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CHENOPODIN AS A TEMPLATE FOR THE DESIGN AND SYNTHESIS OF ANTIVIRAL AND ANTIBACTERIAL PEPTIDES

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DEDICATORIA

Este trabajo está dedicado a mi abuela y a mi familia que siempre estuvieron y están para apoyarme.

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RESUMEN

El enfoque basado en plantillas se ha aplicado con éxito al diseño de nuevos antimicrobianos basados en péptidos antimicrobianos (AMPs). La Chenopodina es una proteína de almacenamiento abundante de la semilla de quinoa (*Chenopodium quinoa*), una planta andina con altas propiedades nutricionales y bioterapéuticas. Aquí, utilizamos estrategias asistidas por ordenador y basadas en la física, incluyendo las herramientas AMPA, AMPfun, HLPredFuse y PED2D, para seleccionar cuatro péptidos candidatos inspirados en la estructura primaria de esta globina vegetal de tipo 11S para su cribado biológico, incluyendo actividades antibacterianas y anti-SARS-CoV2. Dos péptidos reproducen fragmentos naturales de 14 aminoácidos de la Chenopodina, denominados Chen1 (YGVRGRGRIQIVNA) y Chen2 (AHSIIYGVRGRGRI), y fueron diseñados dos péptidos de la misma longitud basados en la secuencia de Chen1. Sus dos aminoácidos que contienen cadenas laterales amidas fueron sustituidos por arginina (ChenR) o triptófano (ChenW), generando péptidos catiónicos e hidrofóbicos de ingeniería, respectivamente. La evaluación de estos péptidos sintéticos de 14 polímeros frente a *Staphylococcus aureus, Escherichia coli*, mostró que Chen1 no es activo (hasta 512 µM), mientras que los otros péptidos presentan efectos en el rango micromolar, Chen2 (64 - 128 µM), ChenR (16-128 µM), ChenW (8 µM). La sustitución química de la glutamina y la asparagina por aminoácidos con cadenas laterales catiónicas o aromáticas favoreció significativamente el efecto antibacteriano. Los péptidos no mostraron una lisis eritrocitaria significativa en el rango de MIC. ChenW es el péptido más activo, pero también el de mayor tendencia hemolítica. El análisis mediante microscopía de fluorescencia puso de manifiesto la naturaleza membranolítica de los péptidos bioactivos derivados de Chenopodina. Se observó una elevada captación del colorante impermeable de intercalación de ácidos nucleicos, el yoduro de propidio, cuando *S. aureus* se incuba con Chen2, ChenR y ChenW. Por último, Chen2 y ChenR tienen la capacidad de mantener la viabilidad de las células Calu-3 infectadas por SARS-CoV2 en una proporción > 0.8, lo que demuestra la acción dual y dinámica de los AMPs en la lucha contra infecciones bacterianas y antivirales. Nuestros resultados confirman que la Chenopodina es una plantilla útil para el diseño biológico asistido por ordenador de péptidos no tóxicos, activos en la membrana, antibacterianos y antivirales.

Palabras claves: péptidos, antimicrobianos, membranolíticos, antivirales y Chenopodina.

ABSTRACT

The template-based approach has been successful applied to design of novel antimicrobial peptides (AMPs) based antimicrobials. Chenopodin is an abundant storage protein of quinoa seed (*Chenopodium quinoa*), an Andean plant with nutritional and biotherapeutic properties. Here, we used computer-aided and physicochemical-based strategies, including AMPA, AMPfun, HLPredFuse and PED2D tools, to select four candidate peptides inspired on the primary structure of this 11S-type vegetal globulin for biological screening, including antibacterial and anti-SARS-CoV2 activities. Two peptides reproduce natural fragments of 14 amino acids from Chenopodin, named as Chen1 (YGVRGRGRIQIVNA) and Chen2 (AHSIIYGVRGRGRI), and two engineered peptides of the same length were designed based on Chen1 sequence. Their two amino acids containing amide side-chains were replaced by arginine (ChenR) or tryptophan (ChenW) generating engineered cationic and hydrophobic peptides, respectively. The evaluation of these 14-mer synthetic peptides towards *Staphylococcus aureus*, *Escherichia coli*, showed that Chen1 is not active (up to 512 µM), while other peptides show antimicrobial effect at the micromolar range: Chen2 (64 – 128 µM), ChenR (16-128 µM), ChenW (8 µM). The chemical substitution of glutamine and asparagine by amino acids with cationic or aromatic side-chains significantly favored the antibacterial effect. The peptides did not show significant erythrocyte lysis at the MIC range. ChenW is the most active peptide, but also with the greatest hemolytic tendency. Analysis by fluorescence microscopy highlighted the membranolytic nature of bioactive peptides derived from Chenopodin. A high uptake of impermeable nucleic acid intercalating dye, propidium iodide, is observed when *S. aureus* is incubated with Chen2, ChenR and ChenW. Finally, Chen2 and ChenR have the ability to maintain viability of Calu-3 cells-infected SARS-CoV2 at a ratio > 0.8, demonstrating the dual and dynamic action of AMPs in combating bacterial and viral infections. Our findings confirm Chenopodin as a useful template for the biological computer aided design of non-toxic, membrane-active, antibacterial, and antiviral peptides.

Keywords: peptides, antimicrobial, membranolytic, antiviral and Chenopodin.

INTRODUCTION

Infectious diseases represent a serious and challenging problem in the global scenario and modern medicine with epidemiological, economic and health impact, which were clearly evidenced during the COVID-19 pandemic [1,2]. Until 2022, the SARS-CoV2 virus produced 579,092,623 confirmed cases and 6,407,556 deaths [3]. It was also reported that 4% to 15% of hospitalized patients acquire bacterial infections that increase the mortality rate [4–6]. In an era of global change, the emergence of new diseases, such as the coronavirus (SARS-CoV2), large-scale outbreaks and multidrug resistant bacteria constitute a relevant problem that requires immediate solutions [7,8]. Biodiversity-inspired compounds have successfully assisted in these current obstacles facing infectious diseases, particularly plant-based bioactive proteins and peptides [9,10].

