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Antimicrobial activity of synthetic Dq-3162, a 28-residue ponericin G-like dinoponeratoxin from the giant ant *Dinoponera quadriceps* venom, against carbapenem-resistant bacteria

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ABSTRACT

The predatory giant ant *Dinoponera quadriceps* is one of the largest venomous ants on Earth. The venom of *D. quadriceps* comprises a rich blend of bioactive peptides that includes structures related to at least five classes of antimicrobial peptides. In the present study, two representative synthetic peptides, sDq-2562 and sDq-3162, belonging to the ponericin-like dinoponeratoxin family, were evaluated for their microbicide activity against antibiotic-resistant bacteria. The most effective peptide, the 28-residue sDq-3162 displayed a significant bacteriostatic and bactericidal effect with minimal inhibitory concentrations (MICs) between 5 μM and 10 μM (15.6 μg mL^{-1} and 31.2 μg mL^{-1}), according to the strain of drug-resistant bacteria tested. In combination with conventional antibiotics, sDq-3162 displayed *in vitro* synergistic effects, reducing the MICs of antibiotics for more than 2-log against clinical isolates of carbapenem-resistant *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, with low cytotoxicity to human erythrocytes, *in vitro*. Since the development of molecules to circumvent the spread of antibiotic-resistant bacteria is demanding, ant venom peptides arise as useful molecular resources to contribute with the antimicrobial arsenal and therapeutic strategies to fight clinically relevant microbial infections.

1. Introduction

In recent decades, bacterial resistance to antimicrobials has raised a growing medical problem worldwide. The emergence of multidrug resistant microbes as result of misuse and overuse of antibiotics, as well as the lack of new effective antimicrobials have culminated with the present-day antibiotic crisis (Ventola, 2015). Faced with this emerging global concern, researchers have investigated numerous natural products from plants and animals for novel antibiotic substances (Moloney, 2016). Among these natural products, antimicrobial peptides (AMPs) arise as promising candidates for adjuvant antibiotic therapies, due to their biological, mechanistic and physiochemical properties (Chen and Lu, 2020). AMPs are gene-encoded products related to the innate immune system of organisms from all kingdoms, which are expressed constitutively or inductively in response to invading pathogens. In general, AMPs are variable in length and structures, prevailing short sequences (<50 amino acids), mostly cationic and amphipathic, which kill microbial pathogens by distinct mechanisms but usually involving initial plasma membrane disruption (Brogden, 2005). Some AMPs are able to enter into the cells and bind to intracellular targets such as nucleic acids and cellular organelles, and, consequently, potentialize the antimicrobial effect by halting DNA and protein metabolism, and inducing cell death by apoptosis in certain cases (Andrea et al., 2007; Brogden, 2005; Lee and Lee, 2015). Other AMPs are able to connect the innate and adaptative immunity by functioning as immunomodulators (Choi et al., 2012). In reason of the rapid membrane-disrupting effect of this class of bioactive peptides, AMPs are considered advantageous in the present and future drug development of antibiotic therapy. Moreover, they have broad-spectrum of antimicrobial activity and impair or diminish the chance of emergence of drug-resistant microbes compared to conventional antibiotics (Gordon et al., 2005; Li et al., 2012).

Venoms of native animals and their components have been prospected and used for therapeutic purposes in folk medicine (Utkin, 2015), with some examples of venom-derived peptides and templates

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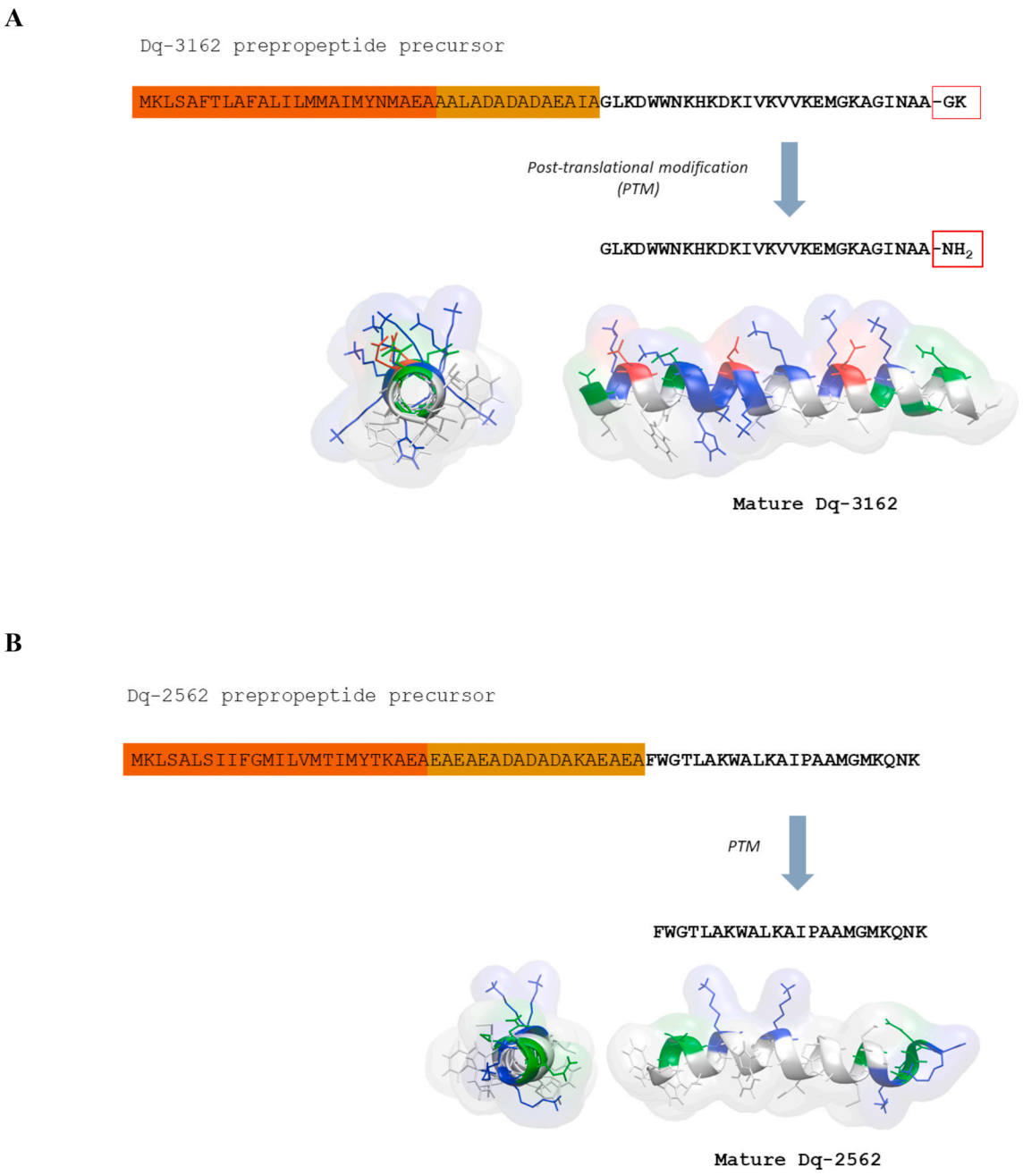
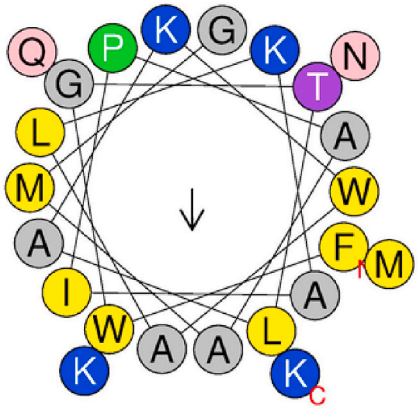
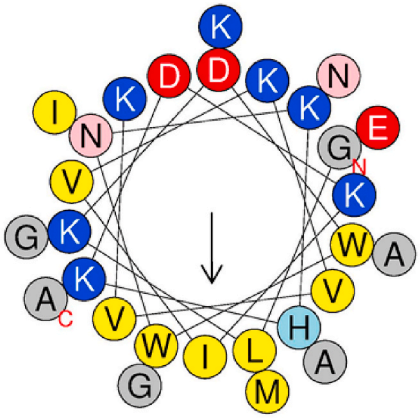


