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Article

Evaluation of the Antibacterial Activity of New Dermaseptin Derivatives against *Acinetobacter baumannii*

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Abstract: Healthcare-Associated infections (HAIs) affect more than 100 million patients each year worldwide. These infections are mainly caused by multi-resistant bacterial strains (MDR) such as *Acinetobacter Baumanii* which is responsible of high mortality rate in immunocompromised individuals. However, the treatment is further aggravated by the abuse of antibiotics, the lack of vaccine and the emergence of resistance posing a huge challenge for the discovery of new therapeutic drugs to overcome this nosocomial infection. In this context, antimicrobial peptides with potential antibacterial proprieties could be an alternative. In this research, we describe the synthesis and the bioactivity of dermaseptins and their derivatives against *Acinetobacter baumanii*. The cytotoxicity of these DS was investigated on the HEp-2 cell line by the MTT cell viability assay. Thereafter, we studied morphological alterations caused by the action of one of the active peptides on the bacterial membrane using Atomic Force Microscopy (AFM). The cytotoxicity of dermaseptins was concentration-dependent at microgram concentrations. It was observed that all tested analogs exhibit antibacterial activity with MICs ranging from 3.125 to 12.5 µg/mL and MBCs ranging from 6.25 to 25 µg/mL. Microscopic images obtained by AFM revealed morphological changes on the surface of treated bacteria caused by K₄S4(1-16), as well as significant surface alterations. Overall, these findings demonstrate that DS might constitute new lead structures for the development of potent antibacterial agents against *Acinetobacter* infections.

Keywords: dermaseptin b2; dermaseptin s4; analogs; *acinetobacter baumanii*; healthcare-associated infections; antibacterial activity

1. Introduction

Healthcare-Associated infections (HAIs), also known as nosocomial infections are infections acquired within a healthcare establishment, which were not initially present or incubating at the time of patient admission. They generally manifest 48 hours or more after hospitalization [1]. HAIs can be transmitted by patient contact with healthcare personnel, patients contact, medical devices, or procedural interventions by the means of hand contact, oral transmission, parental, aerial and vector borne [2]. These infections are caused by various microorganisms, such as multidrug-resistant bacteria (MDR) and other agents responsible for their occurrence. World Health Organization (WHO) data estimates that HAIs lead to approximately 40,000 deaths per year. These nosocomial infections have rates of up to 25% in developing countries unlike developed countries which show lower values of around 5 and 15% [3,4]. HAIs represent important public health concerns requiring global attention, given their impact on morbidity and mortality rates, as well as the considerable associated cost of medical care. Additionally, Cases of nosocomial COVID-19 infections have been identified and acquired in many medical facilities around the world [5]. Most of HAIs are attributed to catheter-associated urinary tract infections (CAUTIs), hospital-acquired pneumonias (HAPs), bloodstream infections (BSIs), and surgical site infections (SSIs) [6].



Acinetobacter baumannii, a Gram-negative coccobacillus, is the predominant human pathogen in hospital settings presents considerable viability on human hands, contributing to significant cross-contamination rates in nosocomial infections due to this characteristic [7]. Nowadays, *A. baumannii* holds the highest rank on the WHO priority pathogen list as "critical," highlighting its importance as a nosocomial causative pathogen, particularly in cases where it presents resistance to carbapenem, the "last resort" antibiotic [8]. Previous study has been reported the *Acinetobacter baumannii* as a responsible agent of ocular infections. Ophthalmic complications in burn victims have been observed due to corneal injuries or an associated trauma. Acinetobacter ophtalmic infections are more common in patients requiring respiratory assistance [9]. Therefore, this multi-drug resistant bacillus is the top priority for antibiotic research and development efforts due to their ineffectiveness. The evolution of antimicrobial resistance of Acinetobacter strains to antibiotics began in 1932 [10]. These antibiotics include: beta-lactams [11], Carbapenems [12], Colistin [13], Tigecycline [14] and quinolones [15].

For years, the medical field has witnessed the emergence of new resistance mechanisms in *A. baumannii* against antimicrobial treatments, leading to a reduction in effectiveness of therapeutic interventions. Significant research has been conducted on the development of preventive vaccines. Several promising vaccine methods have been studied in preclinical trials over the past decade, showing varying degrees of success. However, to our knowledge, none of these investigated candidates has progressed beyond preclinical evaluation [16]. For these reasons, the development of new drugs for HAIs caused by *A. baumanii* is immediately required, preferably employing compounds effective in low concentrations, non-toxic and selective against this bacteria. Antimicrobial peptides, especially dermaseptins could be potential candidates for such assays. Cationic antimicrobial peptides (AMPs) have attracted considerable attention in the search of new therapeutic options. These small peptides, composed of 5 to 100 amino acid residues, present a wide range of molecular weights (<10 kDa) [17,18]. Various organisms produce these peptides through their innate immune system; they are derived from various sources such as: macrophages, neutrophils, epithelial cells, haemocytes, fat body, reproductive tract, and similar origins [19]. These PAMs are potentially active against a broad spectrum of microorganisms against bacteria, fungi, eukaryotic parasites and/or viruses [19]. The majority of these peptides are positively charged, amphipathic and act according to various mechanisms: the formation of pores in the bacterial membrane, dissolving the membrane, or targeting crucial bacterial processes like cell wall synthesis or protein production [20]. Among these antimicrobial peptides are Dermaseptins, a family of cationic AMPs initially identified in the skin of South American *Phyllomedusa* frog. These DS are secreted by amphibians' skin as part of their defense mechanism against microbes. These polycationic-amphipathic peptides are linear and generally composed of 28–34 amino acids, exhibiting significant variability between different peptides. Nevertheless, in apolar solvents, they tend to adopt an amphipathic α -helices [21]. The Dermaseptins exhibit substantial variation in both their peptide sequences and lengths. However, they share structural similarities, such as the presence of conserved Trp residue at position 3, a conserved sequence of AA(G)KAALG(N)A in the middle region ,and a net positive charge [22]. Overall, DS-S peptides isolated from the secretions of *Phyllomedusa sauvagei* have not been widely used for various human antimicrobial applications. These applications are particularly interesting due to their ability to effectively eliminate microbes without encountering resistance and their rapid mechanism for killing pathogens [23]. So far, a total of thirteen dermaseptins (DS1 to 13) have been discovered and characterized [24]. Considering the existing data regarding dermaseptins' action against bacteria, we can identify two distinct types of mechanisms: The initially proposed mechanism involves the "barrel-stave" model; it is mediated by the attachment of dermaseptins to membrane phospholipids. This interaction disrupts the osmotic balance of the cell, leading to membrane permeabilization. Subsequently, transmembrane pores or channels form, eventually causing membrane rupture. Another second mechanism, called the "carpet-like" mechanism, involves the binding of positively charged lytic peptides to the negatively charged surface. This destruction method allows complete surface coverage, leading to the impregnation and disintegration of the membrane [25].