According to Food and Agriculture Organization of United Nations (FAO), quinoa total protein provides of the requirements of the 9 essential amino acids, which cannot be synthesized in human cells and play an important role for maintaining good health and normal functioning [11]. Some quinoa proteins and peptides have been recognized by their antioxidant, anti-diabetic, antihypertensive and antibacterial properties [12,13]. Lower molecular weight peptides generated during alcalase, and trypsin-driven hydrolysis have the ability to scavenge free radicals and an increased antioxidant action. On the other hand, these biomolecules act as natural inhibitors of the α-glucosidase enzyme, which is responsible for carbohydrate absorption, controlling metabolic conditions, such as postprandial hyperglycemia and type 2 diabetes mellitus [14,15].

The most abundant protein in quinoa seeds is Chenopodin, an oligomeric 11S globulin with lectin-like property, a relative mass of 320 kDa and isoelectric point (pI) of 6.58, which represents 37% of its biochemical composition [16–18]. A previous study has demonstrated the antimicrobial nature of isolated quinoa seeds against *Escherichia coli, Pseudomonas aeruginosa* [19]. Other investigations have also showed its *in vivo* anti-inflammatory, *in vitro* immunomodulatory and hemagglutination properties [16,20,21]. This multifunctional molecule presents a hexameric quaternary structure composed by identical subunits formed by an alpha and β-chains connected by a disulfide bridge [18,22]. These polypeptide subunits differ by composition, length, size and charge. The

β-chain exhibits a lower molecular weight and a rich composition in positively charged amino acid residues for which reason it is known as the basic subunit [18].

Cationic lytic peptides inspired by molecular scaffolds from natural sources have been proposed as a fundamental basis for the development of next-generation of effective antibiotics and antiviral agents [23]. Positive and hydrophobic amino acids, such as arginine, lysine, leucine, and tryptophan are key elements of membranolytic and peptide-based drugs available in pharmaceutical market [24,25]. Historically, quinoa protein is identified as a promising precursor of biotherapeutic short peptides with health benefits [26]. These functional and biochemical characteristics turn quinoa protein into a potential candidate for the design of nutraceutical products and functional foods [27]. The discovery of therapeutic entities is a slow, long and costly process originally based on wet-laboratory approaches [25]. However, this field has developed more rapidly due to the valuable assistance of computational/bioinformatic tools and the progress of artificial intelligence applications [28,29]. Therefore, in this current study, an *in-silico* library of four 14-mer peptide sequences were generated by computational screening, using the basic subunit of Chenopodin protein as a structural template.

MATERIALS AND METHODS

Computer-aided design of cationic peptides based on Chenopodin

Screening of Chenopodin sequence and prediction of toxicity, structure and biological activity

AMPA software (http://tcoffee.crg.cat/apps/ampa/do) was used to screen the Chenopodin protein (UniProtKB - Q6Q384_CHEQI) for antibacterial candidate peptides for chemical synthesis. The antimicrobial region was selected as source of peptide fragments, which were posteriorly examined using bioinformatics tools such as: AMPfun (http://fdblab.csie.ncu.edu.tw/AMPfun/run.html) and AMP Scanner (www.ampscanner.com) to predict antimicrobial activity; HLPpred-Fuse (http://thegleelab.org/HLPpred-Fuse/), HAPPENN (https://research.timmons.eu/happenn) and HemoPred (http://codes.bio/hemopred/) to

test for hemotoxicity. Four novel peptides with high *in silico* antimicrobial potential were identified by physicochemical-guided design and employed in the experimental phase. Briefly, two peptides, Chen1 (YGVRGRGRIQIVNA) and Chen2 (AHSIIYGVRGRGRI), mimic short molecular fragments of parent protein (Chenopodin), and two engineered cationic/hydrophobic peptides, which were designed based on Chen1 replacing amide side-chains amino acids by arginine (ChenR) or tryptophan (ChenW). Finally, the charge, hydrophobicity, isoelectric point (pI) and mass of the peptides were estimated using PepDraw (http://www.tulane.edu/~biochem/WW/PepDraw/). In addition, the secondary structure was investigated with I-TASSER and supplemented with information provided by PED2D (https://webs.iiitd.edu.in/raghava/pep2d/submit.html).

Chenopodin-directed peptide synthesis, purification and molecular mass determination

These computationally selected 14-mer peptides were obtained using the Fmoc solid-phase chemical synthesis technique with the Liberty Blue automated microwave peptide synthesizer (CEM Corporation) with >90% purity. The main solvent used was N, N'-dimethylformamide, and 0.192 g of Rink Amide Novabiochem resin, 0.52 mmol/g substitution at filling. After removing the protective groups with piperidina, synthetic peptides were cleaved from resin using 95% trifluoroacetic acid, 2.5% triisopropylsilane, and 2.5% water. The final products were washed in cold ethyl ether and freeze-dried for 24 hours at -80°C at 0.09 mT pressure [30]. Purity was analyzed by reverse-phase high performance liquid chromatography (RP-HPLC) and peptides molecular weight was confirmed by matrix-assisted laser ionization/desorption time-of-flight mass spectrometry (MALDI-TOF MS).

Biological evaluation

Antibacterial activity

Broth microdilution protocol previously employed by Proaño-Bolaños et al. [31] was taken into account to determine the minimum inhibitory concentration against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. Briefly, lyophilized peptides were solubilized in dimethyl sulfoxide (DMSO). Each micro-organism reached logarithmic phase and was diluted to 1×10^6 CFU/ml. Then 198 µl of

each microorganism and 2 µl of the peptide solutions were dispensed into 96-well plates. Ten different peptide concentrations (1, 2, 4, 8, 16, 32, 64, 128, 256, 512 µM) were assessed. As control, 2 µl of DMSO was used instead of the peptide and 198 µl of the microbial culture media, and for the negative control, Mueller Hinton Broth was used in the absence of microorganisms. Three independent experiments in triplicate were performed for each concentration. The plates were incubated at 37°C for 18 h and the inhibition of microbial growth was measured spectrophotometrically at 600 nm using a GloMax multiplex detection system (Promega, Madison, WI, USA). Finally, 10 µl from the MIC value wells were cultured on Mueller Hinton Agar. It was incubated at 37°C for 18h to identify, the concentrations with no microbial growth that were considered as the Minimum Bactericidal Concentration (MBC).

