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Predicting antimicrobial peptides from eukaryotic genomes: *In silico* strategies to develop antibiotics

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ABSTRACT

A remarkable and intriguing challenge for the modern medicine consists in the development of alternative therapies to avoid the problem of microbial resistance. The cationic antimicrobial peptides present a promise to be used to develop more efficient drugs applied to human health. The *in silico* analysis of genomic databases is a strategy utilized to predict peptides of therapeutic interest. Once the main antimicrobial peptides' physical–chemical properties are already known, the correlation of those features to search on these databases is a tool to shorten identifying new antibiotics. This study reports the identification of antimicrobial peptides by theoretical analyses by scanning the *Paracoccidioides brasiliensis* transcriptome and the human genome databases. The identified sequences were synthesized and investigated for hemocompatibility and also antimicrobial activity. Two peptides presented antifungal activity against *Candida albicans*. Furthermore, three peptides exhibited antibacterial effects against *Staphylococcus aureus* and *Escherichia coli*; finally one of them presented high potential to kill both pathogens with superior activity in comparison to chloramphenicol. None of them showed toxicity to mammalian cells. *In silico* structural analyses were performed in order to better understand function–structure relation, clearly demonstrating the necessity of cationic peptide surfaces and the exposition of hydrophobic amino acid residues. In summary, our results suggest that the use of computational programs in order to identify and evaluate antimicrobial peptides from genomic databases is a remarkable tool that could be used to abbreviate the search of peptides with biotechnological potential from natural resources.

1. Introduction

The growing number of the pathogen's resistance mechanisms to conventional drugs significantly increased in the last decade, in part because of the increase of the immune-compromised patients [5]. In some cases due to the resistance problem, only few drugs present the potency necessary to treat these opportunistic infections. Unfortunately, some of these drugs, such as amphotericin B, have the disadvantage of excessive toxicity, which could limit its use by patients receiving other therapies with toxic drugs, *i.e.* anticancer therapy [16]. The development of alternative antibiotic therapies able to circumvent this problem is one of the intriguing challenges of the modern medicine. For this purpose, antimicrobial peptides represent a promise to be used as antifungal and bactericidal agents since episodes of natural resistance to these peptides are not frequent [2,18,23].

Antimicrobial peptides can be found in all forms of life, from bacteria and fungi to plants, invertebrates and vertebrates [23]. They can be produced from secondary metabolites or, as most of them, encoded by genes conserved throughout evolution [3,44]. Despite some exceptions [32], usually, these peptides have the common features of being present on a cationic surface and also forming amphipathic structures [34,37].

Among the strategies used to identify these peptides, the technique to predict peptide sequences directly from genomic or transcriptome databases is currently used [19]. The genomic and

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transcriptome databases are valuable sources to identify gene sequences involved in the biosynthesis of antibiotics [4]. The *in silico* analysis of protein sequences or direct into the genes databases are strategies used to predict peptides of therapeutic interest [31]. The search for peptides using this strategy is performed by using sophisticated computational programs that scans the databases, correlating the antimicrobial peptide features previously described in the literature on the amino acid sequences. Since the main characteristics of antimicrobial peptides are already known, the pursuit of these similarities *in silico* in these databases is a tool to shorten the identification and selection of new antibiotics [14,17].

In order to certify the *in silico* identified peptides, the selected sequences must be synthesized by chemical synthesis and evaluated *in vitro* against selected microorganisms aiming to explore the antimicrobial potential [4,23]. Besides, knowing these sequences, it is also possible to make chemical variations in their amino acid residues, changing the primary structures in order to improve their antimicrobial activity [31].

The present study reports on the use of novel software to identify antimicrobial peptide sequences on the fungus *Paracoccidioides brasiliensis* transcriptome and on the human genome databases. The selected sequences were biochemically synthesized and *in vitro* tested against fungi and bacteria. Furthermore, *in silico* structural analyses were also conducted.

2. Materials and methods

2.1. Peptides selection

2.1.1. In silico identification of antimicrobial peptides

The peptides were obtained from genome databank by using a script that takes in consideration peptide length, total charge surface and hydrophobic moment (data not published). Among hundred peptides, 13 were selected since it fitted to properties described in APD2 databank as antimicrobial peptides [47]. The criteria used to design this software took into consideration some antimicrobial characteristics such as the presence of positively charged amino acid residues, low molecular weight, and the balance between cationic charge and hydrophobicity. The databases used to identify these sequences were the human genome (http://genome.gov) and transcriptome of the human pathogenic fungus *P. brasiliensis* (https://dna.biomol.unb.br/Pb/). Several potential antimicrobial peptide sequences were identified in both databases and four of them, two from each database, based on better antimicrobial characteristics, were selected and chemically synthesized.

2.1.2. Peptides synthesis

The peptides were synthesized by the 9-fluoroenylmethoxycarbonyl technique [22] using an automated bench top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system; Shimadzu, Tokyo, Japan). The synthesized peptides were then re-purified with a semi-preparative reverse-phase C-18 (5 μm, 300A, Vydac 218TP510, Hesperia, USA) in a high-pressure liquid chromatography (HPLC) system (Shimadzu Co., Japan). The HPLC fractions were eluted in 60 min in linear gradient water and acetonitrile (JT Baker, Mexico), both containing 0.1% trifluoroacetic acid (TFA, JT Baker, Mexico). RP-HPLC experiments were monitored at two different wavelengths (216 and 280 nm). The purity of peptides was assessed by analysis of the molecules present in the fractions using mass spectrometry MALDI-TOF/TOF Ultraflex II (Bruker Daltonics, Germany). The purified peptides were lyophilized and stored at −70 °C until used. The peptides were identified as P1 and P4 from the human genome and P2 and P3 from *P. brasiliensis* transcriptome.