Dermaseptins S have generated substantial interest due to their potential as a clinical approach against microbial resistance to existing antibiotics and antifungals. Certain dermaseptins exhibit an impressive ability to efficiently, rapidly, and irreversibly inhibit microbial cells, without causing toxicity on mammalian cells [23]. Besides their large spectrum of activity, DRS are not hemolytic [24], excepting for dermaseptin S4, which demonstrates a potent hemolytic effect [26]. In the same context, DRS-B2, extracted from the secretion of *Phyllomedusa bicolor* [27], is alternatively referred to as adenoregulin due to its capacity to modulate the binding of adenosine A1 receptor agonists [28]. Among the peptides of DRS family, DRS-B2 stands out for its highest level of activity, making it the subject of extensive research. This amphipathic (+3) cationic polypeptide composed of 33 amino acids, with a molecular weight of approximately 3180 Da, and a tryptophan residue at position 3, and six lysine residues [29]. This α -helical peptide has the ability to disrupt the membranes of a wide range of microorganisms, such as bacteria, yeast, fungi, and protozoa. However, its specific mechanism of action remains unclear [30]. There are reports of administration of (DRS-Bs) to humans in non-experimental settings; comprehensive data on the pharmacokinetics, efficacy, and safety in humans are currently unavailable [31].

To our knowledge, no studies have been carried out to evaluate the antibacterial effect of the native B2 and our derivatives from both dermaseptin families against *Acinetobacter baumanii*. Thus, in the current research, we report for the first time the *in vitro* antibacterial activity of these peptides against nosocomial infections. Dermaseptin derivatives were synthesized, purified and evaluated for their antibacterial activity. Their cytotoxicity towards Hep-2 cells was evaluated and then the atomic force microscopy (AFM) was performed to describe morphological changes on bacteria.

2. Materials and Methods

2.1. Synthesis, Purification, And Preparation of Peptides

Peptides were prepared by stepwise solid phase synthesis using Fmoc polyamide-active ester chemistry on a Milligen 9050 pepsynthesizer. All Fmoc-amino acids were from Milligen-Waters (France). 4-(Hydroxymethyl) phenoacetic acid-linked polyamide/kieselguhr resin (pepsin kA), Fmoc-aminoacidpentafluorophenyl (Pfp), and 3-hydroxy- 2,3-dehydro-4-oxo-benzotriazine (Dhbt) esters were from Milligen/Bioresearch. Cleavage of peptidyl-resin and side chain deprotection were carried out using 5 mg of peptidyl-resin in 1 ml of a mixture composed of trifluoroacetic acid, para-cresol, thioanisol, water, and ethyl methyl sulfide (82.5%, 5.5%, and 2.5% (v/v)) for 2 hr at room temperature. After filtering to remove the resin and ether extraction, the crude peptides were purified by a combination of Sephadex gel filtration, ion exchange chromatography, and preparative high performance liquid chromatography (HPLC). The homogeneity of synthetic peptides was assessed by analytical HPLC, amino acid analysis, solid phase sequence analysis, and mass spectrometry [21]. All peptides were stored frozen as stock solutions at 3.5 mM in double-distilled water at -20°C.

2.2. Calculation of peptide physicochemical and structural parameters

Properties of our peptides such as Length, net charge (Z) and Molecular Weights (MW) were calculated using BACHEM peptide calculator tool. Both hydrophobicity (H) and hydrophobic moment (μ H) were calculated using Heliquest software [32]. The total trend of aggregation (aggregation) in aqueous solution was predicted using TANGO software [33]. While the helicity (α -helix %) of each peptide was calculated using AGADIR software [34].

2.3. Bacterial strains and inoculum standardization

The bacterial strain used in the present study is from the stock culture of the microbiology laboratory of the Federal University of the Delta of Parnaíba - UFDPar, Parnaíba - PI. The strain used in the tests is: *Acinetobacter baumannii* clinical specimen MDR (multidrug resistant). For the study, these isolates were subjected to susceptibility test according to the standards of the Clinical Laboratory Standards Institute [35]. Before performing all experiments, the selected strains were seeded in Petri dishes containing Mueller-Hinton agar (DifcoTM), and then, under aerobic conditions,

they were incubated in a bacteriological oven for 24 hours at 35 ± 2 °C. After this time, the colonies grown alone were collected with a disposable bacteriological loop and suspended in sterile saline solution (0.85% NaCl (w/v)), in order to reach an absorbance pattern between 0.08 and 0.13, at 625 nm in UV-vis spectrophotometer (Shimadzu, Japan), thus corresponding to 0.5 McFarland scale (1 - 2 x 10⁸ CFU/mL), as recommended by CLSI. Once standardized, the bacterial suspension obtained was subsequently used to prepare the bacterial inoculum used in the execution of Minimum Inhibitory Concentration (MIC) determination protocols [35].