Antiviral activity

The human lung epithelial Calu-3 cell lines (ATCC®HTB-55™) used in this study were cultured in Dulbecco's modified Eagle medium (DMEN) high in glucose and supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, 1% (v/v) penicillin/streptomycin, 1% (v/v) L-glutamine. Cells were cultured at 37°C and 5% CO_2, and SARS-CoV2 viral particles were stored at -80°C with stock of 1,7 x 10^6 PFU/mL.

MTT assay

The antiviral properties of Chenopodin-derived peptides were determined *in vitro* cell viability assay. Calu-3 cells were cultivated in sterile 96-well plates at a density of 2x10^4 Calu-3 cells (70% confluence) two days before infection with SARS-CoV-2 (moi: 0.1, 4°C for 2 h). They were fixed in 4% formaldehyde/PBS for 15 min at room temperature and then washed three times with PBS. Then 0.5 mL of DMEN was added and incubated at 37°C and 5% CO_2 atmosphere. The cells were then subjected to the action of Chenopodin-derived peptides at different concentrations (1, 10, 10^2, 10^3, 10^4, 5×10^4 and 10^5 nM and incubated for 24 hours at 37°C in 5% CO_2. The drug Nafamostat (NT) was used as a control. Finally, the medium was changed and MTT (0.5 mg/ml) was added. Then 20% SDS was added and absorbance was measured at 570 nm in the plate reader (Glomax DS, Promega) to report cell viability [32]. In addition, viral RNA was extracted for subsequent RT-PCR analysis as described by Bakovic et al. [33].

Hemolytic activity

Peptide-induced red blood cell membrane lysis was investigated using the approach outlined by Proaño-Bolaños et al. [31]. Lyophilized aliquots of pure peptides were solubilized in DMSO to obtain the same concentrations previously used to evaluate antibacterial properties. Peptide solutions were incubated for 2 hours at 37 °C with a 1:1 (v/v) dilution of 4% erythrocytes (O+), totalizing 200 µl. The cells were then dispersed in a 96-well plate after being centrifuged at 1000 rpm for 5 minutes. The percentage of hemolysis was measured using a GloMax multiplex detection system (Promega, Madison, WI, USA) at 550 nm. Three independent experiments were carried out in triplicate. Negative and positive controls were PBS and 2% (v/v) Triton X-100, respectively. The maximum percentage hemolysis (100%) was defined as the absorbance monitored by the effected induced by Triton X-100. This was calculated using Equation 1.

$$\%\, hemolysis = \left(\frac{A-Ao}{A\chi-Ao}\right) \qquad (1)$$

This formula includes the absorbance of the peptide (A), the absorbance of the negative control (Ao) and the absorbance of the positive control ($A\chi$).

Insights into mechanism of action

The experimental protocol described by Valdivieso-Rivera et al. [34] was utilized to figure out the possible peptide's molecular mode of action. Peptide dilutions were made using MIC concentrations that had been determined previously and cultured with log-phase bacteria for 30 min before centrifugation at 3000xg for 15 min. The pellet was stained for 15 min in the dark at 0°C with propidium iodide (PI, 120 mM) and 4',6-diamidino-2-fenilindol (DAPI, 1 µM). Bacteria treated with 10% sodium dodecyl sulfate (SDS) were employed as positive control; and microorganisms without peptide incubation as a negative control. Finally, membrane damage was visualized using a Nikon Eclipse Ni microscope and a combination of fluorescence and differential interference contrast (DIC). Fluorescent micrographics were processed using ImageJ software.

Statistical analysis

The results were analyzed by two-way analysis of variance (ANOVA) and Tukey's test, using the OriginPro 9.0 Software (Origin Lab). The level of significance was established with $p < 0.05$.

RESULTS

In silico screening

Bioinformatic analysis of the basic subunit of Chenopodin revealed a short region composed by 14 amino acid residues (73-86 aa), which can play a role as molecular determinant for the antibacterial potential of the vegetal protein (Fig. 1). We termed this sequence Chen1. Additionally, we selected the region 68-81 aa from the natural protein, that was named Chen2. Two peptides were constructed based on the primary structure from Chen1, in which asparagine and glutamine were replaced by tryptophan (ChenW) and arginine (ChenR), respectively. On the other hand, peptides have a probability ranging from 64.22% to 85% of acquiring a random coil secondary structure according to the PED2D predictor (Supplementary material Table 6S). However, in some peptide regions the β-sheet structure is manifested in a lower percentage, which can be observed in the simulation shown by I-TASSER (Fig. 2).