2.2. In vitro hemocompatibility study

Fresh heparinized blood of Swiss mice was used to investigate the *in vitro* hemolytic activity of the peptides according to Italia and collaborators [26] with minor modifications. The red blood cells (RBCs) were obtained by centrifugation of the whole blood at 3000 rcf for 15 min. The supernatant was discarded and the RBCs were washed thrice with saline solution (NaCl 0.9%). The working solution was prepared containing three parts of RBCs mixed with 11 parts of saline solution. For the assay, a dilution of 100 μl of the working solution to 1 ml of the samples was prepared. The treatment groups were the RBCs (working solution) mixed with: (a) distilled water (positive control, 100% hemolysis); (b) saline solution (negative control, minimum hemolysis); and (c) samples of the peptides P1, P2, P3, or P4 at concentrations of 64, 128, and 256 μg ml^{-1}. The samples were incubated at 37 °C for 6 h and at time intervals of 30 min, 3 h and 6 h, they were centrifuged at 3000 rcf for 15 min. The supernatant was collected and maintained for 30 min at room temperature to oxidize hemoglobin and the absorbance of Oxy-Hb was determined by spectrophotometry at 540 nm. The percentage of hemolysis was calculated based on the assumption that 100% RBC lysis resulted from mixing of RBCs with distilled water.

2.3. Antimicrobial investigation

Antimicrobial activity of the peptides against Gram-positive, Gram-negative bacteria and fungi was determined by the broth microdilution assay in accordance with the methods developed by the National Committee on Clinical Laboratory Standards (NCCLS) [11] with some modifications.

2.4. Antifungal tests

2.4.1. Strains and growth conditions

The human pathogenic fungus *P. brasiliensis*, isolates Pb01 and Pb18, were obtained from the fungi collection of Molecular Biology, Universidade de Brasília, and cultivated in Brain Heart Infusion culture medium (Merck, Germany) at 36 °C in rotary shaker (220 rpm) for 5 days before the tests. The *Candida albicans* clinical isolate was provided by Sabin Laboratory, Brasília, DF, and was grown in culture medium Sabouraud agar (Acumedica, USA) at 37 °C overnight before performing the assay.

2.4.2. Determination of the minimum inhibitory concentration (MIC)

Two different protocols were used to test the *in vitro* activity of the peptides against fungi in order to investigate the influence of the incubation time on the assay.

Protocol I was used to test the peptides fungal activities against *P. brasiliensis* and *C. albicans*. The methodology used to determine the MICs was adapted from the antifungal protocol NCCLS [11]. The peptides P1, P2, P3, and P4 were serially diluted from 2 to 256 μg ml^{-1} in culture medium Muller-Hinton for *C. albicans* and RPMI1640 for *P. brasiliensis*. A 2-fold dilution series of peptides was prepared and serial dilutions (50 μl) were added to 50 μl of cell suspension of *C. albicans* (2×10^4 viable cells ml^{-1}) or *P. brasiliensis* (2×10^5 viable cells ml^{-1}) in 96-well microtiter polypropylene plates (Corning). The plates were incubated at 36 °C during 24 h for *C. albicans* and 6 days for *P. brasiliensis*. The differences in the incubation time and the smaller amount (10 times) of cells used for *C. albicans* than for *P. brasiliensis* were due to the growth characteristic differences observed for each fungus. The growth inhibition was determined by measuring absorbance at 595 nm with a Model 450 Microplate Reader (Bio-Rad) after the incubation times. The lowest concentration of peptide that completely inhibited growth of

the fungi was defined as the minimal inhibitory concentration. The MICs were calculated by the average values obtained in triplicates on three independent measurements.

Protocol II was used only for *P. brasiliensis*, in which the incubation time was 12 h and the methodology was adapted from Travassos and collaborators [42]. The peptides P1, P2, P3, and P4 were serially diluted from 16 to 500 $\mu g\,ml^{-1}$ in phosphate buffer saline (PBS, pH 7.2). A 2-fold dilution series (100 μl) were added to 100 μl of 2×10^4 viable cells of the *P. brasiliensis* in 500 μl plastic tubes. The tubes were incubated at 36 °C in rotatory shaker (100 rpm) during 12 h. After this period, 100 μl of each tube were plated in solid medium Brain Heart Infusion (BHI, Acumedia®, USA) supplemented with 4% (v/v) horse serum (Gibco, USA), 5% (v/v) supernatant of the culture filtrate of the isolate Pb192 and 40 $mg\,l^{-1}$ gentamycin (Schering-Plow, USA). The filtrate was prepared according to methodology described previously [42]. The growth of colony-forming units was observed for 21 days. The lowest concentration of peptide that completely inhibited growth of the fungi was defined as the minimal inhibitory concentration. The MICs were calculated by the average values obtained in triplicates on three independent measurements [36]. The experimental controls used in both protocols were amphotericin B (Sigma–Aldrich, USA) and for protocol II the killer peptide (KP) as control was also used.