2.4. Antibacterial experiments

In accordance with the standards recommended by CLSI [35], the antibacterial potential of peptides was evaluated with the method of determining the Minimum Inhibitory Concentration (MIC), through broth microdilution method. Using a 96-well microdilution plate (KASVI), the antibacterial effect was analyzed against the bacterial strains. Therefore, 5 μ L of each peptide were added to the first line of the microtiter wells containing 195 μ L Mueller-Hinton broth followed by 2-fold serial dilutions with final concentrations ranging from 25 μ g/mL to 0.19 μ g/mL for all peptides. The volume of the bacterial inoculum was equal to 50 μ L, it was added to the test wells with the M-H broth at the beginning of the experience to give the final concentration of 5 x 10⁵ CFU/mL and to reach the final volume of 100 μ L in each well after discarding the last additional volume of 100 μ L following the serial dilution. MIC was defined as the lowest concentration of an antibacterial agent expressed in mg/L (μ g/mL) which, under strictly controlled in vitro conditions, completely prevents visible bacterial growth after incubation for 24 h at 37°C in aerobic conditions. The MBC is complementary to the MIC; the MBC demonstrates the lowest concentration of antimicrobial agent that inhibited growth of bacterial colonies on the agar. MBC was verified by seeding 10 μ L of the wells that showed results equal to or greater than the MIC onto Mueller-Hinton Agar (MHA), with the assistance of the Drigalski spatula. All tests were performed in triplicate. In order to guarantee the quality and safety of the protocols of this study, the manipulation of bacterial strains was performed under recommended aseptic conditions. In addition, all procedures for the execution of the experimental protocols were performed in a class II B2 biological safety cabinet (Buzattos, MG, Brazil).

2.5. Cell Culture

The HEp-2 cell line contains HeLa marker chromosomes and was derived via HeLa contamination. It was obtained from the American Type Culture Collection (ATCC, USA). Cells were routinely maintained in a humidified atmosphere of 5% CO₂ at 37 °C. The Culture Medium Dulbecco's Modified Eagle Medium (DMEM) was supplemented with 1% L-glutamine, 1% penicillin/streptomycin and 10% (v/v) heat inactivated fetal bovine serum (FBS).

2.6. MTT assay and Cytotoxicity analysis

The cytotoxicity test consists of measuring the viability of cells in culture when they are brought into contact with the peptides to be tested. Cytotoxicity is determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) colorimetric assay on cultured cells (HEp-2 lines). The lyophilized peptides are diluted in distilled water to obtain a final concentration of 1 mg/ml. 1/2 dilutions of the different peptides were then prepared in Eppendorf tubes with the fresh medium DMEM. These concentrations range from 100 μ g/ml to 1.5 μ g/ml. The HEp-2 cells in suspension (MEM medium with 10% FCS) are distributed in 96-well plates, at a rate of 100 μ l containing 10⁵ cells/well, and incubated in the oven at 37°C at 5% CO₂ for 24 hours. Subsequently, the medium is removed and replaced with 50 μ l DMEM medium with 2% FCS. 50 μ l of each peptide are added to all wells, and a 1/2 dilution series is carried out from 100 μ g/ml to a concentration of 1.5 μ g/ml. The test is carried out in triplicate and in three different times. Untreated cells will serve as a negative control. After 72 hours of incubation, the supernatant is collected and the viability of the cells treated with the peptides is determined by the MTT method. Briefly 50 μ l of the MTT solution

(5 mg/ml) are added to each well. After 4 hours of incubation at 37°C, the optical density (OD) is measured at 570 nm using an ELISA reader (Multiskan EX, Labsystems), after adding 100 µl of DMSO to be able to dissolve the crystals of formazan trained. The results are expressed as a percentage of viability relative to the negative control control without peptide tested, according to the formula: **(Viability percentage = (DO₅₄₄ nm peptide / DO₅₄₄ nm control) × 100)**. The results were expressed in percentage of viable HEp-2 cells and the half maximal cytotoxic concentrations to the HEp-2 CC50 values were calculated with GraphPad Prism® (version 9.0).

2.7. Atomic Force Microscopy (AFM)

With the objective of observing possible morphological alterations caused by the action of an active peptide from dermaseptin families, bacterial growth control and bacteria treated with K₄S4 (1-16) were evaluated, using the technique of Atomic Force Microscopy (AFM). Therefore, the selected bacterial strain, *Acinetobacter baumannii* MDR, was submitted to this experimental assay. The bacterial inoculum used to obtain the images was 5×10⁵ CFU/mL and, for sample preparation, procedures similar to those described by Araújo et al [36] were used. Briefly, after 24 hours of incubation of the MIC determination assay, 20 µL of culture medium from the wells of the treated and untreated (control) group were deposited on the surface of a glass slide. Then, the samples were submitted to drying in an oven at 37 °C for 10 minutes. After this time, the samples were then carefully washed with distilled water and subjected to drying under the same conditions described above. After this preparation, AFM images of 6 × 6 µm were obtained using the model TT-AFM microscope (AFM Workshop - USA), in intermittent contact mode (tapping mode), using TAP300-G10 tips (TED PELLA, INC.), with a resonance frequency of approximately 239 kHz. Multiple areas (n=10, 0.3 × 0.3 µm) of each sample were examined in order to verify the average roughness of treated and untreated bacteria, using the program Gwyddion 2.60.

2.8. Statistical Analysis

The statistical analyses were performed with the GraphPad Prism 9.0 software (GraphPad software Inc.) for cell viability assays. The difference between the average roughnesses (Ra) was statistically analyzed using T Test, with GraphPad Prism 8.0 software. Differences were considered as statistically significant when p < 0.05.