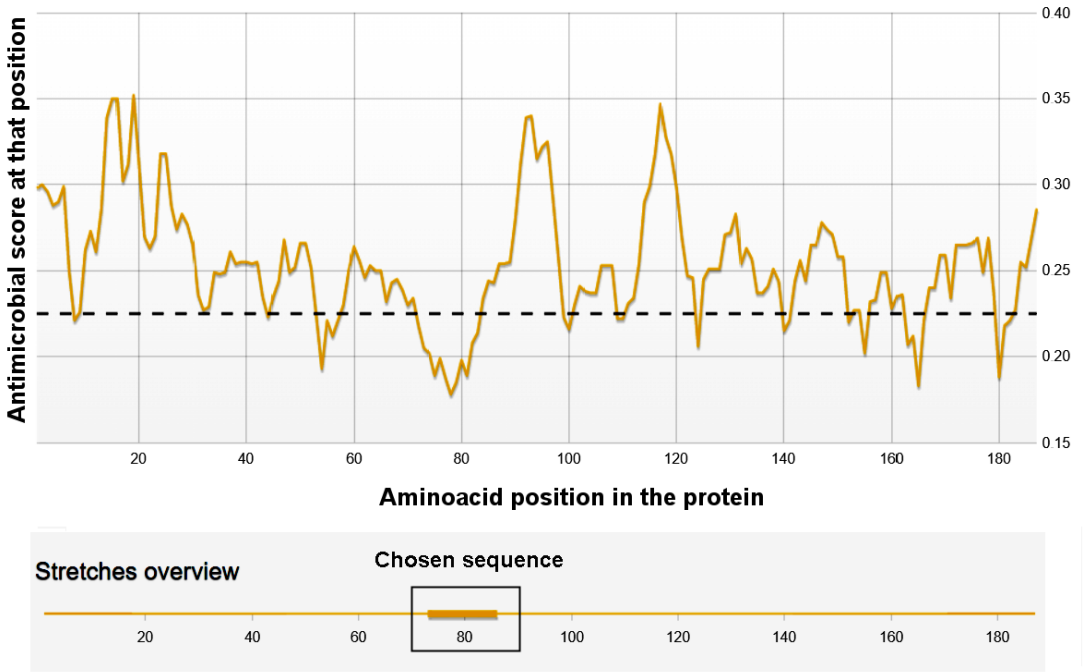

Figure 1. Antimicrobial screening of the Chenopodin sequence using the AMPA tool.
Elaborated by: Feijoo, 2022.

This protein has an antimicrobial region named Chen1, which was selected for the design of three novel Chenopodin-derived peptides. Briefly, four 14-mer

sequences were chosen for synthesis after *in silico* screening for their antibacterial potential against bacteria, viruses and hemolytic tendency.

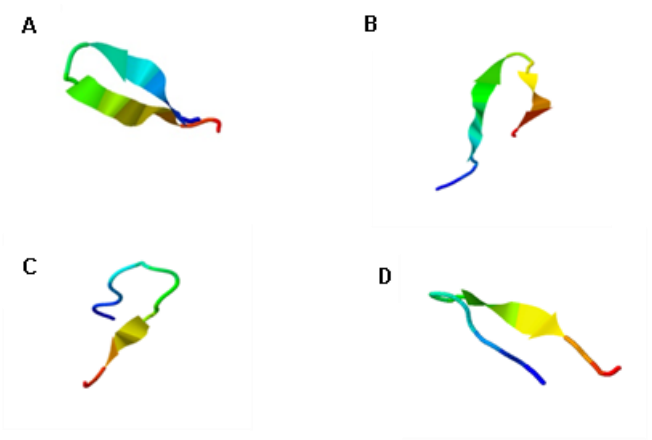

Figure 2. Secondary structure of Chen1(A), Chen2(B), ChenR(C) and ChenW(D).
Elaborated by: Feijoo, 2022.

These sequences have a positive charge, a basic pI, and a high hydrophobicity score (Table 1). Peptides derived from natural sequence of Chenopodin (Chen1 and Chen 2) present a lower molecular weight than engineered peptides. ChenR shows the highest charge (+5) and pI (12.66), while ChenW exhibits the lowest hydrophobicity value (+9.23). Computational screening predicted that these cationic hydrophobic peptides are AMPs, with high ability to harm Gram-positive, Gram-negative bacteria and viruses, and poor hemolytic activity (Supplementary material Table 1S and 2S). Based on the foregoing, four peptides were further synthesized, purified, characterized, and tested *in vitro.*

Table 1. Physicochemical characteristics of the Chenopodin-derived peptides. pI, charge and hydrophobicity were determined using PepDraw.

Peptides	Sequences	Length (aa)	pI	Charge	Hydrophobicity (Kcal * mol-1)	Mass (Da)
Chen1	YGVRGRGRIQIVNA	14	11.71	+3	+15.03	1557.88
Chen2	AHSIIYGVRGRGRI	14	12.19	+3	+15.54	1553.88
ChenR	YGVRGRGRI**R**IV**R**A	14	12.66	+5	+17.03	1627.98
ChenW	YGVRGRGRI**W**IV**W**A	14	12.19	+3	+9.23	1687.93

Elaborated by: Feijoo, 2022.

Synthesis and characterization of peptides

The four synthetic peptides showed a high purity level (>90%) as assessed by RP-HPLC. This is reflected in the m/z signals of the MALDI-TOF mass spectra of the four peptides (Fig. 3). The masses of the peptides agree to the theoretical masses obtained from the *in silico* analysis, which indicates a correct procurement of the peptides of interest.

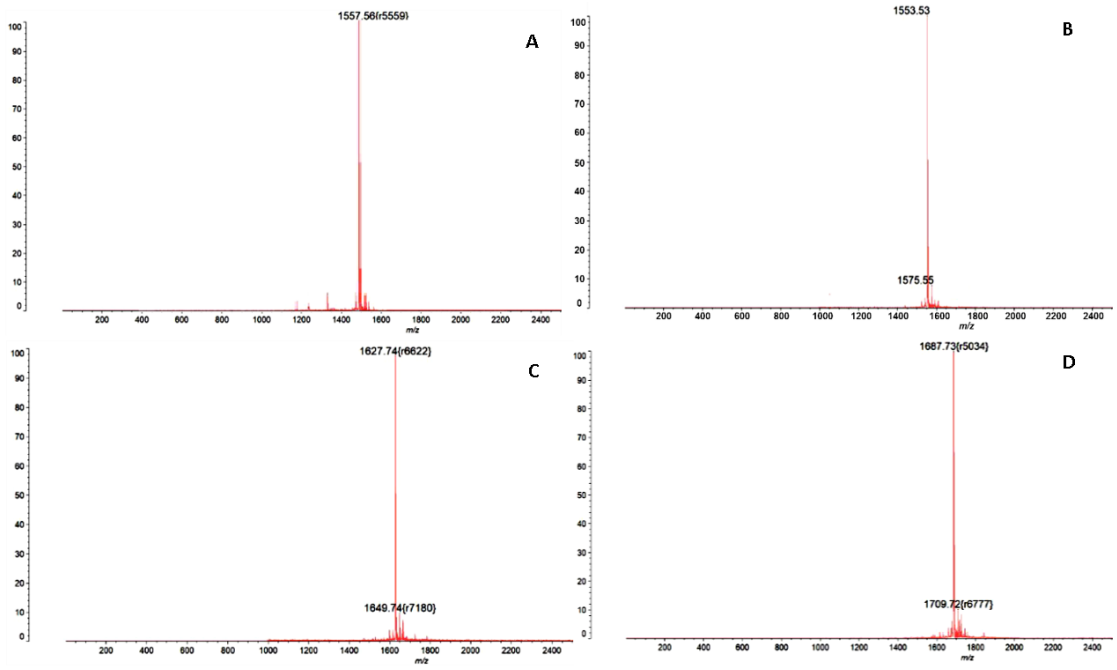

Figure 3. Mass spectra of Chenopodin-derived peptides. (A) Chen1, m/z=1557.56 Da. (B) Chen2, m/z=1553.53 Da. (C) ChenR, m/z=1627.74 Da. (D) ChenW, m/z=1687.73 Da. **Elaborated by:** Feijoo, 2022.