2.5. Antibacterial tests

2.5.1. Bacterial strains and growth conditions

The antibacterial activity was evaluated against human pathogenic bacteria *Escherichia coli* ATCC8739 and *Staphylococcus aureus* ATCC25923, both obtained from the American Type Culture Collection (ATCC). Briefly, the bacterial cultures were grown in Lysogeny Broth (LB) medium, pH 7.0, at 37 °C until they reached the exponential phase.

2.5.2. Determination of the antibacterial activity

The method used to study the antibacterial activity of the peptides was based on the broth microdilution assay. The culture for the assay was prepared by diluting 1:11 the bacteria obtained on the exponential phase. The peptides P1, P2, P3, and P4 were serially diluted from 2 to 256 $\mu g\,ml^{-1}$ in LB medium. A 2-fold dilution series (100 μl) were added to 10 μl of approximately 5×10^6 CFU of bacteria in each well of a 96-well polypropylene plate. The plates were incubated for 4 h at 37 °C and the peptides antibacterial activities were observed in every 30 min by measuring the absorbance in a plate reader (Bio-Rad 680 Microplate Reader) at 595 nm. The controls utilized were distilled water and chloramphenicol 60 $\mu g\,ml^{-1}$.

2.6. Molecular modeling

Primaries sequences were obtained from initial selection previously described in this section. All of them being of synthetic peptide amidate. PSI-BLAST was used for templates data mining [48]. For P1 and P2 peptides models, it was possible to obtain templates by homology method (pdb: 2jx6 and 1id3), showing 55 and 88% of identity respectively [45,48]. Fifty models for each peptide were constructed by using Modeller v9.8. These models were evaluated by satisfaction of spatial restraints, considering energy minimization, which was conducted by default parameters. P3 and P4 do not show significant homology to any peptide with structures previously elucidated. For these last I-Tasser server was utilized in construct models combining *ab initio* and threading methodologies. Models validation was realized by using C-score and TM-score parameters. C-score is based on the significance of threading template alignment and varies between −5 and 2 and positive values indicate better quality of predicted models. TM-score standards were used for measuring similarities between two structures, which are usually used to measure the accuracy of model when the native structures are known. Models with TM-score higher than 0.5 indicate a model with correct topology. Predicted P1, P2, P3 and P4 tridimensional models were evaluated using PROCHECK for analysis of stereochemical quality. In addition RMSDs were calculated for superposition of Cα traces and backbones onto the templates structures through the program 3DSS. The peptides structures were visualized and analyzed on Delano Scientific's PYMOL (http://pymol.sourceforge.net/).

2.7. Statistical analysis

All data were analyzed by Student's test and ANOVA. *P* values below 0.05 were considered significant.

3. Results

3.1. In silico identification of antimicrobial peptides

Using a software designed by us to identify antimicrobial peptide sequences in the transcriptome and genome databases, it was possible to abbreviate and find out the search for these molecules. This software was used to scan the transcriptome of the human pathogenic fungus *P. brasiliensis* and the human genome to find amino acids sequences that presented antimicrobial characteristics according to algorithms previously designed to identify, among other characteristics, the presence of specific amino acids residues. Data presented here are part of a research line including the sequencing of the *P. brasiliensis* transcriptome focusing on further molecular drug targets identification. In this view, *P. brasiliensis* database was explored in order to find novel antimicrobial peptides since few is known about the presence of such compounds in this species. Nevertheless in last few years the presence of antimicrobials in pathogens has been widely described due to necessity of pathogenic fungi to develop defense mechanisms to compete and survive to the presence of other microorganisms [17,21].

After performing the scan on the genomic databases, some possible amino acids sequences with the desired characteristics previously defined were identified. Of these, we selected the four most promising that contained the higher algorithms score previously developed (data not shown) and also that have higher fitness to APD2 best scores for antimicrobial peptides [47], such as presence of positively charged amino acid residues, peptide length and the balance between cationic charge and hydrophobicity. They were then chemically synthesized, purified, sequences confirmed by MALD-TOF/TOF and investigated *in vitro* for hemocompatibility and antimicrobial activity. The selected amino acid sequences were submitted to a Blast analysis and no significant hits were identified for the peptides P1 and P4 from the human genome. Similarities were found for the peptides P2 and P3, from the *P. brasiliensis* transcriptome, for the histone h2 and a ribososomal protein S12, respectively, of several fungi species. Nevertheless, no identity was observed for peptides reported here with antimicrobial peptides classes previously described.

3.2. In vitro hemocompatibility study

In order to investigate whether the peptides could cause some hemolytic effect, they were incubated for 0.5 h, 3 h, and 6 h with the red blood cells (RBCs) in saline solution (NaCl 0.9%) phosphate buffer saline (pH 7.2). The pattern of hemolysis resulting from the incubation of RBCs with the peptides P1, P2, P3, and P4, are depicted in Fig. 1. Since no differences were observed between peptide concentrations tested (64, 128, and 256 $\mu g\,ml^{-1}$) or between the times