3. Results and Discussion

3.1. Design of dermaseptin S4 and B2 derivatives

In our study, the peptides were designed by making three main steps of structural modifications: substitutions and/or deletions to S4 and B2 sequences (Table 1). K₄K₂₀S4 is a derivative that was formed by replacing methionine (M) in position 4 and asparagine in position 20 with a lysine (K) and designating it as M4K and N20K. Peptides K₄S4(1-16) have the same M4K substitution with the deletion of 12 C-terminal residues. The K₃K₄B2 derivative was obtained by a double substitution, a tryptophan residue (W) with a lysine (K) at position 3(W3K) and a serine residue (S) with a lysine at position 4 (S4K) of B2. The one-letter code was used to identify the amino-acid sequence of these peptides, taking as reference the sequence and length of the native peptides, dermaseptins S4 and B2 (Table 1).

Table 1. Structural and physicochemical properties of dermaseptins and their derivatives.

Peptides	Sequence*	Parameters**						
		Length	MW	Net Charge	H	Aggregation	µH	α-Helix %
S4 (Native)	ALWMTLLKKVLKAAAKAALNAVLVGANA	28	2.850	+4	0.544	183.33	0.248	16.55
K ₄ K ₂₀ S4	ALWKTLKKVLKAAAKAALKAVLVGANA	28	2.861	+6	0.451	112.02	0.246	11.8
K ₄ S4(1-16)	ALWKTLKKVLKAAAK	16	1.782	+5	0.426	0	0.526	2.41
B2 (Native)	GLWSKIKEVGKEAKAAAKAAGKAALGAVSEAV	33	3.181	+3	0.199	9.681	0.204	10.02

K₃K₄B2	GLKKKIKEVGKEAAKAAAGKAALGAVSEAV	33	3.164	+5	0.072	9.681	0.159	9.85
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*The sequences are shown using the one letter code for the amino acids. **Parameters: MW (kDa); H: Hydrophobicity; Aggregation: total trend of aggregation; μ H: Hydrophobic moment; α -Helix %: Helicity.

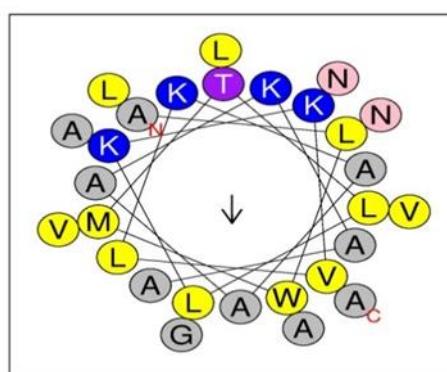
The first modification step was designed to increase hydrophilic characteristics and decrease hydrophobicity. Since S4 and B2 exhibited some levels of functional activity with cationic residues, our initial focus in modifying this peptide was to enhance its hydrophilic properties by adding basic amino acids such as lysine and the choice of lysine was also made to prevent an increase in cytotoxicity. In fact, it was previously shown that increasing the net positive charge and decreasing the hydrophobicity of dermaseptin S4 led to decreased hemolytic activity while maintaining high biological activity [26,37,38]. The S4 sequence is minimized by truncating 12 amino acids in the secondary structure. It has been reported that the use of AMPs as therapeutic agents faces challenges attributed to their long peptide sequences, limited efficacy, instability, systemic toxicities, and the potential to compromise innate defense immunity of the host. These factors have hampered the progress and clinical implementation of AMPs [39]. Several approaches have been created in the design of analogous peptides to surmount these obstacles. These methods include motif hybridization, aimed at enhancing antimicrobial efficacy and functionality [40], truncation/substitution intended to reduce toxicity [41], and de novo design, aimed at shortening peptide length and eliminating host defense immunogenicity [42]. Previous data suggested that the N-terminal domain of dermaseptin demonstrates selectivity during the interaction with the bacterial cell membrane, while the C-terminal helix mainly exhibits nonspecific membrane lytic activity [43,44]. Previous studies on N-terminal peptide fragments of dermaseptins revealed that truncated peptides ranging from 16- to 19- mer, maintain comparable antimicrobial activity. However, shorter sequences containing less than 13 amino acid residues experience a significant reduction in antimicrobial activity [45,46]. In summary, the criteria selection of peptides was examined as a means of defining the structural requirements for biological activity. Regarding B2, to the best of our knowledge it's the first time that the derivative K₃K₄B2 is used. Indeed, modifications made to the native B2 molecule primarily involved truncation, such as the design of a C-terminal truncated analog known as [1-23]-Drs B2. Despite maintaining the net cationic charge of the native peptide B2, this truncated analog was found to be inactive against bacteria [30]. Combining DRS-B2 with alginate nanoparticles (Alg NPs) results in a formulation (Alg NPs + DRS-B2) that creates novel B2 derivatives displaying significant antibacterial efficacy against both sensitive and resistant strains of *Escherichia coli* and *E. coli* to colistin. The antibacterial activity achieved with this innovative formulation exceeds that of DRS-B2 when used alone [29]. Moreover, our synthesized peptides offer the advantage of being configured with D-amino acids. Unlike, peptides in their natural form consist of L-amino acids, which pose a challenge due to their susceptibility to degradation by proteases, thereby limiting their clinical applications owing to poor stability [47]. As previous research pointed out, a feasible approach to address these constraints involves replacing the L-amino acids at the most susceptible site with D-amino acids [48]. Obviously, substituting with D-amino acids does not alter the net positive charge of the native peptide. However, it does impact the structure and function associated with recognizing chiral targets [49, 50].

