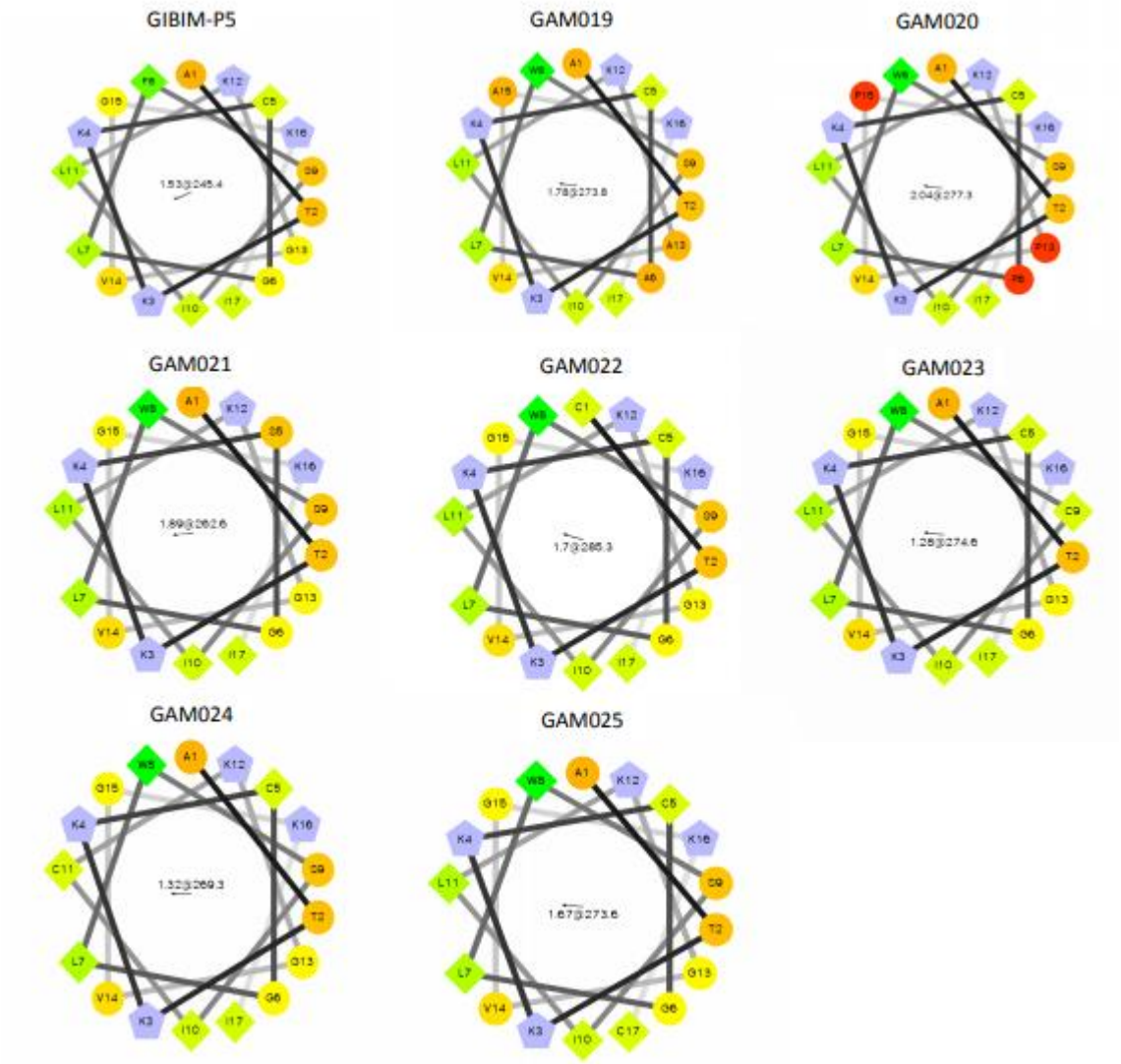


Figure 1. Helical Wheel projections. In this representation, the hydrophobic residues are presented in the form of a diamond, the hydrophilic residues as circles, the positively charged residues have a pentagon shape and the negatively charged ones as triangles. The degree of hydrophobicity and hydrophilicity is represented in colors; the hydrophilic residues are characterized by the color red, the amount of red decreases when descending by hydrophilicity. The hydrophobic residues are represented with the green color, the intensity of the color

decreases with decreasing hydrophobicity. Image generated by Helical wheel projections(Armstrong & Zidovetzk, 2009).