Antimicrobial activity

At concentrations of ≤ 512 µM, Chen1 had no antibacterial effects against *S. aureus* and *E. coli*. The remaining peptides (Chen2, ChenR and ChenW) showed bioactivity against Gram-positive (MIC: 8-128 µM) and Gram-negative bacteria (MIC: 8-64 µM). Therefore, the antibacterial activity of the Chenopodin-inspired peptides predicted by the *in silico* tools was confirmed, with the exception of Chen1. In general, incubation with the synthetic peptides had a greater impact on *E. coli growth* than *S. aureus*. ChenW showed higher inhibition of *S. aureus* and *E. coli* growth, with MIC and MBC values of 8 µM (**Table 2**).

Table 2. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of synthetic Chenopodin-derived peptides. (-) Not determined.

Peptides	**MIC (µM)**		**MBC (µM)**	
	E. coli	*S. aureus*	*E. coli*	*S. aureus*
Chen1	-	-	-	-
Chen2	64	128	128	128
ChenR	16	128	32	256
ChenW	8	8	8	8

Elaborated by: Feijoo, 2022.

Fluorescence Microscopy

Bioactive Chenopodin-inspired peptides showed a membranolytic nature according to fluorescence microscopy assay. *S. aureus* cells incubated with Chen2, ChenR and ChenW showed higher uptake of red fluorescence consistent with PI, indicating membrane rupture (Fig. 4). The peptide ChenW induced the highest red fluorescence values, similarly to effect caused by the positive control (10% SDS detergent). Untreated bacterial cells do not show any membrane damage and retain the blue color corresponding to DAPI.

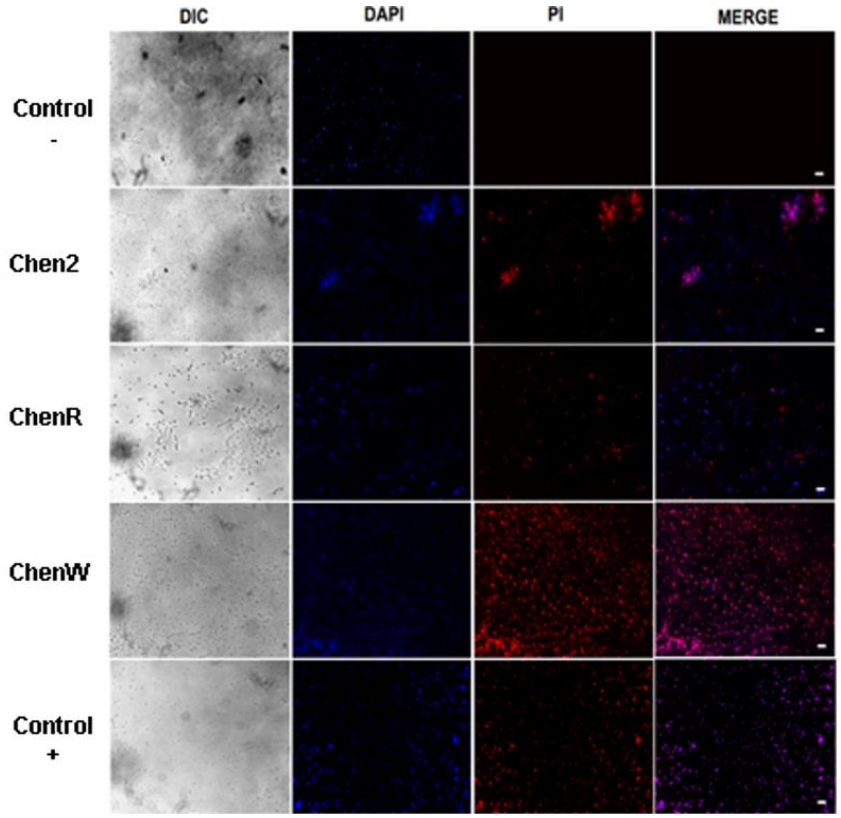

Figure 4. Fluorescence micrographs by dual staining DAPI/PI on *S. aureus* incubated with Chen2, ChenR and ChenW at 1xMIC.
Elaborated by: Feijoo, 2022.

Antiviral activity

The treatment with Chen2 and ChenR peptides have shown to be active against to fight SARS-CoV2 virus in human Calu-3 cells. The assay revealed that the effective concentration to achieve a 50% decrease of the infectious viral titer was EC50: 407.8 nM (0.41 µM) for Chen2 and EC50%: 345.3 nM (0.35 µM) for ChenR. At these concentrations the peptides are non-toxic to the Calu-3 cell line and >50% cell viability is maintained. However, Chen2 and ChenR peptides were shown to be cytotoxic to 50% of lung cells at CC50% concentrations: 6.4508×10^4 nM (64.508 µM) and 9.7356×10^4 nM (97.356 µM), respectively (Fig. 5).