Fig. 1. The precursors and structures of Dq-2562 and Dq-3162, dinoponeratoxins from the giant ant *D. quadriceps* venom. Both *D. quadriceps* mature peptides are products of post-translational modification of longer prepropeptide precursors. (A) Dq-3162 precursor (Genbank accession number P0DSK2) gives rise to an amidated peptide. (B) Dq-2562 precursor (P0DSK0) originates not only mature Dq-2562, by proteolytic cleavage, but also other smaller peptides (not shown). In both peptide precursors, the signal peptide is colored in ruby red and the pro-piece in brick red. The secondary structures were predicted with the PEP-FOLD 2 server (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/) and they are seen along the axis (left) and in lateral view (right) from the N- to C-terminal. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

actually in current use in clinics or under development (Herzig et al., 2020; Pennington et al., 2018)). The venoms, as presently and supposedly known, are produced by animals with the aim of prey capture and defense against competitors or predators and, importantly, they comprise rich sources of biologically and pharmacologically active peptides useful in human pharmacotherapy (King, 2011; Pennington et al., 2018). Thus, venom peptides have been under research focus for discovery and development of compounds of diverse pharmacological classes, including antibiotics; these later as alternatives to the current therapeutic options for anti-infective agents (Andrea et al., 2007; Fratini et al., 2017; Garcia et al., 2013; Rádis-Baptista, 2017).

The venom from ants have been investigated in molecular details in several studies by numerous research groups and the diversity of molecules in the ant venoms has been recapitulated (Aili et al., 2014; Hoffman, 2010). The diversity and structural richness of venom peptides and proteins particularly from ants and arthropods, in general, have been harnessed for application in a range of biomedical fields (Radis-Baptista and Konno, 2020). Overall, the venoms from numerous species of ants consist of complex mixtures of polypeptide toxins (peptides and proteins) and several classes of low molecular weight substances, including sugars, formic acid, biogenic amines, alkaloids and free amino acids (Touchard et al., 2016). Recently, the analysis and description of

Table 1
Structures and physicochemical characteristics of the dinoponeratoxins sDq-2562 and sDq-3162.

Peptide (Synthetic)	Dq-2562 (sDq-2562)	Dq-3162 (sDq-3162)
Systematic name	M-PONTX-Dq3a	M-PONTX-Dq4f
Family	Ponericin W-like	Ponericin G-like
Sequence[a]	FWGTLAKWALKAIPAAMGMKQNK	GLKDWWNKHKDKIVKVVKEMGKAGINAA-NH_2
Nr of residues	23	28
Exp. mol. mass	2561.4	3162.7
pI[b]	11.2	10.8
Net charge[b]	+4.0	+5.1
H[c]	0.509	0.194
μ_H[c]	0.248	0.485
Helical wheel projection[c]		
Hydrophobic face[c]	IAML	LMIWGV

[a] C-terminal amidated (-NH_2) peptide.
[b] Calculated with the "Peptide property calculator" software (http://pepcalc.com).
[c] Calculated with the "Heliquest" software (http://heliquest.ipmc.cnrs.fr/). μ_H, hydrophobic moment; H, hydrophobicity. Hydrophobic residues are labeled in yellow, in the helical wheel plot. N- and C-terminal residues are indicated (adapted from Rádis-Baptista et al., 2020).

the constituents from the venom of giant ant *Dinoponera quadriceps* (order Hymenoptera, family Formicidae) have been published in a series of sequential reports by some of us: the complete analysis of the venom transcriptome uncovered the main components expressed in the venom gland, which include allergens, hydrolytic enzymes (e.g., phospholipases and carboxylesterases), lethal-like toxins and small-size dinoponeratoxins (Torres et al., 2014); the subsequent proteomic study of the polypeptide components of the *D. quadriceps* venom, which corroborated the transcriptomic content and identified nine high molecular weight toxins, such as enzymes, allergens, cytolytic polypeptides and accessory proteins, that together contribute presumably to venom spread, disruption of cell membranes and damage tissues (Ceolin Mariano et al., 2019); the recent description of the low molecular weight composition of the *D. quadriceps* venom, as revealed by the presence of biogenic amines, free amino acids and nucleosides, and bioactive peptides with sequences ranging from 4 to 28 amino acid residues (Radis-Baptista et al., 2020). Among these major *D. quadriceps* venom components, less abundant peptides were disclosed, such as ICK-like and Kunitz-type peptides, in addition to five known classes of AMPs (e.g., dermaseptin-, defensin-, pilosulin-, ponericin- and temporin-like types). The functional of most of these giant ant venom toxins deserves detailed studies, but some dinoponeratoxin variants, with structures similar to ponericins, were showed to possess broad spectrum of activities, including antimicrobial, anti-parasite and histamine-releasing properties (Lima et al., 2018; Radis-Baptista et al., 2020). The activities of dinopoenratoxins were also characterized from the venom of other populations of *D. quadriceps* and distinct species of *Dinoponera*, namely *D. australis* (Cologna et al., 2013; Johnson et al., 2010). Dinoponeratoxins are linear, ponericin-related peptides, ranging in length from 11- to 28- amino acid residues, which result from the post-translational cleavage of longer transcript precursors (Torres et al., 2014). As for conceived for other animal venoms and their components, despite the deleterious effects that insect venoms and toxins might cause in prey or victims, the potential beneficial pharmacological properties of *D. quadriceps* crude venom and components have been evidenced in a number of studies. For instance, with regard to *D. quadriceps* crude venom and venom fractions, the neuroprotective and neurotoxic effects (Lopes et al., 2013), the antibacterial activity against *Staphylococcus aureus* (Lima et al., 2014), the pro- and anti-convulsant effects in mice model (Noga et al., 2015, 2016), the anti-inflammatory, anticoagulant and antiplatelet activities (Madeira et al., 2015) and the anti-parasite effect (Lima et al., 2016) have been reported. The antibacterial, antifungal and trypanocidal activities of *D. quadriceps* venom components were reported for natural and synthetic dinoponeratoxin peptides (Cologna et al., 2013; Dodou Lima et al., 2020; Lima et al., 2018).