Taking into account this seven analog peptide was designed from GIBIM-P5 to determine the effect the α-helix structure and the Cys redox potential on the antimicrobial activity. In all the analogue peptides the Phe residue was changed by Trp because this residue usually increases the antimicrobial activity (Zhu, Ma, Wang, Chou, & Shan, 2014) . In GAM019 design Gly residues were changed by Ala to increase the α-helix stability of this peptide having into account reports about the low propensity of Gly to be in helix and the stabilizing effect of Ala in helix. On the contrary, in GAM020 these Gly were changed by Pro to disrupt the a-helix structure (Zhang, Benz, & Hancock, 1999). In order to determine the effect of S and its redox potential in the antimicrobial activity Cys was changed by Ser in GAM021. In GAM022 and GAM023 another Cys was included in the same side of the helix changing Ala and Ser by Cys, respectively. In GAM024 and GAM025 also an additional Cys were included changing Leu11 and Ile17 but in the opposite side of the helix. These peptides were synthesised with the N-terminus and C-terminus modified by acetylation and amidation, respectively. These purified peptides presented one peak in the HPLC chromatogram and by maldi-tof presented the expected molecular weight (Table 1). Peptides GIBIM-P5, GAM019, GAM020, GAM021, GAM022, GAM023, GAM024 and GAM025 were 17 amino acids in length with four positive charges and the molecular weight in the range of 1760 to 1970 Da (Table 1). According to the HPLC analysis the purity was higher than 90%.

Table 1. Physicochemical characterization of peptide GIBIM-P5 and its analogues.

Peptide	Sequence	number of amino acids	MW(Da)	m/z	Holding time (min)	Net charge at pH 7.0	Isoelectric Point (PI)	Instability index	GRAVY	molar extinction coefficient
GIBIM-P5*	ATKKCGLFSILKGVGKI-NH2	17	1763.22	1763.02	ND	4	10.04	-4,23	0.56	-
GAM019	Ace-ATKKCALWSILKAVAKI-NH2	17	1885.39	1886.77	10.7	4	11	24.68	0.73	5500
GAM020	Ace-ATKKCPLWSILKPVPKI-NH2	17	1963.51	1962.5	9.94	4	11	54.24	0.13	5500
GAM021	Ace-ATKKSGLWSILKGVGKI-NH2	17	1827.24	ND	ND	4	10.4	9.70	0.15	5500
GAM022	Ace-CTKKCGLWSILKGVGKI-NH2	17	1875.38	1875.73	9.53	4	10.5	28.88	0.38	5625
GAM023	Ace-ATKKCGLWCILKGVGKI-NH2	17	1859.38	1860.42	9.64	4	10.5	9.70	0.54	5625
GAM024	Ace-ATKKCGLWSICKGVGKI-NH2	17	1833.30	ND	ND	4	10.5	3.36	0.27	5625
GAM025	Ace-ATKKCGLWSILKGVGKC-NH2	17	1833.30	ND	ND	4	10.5	14.69	0.23	5625

[a] Grand Average of Hydropathicity
* Peptide design basis(Jenniffer Cruz et al., 2018)
\+ Peptides discarded by null activity
ND non determined

The circular dichroism spectra of peptides GAM019, GAM022 and GAM023 present the minimum characteristic of the α-helical structure at 205 and 222 nm (Avitabile, D'Andrea, & Romanelli, 2015); on the contrary the CD-spectrum of peptide GAM020 was characteristic of a beta sheet secondary structure (Figure 2). Using these CD-spectra the helical content of these peptides was calculated using the molar ellipticity to 222 nm (Urquiza et al., 2010) showing that the peptide GAM019 and GAM022 had the highest percentage of helicicity with 33.7% and 14.9% and the peptides GAM023 and GAM020 had the lowest percentage of helix of 13.4% and 2.9%, respectively.