3.2. Structural and physicochemical properties of peptides

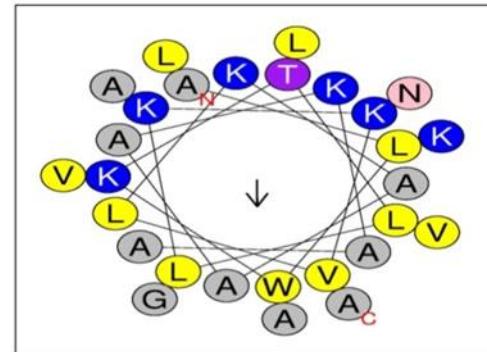
According to Heliquest, we noted that K₄K₂₀S4 peptide had the highest value of Hydrophobicity (H) among S4 peptide derivatives (0.451), whereas K₄S4(1-16) had the lowest value (0.426) (Table 1). In general, all peptide derivatives of dermaseptin S4 had a lower H than the native peptide (S4). This is also confirmed for dermaseptin B2 and its derivative, where the H value is higher (0.199) than K₃K₄B2 value (0.072). This hydrophobicity is a crucial physicochemical characteristic of peptides. Typically, it is determined by analyzing the peptide's sequence [51]. It plays an important role on their conformational modifications, stability and their molecular interactions [52]. The μ H ranged from 0.159 to 0.526, where K₄S4(1-16) showed the highest value (μ H = 0.526) and K₃K₄B2 showed the lowest (μ H = 0.159) (Table 1). The hydrophobic moment μ H is established through the calculation of

the vector sum of hydrophobicity values for each individual amino acid [32,53]. This factor significantly influences the interfacial binding of peptides with the membrane [54]. Based on the TANGO algorithm [33], we noticed that dermaseptin S4 presents a greater tendency to aggregation than its derivatives due to the presence of two N-terminal and C-terminal hydrophobic domains (Table 1, Figure 1). These results agreed with Feder et al, who demonstrated that aggregation in dermaseptin S4 and its derivatives occurs through hydrophobic interactions. Indeed, the M₄KN₂₀K substitution in the N-terminal and C-terminal domains induced a decrease in the aggregation tendency of K₄S4, which was in accordance with other studies [26,55]. However, the deletion of C-terminal domain of S4 and the insertion of positive charges in these regions result in a loss of the aggregation. K₄(1-16)S4 had a value of aggregation equal to zero (Table 1). Hence, the deletion of the hydrophobic domains and/or the insertion of positive charges in these regions will probably decrease the aggregation, either due to the absence of such hydrophobic domains or by electrostatic repulsion between cationic residues [26,31]. These results revealed that the aggregation of peptides is influenced not only by their hydrophobicity, but also by their charge distribution. Thus, the introduction of cationic amino acids into one of the hydrophobic domains, and/or the elimination of hydrophobic domains, adversely affects aggregation. Regarding dermaseptin B2, we noticed that neither substitution nor deletion induce a loss or a decrease of aggregation since the native molecule had a very low degree of aggregation (9.681) comparing to S4 (Table 1). Previously, it has been demonstrated that the tendency to aggregation in aqueous solution is another important parameter for antibacterial activity and cell selectivity [56]. This property can be explained by the ability of peptides to form aggregates (oligomers) in aqueous solution, establishing hydrophobic interactions with other monomers. Due to the increase in size and the loss of flexibility, such aggregates are unable to pass through polysaccharide capsules, outer membranes and bacterial cell walls, being unable to interact with the plasma membrane, which is the action target. Consequently, peptides with a high tendency for aggregation are weakly active against bacteria; on the other hand, for a monomeric peptide, it is easier to reach the plasma membrane and present an effective antibacterial activity [57]. Therefore, in designing antimicrobial peptides, it is usually desirable to reduce aggregation to favor antibacterial activity [38,57]. In our study, we noticed that all our peptides' derivatives have a reduced aggregation, which provide them a potential biological activity such as antileishmanial activity.

To estimate the helicity of the studied peptides, we used the AGADIR algorithm [34] which indicated that the native molecule S4 has the greater a helicity (as α -helix %) approximately eightfold greater than K₄S4(1-16), and similar to K₄K₂₀S4. However, DS-B2 and its derivative had the same helicity (10.2 % and 9.85 % respectively) (Table 1). It has been reported that helicity was assessed to investigate the relationships of secondary structure and the selectivity against microbial cells of α -helical antimicrobial peptides [58]. Therefore, the high potency of the dermaseptins may be attributed not only to a higher net positive charge, but also to the stabilization of the helical conformation [59].