Such a body of evidence concerning to the molecular and structural diversity and functionalities of the venom from ants, in general, and *D. quadriceps* giant ant, in particular, incites continuous investigation. The present study aimed at assessing the antimicrobial activity of two synthetic dinoponeratoxins, sDq-2562 and sDq-3162, which are identical to native peptides found in the crude venom, against multi-drug resistant bacteria of Enterobacteriaceae family. The microbicide effect was evaluated on clinical isolates of multi-drug, carbapenem-resistant *Acinetobacter baumannii*, *Klebsiella pneumoniae, Escherichia coli* and *Pseudomonas aeruginosa*, which are listed in the critical priority directives by the World Health Organization (WHO). Notably, the ponericin-like, sDq-3162 peptide demonstrated an important bactericidal activity against these life-threatening carbapenem-resistant Enterobacteriaceae either individually or in additive and synergistic combinations with conventional antibiotics.

2. Materials and methods

2.1. Peptides

Synthetic peptides, sDq-2562 and sDq-3162, which are identical to

native dinoponeratoxin sequences expressed in the crude venom, were commercially prepared by solid phase synthesis (GenScript Biotech Corp, Piscataway, NJ, USA), purified by HPLC and characterized with analysis by mass spectrometry, as previously described (Radis-Baptista et al., 2020). The origin, structural and physicochemical characteristics of native and synthetic dinoponeratoxins are summarized, respectively, in Fig. 1 and Table 1. The lyophilized peptides were separately weighed and solubilized in sterile deionized water to obtain a stock solution at 1 mM, which was stored in aliquots at - 20 °C until required.

2.2. Antibacterial activity

2.2.1. Bacteria

The clinical, multi-drug resistant bacterial strains of Enterobacteriaceae family, i.e., *Acinetobacter baumannii* (strain AB1), *Klebsiella pneumoniae (KP1)*, *Escherichia coli* (EC16, EC18 and EC23) and *Pseudomonas aeruginosa* (PA14 and PA19) used in this study were from the Hospital Santa Casa de Misericórdia de Sobral (Ceará, Brazil) and by LabLuz Laboratory (Fortaleza, Ceará, Brazil). The bacteria isolates were identified with CHROMagar (CHROMagar Company, Paris, France) and the automated system VITEK® 2 (BioMérieux, France) and/or BD Phoenix (New Jersey, USA). The antibiotic sensitivity and resistance of these bacteria strains were determined by testing several conventional antibiotics and carbapenems, according to the microdilution method in culture broth, as recommended by the Clinical and Laboratory Standard Institute, CLSI (Clinical and Laboratory Standards Institute, 2018). The summary of the bacteria strains and their antibiotic profiles of resistance and sensitivity is shown in Supplementary Table 1.

2.2.2. Determination of minimum inhibitory concentration and minimum lethal concentration of sDq-2562 and sDq-3162

The minimum inhibitory concentration (MIC) of the sDq-2562 and sDq-3162 peptides were determined by means of the microdilution method in culture broth, as recommended by the CLSI (Clinical and Laboratory Standards Institute, 2018). Firstly, to standardize the microbial inoculum, bacteria strains were initially cultured in Brain Heart Infusion (BHI) broth at 37 °C, for 24 h. Aliquots of this inoculum were transferred to test tubes with sterile 0.85% saline to obtain a cell density equivalent to 0.5 McFarland standard (10^8 colony forming unit, CFU mL^{-1}). Then, the standardize microbial inoculum was further diluted to obtain a final bacterial suspension of 10^6 CFU mL^{-1} that was used in the determination of antimicrobial activity. Following, in 96-well, flat-bottom sterile and capped microplates, the peptides diluted in 100 μL BHI broth (initial concentrations ranging from 0.9 μM to 20 μM) were combined with 100 μL of the standardized microbial inoculum (10^6 CFU mL^{-1}). Controls of the medium sterility (culture medium only) and of bacterial growth (bacteria suspension in culture medium) were included in the MIC determination. The MICs of conventional antibiotics, namely meropenem, imipenem, gentamicin, hygromycin, kanamycin, tetracycline, chloramphenicol, ampicillin and novobiocin (Sigma-Aldrich, St. Louis, MO, USA) were also determined to certify the drug-resistance phenotype of the clinical strains. The microplates were incubated at 37 °C, for 24 h and the bacterial growth or its absence was inspected by eyes and the results scored. The MIC was considered as the lowest concentration of the peptide and/or antibiotics capable of inhibiting the bacterial growth, as evidenced by the absence of turbidity (microbial growth).

To determine the minimum lethal concentration (MLC), aliquots of 5 μL from each individual well in the MLC determination that did not show visible bacterial growth were plated on plate count agar and incubated at 37 °C, for 24 h. Colonies that grown on the agar surface was enumerated and the MLC was considered the lowest concentration of the peptide causing at least 99.9% of cell death in relation to the initial inoculum (Shanholtzer et al., 1984; Taylor et al., 1983). The peptide sDq-3162 that showed an effective antimicrobial activity against bacteria strains that were resistant to a minimal of two carbapenem antibiotics (imipenem and meropenem) were selected for the subsequent tests and study. The MIC and MLC were performed in triplicate.