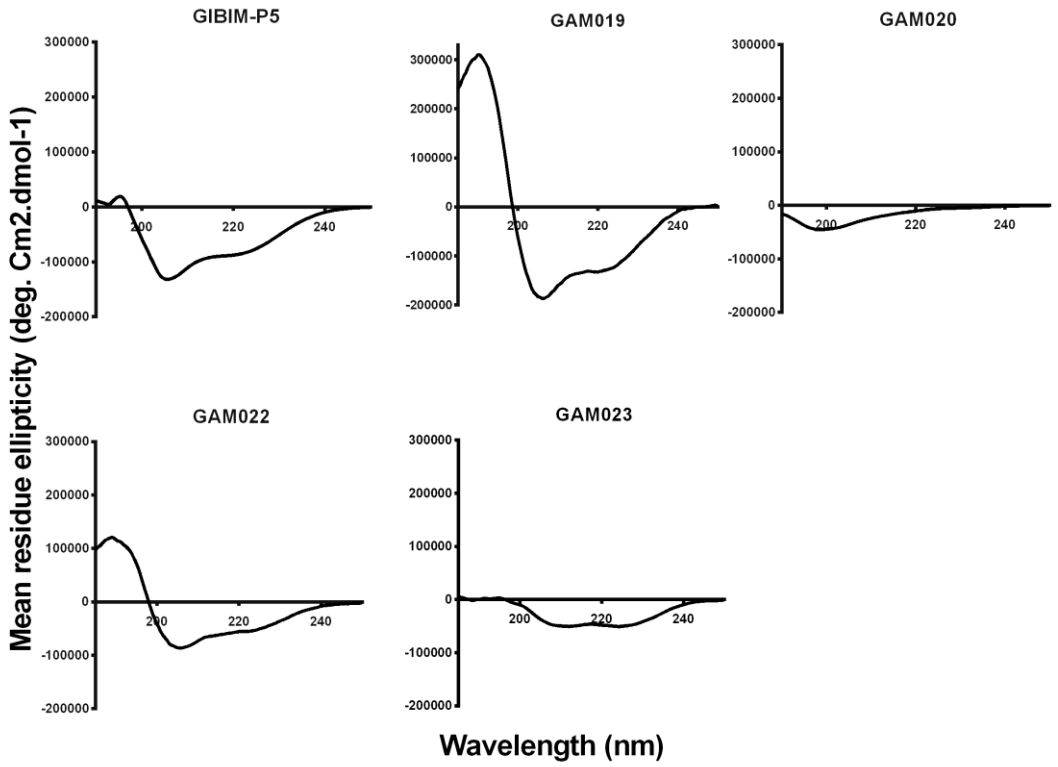


Figure 2. CD spectra of GIBIM-P5 and its analogs. Taken in solution 30% TFE and at a concentration of 1 mg/Ml

The in vitro effect of these seven peptides against *P. aeruginosa*, SARM and *E. coli* O157:H7 in a range of peptide concentration between 2 and 64 ųM were determined recording the kinetics of growth of these bacteria during 8 hours. GAM019, GAM022 and GAM023 significantly inhibited the growth of these three strains at low peptide concentrations. GAM020 also inhibited the bacterial growth, but a higher peptide concentration than the former peptides. On the other hand GAM021, GAM024 and GAM025 did not inhibit the growth of these three strains in the range of tested concentrations. The effect on the growth of these peptides against *P. aeruginosa*

and SARM at 16 ųM and against *E. coli* O157:H7 at 30 ųM is shown (Figure 3). GAM021, GAM024 and GAM025 were discarded for further studies having into account that they did not inhibit significantly the growth of these three bacterial strains.

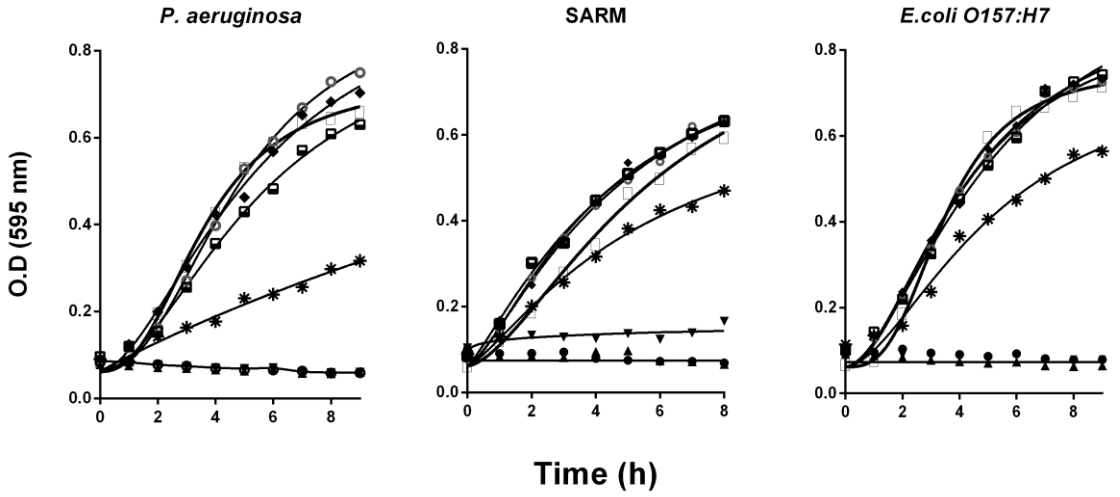


Figure 3. Peptide antimicrobial activity against P.aeruginosa, SARM and E.coli O157:H7.In presence or absence of 16 ųM, 16 ųM and 30ųM peptide, respectively. The OD of 200 ųl of bacterial culture in a 96 well plate in the presence or absence of peptides were measured every hour during 8 hours as bacterial growth indicator (o) Control, (•) GAM019, (*) GAM020, (□) GAM021, (▲) GAM022, (▼) GAM023, (♦) GAM024, (▪) GAM025.