(a) S4

(b) K₄K₂₀S4

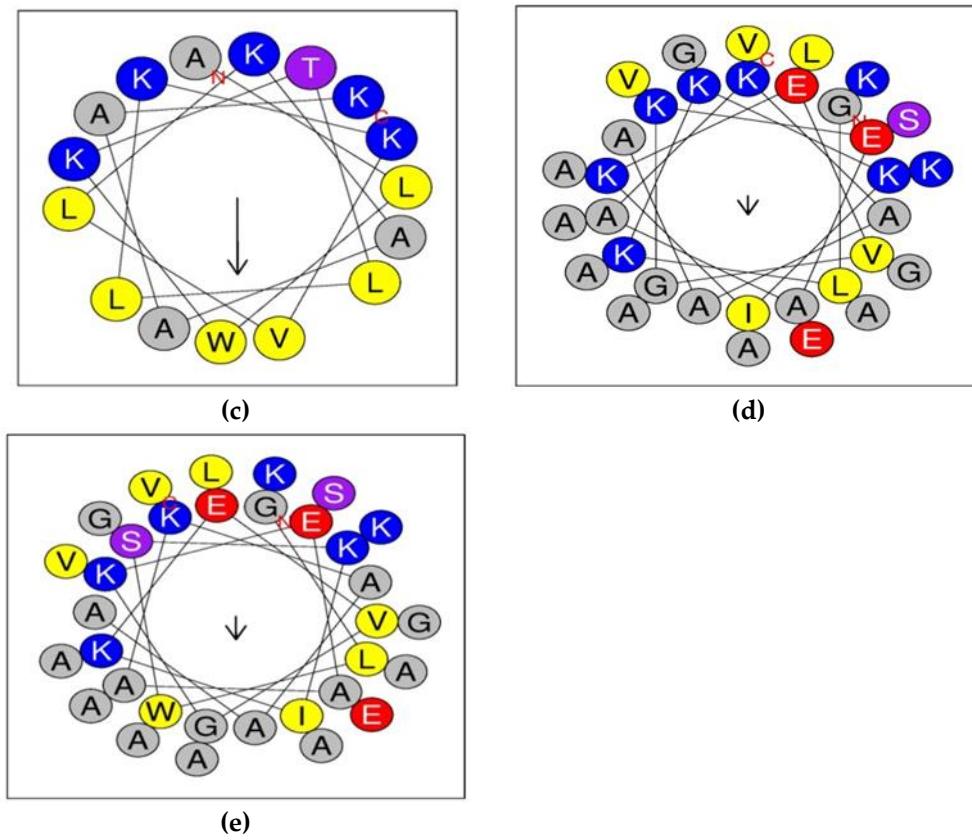


Figure 1. Helical structure of dermaseptines and their derivatives. These peptides were represented as the 2-dimensional axial projection of an ideal α -helix. A) Helical structure of the native S4; B) Helix structure of K₄K₂₀S4; C) Helical structure of K₄S4(1-16); D) Helical structure of K₃K₄ B2; E) Helical structure of the native B2. The figures use the one-letter code for amino acids. Figure built using Heliquest software (Gautier et al, 2008).

3.3. *In vitro* toxicity of dermaseptin and derivatives against HEp-2 Cells

Considering that the respiratory tract is the most commonly linked site for *A. baumannii* infection [60]. HEp-2 cells originating from the respiratory tract exhibit higher susceptibility to *A. baumannii* cell invasion compared to HeLa cells that were not derived from the respiratory tract [61]. This cell line contains HeLa marker chromosomes and was derived via HeLa contamination. Thus, all our peptides were evaluated for their cytotoxicity on HEp-2 cells using the MTT viability assay and the results are shown as survival rates after 72 h of treatment with the compounds (Table 2).

Table 2. Antimicrobial activities and dose-dependent effects of different dermaseptins and their derivatives.

Peptides	CC ₅₀ Hep-2 cells (μ g/ml)	Acinetobacter <i>baumannii</i> MIC (μ g/ml)	Acinetobacter <i>baumannii</i> MBC (μ g/ml)
S4	16.51	12.5	25
K ₄ S4(1-16)	68.9	6.25	12.5
K ₄ K ₂₀ S4	75.71	3.125	6.25
B2	30.4	12.5	25
K ₃ K ₄ B2	61.25	6.25	12.5
meropenem	ND	32	64

CC₅₀: peptide concentration that causes 50% cytotoxicity in HEp-2 cells for dermaseptin S4 and derivatives (μ g/ml); ND, not determined; MIC: Minimum Inhibitory Concentration (μ g/ml); MBC: Minimal Bactericidal Concentration (μ g/ml).

Considering our results, the tested cationic derivatives exhibit variable toxicity towards Hep-2 cells. Cells were exposed to increasing concentrations of peptides ranging from 1.5 to 100 μ g/ml. The cytotoxicity of dermaseptins was concentration dependent (data not shown), and peptide 50% cytotoxic concentration (CC₅₀) values were determined (Table 2). The highest cytotoxicity values for all S4 and B2 derivatives were recorded at concentrations higher than 61.25 μ g/ml (CC₅₀). Interestingly, results showed that shortening the peptide in the C-terminal extremity of K₄S4(1-16) which present an CC₅₀ equal to 68.9 μ g/ml; and increasing its positive charge with different substitutions (K₄K₂₀S4 or K₃K₄B2) led to peptides with low toxicity, with CC₅₀ of about: 75.71 μ g/ml and 61.25 μ g/ml respectively. Belaid et al demonstrated that maximal non-cytotoxic concentrations were 32 μ g/ml for dermaseptin S1, S2; 16 μ g/ml for dermaseptin S3 and S4, and 64 μ g/ml for dermaseptin S5 to HEp-2 cells [62]. Besides, the study of Gourkhede et al indicate that other AMPs like Cecropin A (1-7)-Melittin and lactoferricin (17-30)a exhibited negligible cytotoxicity at lower concentrations (1X and 2X MIC); however, at 4X MIC, slightly higher cytotoxicity was observed, given that MIC values are equal to 64 μ g/ml and 128 μ g/ml respectively [63]. Sruthy et al confirm that at the highest concentration tested of histone H2A-derived antimicrobial peptide (200 μ M), growth inhibition of 89% was observed in HEp2 cell lines [64]. Another study conducted by Hazime et al. tested the cytotoxic effects of DRS-B2, and also that of the new formulation (Alg NPs + DRS-B2) on the human erythrocytes and eukaryotic cell line types HT29 (human) and IPEC-1 (animal) and their safety have been verified [29]. Zairi et al. found that dermaseptin K₄S4 exhibited an enhanced toxicity profile, against human endometrial epithelial cells displaying lower sensitivity to dermaseptins' toxic effects compared to other cell types [65]. While, dermaseptin S4 and its derivatives demonstrate significant cytotoxicity against SW620 cell line, it remains challenging to discern their cellular selectivity or mode of action [66]. Moreover, Lorin et al demonstrated that dermaseptin K₄S4(1-16)a exhibited comparable effect on both HeLa P4-CCR5 cells and primary PBMCs, showed no toxicity in mice, and revealed its reduced cell toxicity at high concentrations [67]. All this work has shown that synthesized and modified peptides are less toxic to HEp-2 cells compared to the native molecules S4 and B2.