2.2.3. Determination of bacterial cell viability exposed to sDq-3162

To determine the level of interference on bacterial growth and cell killing, 100 μL of peptide solution were added in microtubes with 100 μL of the bacterial inoculum (10^8 CFU mL^{-1}, 0.5 McFarland standard) to reach final concentrations equivalent to the peptide MIC and 2 × MIC. The microtubes were incubated at 37 °C, without agitation, then aliquots of 50 μL were removed at eight-time intervals (0, 0.5, 1, 2, 4, 8, 16 and 24 h) and transferred to 96-well Corning® black flat bottom microplates. Thereafter, 50 μL of the BacTiter-Glo® reagent (Promega, Madison, WI, USA) were added to wells and the plates incubated for 5 min, under gentle agitation. Luminescence was measured with the Synergy HT multiple detection microplate reader (BioTek Instruments Inc., Winooski, VT, USA). Bacterial suspensions not exposed to sDq-3162 but to carbapenem antibiotics of current use (imipenem, meropenem or gentamicin) were used as controls. Wells containing only BHI broth culture medium were used to obtain the background luminescence. The bacterial cell viability assay was performed in triplicate.

2.2.4. Antimicrobial activity of sDq-3162 in combination- with conventional antibiotics

2.2.4.1. Checkerboard test. The antimicrobial activity of sDq-3162 in combinations with conventional antibiotics meropenem, imipenem and gentamicin on the viability of *K. pneumoniae* KPC1, *P. aeruginosa* PA14, *A. baumanii* AB1 strains was evaluated using the checkerboard test (Lorian, 2005). In 96-well clear flat bottom microplates, 50 μL-aliquots of the peptide solution and 50 μL of each antibiotic, diluted in BHI broth to final concentrations equivalent to 0.5, 0.25, 0.125 and 0.0625 × MIC, were combined and mixed with 100 μL of microbial suspension (10^6 CFU mL^{-1}). For antibiotics that showed MICs above 16 μg mL^{-1}, sub-inhibitory concentrations (sub-MIC) were then tested (i.e., 1, 2, 4 and 8 μg mL^{-1}), in order to assess the potential synergistic effects in concentration ranges that are inferior to the effective inhibitory concentrations to overcome drug-resistance bacteria but that are effective to kill sensitive bacteria. The microplates were incubated at 37 °C for 24 h and the microbial growth was inspected by eyes and the results scored. The assay was performed in triplicate.

The effect of sDq-3162 in combination with carbapenem antibiotics was estimated by the fractional inhibitory concentration index (FICI) (EUCAST, 2003; Shin and Lim, 2004), calculated according to the following equation:

$$FICI = FIC_{SDq} - 3162 + FIC_{ATB} \quad (1)$$

$$FICI = [SDq - 3162] \,/\, MIC^{SDq-3162} + [ATB] \,/\, MICATB \quad (2)$$

where.

$FIC^{SDq-3162}$ is the fractional inhibitory concentration of the peptide; and.

FIC^{ATB} is the fractional inhibitory concentration of the antibiotic.

[SDq-3162] is the lowest concentration of peptide in combination that displays the antimicrobial activity;

[ATB] is the lowest concentration of the antibiotic in combination that displays the antimicrobial activity; $MIC^{SDq-3162}$ is the MIC of the peptide alone; MIC^{ATB} is the MIC of the antibiotic alone.

The FICI was interpreted as: synergistic effect whether FICI $\leq$0.5; additive effect if 0.5 < FICI <1.0; and antagonistic effect when FICI >1.0 (EUCAST, 2003).

2.2.4.2. Time-kill kinetics of sDq-3162 in combination with antibiotics. For the time-kill kinetics, the antibiotic-resistant strains susceptible to the synergistic combinations of sDq-3162 and conventional antibiotics were selected for the subsequent evaluation, according to experimental

data obtained from the checkerboard tests. In brief, sDq-3162 in combination with carbapenem antibiotics, at their subinhibitory concentrations (sub-MICs, i.e, 1–8 μg mL^{-1}), in a volume of 100 μL, were mixed with 100 μL of bacteria inoculum (10^8 CFU mL^{-1}, 0.5 McFarland standard) in sterile microtubes. The mixtures were incubated at 37 °C and 50 μL-aliquots were taken at the eight-time intervals (0, 0.5, 1, 2, 4, 8, 16 and 24 h). The aliquots were transferred to individual wells of 96-well Corning® black flat bottom microplate and the same volume (50 μL) of BacTiter-Glo® reagent (Promega, Madison, WI, USA) was added. The microplate was incubated for 5 min, under gentle agitation. Luminescence was measured with the Synergy HT multiple detection microplate reader (BioTek Instruments Inc., Winooski, VT, USA), as aforementioned. Untreated bacterial cells and treated only with either the peptide or the antibiotics were used as controls. Wells containing only BHI broth were used to obtain the luminescent signal background. The assay was performed in triplicate.

2.3. Hemolytic activity

The hemolytic activity of the sDq-3162 alone or in combination with antibiotics was determined according to a procedure previously described (Falcao et al., 2014). In brief, fresh human blood samples from healthy donors were collected in Vacutainer tubes (BD, Beckton Dickinson, São Paulo, Brazil) and centrifuged at 1000×*g*, for 10 min at 4 °C. The plasma was removed, the pellet of red blood cells washed three times with phosphate-buffered saline (PBS, 35 mM phosphate, 150 mM NaCl, pH 7.4) and resuspended in PBS to make an 8% (v/v) suspension of red blood cells. Aliquots of 100 μL of this suspension was transferred to microtubes containing 100 μL of sDq-3162 solution with concentrations ranging from 0.3125 μM to 40 μM. The final concentrations of components in the hemolytic test were: 4% (v/v) of human red blood cells, 0.1562 μM–20 μM of sDq-3162 and sub-MICs (1–8 μg mL^{-1}) of conventional antibiotics. After incubation at 37 °C for 60 min, under gentle agitation in an orbital shaker, the microtubes were centrifuged at 1000×*g* for 2 min, and the supernatant transferred to 96-well clear flat bottom microplates. The hemoglobin released from red blood cells, as result of exposure to peptide and/or antibiotics, was determined by measuring the optical density at 540 nm (OD^{540nm}) in a multiple detection microplate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA). Untreated red blood cell suspension (4% v/v, in PBS) and treated with 1% Triton X-100 were used as negative and positive controls, respectively. All tests were performed in triplicate. The percentage of hemolysis was determined as follow:

Hemolysis (%) = [OD^{540nm} (treated) - OD^{540nm} (untreated)]/[OD^{540nm} (tritonX-100) - OD^{540nm} (untreated)] x 100.

Finally, the concentration of peptide that lyses 50% of human erythrocytes (LC50) was determined by plotting the percentage of hemolysis in relation to the concentration of peptide (in μM).