When Cys residue in GAM019 was changed by Ser to generate GAM021 the antibacterial activity against these three strains was lost, suggesting that the Cys is critical for this activity. Considering that Cys residues in biological environment are in equilibrium between the oxidized and reduced states, peptides were oxidized and reduced to determine how the redox state of these peptides affect their biological activity. Cys residues were oxidized by air, reduced using 2-mercaptoethanol (BME) or changed by Ser that has an Oxygen instead of Sulfur atom. The

oxidation and reduction of Cys were followed by the changes in the UV spectrum in the region between 260 and 285 nm and structural changes involving aromatic residues in the range of 285 to 295 nm (Schmid, 2001). In the oxidized GAM019 and GAM023 the absorbance increases in the range of 285 to 295 nm compared to native and reduced peptides that were similar. On the other hand, there were no differences in this region among native, reduced and oxidized for GAM020 and GAM022 (Figure 4).

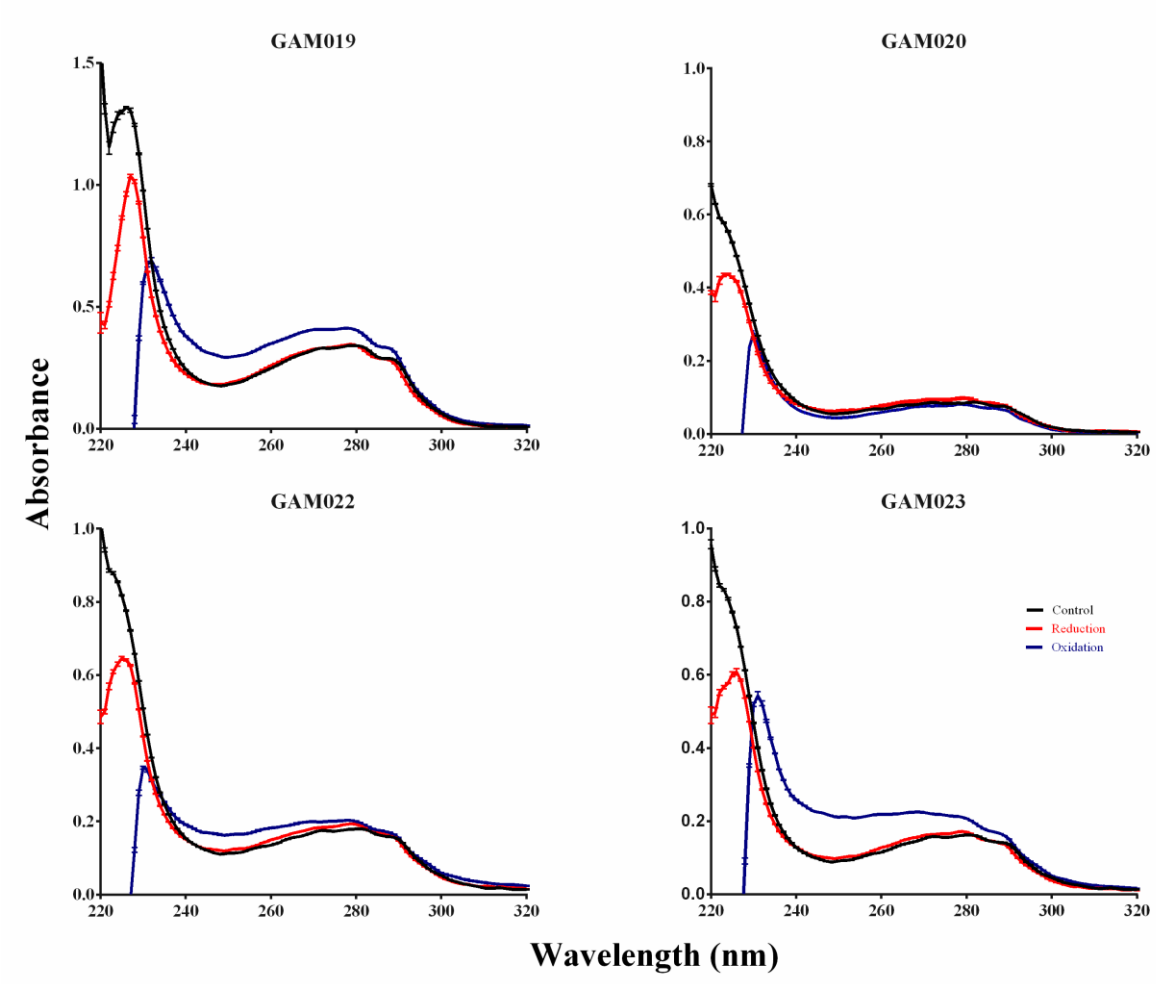


Figure 4. UV-vis spectra of peptides. To determine changes due to the peptide oxidation and reduction procedures the UV-spectra were recorded for oxidized, reduced and native peptides in the wavelength range of 220 and 230 nm.