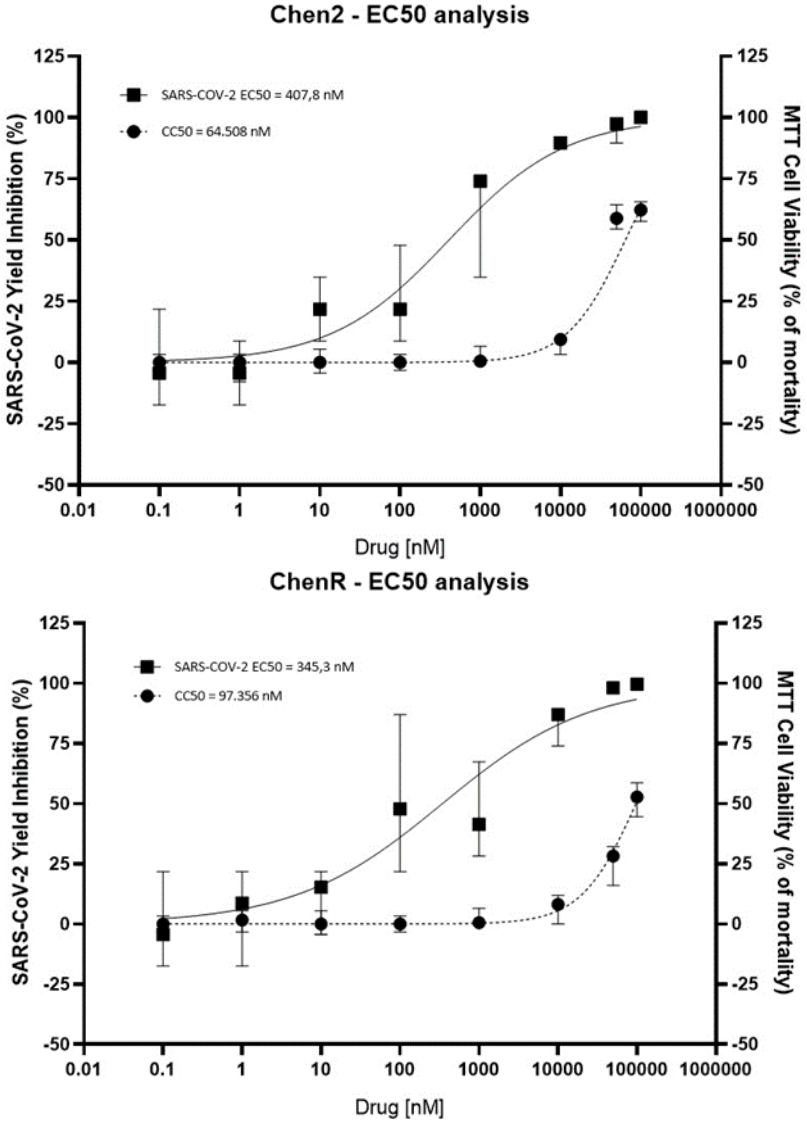

Figure 5. Effect of Chen2 and ChenR in Calu3 cells infected with SARS-CoV-2.
Elaborated by: Feijoo, 2022.

Hemolytic action

The HLPredFuse and HAPPENN predictors suggest that Chen W is most likely to be hemolytic with values of 0.5 and 0.01 respectively, while Chen1, Chen2 and ChenR have no hemolytic activity. On the contrary, the HemoPred classifier indicates the possible hemolytic action of Chen2 (Supplementary material Table 3-5S). However, *in vitro* assays showed that Chenopodin-derived peptides have a low hemolytic tendency, particularly at MIC concentrations. Our results evidenced that the peptides caused 3-18% red blood cell membrane damage at the range evaluated. Among the Chenopodin-based peptides, ChenW caused a larger proportion of hemolysis at concentrations over 32 µM (Fig. 6). Therefore, these *in vitro* data is partially consistent with the bioinformatic screening performed in on line web tools, such as HLPredFuse and HAPPENN.

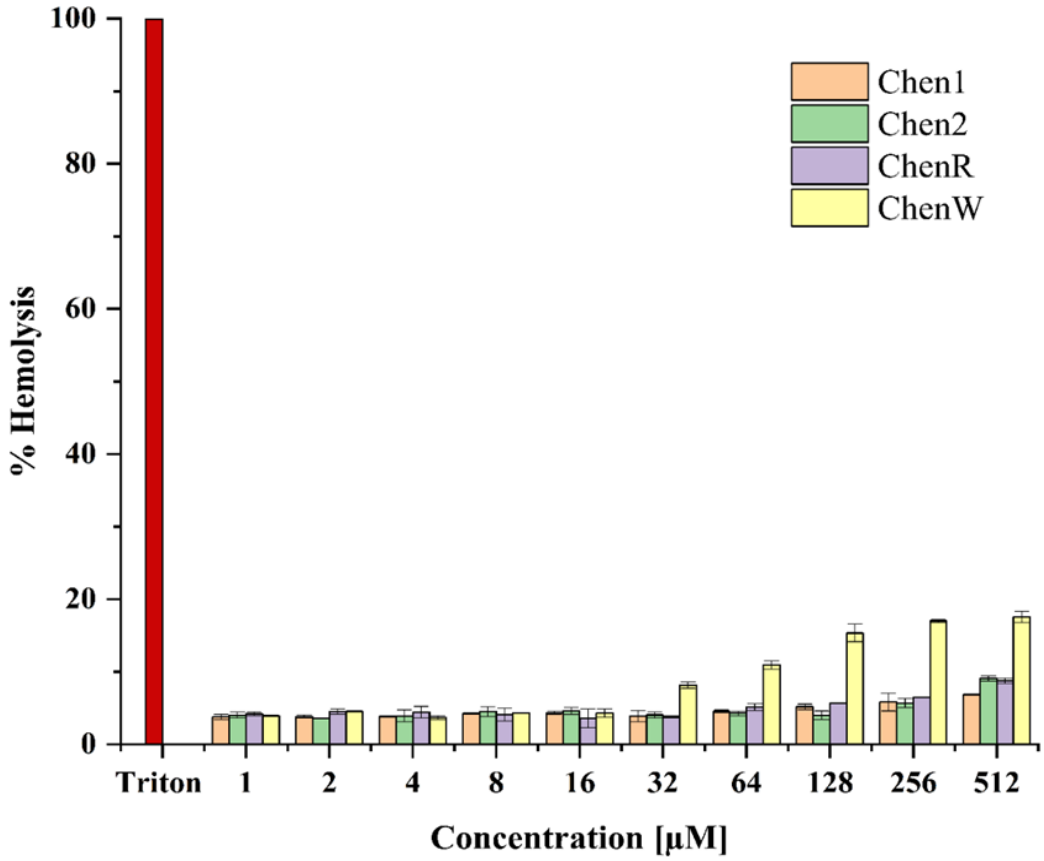

Figure 6. Evaluation of hemolytic activity of Chenopodin-based peptides. The positive control (Triton X-100) was considered as 100% hemolysis.
Elaborated by: Feijoo, 2022.