3.4. Antibacterial activity of dermaseptins derivatives against *Acinetobacter baumannii*

The susceptibility of bacteria to dermaseptin S4 and B2 derivatives was assessed by measuring the peptide Minimum Inhibitory Concentration (MICs) against the clinical isolate: *Acinetobacter baumannii*, the multidrug-resistant gram-negative bacteria. The resulted data are summarized in (Table 2). The data shows that all peptides tested inhibited *A. baumannii* growth was dependent on the nature of the peptide and the highly charged molecules were the most active. The MICs ranged from 3.125-12.5 μ g/ml. Thereby, demonstrating the potential of these peptides as antibacterial agents. Thus, in Table 2 we can notice that the most effective peptide is K₄K₂₀S4, this peptide displayed potent antibacterial activity with MICs of about 3.125 μ g/ml. Likewise, The mono-substituted truncate peptide K₄S4(1-16) present a nearly homogenous potency with a MICs value equal to 6.25 μ g/ml. The truncation of the C-terminal extremity results in a less toxic peptide and did not affect its potency. As Kustanovich suggested, the interaction with cell membrane of N-terminal domain mainly depends on the net charge state while the C-terminal domain also contributes to the binding affinity [38]. The native dermaseptin S4 has a MICs of 12.5 μ g/ml and was the less potent against *A. baumannii*, but S4 presents the same MICs value as the native B2. The analog K₃K₄B2 is likewise more active compared to the native B2, the peptide exhibits a MICs equal to 6.25 μ g/ml (same value as K₄S4(1-16)). The increase of the positive charge for the derivatives of both of dermaseptin S and B is essential to increase their antibacterial activities. Therefore, K₄K₂₀S4 and K₃K₄B2 are the most active analogs against *Acinetobacter baumannii* since they have the lowest MICs values. In the same context, our result showed that native molecules such as B2 and S4 remain less actives comparing to their cationic analogs.

After determining the MIC, the Minimal Bactericidal Concentration (MBC) was assessed against *A. baumannii* MDR. Meropenem was used as antibacterial control drug. The solvent saline loaded served as negative control. However, meropenem which is used as a reference presented an MIC of

32 $\mu\text{g}/\text{ml}$ and an MBC value of 64 $\mu\text{g}/\text{ml}$. Meropenem, belonging to the carbapenem class of beta-lactam antibiotics, exhibits a broad spectrum of activity and minimal toxicity. This antimicrobial agent provides effective coverage against various microorganisms, making it a valuable and frequently prescribed treatment for managing severe and nosocomial infections [68]. Meropenem targets Gram-positive and Gram-negative bacteria as well as anaerobic bacteria. Similar to other carbapenems, meropenem disrupts the synthesis of bacterial cell walls, inhibiting growth and leading to cell death [69]. Inadequate concentrations of meropenem may result in treatment failure and heighten the risk of microbial resistance emergence [70].

MBC showed us results equal to or greater than the MIC values, values ranging from 6.25 $\mu\text{g}/\text{ml}$ to 25 $\mu\text{g}/\text{ml}$ for both dermaseptins derivatives. The highest MBC found (25 $\mu\text{g}/\text{ml}$) present the native molecules S4 and B2 values. The lowest MBC (6.25) was for K₄K₂₀S4. For K₄S4(1-16) and K₃K₄B2, MBC is equal to 12.5 $\mu\text{g}/\text{ml}$. However, it has been reported in a previous study of Jiang et al, that some dermaseptins S4 and Piscidin 1 and their derivatives (piscidin 1 (I9K) and dermaseptin S4 (L7K, A14K)) were active against the gram-negative pathogen *A. baumannii* (11 strains) [71]. In the case of D-dermaseptin S4, the geometric mean of MIC values for *A. baumannii* decreased from 1.8 μM for D-dermaseptin S4 to 1.1 μM for D-dermaseptin S4 L7K, A14K indicating a small improvement in antimicrobial activity [71]. D-dermaseptin S4 L7K, A14K, the most selective peptide of dermaseptin S4 analogs. For *A. baumannii*, the therapeutic index improved by 730-fold from 0.3 for native D-dermaseptin S4 to 219 for this analog [71]. To the best of our knowledge, it's the first time that the antibacterial activity of dermaseptin B2 and its derivative against *Acinetobacter baumannii* was evaluated. Previous study demonstrated that DRS-B1 and S1 exhibit *in vitro* activity against both gram-positive and gram-negative bacteria, demonstrating varied specificities [72]. Furthermore, The literature shows that derivatives of DRS-S4, DRS-CA1, DRS-DU1 and DRS-PH present *in vitro* activity against *Staphylococcus aureus* (including the methicillin resistant strain), *Pseudomonas aeruginosa* and *E. coli*, even when they are formed in biofilm [45,73-75]. Due to Zairi et al, DRS-S4 derivatives have been shown to be less cytotoxic than conventional antibiotics [73].

Understanding the peptide-membrane interaction and resistance mechanisms of *Acinetobacter baumannii* is crucial for the development of new antimicrobial agents or other alternative tools to combat this public health challenge. The mechanisms of drug resistance can be broadly categorized into several groups, such as drug inactivation or alteration, modification of drug binding sites or targets, alterations in cell permeability leading to decreased intracellular drug accumulation, and the formation of biofilms [76]. AMPs are known for their primary interaction with the bacterial cytoplasmic membrane, affecting both membrane integrity and electrical potential [77]. The Gram-negative bacteria consist of two layers of membranes; at the moment of interaction AMPs must first penetrate the outer membrane before exerting their effects on the cytoplasmic membrane or further acting on bacteria [78]. While interacting with the membrane, AMPs might additionally influence the trans-membrane voltage, referred to as the membrane potential, which typically regulates ATP synthesis, membrane transport, and cell division [78]. On the other hand, Jiang et al suggest that AMPs interact with the negatively charged prokaryotic cell membranes employs a detergent-like mechanism (also known as the carpet mechanism [79], where antimicrobial activity does not necessitate trans-membrane insertion [71]. the cationic peptides can align parallel to the bacterial membrane surface, where the positively charged residues on the polar face interact with the negatively charged phospholipid headgroups of the bilayer and the ϵ -amino group of the Lys side chain of the "specificity determinant(s)" can be long enough to prevent hydrophobicity of the bilayer when lying parallel to the membrane surface, even if they are on the nonpolar face of the AMP. These peptides conserve their ability to disrupt the lipid bilayer, leading to cytoplasmic leakage and cell death [71].