The formation of disulfide bridges is associated with an increase in absorbance at 260 nm [39]; oxidized GAM019, GAM022 and GAM023 increased the absorbance at 260nm suggesting the formation of disulfide bonds intercatenary in GAM019 and inter and/or intra catenary in GAM022 and GAM023. Oxidized, reduced and native GAM020 did not show changes at this wavelength.

The kinetics of bacterial growth in the presence of different peptide concentrations were performed to determine the minimum inhibitory concentration (MIC_{99}). For *P. aeruginosa* and MRSA the peptide concentrations used were 16, 8, 4 and 2 ɥM and for *E. coli* O157:H7 were 30, 15, 7.5 and 3.8 ɥM; the percentages of bacterial growth inhibition at these four peptide concentrations were used to calculate MIC_{99} by plotting percentages of bacterial growth inhibition versus peptide concentration and using linear regression (Table 2). In general, there were no differences in the MIC99 among the same peptide oxidized, reduced and native; except that the MIC99 of GAM019 against *E. coli* O157:H7 is higher for the oxidized than native or reduced and the MIC_{99} of GAM020, GAM022 and GAM023 against *P. aeruginosa* was lower for native than for reduced or oxidized. These four peptides presented lower MIC99 for *P. aeruginosa* and higher for *E. coli* O157:H7. The MIC_{99} of GAM019 was less than 2 μM for *P. aeruginosa* and 4.32 μM for SARM; being the lowest MIC_{99} obtained with the tested peptides. GAM022 and GAM023 also inhibit *P. aeruginosa* and SARM but their MIC_{99} is at least three

times higher than those with GAM019. The MIC_{99} against *E. coli* was around 30 μM and similar for GAM019, GAM022 and GAM023. The MIC_{99} of GAM020 was higher than the MIC99 of GAM019 at least 15 times against *P. aeruginosa*, at least 20 times against SARM and at least 4 times against *E. coli* O157:H7 to have a minimum inhibitory concentration (MIC).

Table 2. Antimicrobial activity of oxidized, reduced and native peptides.

Minimum Inhibitory Concentration MIC_{99} (μM)				
Microorganism		*P. aeruginosa*	*E. coli O157:H7*	*SARM*
peptide	condition			
GAM019	Native	1.48	29.34	4.32
	Reduced	1.67	28.23	5.33
	Oxidized	1.85	60.91	5.46
GAM020	Native	28.98	132.65	91.00
	Reduced	50.50	112.32	100.82
	Oxidized	58.97	133.77	100.85
GAM022	Native	7.64	32.33	15.43
	Reduced	15.94	32.56	15.78
	Oxidized	17.89	40.07	16.18
GAM023	Native	7.05	30.89	16.04
	Reduced	10.95	30.40	13.84
	Oxidized	13.30	34.63	15.84

Peptides containing Cys residues and having hydrophobic residues in aqueous solutions could form aggregates to assemble nano- or microparticles exposing on their surface the peptide charges. The formation, characteristics and stability of these peptides particles could affect the biological activity of these peptides. To know whether or not these peptides form charged particles, peptide solutions were analyzed by dynamic light scattering (DLS) in the Malvern zetasizer nano Z590 (Lorber, Fischer, Bailly, Roy, & Kern, 2012), to determine the size of the particles and the zeta potential of these particles (Table 3).

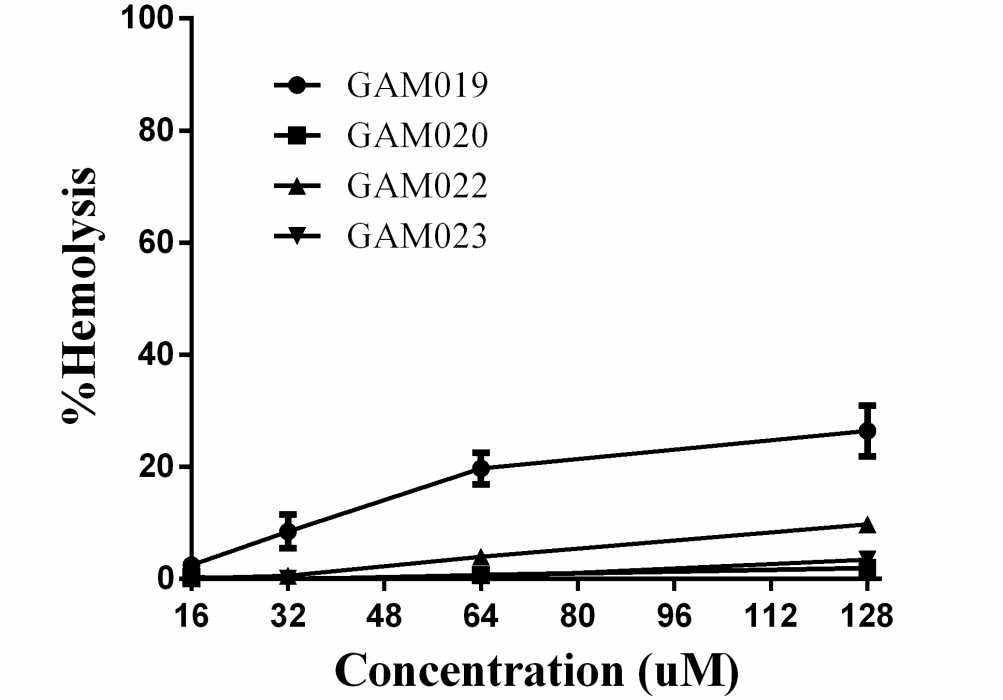
Table 3. Size and zeta potential of the evaluated in normal, reduced and oxidized peptides