3. Results and discussion

3.1. Peptide structure and properties

The *D. quadriceps* venom peptides evaluated in this study for their antimicrobial activity against antibiotic-resistant bacteria comprised two synthetic dinoponeratoxins; the 23-residue sDq-2562, related to the ponericin W and the sDq-3162, an amidated 28-residue ponericin G-like peptide, of which the sequence is derived from a longer precursor with 30 amino acid residues (Fig. 1). The amino acid sequences and physicochemical properties of these peptides, as well as the helical wheel projection are presented and summarized in Table 1. These two dinoponeratoxins were selected in reason of a previous characterization of the multiple biological activities of the low molecular mass peptides of *D. quadriceps* venom (Radis-Baptista et al., 2020). The physicochemical properties of venom-derived peptides allow one to get a quick look at and predict how these peptide sequences might potentially interact with plasma membranes, in terms of affinity and ability to cause membrane perturbation. The isoelectric point (pI) indicates the net charge of a given molecule at physiological pH of a solution or biological fluid and, as calculated for sDq-2562 and amidated sDq-3162 they are, respectively, + 4.0 and +5.1. Net positive charge (cationicity) is an important attribute of peptides that interact with negatively charged components on the cell surface, such as lipopolysaccharides and phospholipids that make up the membranes of Gram-negative bacteria (Andrea et al., 2007). The hydrophobic moment measures the amphipathicity of the peptide and is a predictive parameter of α-helical formation, affinity for lipid membranes and ability for membrane translocation (Dathe and Wieprecht, 1999; Eisenberg et al., 1982; Phoenix and Harris, 2002). Peptide structures that target membranes and are surface active, transmembrane or lytic α-helices, show frequently cut-off boundaries confined by hydrophobicity (H) between 0.1 and 0.6 and $\mu H > 0.2$. In Table 1, the hydrophobic faces of these two dinoponeratoxin are also shown. Based on these parameters and model prediction, the peptides sDq-2562 (H = 0.509, μH = 0.248) and sDq-3162 (H = 0.194, μH = 0.485) comprise dinoponeratoxins that tend to form amphiphilic, membrane-interacting α-helical structures. In fact, the antifungal activity of these two dinoponeratoxins and other related peptides of this group involves the permeabilization of plasma membrane of pathogenic fungi, as seen by the uptake of a cell impermeant fluorescent dye that penetrates into *Candida* cells with peptide-damaged cytoplasmic membranes (Dodou Lima et al., 2020). Indeed, disruption of plasma membrane is the main mechanism of action of numerous amphipathic α-helical AMPs from diverse sources, including from arthropod venoms (Cesa-Luna et al., 2019; Dathe and Wieprecht, 1999; Dos Santos Cabrera et al., 2019; Yacoub et al., 2020).

Table 2
Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) of the dinoponeratoxins sDq-2562 and sDq-3162 for Gram-negative bacteria.

Clinical strains	Peptide μM (μg mL^{-1})			
	sDq-2562		sDq-3162	
	MIC	MLC	MIC	MLC
Escherichia coli EC16	–	–	5 (15.6)	10 (31.2)
Escherichia coli EC18	–	–	10 (31.2)	10 (31.2)
Escherichia coli EC23	–	–	10 (31.2)	10 (31.2)
Acinetobacter baumannii AB1	–	–	10 (31.2)	10 (31.2)
Klebsiella pneumoniae KP1	–	–	5 (15.6)	5 (15.6)
Pseudomonas aeruginosa PA14	–	–	5 (15.6)	10 (31.2)
Pseudomonas aeruginosa PA19	–	–	5 (15.6)	10 (31.2)

Note: hyphen, "-": inactive (up to 20 μM, 62.4 μg mL^{-1}).

3.2. Antimicrobial activity of sDq-2562 and sDq-3162 against antibiotic-resistant bacteria

In a previous trial, the dinoponeratoxins sDq-2562 and sDq-3162 demonstrated antimicrobial activity against some strains of bacteria and yeasts, as aforementioned (Radis-Baptista et al., 2020). Recently, the antifungal activity of four dinoponeratoxins that included sDq-2562 and sDq-3162 was studied and the action spectrum broadened against pathogenic species of *Candida* (Dodou Lima et al., 2020). Here, the aim was to access the effect of these two most active *D. quadriceps* dinoponeratoxins, sDq-2562 and sDq-3162, against clinical isolates of bacteria that are resistant to carbapenems and other antibiotic classes of current use in clinics (Supplementary Table 1). Although, sDq-2562 was shown to have bactericidal and fungicide activity, as observed in previous works, it was inactive against the tested strains of multi-drug resistant bacteria (Table 2). In contrast, SDq-3162, which has greater cationicity and hydrophobic moment, demonstrated effective bacteriostatic and bactericidal activity against all tested strains of carbapenem-resistant bacteria (Table 2). SDq-3162 displayed an important activity against

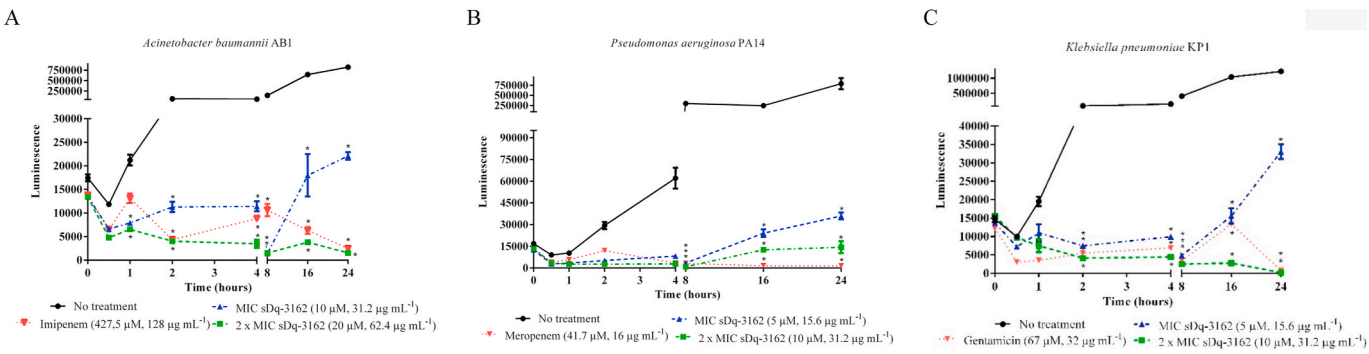


Fig. 2. Time-exposure effect of sDq-3162 peptide on the cell viability of carbapenem-resistant Gram-negative bacteria.