DISCUSSION

Peptides are attractive scaffolds for the future search and generation of anti-infective drugs [35,36]. From the study of natural sources, such as plants and venom samples, the pharmaceutical market has gained new prototypes, as well as expanded treatment regimens [37,38]. Advances in data-driven methods for predicting bioactivity constitute a promising strategy to optimize the design of plant AMPs by reducing their length, MIC, and host toxicity [39]. Based on this context, four structurally similar Chenopodin-inspired peptides were evaluated in this study.

The basic subunit of Chenopodin is rich in amino acids residues frequently found in typical AMP sequences [40]. Most of them have short lengths with hydrophobic cationic residues that form amphipathic structures [41]. AMPA software mapped a positive charge sequence made up of 14 amino acids (Chen1). This region was selected for *in silico* and *in vitro* screening. Additionally, we evaluated the *in silico* potential of a Chenopodin-based peptide of the same length (Chen2) that includes the last 10 residues of Chen1. Finally, two engineered peptides based on Chen1 were designed, replacing the amide chain amino acids with arginine (ChenR) or tryptophan (ChenW).

The computational approach of the AMPfun program suggested that the four designed peptides are antimicrobial. However, *in vitro* screening of antibacterial activity revealed that Chen1 was not active at the concentrations evaluated against *S. aureus* and *E. coli*. On the other hand, this result is consistent with AMP scanner predictor. The experimental study of new peptides predicted by bioinformatic tools is fundamental, as it expands the data on the functionality of the peptides and provides valuable information for the improvement of the artificial intelligence methods used for antimicrobial screening. Other investigations have also reported discrepancies between *in silico* and *in vitro* results. For instance, Fathin et al. [42] reported the computational design of a 10-mer peptide (LVSARIRCPK) with broad spectrum activity and 87% antibacterial action. Contrary to predictions, no antibacterial activity was observed in the *in vitro* experiment. On the other hand, the other three peptides (Chen2, ChenR and Chen W) showed activity against Gram-positive and Gram-negative, confirming the initial predictions. Chen1 shared 10 amino acids with Chen2. The difference of activity may be due to the presence residues of asparagine (N) and glutamine (Q) located at C-terminal, which are hydrophilic polar

amino acids that can affect interactions with target membranes [43,44]. There are several examples where the presence of these amino acids compromises the antimicrobial activity of the peptides, for example: in the peptide P4bPP21NNN derived from *Moringa oleifera* seed, two proline (P) were replaced by asparagines (N), which are more hydrophilic causing the antibacterial activity to be annulled [45]. Similarly, in the CT-K3K7 peptide the amino acid N was replaced by lysine (K) decreasing its MIC from 12.5 µg/ml to 3.125 µg/ml [46]. Finally, Peña-Carrillo et al. [30] mention that the presence of N in the pBmje peptide sequence could influence the lack of antibacterial activity *against E. coli* and *S. aureus*. In summary, these investigations highlight how the presence and position of this type of amino acid affect the antibacterial potential.

Chen-2 reproduces an original fragment (AHSIIYGVRGRGRI) from Chenopodin, suggesting their determinant role for antibacterial activity of this vegetal protein. The use of shorter molecules benefits the costs of synthesis and optimization of new antibiotics. Chen2, ChenR, and ChenW peptides are more active against *E. coli* than *S. aureus*. Similarly, Porto et al. [39] reported the affinity of Guavanin-2 peptide with *E. coli* membrane with a MIC of 6.25 µg mL^{-1} while against *S. aureus* it has a MIC of 100 µg mL^{-1}.

Engineering the primary structure of Chen1 produced bioactive sequences against both bacteria. Generally, cationicity and hydrophobicity provide peptides a higher potential of fighting pathogenic organisms due to increasing the interactions with elements of its membranes [47]. Arginine and tryptophan have been pointed out as key elements to understand the AMPs mode of action, which involves predominantly the membrane attraction, binding, insertion, and permeability [48,49]. The amino acid substitution employed have been successfully used in earlier studies. In this line, the peptides with three or four W residues, short lengths, balancing their charge and hydrophobicity through R and V residues, combat ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter* species) and *E. coli* pathogens at MICs <10 µM, with a toxicity to human erythrocytes of 25%, which was negligible and harmless in mouse experiments [50].

The antimicrobial and hemolytic activity no coupled to each other. Toxicity has been a critical bottleneck for the effective clinical translation of peptide-based drugs [25]. It has been demonstrated that some antimicrobial peptides can cause erythrocyte lysis, such

as melittin (HC50 = 1.7 µM) and Pis-1 (HC50 = 11 µM) [51]. In this studio, *in silico* tools predict non-toxicity or low hemolytic tendency of Chenopodin-derived peptides. These results were confirmed by *in vitro* assays. Peptides did not show erythrocyte membrane lysis in the range of antimicrobial activity. ChenW showed the highest propensity for hemolysis, according with *in silico* results. Feng et al. [52], peptides with a higher number of W residues will produce greater damage to red blood cells. This study showed that dCATH-derived peptides with one W residue has reduces hemolytic activity. Staubitz et al. [53] showed that replacing five W residues with Phe contributed to decrease the hemolytic activity of the peptide while preserving its antimicrobial activity. On the other hand, Chen2 yielded hemolytic activity according to the HemoPred predictor, which was not observed *in vitro*.

The most common secondary structure of AMPs is α-helical [54], followed by β-sheet, as shown for magainin and protegrin-1 respectively [55,56]. In contrast, Chenopodin-derived peptides exhibited a secondary structure of random coiled-coil shape. Peptides with this type of structure, such as hLF1-11 and DPK-060, have been characterized as antimicrobial activity against Gram-negative and Gram-positive bacteria, as well as fungi, through membrane disruption [57]. Usually the negatively-charge environment affects their shape maximizing the interactions that favor the activity [58,59].