Peptide	Zeta potential (mV)			Size (nm)		
	Native	Reduced	Oxidized	Native	Reduced	Oxidized
GAM019	77.1 ± 3.5	63.7 ± 1.8	50.2 ± 4.5	0,5 ± 0.3	1.12 ± 1.0	86.1 ± 24.7
GAM020	42.3 ± 3.5	-0.2 ± 0.4	77.7 ± 0.4	0,1 ± 0.1	1.0 ± 0.6	228. 2 ± 8.6
GAM022	28.3 ± 2.2	23.8 ± 1.5	75.2 ± 3.5	0.9 ± 0.8	0.1 ± 0.1	72.5 ± 10.7
GAM023	70.9 ± 1.8	38.7± 4.5	76.6 ± 1.4	0.5 ± 0.3	0 ± 0	0.5 ± 0.4
BUFFER	-2.81 ± 1,9	1.4 ± 0.7	41.3 ± 0.9	0 ± 0	0 ± 0	0 ± 0

The sizes of native and reduced were around 1 nm peptides that according to previous studies, support the idea of having monomeric or dimeric helical peptide molecules in solution, except for native GAM020, reduced GAM022 and GAM023 that could be unfolded monomeric molecules (Zagrovic, Jayachandran, Millett, Doniach, & Pande, 2005) On the other hand, the oxidized peptides aggregate into large nanoparticles with sizes between 70 and 230 nm. In general, the surface charge of these peptide particles determined as zeta potential was positive between 24 and 78 mV, as expected; except for the reduced GAM020.

To determine the effect of GAM019, GAM020, GAM022 and GAM023 on the integrity of the eukaryotic membrane, human erythrocytes were incubated with these peptides at different peptide concentrations from 16 to 128 μM (Figure 5). These peptides up to 128 μM did not exhibit significant hemolytic activity. Peptide GAM019 exhibited 26% hemolysis at 128 μM, which is 64 times higher than the MIC_{99} against *P.aeruginosa* and MRSA and 4 times higher

than the MIC_{99} against *E.coli* O157:H7. GAM020 and GAM022 showed less than 10% hemolysis at 128 μM which the highest concentration evaluated. GAM023 showed 15% hemolysis at 128 μM, which is 32 times greater than the MIC_{99} against *P. aeruginosa* and MRSA.


100
80
60
40
20
0
%Hemolysis
GAM019
GAM020
GAM022
GAM023
16
32
48
64
80
96
112
128
Concentration (uM)