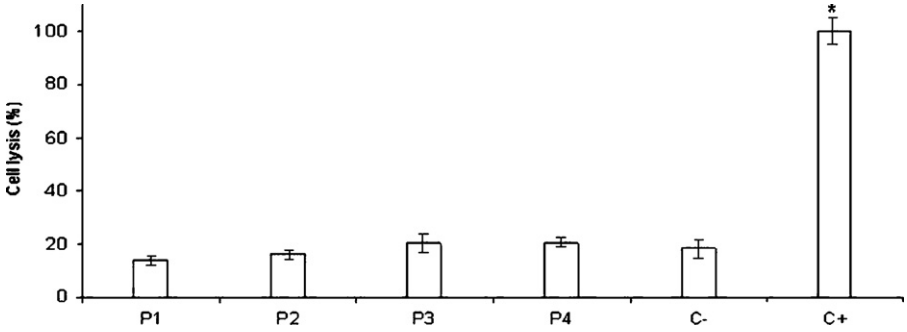

Fig. 1. The *in vitro* percentage of red blood cells (RBCs) lysis pattern following incubation with the peptides P1, P2, P3, or P4 at concentration of 256 μg ml^{-1} for 6 h. The C$^-$: saline solution, pH 7.2 used as negative control (minimum lysis); C$^+$: distilled water, used as positive control (100% of lysis). *$P \leq 0.05$.

observed (0.5 h, 3 h, 6 h), only results for the highest concentration (256 μg ml^{-1}) and for the most extended incubation time (6 h) are presented here. The distilled water was used as positive control and considered to cause 100% hemolysis due to the rupture caused by the osmotic pressure on the RBCs. The saline solution was used as negative control which causes a minimum osmotic pressure across the cell membrane of RBCs maintaining the integrity of cell membrane. None of the peptides presented hemolytic effect when compared to the positive control. The peptides P3 and P4, that presented the higher levels of hemolysis, did not show significant difference even when compared with the saline solution. The data therefore, indicate that the predicted peptides did not cause RBCs lysis.

3.3. Antimicrobial investigation

3.3.1. Antifungal tests

The peptides P1, P2, P3 and P4 were tested in order to investigate the *in vitro* antimicrobial activity against the human pathogenic fungi *C. albicans* and *P. brasiliensis* isolates Pb01 and Pb18. Two different protocols were used, which differ from each other on the incubation time used, as described in the Materials and Methods section. Table 1 shows that two of four selected peptides exhibited antifungal activity against *C. albicans*, determined by the minimum inhibitory concentration (MIC) of 82 μM and 133 μM for peptides P1 and P2, respectively. The MIC indicates the required amount of the active compound to kill or inhibit the growth of the microorganisms. The control for the assay used was amphotericin B, MIC 0.5 μM. Another control used against this pathogen was the killer peptide (KP), which presented MIC value of 1 μM. Moreover, the four peptides tested exerted no detectable antifungal activity against *P. brasiliensis* even at the highest concentration (256 μM) utilized in the assay for both of the protocols as indicated in Table 1. Considering that the incubation time could be influencing on the peptide activity by its degradation, the Protocol II was used. This protocol was adapted from another assay to test the peptide KP, also used as control here, against *P. brasiliensis* in which the incubation time was only for 12 h. The MIC established for *P. brasiliensis* isolate Pb01 was 32 μM and for Pb18 was 16 μM. However, even with this short incubation time no antifungal activity was detected for the predicted peptides against *P. brasiliensis*.

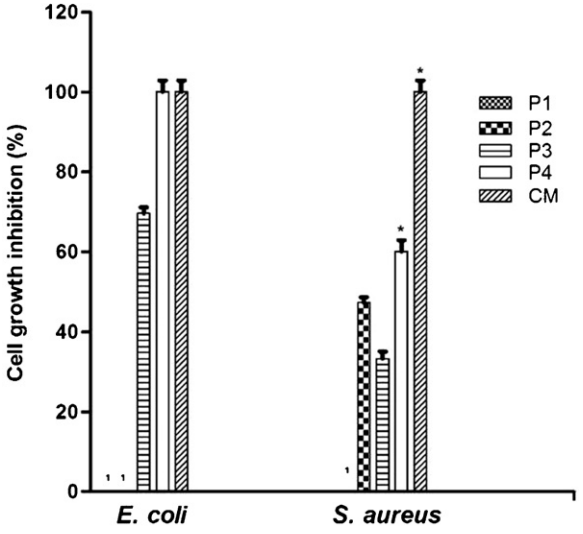

Fig. 2. The inhibitory activity on bacterial cell growth induced by the peptides P1, P2, P3, and P4 at concentration of 150 μM and the antibiotic chloramphenicol (Cm) at 185 μM. *Statistically no difference was observed between P4 and Cm for *E. coli*. [1]No inhibition was detected in P1 for *E. coli* and *S. aureus* and P2 for *E. coli*. $P \leq 0.05$.

3.4. Bactericidal evaluation

The microdilution assay was performed in order to determine the ability of the selected peptides from the genomes to kill or to inhibit the growth of the Gram-negative bacteria *E. coli* and the Gram-positive bacteria *S. aureus*. According to Fig. 2, considering the ability of all the peptides to kill or inhibit the growth of *E. coli* and *S. aureus*, the best activity was exhibited by the peptide P4 with the inhibition of nearly 100% for *E. coli* and 60% for *S. aureus* at concentration of 150 μM. The peptide P1 did not show any antimicrobial activity against both bacteria tested and the peptide P2 showed inhibition only for *S. aureus* (46%) at concentration of 133 μM. The

Table 1
The *in vitro* antifungal activity investigation of peptides selected from the transcriptome of *P. brasiliensis* and from the human genome against *C. albicans* and *P. brasiliensis* Pb01 and Pb18. The minimum inhibitory concentration (MIC, μM) was investigated using two different protocols (as described in Section 2).