Our study aimed also at improving the antibacterial activity of our peptides and has focused on the morphological alterations due to the interaction of our modified peptide K₄S4(1-16) to the outer membrane of *Acinetobacter baumannii*.

3.5. The morphological effect of K₄S4(1-16) on the treated bacteria

In order to understand how our cationic peptide eliminates bacteria, we must consider its effect on the outer membrane found in Gram-negative bacteria. Here, we use atomic force microscopy (AFM) to directly investigate K₄S4(1-16) interaction with the outer membrane of *Acinetobacter baumannii* and characterize the biophysical consequences of K₄S4(1-16) treatment. However, Gram-negative bacteria are complex organisms with two membranes and molecular machinery dedicated to maintaining membrane stability. The images show the morphology of the bacteria before and after treatment of the clinical strain of *Acinetobacter* MDR with the derivative. AFM provides high resolution images, AFM imaging reveals that the membrane becomes pitted, more flexible and more adhesive after K₄S4(1-16) treatment (Figure 2), it shows also changes in the appearance in the cell envelope of treated bacteria, the changes become more pronounced including more variable cell shape with shrinkage and membrane disruption. The cell differs considerably from the coccobacilli-shaped cell observed without peptide treatment. Therefore, this cationic peptide appears to have a highly disruptive effect. This interaction causes changes in elasticity and adhesion as well as increased roughness on the cell surface after treatment, from 25.77 nm to 43.41 nm, compared to the smooth surface of the non-treated bacteria (Figure 3).

These changes observed during AFM may be caused by a disruption of membrane integrity, which could lead in cell osmolarity without the occurrence of lysis in the observed images [36]. Few studies have focused on the membrane changes observed in treated *A. baumannii*. Eales et al demonstrated that atomic force microscopy (AFM) revealed significant alterations in both the size and surface conformity of *A. baumannii* cells when treated with different peptide concentrations (Bicarinalin and BP100) equal to or surpassing the minimum bactericidal concentration (MBC) [80].

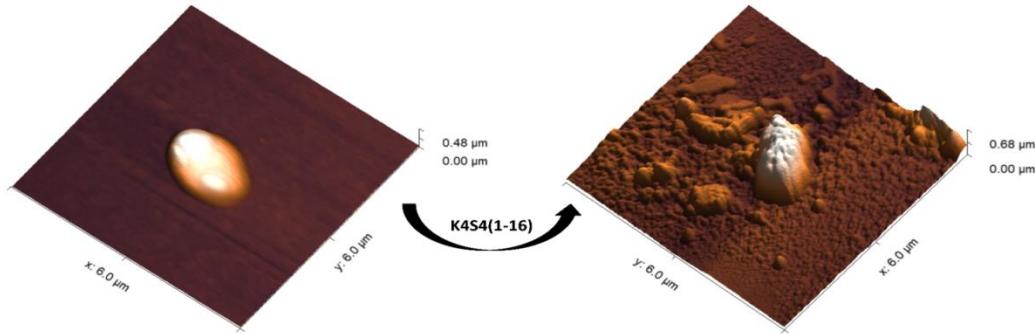


Figure 2. AFM images of *Acinetobacter baumannii* before and after treatment with K₄S4 (1-16) peptide.

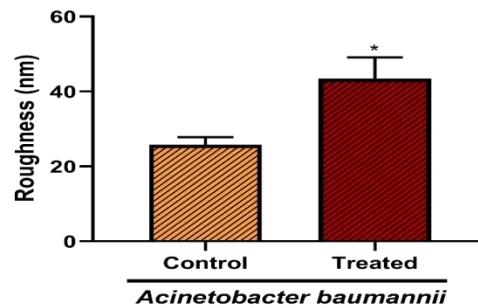


Figure 3. Average roughness of *Acinetobacter baumannii* before (control) and after treatment with K₄S4 peptide (1-16) by AFM technique.

4. Conclusion

In conclusion, the present findings showed that our dermaseptines from the family of S4 and B2 have significant and selective antibacterial effects against *Acinetobacter baumannii*. This study also revealed that the cytotoxicity of these modified peptides was concentration dependent. We conclude that the bi-substituted peptide, K₄K₂₀S4, which has the highest CC₅₀ (75.71 μg/ml), the highest net

positive charge (+6) and the lower values of MIC and MBC (3.125 µg/ml and 6.25 µg/ml respectively), is the best candidate against antibacterial activity. Collectively, these small molecules may have the potential for being safe antibacterial compounds. Furthermore, AFM images obtained revealed morphological changes and alterations as well as increased roughness.

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Abbreviations

HAIs : Healthcare-Associated infections
MDR: Multi-resistant bacterial strains
MTT: (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)
WHO: World Health Organization
CAUTIs: Catheter-associated urinary tract infections
HAPs: Hospital-acquired pneumonias
BSIs: Bloodstream infections
SSIs: Surgical site infections
AMPs: Antimicrobial peptides
DS: Dermaseptin
AFM: Atomic force microscopy
Pfp: Fmoc-aminoacidpentafluorophenyl
Dhbt: 3-hydroxy- 2,3-dehydro-4-oxo-benzotriazine
HPLC: High performance liquid chromatography
MHA: Mueller Hinton Agar
DMEM: Modified Eagle Medium
FBS: Fetal Bovine Serum
Alg NPs: Alginate nanoparticles

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