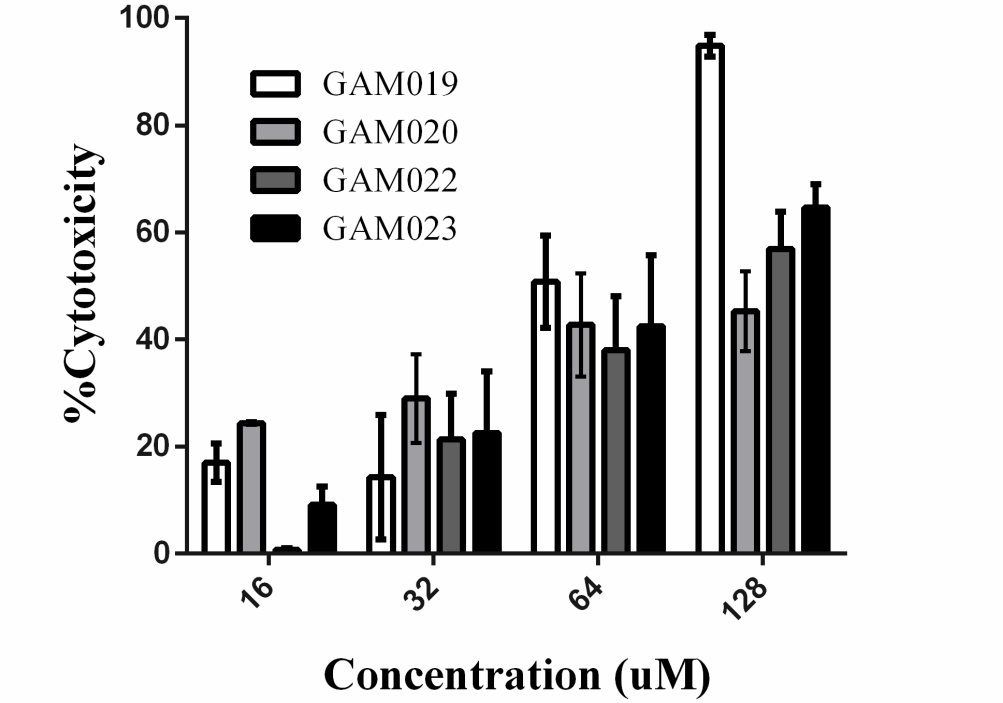

100
80
60
40
20
0
%Cytotoxicity
GAM019
GAM020
GAM022
GAM023
16
32
64
128
Concentration (uM)

Figure 5. Hemolytic and cytotoxic activity. Human erythrocytes in phosphate buffer at pH 7.2 were incubated with GAM019, GAM020, GAM022 and GAM023 at four concentrations in 96 well Microplates and the released hemoglobin was measured by recording the absorbance at 280 nm. The percentage of cytotoxicity of the peptides in eukaryotic cells was evaluated in Cos7 cells at concentrations from 128 to 16 μM.

The peptide activity on the viability of eukaryotic cells was evaluated in *Cos7* cells, using the neutral red method and four different peptide concentrations (Figure 5). In general the effect was peptide dose dependent; GAM019 showed 97% cytotoxicity at 128 and <20% at 32 and16 μM. GAM020 showed 50% cytotoxicity at 128 ɥM and 23% cytotoxicity at 16 ɥM. GAM022 at 128 μM, presented 55% cytotoxicity and at 16 μM did not present any detectable effect on the viability of these cells. GAM023 displayed 67% cytotoxicity at 128 ɥM and at 16 ɥM the cytotocity was <20%. The concentrations at which these peptides presented cytotoxic effect were significantly higher than the MIC_{99} of these peptides against the three studied bacterial strains; additionally, the 24 hours of incubation time with the eukaryotic cell was greater than the 8 hours of incubation time with the bacteria.

The therapeutic index of these peptides was calculated using the bacterial MIC_{50} and the concentration causing 50% eukaryotic cell cytotoxicity. The therapeutic index for these peptides were between 2.1 for GAM020 against *E. coli* and 88 for GAM019 against; in general the therapeutic index was higher for the analogue peptides designed in this study than the initial peptide GIBIM-P5, except for GAM020 against *P. aeruginosa* (Table 4).

Table 4. Therapeutic Index of the antibacterial peptides**.**

Peptide	Microorganism	Concentration (ɥM)			Therapeutic Index (CC50/MIC50)
		MIC50	CC50	HEM50	
GIBIM-P5*	*P. aeruginosa*	5.0			20.0
	E.coli O157:H7	50.0	>100.0	~100.0	2.0
	SARM	50.0			2.0
GAM019	*P. aeruginosa*	0.8			88.0
	E.coli O157:H7	16.0	66.0	232.1	4.1
	SARM	2.7			24.6
GAM020	*P. aeruginosa*	13.7			10.1
	E.coli O157:H7	66.4	139.0	2833.2	2.1
	SARM	44.7			3.1
GAM022	*P. aeruginosa*	4.5			23.4
	E.coli O157:H7	19.0	105.0	578.1	5.5
	SARM	8.6			12.2
GAM023	*P. aeruginosa*	3.9			23.1
	E.coli O157:H7	19.2	92.0	1562.5	4.8
	SARM	7.7			11.9

4. Discussion

The analogue peptides GAM019, GAM022 and GAM023 presented higher antibacterial and lower cytotoxicity to eukaryotic cells than GIBIM-P5 from which they were derived. The antibacterial activity was associated with the ability of these peptides to form helical structures because of higher peptide helical content (determined by the circular dichroism spectra) higher the antibacterial activity. The peptide helical content was GAM020 (generated changing Gly by Pro) < GAM021 (generated changing Gly by Ser) < GIBIMP5 (original peptide) < GAM019

(generated changing Gly by Ala). GAM020 presented the lowest antimicrobial activity of these peptides, probably because the drastic change in the secondary structure as determined by CD caused by the inclusion of prolines within its primary structure; moreover proline is not able to form a backbone hydrogen bond with its imino group and the psi and phi dihedral angles to form an α-helix are of high energy in this residue [14]; in fact the MIC_{99} increased when the Cys residue that is located next the Pro residue in this peptide is reduced or oxidized. The antibacterial activity was not only associated to the net charge of the peptide, because GAM020 and GAM019 having similar cationic charge but the antibacterial activity was lower for GAM020 than for GAM019. Moreover, the negative charge of GAM020 nanoparticles may be associated with the secondary structure that it adopts in aqueous solution and the counter-ion iodide of the iodoacetamide used in the reduction reaction.