	P1	P2	P3	P4	AMB	KP
Candida albicans	82 μM[b]	133 μM[b]	N.D.[b]	N.D.[b]	0.5 μM	1 μM[c]
Paracoccidioides brasiliensis Pb01	N.D.[a]	N.D.[a]	N.D.[a]	N.D.[a]	0.5 μM	32 μM
Paracoccidioides brasiliensis Pb18	N.D.[a]	N.D.[a]	N.D.[a]	N.D.[a]	0.5 μM	16 μM

[a] N.D. = no detectable antifungal effect until 256 μg ml^{-1} using both protocols.
[b] MIC determined using protocol I and N.D. = activity not detected until 256 μg ml^{-1}.
[c] Obtained from another assay. Molecular weight (Da): P1 = 1756.12; P2 = 1918.20; P3 = 1612.92; P4 = 1697.03; KP (*killer peptide*) = 998.47; AMB (amphotericin B) = 924.08.

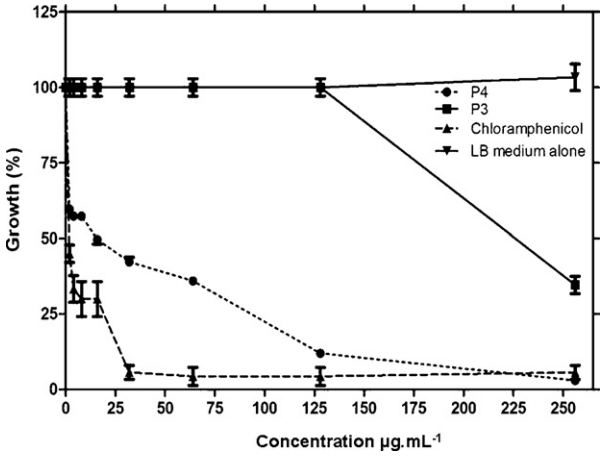

Fig. 3. Antibacterial activity of peptide P4 at concentrations of 10, 75 and 150 μM against *E. coli*. At 150 μM it was observed a 97% on growth inhibition, 86.6% at 75 μM and 48% at 10 μM. Cell growth inhibition was estimated by measuring the absorbance change at 595 nm each 30 min during 180 min. C^+: positive control, LB medium alone; C^-: negative control, chloramphenicol at concentration of 185 μM. Molecular mass (Da): P1 = 1756.12; P2 = 1918.20; P3 = 1612.92; P4 = 1697.03; chloramphenicol = 323.13.

peptide P3 exhibited antimicrobial inhibition of 66.8% for *E. coli* and 34% for *S. aureus* at concentration of 150 μM for both peptides.

Fig. 3 shows the growth inhibition in function of time and concentration exposure of *E. coli* incubated with the peptide P4, which presented the best antimicrobial activity against these two bacteria. For *E. coli* (Fig. 3), the P4 presented the same antibactericidal activity (97.3%) observed for chloramphenicol, although in a small peptide amount (150 μM) than used for this antibiotic (185 μM). Moreover, at concentration of 10 μM a 48.2% of growth inhibition was observed, using about twenty times less peptide than antibiotic. For the *S. aureus* (data not show), as observed for *E. coli*, the best antifungal activity was also obtained by the peptide P4 with a growth inhibition of 60.7% at concentration of 150 μM *versus* 95% growth inhibition presented by the chloramphenicol at 185 μM. In half of this concentration (75 μM), P4 presented a 42.8% of growth inhibition.

3.5. Molecular modeling analyses

After the construction of models (Fig. 4) it was observed that all four peptides were structurally organized in α-helix conformation, as observed for several antimicrobial peptides previously reported [10,28,45] and listed in the publicly available databases such as Swissprot and TrEMBL (http://www.expasy.org/sprot/sprot-top.html), AMSDd (http://www.bbcm.univ.trieste.it/~tossi/pag1.htm), APD (http://aps.unmc.edu/AP/main.html) and ANTIMIC (http://research.i2r.a-star.edu.sg/Templar/DB/ANTIMIC/). A Procheck summary of all peptides showed that 100% of amino acid residues are in most favorable region for helix formation (Table 2). Structural differences between the template structures and predicted three-dimensional structure of the peptides model were calculated by superimposition of Cα traces and backbones onto the templates structures. The RMSD values between the structures experimentally resolved and modeled *in silico*, were calculated for P1, P2, P3 and P4. Cα traces, and the main chain atom were measured at 0.97, 0.59, 0.90 and 0.50 Å respectively. The RMSD values and low variability among the experimental structure templates and the structure modeled reflect conservation in most regions and emphasize a similar folding pattern among these antimicrobial peptides. This result indicated that the constructed peptides model presented its amino acid residues of the average of the observed parameters. On the other hand, the structure of the lateral chains was considered to be well located, when compared with the experimental structures with the same