Membranes are important biological and functional barriers for cells and promising drug targets [40]. The antibacterial properties of many AMPs consists in membrane-damage events [60,61]. The effect of peptides on *S. aureus* membranes has been monitored by fluorescence microscopy studies using PI, which is an indicator of membrane damage because cannot penetrate viable membranes [62], and DAPI, which is permeable and stains DNA, is a sign of intact membranes [63] Our results suggested that Chen2, ChenR and ChenW peptides act by membrane permeabilization mechanism, manifested by increased red fluorescence in bacterial cells such signal is indicative of membrane destabilization. This is consistent with the blue fluorescence of DAPI and the absence of PI in untreated cells, since PI cannot penetrate intact bacterial membranes. This effect has been studied in Liu et al. [64] where GL-22 and GL-29 peptides showed damage against *S. aureus* by a membranalytic mechanism represented by PI staining of bacterial membranes observed by fluorescence microscopy. On the other hand, it has been observed that bacteria tend to cluster together due to instability caused by reduced membrane potential [65].

Dual acting-peptides has gained a lot of attention, especially during the pandemic. Peptide repositioning has been converted into a strategy to explore other benefits and find new applications. Some AMPs, have shown efficiency in reducing SARS-CoV2 infection [66]. Several peptides have been tested *in vitro* against SARS-Cov2 [67–69]. Brilacidin acts at the viral entry, thus altering the integrity of the virus. Bakovic et al. [33] concluded that brilacidin inhibits 61% of virus titer at EC50% of 20 µM while affecting 50% of lung cells Calu-3 cells at CC50% of 241 µM. Other peptides such as gramicidin S (from *Bacillus brevis*) and melitin (from bee venom) have a potential to maintained Vero cells infected with SARS-CoV2, as it can decrease the viral load at 95% to 99% with EC50 values of 1.571 µM and 0.656 µM, respectively [70]. Gramicidin S has been shown to exhibit cellular cytotoxicity (CC50%) at 18.7 µg/ml in HT-29 cells [71], while melittin has CC50% at 6.45 µg/ml in C654 cells [72]. In contrast, Chen2 and ChenR requires a concentration of CC50%: 6.4508×10^4 nM (64.508 µM) and 9.7356×10^4 nM (97.356 µM), respectively, to maintain 50% of viable cells. Furthermore, Chen2 inhibits SARS-CoV2 viral titer at EC50% of 0.41 µM while ChenR at EC50% of 0.35 µM. At this concentration there is a low percentage of hemolysis, so it's possible use as an antiviral agent can be considered. However, strategies can be used to avoid hemolytic damage or peptide toxicity such as: peptide modifications [70], use of optimized nanoformulations [73] or polymeric nanoparticles [74].

Taken together, our template-based design approach evidenced that the primary structure of Chenopodin serves as a starting point for further refinement and lead optimization of new membrane disrupting peptides with antibacterial and antiviral properties. Future studies should access the serum stability and toxicity towards other cell types of bioactive Chenopodin-derived peptides.

CONCLUSION

In conclusion, we identified a sequence (Chen2) that reproduces the antibacterial effect of Chenopodin. Engineered peptides were also active against *E. coli* and *S. aureus*, demonstrating the utility of amino acid substitution to enhance activity of therapeutics peptides. The action of these Chenopodin-inspired molecules is based on membrane disruption. Chen2 and ChenR may be regarded promising agents for further evaluation of their antiviral potential *in vitro* and *in vivo*. Overall, this investigation reveals clues for engineering peptides and opens new research avenues to explore the biotherapeutic potential of vegetal proteins as privileged scaffolds and rich source of shorter dual-acting molecules with membrane-damaging mechanisms.

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SUPPLEMENTARY MATERIAL

Table 1S. Prediction of the antimicrobial activity of Chenopodin-derived peptides using Ampfun software.

ID	AMP	Antiparasitic	Antiviral	Anticancer	Targeting mammals	Antifungal	Gram + bacteria	Gram - bacteria
Chen1	0.9633	0.1182	0.625	0.1581	0.0667	0.4678	0.675	0.5362
Chen2	0.997	0.0545	0.5833	0.1925	0.0667	0.7167	0.8083	0.6232
ChenR	0.9884	0.1	0.5667	0.1648	0.1	0.5763	0.7583	0.5362
ChenW	0.9829	0.0636	0.5917	0.1058	0.0667	0.3664	0.6833	0.7213

Elaborated by: Feijoo, 2022.

Table 2S. Prediction of the antimicrobial activity of Chenopodin-derived peptides by AMP Scanner (www.ampscanner.com.) software.

ID	Predicted Class	Prediction Probability
Chen1	Non-AMP	0.1207
Chen2	AMP	0.9718
ChenR	AMP	0.9358
ChenW	AMP	0.9872

> 0.5 = Predicted AMP

Elaborated by: Feijoo, 2022.

Table 3S. Prediction of the hemotoxicity of peptides derived of Chenopodin protein by Hlppred-fuse.

ID	High or low	Predicted probability of High
Chen1	low	0.341666666667
Chen2	low	0.384375
ChenR	low	0.384375
ChenW	high	0.539583333333

Elaborated by: Feijoo, 2022.

Table 4S. Prediction of the hemotoxicity of peptides derived from Chenopodin protein by HAPPENN.

ID	Predicted probability
Chen1	0.007
Chen2	0.003
ChenR	0.004
ChenW	0.010

Elaborated by: Feijoo, 2022.

Table 5S. Prediction of the hemotoxicity of Chenopodin-derived peptides by HemoPred.

ID	Status
Chen1	Non-hemolytic
Chen2	Hemolytic
ChenR	Non-hemolytic
ChenW	Non-hemolytic

Elaborated by: Feijoo, 2022.

Table 6S. Prediction of the secondary structure of Chenopodin-derived peptides by PED2D.

ID	Secondary Structure	Helix %age(H)	Sheet %age(E)	Coil %age(C)
Chen1	CCCCCCCCEEHCCC	7.14	14.29	78.57
Chen2	CCEECEECECCCCC	0.00	35.71	64.29
ChenR	CCCCCCCCEECCCC	0.00	14.29	85.71
ChenW	CCCCCCCEEEECEC	0.00	35.71	64.29

Elaborated by: Feijoo, 2022.