carbapenem-resistant *A. baumannii*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* with MICs between 5 μM and 10 μM (15.6 and 31.2 μg mL^{-1}). This is an interesting finding, since these carbapenem-resistant Enterobacteriaceae strains comprise a group of Gram-negative bacteria that figures in the list of critical global priority by the WHO for the discovery of new anti-infective drugs and therapeutics (World Health Organization, 2017). In addition to the determination of peptide MIC and MLC, the bactericidal and bacteriostatic activity were validated by assessing the time-exposure effect of sDq-3162 on bacterial cell viability. The effect of antimicrobials on the bacterial viability reveals how fast such agents act to inactivate or kill microorganisms (Dodou et al., 2017). The microbial viability was evaluated with the BacTiter-Glo® reagent, in which the luminescent signal produced in the assay is proportional to the amount of intracellular ATP and, consequently, to the number of metabolically active cells and, thus, the number of viable bacterial cells. The active sDq-3162, at a concentration equivalent to its MIC, was able to reduce the microbial viability of carbapenem-resistant clinical strains of *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* within the first 30 min of exposure, maintained the inhibition of bacterial growth by 8 h, as experimentally observed (Fig. 2A–C). After 8 h, the bacterial growth was recovered, but this might be related to the disproportional peptide to cell ratio; in this particular assay, the initial inoculum in this test (10^8 CFU mL^{-1}) required a inoculum that was 100 times greater than the inoculum in the usual MIC assay (10^6 CFU mL^{-1}). At a concentration equivalent to twice the MIC (2 × MIC), the growth inhibition of all strains was maintained for 24 h (Fig. 2A–C).

The presence of antimicrobial peptides in the venom and venom gland of poisonous and venomous animals are intriguing and it remains an apparent open question. A number of AMPs are non-selective membranolytic molecules, so it is speculated that AMPs might exacerbate tissue damage upon envenomation, by one side, as known from insect and cnidarian cytolytic toxins (Hernández-Matehuala et al., 2015; Lima and Brochetto-Braga, 2003; Santos et al., 2016). On the other side, AMPs are known components of the innate immune defense, as aforementioned, that promote host protection against a variety of pathogens (Wu et al., 2018; Yacoub et al., 2020). Hymenopterans, in particular, that prey on other arthropods may delivery AMPs in the venom to protect themselves from ingesting dead insect bodies and preventing infection (Konno et al., 2001; Moreau, 2013). Moreover, venom peptides from ants with antimicrobial activity might have multiple biological activities, ecological roles and functionalities, as exemplified by myrtoxin-Mp1a from the jack jumper ant *Myrmecia pilosula* (Dekan et al., 2017), as well as other pilosulins and ponericins (Orivel et al., 2001; Wanandy et al., 2015). No matter the definitive answer, AMPs that interact with and quickly disrupt microbial plasma membrane are effective anti-infective agents, not only in natural realm but also they are promising candidates for further development in pharmaceutical biotechnology, in face of the current needs to counteract the emergence and rapid dissemination of multi-drug resistance phenotype seen in pathogenic microorganisms (Avci et al., 2018). Indeed, it was recently demonstrated that bicarinalin, a cationic antimicrobial peptide from the venom of the myrmicine ant *Tetramorium bicarinatum* was active and as effective as the general antibiotics against antibiotic-resistant *Helicobacter pylori*, therefore, showing a promising profile for further development and therapeutic use (Guzman et al., 2018).

3.3. Bactericidal activity of combinations of sDq-3162 and conventional antibiotics

Carbapenems are β-lactam antibiotics that exert their effect by inhibiting the formation of peptidoglycan and, consequently, the bacterial cell wall. Meropenem is slightly more active against Gram-

Table 3
Synergistic antimicrobial activity of sDq-3162 in combinations with conventional antibiotics on the growth of carbapenem-resistant Gram-negative bacteria.

Antibiotics	Strain	[sDq-3162]	MIC sDq-3162	FIC sDq-3162	[ATB]	MIC ATB	FIC ATB	FICI	Effect	Reduction [ATB]
Gentamicine	AB1	5 (15.6)	10 (31.2)	0.5	0.5 (0.24)	8 (3.84)	0.0625	**0.5625**	Additive	16 x
	KP1	0.312 (0.98)	5 (15.6)	0.0625	1 (0.48)	32 (15.4)	0.0312	**0.0937**	Synergic	32 x
	PA14	2.5 (7.8)	5 (15.6)	0.5	1 (0.48)	128 (30.8)	0.0078	**0.5078**	Additive	128 x
Imipenem	AB1	2.5 (7.8)	10 (31.2)	0.25	1 (0.60)	128 (76.8)	0.0078	**0.2578**	Synergic	128 x
	KP1	2.5 (7.8)	5 (15.6)	0.5	1 (0.60)	128 (76.8)	0.0078	**0.5078**	Additive	128 x
	PA14	2.5 (7.8)	5 (15.6)	0.5	1 (0.60)	128 (76.8)	0.0078	**0.5078**	Additive	128 x
Meropenem	AB1	2.5 (7.8)	10 (31.2)	0.25	1 (0.38)	128 (48.6)	0.0078	**0.2578**	Synergic	128 x
	KP1	0.312 (0.98)	5 (15.6)	0.0625	1 (0.38)	16 (6.08)	0.0625	**0.125**	Synergic	16 x
	PA14	1.25 (3.9)	5 (15.6)	0.25	4 (1.52)	16 (6.08)	0.25	**0.5**	Synergic	4 x

Note: Clinical strains "AB1", *Acinetobacter baumannii*; "KP1", *Klebsiella pneumoniae*; "EC16", *Escherichia coli*; "PA14", *Pseudomonas aeruginosa*.
[Dq3162], the MIC of peptide in association; MIC_{Dq3162}, the MIC of isolated peptide.
[ATB], the MIC of antibiotic in association; MIC_{ATB}, MIC of isolated antibiotic.
FIC_{Dq3162} = [Dq3162]/MIC_{Dq3162} FIC_{ATB} = [ATB]/MIC_{ATB}.
FICI = FIC_{Dq3162} + FIC_{ATB}.
FICI ≤ 0.5, synergism; FICI > 0.5 and <1.0, additive; FICI ≥ 1.0, antagonism.