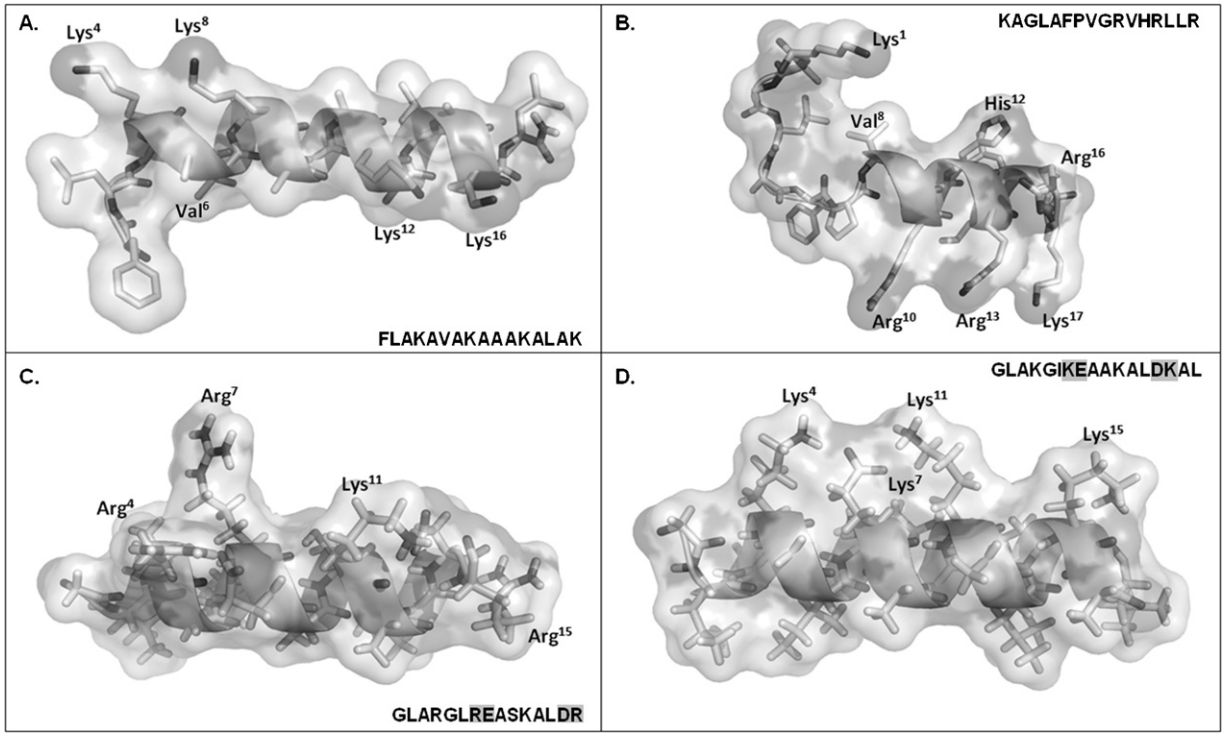

Fig. 4. Tridimensional models constructed by using Modeller and I-Tasser servers. All the models are represented in cartoon and surface demonstrating in red residues negatively charged and blue residues positively charged. Side chains are presented at stick format. Residues involved with pathogen interaction were named. (A) P1 antifungal peptide, (B) P2 antifungal and antibacterial peptide, (C) P3 antibacterial peptide and (D) P4 antibacterial peptide models. Binding motifs that determine specific activity against bacteria are highlighted in gray. The structures were visualized and analyzed using Pymol program.

Table 2
Physicochemical properties of peptides P1, P2, P3 and P4.

Peptides	Sequence (—NH_2)	Molecular mass (Da)	Total hydrophobic ratio (%) [a]	Boman index (kcal mol^{-1}) [b]	Ramachandran plot (%) [c]	C-score [d]	TM-score [d]
P1	FLAKAVAKAAAKALAKAL	1755.13	77	−0.88	100	–	–
P2	KAGLAFPVGRVHRLLRK	1917.21	47	1.71	100	–	–
P3	GLARGLREASKALDR	1611.94	40	3.14	100	0.06	0.68 ± 0.12
P4	GLAKGIKEAAKALDKAL	1696.04	52	0.41	100	0.28	0.73 ± 0.11

[a] Percentage total of hydrophobic amino acid residues in the peptide.
[b] Index generated by Antimicrobial Peptide Database (APD) that indicate protein-binding potential [51].
[c] Percentage of the amino acid residues in favorable regions for secondary structure formation.
[d] Value obtained through of I-TASSER server for protein structure predictions that are based on multiple-threading alignments.

resolution. Moreover, probable residues involved with pathogen interaction and their physicochemical properties will be discussed below.

4. Discussion

The emerging incidence of antimicrobial resistance mechanisms developed by microbial pathogens remains a serious worldwide problem to public health. This threat is increasing mainly in immunocompromised patients, including those with AIDS, undergoing anticancer chemotherapy or therapy after organ transplants. The opportunistic pathogenic fungi, such as *Candida* sp., can invade various tissues and cause systemic infections, which are considered life-threatening for the patient [12]. Also, the infectious diseases caused by antibiotic-resistant bacteria have contributed to make the situation worst, especially in those patients, for which the success of treatment with currently available drugs is becoming less efficient [8,16,27]. Thus, there is an urgent need to develop alternative antibiotic therapies to change this situation.

Antimicrobial peptides are attractive to be used as antimicrobial agents since episodes of natural resistance episodes are rare and they could show low toxicity effects to the mammalian cells [23]. These compounds can be obtained from a variety of sources, including frog's skin, vertebrate and invertebrate animals, plants and microorganisms, presenting a broad range of activity [6]. Despite the inexorable source of peptides, their obtainment from identification to purification is a very long and expensive approach.