The activity of the peptide decreased drastically when the cysteine was changed by serine (that specifically is the change of Sulfur by Oxygen) to generate GAM021, suggesting that this amino acid is critical the antibacterial activity. One of the basic differences between serine and cysteine in its redox capacity, which can be modulated by oxidizing and reducing cysteine. However, no significant differences were found in the antibacterial activity of the oxidized, reduced, or native peptides. Another explanation could be the differences in the propensity to helix between Ala and Ser, associated with the differences in free energy helix formation that is higher for Cys than for Ser (Echeverria & Mario Amzel, 2010).

Having into account that the Cys residue was critical for the antibacterial activity and additional Cys was included in GAM022 to GAM025; in different sides of the helix and the

effect on the antibacterial activity determined. In GAM022 and GAM023 the additional Cys residue was located at the same helix side of the initial Cys residue. On the contrary, GAM024 and GAM025 the additional Cys residue was located at the opposite helix side of the initial Cys residue. GAM024 and GAM025 presented significant low antibacterial activity than GAM022 to GAM023 suggesting that the Cys residue location on the helix but no the Cys presence is critical in their antibacterial activity.

In general, the oxidized peptide showed similar antibacterial activity compared to either the reduced or native peptide despite the size of the peptide particles increased under oxidation conditions indicating peptide agglomeration; in GAM019 and GAM022 probably due to formation of intercatenary disulfide bonds. In general peptides in the native state appear to be stable and do not tend to form aggregates and oxidized, reduced and native peptide particles were positive charge on their surface that facilitates a probable interaction with the bacterial membrane negatively charged. This suggests that in this case that the antibacterial activity is not associated to the size of the peptide particles and there was no found correlation between the density of peptide surface charge and antibacterial activity, except that most of them presented positive surface charge.

The differences in the antibacterial activity not only among GAM019, GAM022 and GAM023 but also among *P.aeruginosa*, SARM and *E. coli* O157: H7 strongly suggest that the specificity of this peptide can be modulated by modifying the peptide sequence; for example the MIC_{99} against *P.aeruginosa* of the native GAM019 and native GAM022 were 1.5 and 7.6, respectively, five times higher.

GAM019, GAM020, GAM022 and GAM023 showed no significant hemolytic activity at 128 μM, suggesting that these peptides interacted specifically with these bacteria, probably through some molecules present in these bacteria but not in eukaryotic cells. This suggests that the mechanism of action is not only the disruption of the membrane structure. In fact, other studies in our lab showed that the peptide changed the pattern of bacterial protein murG expression (manuscript in preparation).

GAM019, GAM022 and GAM023 only exhibited cytotoxicity at concentrations higher than the MIC_{99} of these peptides against *E. coli* O157: H7, *P.aeruginosa* and SARM. Moreover, this effect was seen after 24 hours of incubation with these peptides because *Cos-7* cells present doubling time higher than 35 hours. Under this condition the low cytotoxicity of these peptides is very important because it is expected that long times of incubation with these peptides increased the probability of cell damage. With these data the therapeutic index of GAM019 is 88, 24.6 and 4.1 for *P.aeruginosa*, MRSA and *E.coli* O157:H7 respectively, which are greater than the therapeutic indexes calculated for GIBIM-P5, which demonstrates that the modifications of the sequence of GIBIM-P5 generated peptides with higher antibacterial activity and lower cytotoxicity.

The therapeutic index of GAM019, GAM022 and GAM023 for *P.aeruginosa* and SARM was over 10 that is considered safe to be used as a possible drug and support the potential of these peptides to be used as a possible treatment of infections associated with these pathogenic microorganisms. On the other hand these peptides despite of having antibacterial activity against *E. coli* O157:H7 their therapeutic index was lower than 10 which is considered insecure to be

used as a possible drug(Deepa, Vandhana, Jayanthi, & Krishnakumar, 2012; Fajardo, Est, Ruiz, & Pe, 2017).

5. Conclusions

The α-helix structure of these peptides favors the pathogen-peptide interaction which leads to greater antibacterial activity. GAM019, GAM022 and GAM023 presented high antibacterial activity against *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*, they showed low hemolysis and cytotoxicity at the concentrations to which they are active against these pathogens. These peptides are promising drug candidates against infections caused by the strains evaluated here. However, more studies must be done to determine its potential pharmacological potential.

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