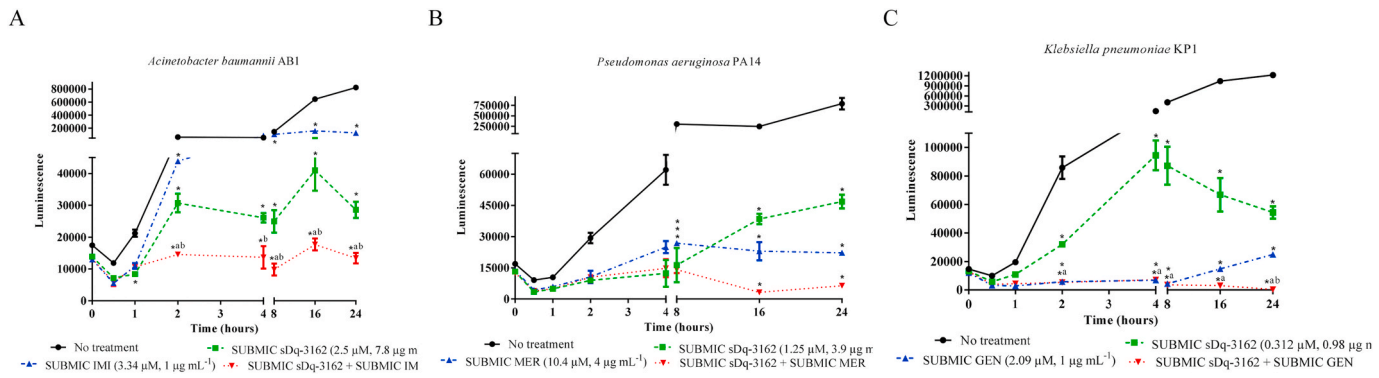


Fig. 3. Time-exposure effect of combinations of sDq-3162 and conventional antibiotics on the viability of carbapenem-resistant Gram-negative bacteria.
Notes. ANOVA and Tukey's multiple comparisons test. *: $p < 0.05$ (compared to untreated bacteria cells); a: $p < 0,05$ (compared to SUBMIC sDq-3162); b: $p < 0,05$ (compared to SUBMIC GEN). IMI, imipenem; MER, meropenem; GEN, gentamicin.

negative bacteria, while imipenem has slightly superior performance against Gram-positive bacteria, but both are highly stable to the hydrolysis by bacterial β-lactamases. These antibiotics are widely used in clinic settings and are among the last therapeutic resorts for the treatment of resistant infections caused by Gram-negative bacteria. Despites these aspects, microbial resistance even to carbapenems is considered major and recurrent threats to public health worldwide, in addition to increase the health costs to treat infectious by multi-drug resistant bacteria (Gales et al., 2002; McCann et al., 2020). These facts highlight the importance of continuous investigation on new isolated antimicrobial compounds, as well as developing strategies to fight drug-resitance, like exemplified by the use of combinations of conventional antibiotics (Ahmed et al., 2014; Podolsky and Greene, 2011).

Take into account the study about drug combination, it was verified by means of the checkerboard test that sDq-3162 (MIC = 10 µM, 31.2 µg mL^{-1}) interacts synergistically with carbapenem antibiotics and inhibits the growth of drug-resistant *A. baumannii*, reducing by more than 2-log the MICs of imipenem, from 427.6 µM (128 µg mL^{-1}, the MIC) to 3.34 µM (1 µg mL^{-1}) and meropenem, from 333.8 µM (128 µg mL^{-1}, the MIC) to 2.6 µM (1 µg mL^{-1}) (Table 3). The combination of the sDq-3162 with meropenem was also synergic to inhibit the growth of *K. pneumoniae* and *P. aeruginosa*, reducing the MIC of meropenem for these bacteria by 16- and 4-fold, from 41.7 µM (the MIC, 16 µg mL^{-1}) to 2.6 µM (1 µg mL^{-1}) and from 41.7 µM (the MIC, 16 µg mL^{-1}) to 10.4 µM (4 µg mL^{-1}), respectively. The combination of sDq-3162 with imipenem was also effective to restrain the growth of *K. pneumoniae* and *P. aeruginosa*, from 427.6 µM (the MICs, 128 µg mL^{-1}) to 3.34 µM (1 µg mL^{-1}) for both strains. In this case, despite the reductive effect of the peptide and antibiotic combination was theoretically considered additive, due to the small changes in the peptide and antibiotic concentrations necessary to kill bacteria, even in this combination a 2-log reduction in the effective inhibitory concentration of the antibiotic was achieved (Table 3). The checkerboard test also indicated that the combination of sDq-3162 and the aminoglycoside gentamicin was additive against antibiotic-resistant *A. baumannii* with a significant reduction of the gentamycin MIC by 1-log, from 16.7 µM (8 µg mL^{-1}) to 1.04 µM (0.5 µg mL^{-1}) and 2-log for *P. aeruginosa*, from 268 µM (the MIC (128 µg mL^{-1}) to 2.09 µM (1 µg mL^{-1}). For *K. pneumoniae*, the combination was synergistic and represented a 32-fold reduction in the antibiotic MIC, from 67 µM (32 µg mL^{-1}) to 2.09 µM (1 µg mL^{-1}) (Table 3). In all these antibiotic and peptide combinations, the MIC of sDq-3162 for antibiotic-resistant bacteria was evidently reduced by 10-fold, as for the combination with gentamicin or meropenem against antibiotic-resistant *K. pneumoniae*. Although gentamicin belongs to a distinct class of antibiotics with a mechanism of action that differs from the carbapenem-antibiotics, it was included in this study because it has a good profile against Gram-negative bacteria and, thus, a low tendence to induce cross- and drug-resistance in bacteria. Gentamicin is an aminoglycoside antibiotic that acts by inhibiting the protein synthesis through the interference with ribosome translation, and is often used to treat infections caused by Gram-negative bacteria that are resistant to other chemotherapeutics (Center of Disease Control, 2019). The combination of antibiotics and peptides that potentiate the action of gentamicin can be considered as a practical and promising alternative against these antibiotic-resistant bacteria that are listed in the WHO's critical priority guide about the multi-drug resistant bacteria (World Health Organization, 2017).

The time-kill kinetics determined with the luminescent reagent in the checkerboard test used here, confirm the effective antibacterial activity of the combination of peptide and conventional antibiotics. The growth inhibition of carbapenem-resistant *A. baumannii* by the peptide and antibiotic combinations was clearly observed, with a sustained bactericidal effect along the period of exposure to the combinations, as for the SDq-3162 and imipenem (Fig. 3 A). The combination of sDq-3162 and meropenem resulted in the growth inhibition of *P. aeruginosa* that was also sustained for 24 h, compared to untreated cells (Fig. 3-B). Finally, the combination of sDq-3162 and gentamicin inhibited the growth of drug-resistant *K. pneumoniae* for 24 h, compared to the peptide alone, after 2 h of treatment, or after 24 h when compared with only the antibiotic (Fig. 3-C). Notably, the combination of sDq-3162 and these antibiotics reduced rapidly the number of viable cells, in a short time span (30 min). Furthermore, the effect on bacteria cells exposed to the combination of peptide and antibiotics lasted for 24 h. Such data demonstrated that the combinations of sDq-3162 and antibiotics displayed a short onset of action with the bactericidal effect sustained for a relatively long period (Fig. 3A–C).