Modern approaches used to identify antimicrobial peptides are proposed to reduce the long-time identification, extraction, purification and characterization of biologically active peptides from natural sources [27,31]. One alternative methodology is to analyze *in silico* genes and proteins from genomic databases to predict and identify amino acid sequences that share similarities and molecular features with natural bioactive peptides, such as cationic residual charge, which have potentially bioactive properties [4].

In this work, the *in silico* approach for identification of antimicrobial peptides was used. A computational program developed by our research group was carried out to identify peptide sequences in the genomic databases of the fungus *P. brasiliensis* [15] and in the human genome. This software identified, initially, thirteen potential antimicrobial peptide sequences, of which the four most promising, that attended the most criteria of the parameters used in the software, two from each databases (P2/P3 present in *P. brasiliensis* and P1/P4 in the human genome), were selected and chemically synthesized for investigation of the antimicrobial activity.

The peptides were tested *in vitro* against the fungi *C. albicans* clinical isolate and *P. brasiliensis*, isolates Pb01 and Pb18. Two of the four selected peptides presented antifungal activity against *C. albicans*. The minimum inhibitory concentration (MIC) exhibited by the peptide P1 was 82 μM and for P2 was 133 μM. Despite the fact that the MIC values obtained against this fungus were higher than those observed for the antifungal amphotericin B (0.5 μM) or for the antimicrobial peptide KP (1 μM) these peptide sequences can still be used to develop new therapeutic agents [27,29]. None of the peptides in the concentrations tested presented antifungal activity for the fungus *P. brasiliensis*. Probably, this could be due to differences observed between these two pathogens on the target of these peptides or because of the *P. brasiliensis* cell wall complexity, which could impede the peptide penetration.

In order to evaluate the antibacterial activity of the transcriptome selected peptides, the microdilution assay was used for *S. aureus* and *E. coli* bacteria. Our present results demonstrate that one of the synthesized peptides, P4, presented a high potential to kill both Gram-positive and Gram-negative bacteria tested. The P4 ability exhibited to inhibit the bacteria growth was superior to that observed for the conventional antibiotic chloramphenicol. It was necessary for 150 μM of the P4 to exhibit the same antibacterial activity elicited by chloramphenicol at 185 μM concentration, resulting in the use of less peptide than antibiotic. Moreover, the peptides P2 and P3 also presented activity to inhibit the *S. aureus* and *E. coli* growth, showing potential to be used as peptide model to develop a potent antibiotic. Another important consideration relies on the fact that, as demonstrated by the hemolytic study, none of the peptides showed toxicity to mammalian cells. This may be an indication that, depending on the modifications made to improve the peptides antimicrobial activity, the chances of developing toxic side effects in a possible therapy using these peptides can be decreased.

Although the potent antibacterial activity for the peptides was observed, they did not present the same effect against fungi. Only two of the peptides, P1 and P2, showed antifungal properties against *C. albicans* with MIC value higher than those obtained for the conventional drugs. Despite the disappointing fact, these peptides should not be disregarded for future use. Due to the incidence of microorganisms' resistance to available therapy, these molecules can be used as a basis for development of more efficient molecules [5,27]. Knowing their sequences, it is possible to make changes in the primary structure envisioning increasing their potency.

A diverse set of synthetic modifications on known peptide sequences have been done to increase their antimicrobial potency. Structural changes on the buforin II increased its activity by the substitution of only a single amino acid residue [35]. The halocidin suchlike were designed and one of them, di-K19Hc emerged as the most promising candidate for the development of a new antibiotic against antibiotic-resistant bacteria, presenting both antifungal and antibacterial properties [33,35].

Finally structural and functional relation was evaluated by *in silico* theoretical analyses. In spite of all peptides showing helical structure and also hydrophobic ratio values of spectra of 20–80%, minor differences between them were evaluated here. Firstly, similar values of hydrophobic ratio and peptides studied here were observed in Antimicrobial Database (APD) for antibacterial, antifungal, antiviral and anticancer peptides [46]. The P1 peptide presented α-helix conformation with the cationic residues of lysine organized in line that favors a membrane-peptide interaction, despite a surprising absence of antibacterial activity. These

data clearly show that the antibacterial activity could be related with other factors in addition to the presence of hydrophobic and cationic residues in the surface. Otherwise, P1 showed a remarkable activity toward pathogenic yeasts (Table 2). These data could be explained by the hydrophobic residues that are also exposed on the structure surface such as Phe[1], Leu[2], Leu[14], Val[6], and Leu[18], which could interact to membrane (Fig. 4). In Table 2 the P1 peptide demonstrates a Boman index value of −0.88 kcal mol^{-1}, which is similar to aurein 2.5, an antifungal peptide from *Litoria aurea* and *Litoria raniformis* (−0.89 kcal mol^{-1}), which shows antifungal activity toward *Candida tropicalis*, *Candida kefyr*, *Candida krusei*, *Candida parapsilosis* and *Candida glabata* [49]. This peptide clearly shows the residues Leu[2], Phe[3], Iso[5], Val[6], Val[9], Val[10], Phe[13] and Leu[16] in the structural surface, showing clear homology with P1 peptide surface. Moreover, others aureins isoforms also presented similar Boman-Index with values ranging from −1.06 to 0.12 kcal mol^{-1}, reinforcing that structures that yield similar Boman index values could present antifungal activity as observed for P1 peptide [38].

The P2 peptide presented a predominant α-helix conformation with cationic residues of arginine (positions 10, 13 and 16), histidine (position 12) and lysine (positions 1 and 17) exposed on the surface in the C-terminal region (Fig. 4). On the other hand, the Pro[7] break the helix formation causing a turn at C-termini region. In opposite side hydrophilic residues some hydrophobic residues are also exposed on surface such as Leu[4], Phe[6], Va[l8], Val[11], and Leu[15]. Another interesting issue consists in the homology (88%) of P2 with histone H2A.1 chain C of yeast nucleosome core structure [45]. The antimicrobial properties of histones have long been recognized despite their low activity [24]. Yonezawa and collaborators [50] related that a structural motif of histone, which strongly binds to DNA, may be used as an antimicrobial peptide to bind to cell membrane. Studies suggested that several phospholipid binding proteins (bovine lung annexins and human serum lipoproteins) and some peptides such as tachyplesin I can bind to DNA [50]. Other result that contributed showed that Boman index obtained for P2 (1.71 kcal mol^{-1}) showed similar values encountered for both antifungal and antibacterial peptides as observed for heliomicin from *Heliothis virescens* with 1.74 kcal mol^{-1} [30]. Moreover *Drosophila melanogaster* andropin and bovine lactoferricin B peptides presented Boman index ranging 0.55–2.75 kcal mol^{-1} that seems to be more active against Gram-positive bacteria and fungi [25,39] corroborating with data reported here.

The P3 peptide presented α-helix conformation with cationic and anionic residues that were exposed on the surface and distributed at N- to C-termini. Some hydrophobic residues such as Leu[2], Leu[6], and Leu[13] are also observed across multiple hydrophilic residues (Fig. 4). Boman index value for P3 was 3.14 kcal mol^{-1}. Similar results were encountered for antibacterial cecropin D-like peptides from *Manduca sexta*, that presented spectra between 1.46 and 3.29 kcal mol^{-1} [13]. Moreover, the P4 peptide presented an α-helix conformation extremely similar to P3, with cationic and anionic residues exposed on the surface and distributed in line favoring electrostatic interaction and hydrogen bounds. On the other hand, hydrophobic residues are also observed in N- and C-terminal boundary such as Leu[2], Iso[6] and Leu[13], Leu[16]. Firstly, Boman index value for P4 was 0.41. Esculentin and brevinins antimicrobial peptides form *Rana esculenta* presented similar properties (0.27–0.75 kcal mol^{-1}) and also showed activity against Gram-positive and Gram-negative bacteria [40]. Moreover, studies demonstrated that the antimicrobial activity is decreased when leucines or isoleucines are changed for charged and glycine residues [1,43].

In summary the peptides here presented showed several physico-chemical properties in common. However, the Val[6] and Val[8] residues observed in P1 and P2, respectively might be important in interaction with fungi. Several studies demonstrate that an amidated valine residue at C-termini showed lethal effects against fungi, as well as a broad spectrum of pathogenic microorganisms [7]. Other physicochemical properties seem to be determinant for antifungal activity such as total hydrophobic ratio. The peptide P1 presented hydrophobic ratio of 77% and residues with positive theoretical charge in pH 7.0 (data not shown). These results are in accordance with biochemical properties obtained from family of basic cysteine-rich plant antifungal proteins from *Brassicaceae* sp. and the antifungal protein from *Aspergillus giganteus* with 60 and 39% hydrophobicity respectively [9,41]. Furthermore, the P2, P3 and P4 peptides presented values of 47, 40 and 52% for hydrophobic ratio, with a clear reduction in comparison with P1, which could explain the reduction/and or absence of antifungal activity. Curiously P2 peptide presented activity against fungi and bacteria indicating the importance of the hydrophobicity, but showing that other physic-chemical properties must be important for activity and specificity. In this view 134 antibacterial peptides randomly evaluated here (data not shown) were obtained in antimicrobial peptides database showing that antibacterial peptides present hydrophobic mean of 46 ± 12% and for charges 4 ± 3 [46] as observed for P2, P3 and P4 indicating that hydrophobicity ratio is also important for bactericidal activity.

On the other hand, another question was drawn. Why were P3 and P4 unable to reduce fungal development? Several theories could be proposed but the presence of binding motifs (RE, DR, KE and DK) in P3 and P4 peptides could shed some light over this issue. These binding motifs are observed in various anionic antibacterial peptides that did not show antifungal activity as well as chromacin, peptide B and enkelytin from bovine and thymosin-β4 and LEK peptides family from *Homo sapiens* [20], leading us to believe that the presence of a cationic residue followed by an anionic one could be important for microorganism selection. Nevertheless studies utilizing mutant peptides could elucidate the importance of those motifs observed here.

5. Conclusions

In conclusion, our results suggest that the development of antimicrobial peptides from genomic databases is an alternative strategy to abbreviate achievement of peptides from natural resources. Even those sequences that do not show effective activity in the first instance, can still be structurally modified to obtain more efficient antimicrobial molecules. Finally, structural *in silico* studies suggested that the presence of interactive ionic binding motifs (charges 4 ± 3), in addition to leucines and isoleucines which could contribute to hydrophobic ratio (around 46 ± 12%), may increase the specific activity for bacteria, playing an important role in the interaction with bacterial membranes.

Conflict of interest

None to declare.

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