The synergistic effect of antibiotic combination is ideal to potentiate the efficacy of conventional antimicrobials. The additive effect despite imperfect could be considered advantageous; in both cases, the peptide contributed to reduce the MIC of antibiotics in the combinations. The antibiotic combination therapy to reduce resistance in bacteria, exploiting synergism and restoring the efficacy of old chemotherapeutics against infectious microbes, has been envisioned to circumvent antimicrobial-resistance in present-day antimicrobial crisis situation (Coates et al., 2020). Thus, membrane-interacting and membranolytic AMPs in combination with aminoglycosides (e.g., gentamycin) and carbapenem antibiotics are promising alternatives to reach a biological effect, hindering the emergence of drug-resistance and decreasing the antibiotic toxicity and their accompanied side effects, which usually occur with higher doses of a single therapeutic substance (Casciaro et al., 2018; Cavalcante et al., 2018; Gupta et al., 2015; Ma et al., 2017; Podolsky and Greene, 2011; Shang et al., 2019). Altogether, sDq-3162 in combination with carbapenem and gentamycin fulfill the concept of synergistic drug combination, in which the combined bactericidal effect of peptide and conventional antibiotics is more effective than the sum of their individual activities (Ahmed et al., 2014; Podolsky and Greene,

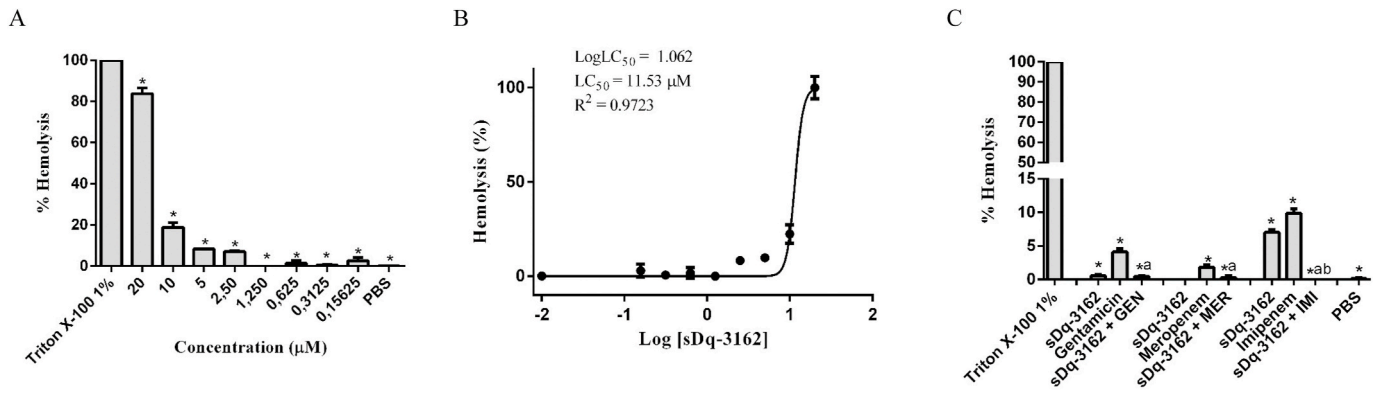


Fig. 4. –Hemolytic effect of sDq-3162 alone and in combinations with conventional antibiotics.

2011).

3.4. Evaluation of percentage of hemolysis as an estimate of peptide cytotoxicity

Venoms from hymenopterans (ants, bees and wasps), in general, contain short peptides that often are cytolytic and hemolytic, apart of being insecticide, microbicide, mast cell-degranulating, neurotoxic, among other activities (Ceolin Mariano et al., 2019; Robinson et al., 2018; Touchard et al., 2016). Moreover, a number of AMPs are not specifically target-selective and, therefore, they are potentially cytoxic to health mammalian cells, making them difficult to be developed into new therapeutics (Shang et al., 2019). The ponericin-like peptide sDq-3162, at concentrations that showed its antimicrobial effect, i.e. between 5 and 10 μM, caused 8% and 18% of lysis to human erythrocytes *in vitro*, respectively (Fig. 4-A), with a LC50 of 11.53 μM (Fig. 4-B). This effect is explained again by the high hydrophobic moment and amphipathicity of sDq-3162. However, when sDq-3162 was combined with carbapenem-antibiotics, the hemolysis was significantly reduced to 1% (Fig. 4-C), i.e., approximately twenty time less than the LC50. Thus, the reduced percentage of hemolysis that resulted from the synergistic combination of sDq-3162 with the current-use antibiotics, as demonstrated here, brings resourceful possibilities for this venom peptide as anti-infective adjuvant. Moreover, these results reinforce the strategy of drug combination with synthetic peptides and conventional antibiotics to treat bacterial infection. This is advantageous not only from the point of view of the bactericidal effect against microbial drug-resistance, but also in terms of reducing the antibiotic toxicity, as well as keeping alive conventional antibiotics.

4. Conclusion

Taking together, the dinoponeratoxin sDq-3162 displays a relevant antimicrobial activity against strains of life-threatening Gram-negative bacteria strains that are resistant to carbapenems and other conventional antibiotics. sDq-3162 substantially potentiates the action of imipenem, meropenem and gentamicin against drug-resistant, clinical-strain of *A. baumannii*, *K. pneumoniae* and *P. aeruginosa*. sDq-3162 exerts its bacteriostatic and bactericidal effects with a rapid onset of action and prolonged duration. The combinations of sDq-3162 with conventional antibiotics were promising not only for improving the action of individual antibiotics and peptide on carbapenem-resistant Enterobacteriaceae, but also for decreasing the toxicity on human red blood cells, as an estimate of low cytotoxicity *in vitro*. Furthermore, the importance of these finding relies on the fact that this study focused on microorganisms listed in the WHO's critical priority guidelines to antibiotic research. Further investigation about the detailed mechanism of action of the peptide individually and in combination with antibiotics will contribute somehow for the development of strategies to circumvent the emergence of antibiotic-resistant microbes. Last but not least, the use of ant venom-derived peptides as templates for the design and development of therapeutics and their adjuvants broadens our resources and possibilities for drug discovery from nature.

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CRediT authorship contribution statement

Hilania V. Dodou Lima: Conceptualization, Methodology, Validation, Visualization, Investigation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Carolina Sidrim de Paula Cavalcante:** Conceptualization, Methodology, Validation, Visualization, Investigation, Formal analysis. **Gandhi Rádis-Baptista:** Conceptualization, Methodology, Supervision, Writing - review & editing, Project administration, Funding acquisition.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2020.08.015.

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