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SHORT COMMUNICATION

Checkerboard testing method indicates synergic effect of pelgipeptins against multidrug resistant *Klebsiella pneumoniae*

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Abstract Multidrug resistant bacteria infections have motivated the search for new therapeutics. The synergic effect of conventional antibiotics and lipopeptides of the pelgipeptin family was evaluated by the Checkerboard method. Results indicate that the association of Pelgipeptin B and C or chloramphenicol has a synergic effect against a multidrug resistant bacteria strain; which may be may be due to the variations on hydrophobicity and mechanisms of action of the molecules tested.

Introduction

The emergence of multidrug-resistant (MDR) bacteria has reduced the efficacy of conventional antibiotics (Chi & Holo, 2018; Doern, 2014; Tanwar, Das, Fatima, & Hameed, 2014). The rapid dissemination of Gram-negative (diderm) bacteria, such as *Klebsiella pneumoniae* and *Escherichia coli*, is responsible for an emerging crisis due to their carbapenemase coding gene that can be carried by plasmids (Ribeiro et al., 2015; Tzouvelekis, Markogiannakis, Psichogiou, Tassios, & Daikos, 2012). In addition, infections caused by β-lactam resistant Gram-positive (monoderm) bacteria (e.g. *Staphylococcus aureus*) have increased in incidence (Croxen et al., 2013; Narasimhaswamy, Bairy, Shenoy, & Bairy, 2017; Tong, Davis, Eichenberger, Holland, & Fowler, 2015; Tzouvelekis et al., 2012). Drug combinations that present synergic effects emerge as a therapeutic option aiming to reduce the drug administration if compared to classical single antibiotic treatments (Chi & Holo, 2018; Montero et al., 2018; Narasimhaswamy et al., 2017). Synergy testing methods uses susceptibility techniques to measure a cumulative efficacy of two or more drugs in which the resulted activity occurs at a lower concentration than the sum of each of them isolated. Synergic results may indicate an increased efficacy, reduced toxicity and adverse effects due to a lower therapeutic dosage administration (Kumar, Siji, Nambisan, & Mohandas, 2012).

The *Paenibacillus* genus is known for producing antimicrobial compounds with broad spectrum activity such as bacteriocins (Baindara et al., 2016; He et al., 2007; Lohans

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et al., 2012) and non-ribosomal lipopeptides (Canova et al., 2010; Cochrane & Vederas, 2016; Ding et al., 2011; Guo, Huang, Yuan, Zhang, & Yousef, 2012; Niu et al., 2013; Qian, Wu, et al., 2012; Selim, Negrel, Govaerts, Gianinazzi, & van Tuinen, 2005; Wen et al., 2011). Pelgipeptins are a group of cyclic cationic lipopeptides that contain the nonproteinogenic amino acid 2,4-diaminobutyric acid (Dab) on its N-terminal position linked to a fatty acid chain and presents antimicrobial activity against monoderm and diderm bacteria (Cochrane & Vederas, 2016; Ding et al., 2011; Wu et al., 2010). Major variations among Pelgipeptin A–D isoforms include a substitution on the second positioned amino acid and on the fatty acid length (Qian, Liu, et al., 2012; Wu et al., 2010). Pelgipeptins A and D present a methyl group linked on its penultimate *(iso)* position of its lipid tail whereas Pelgipeptins B and C show the methyl group on the antepenultimate (*anteiso*-) position of its lipid tail (Cochrane & Vederas, 2016; Kaneda, 1967, 1991; Qian, Liu, et al., 2012). Although the Minimal Inhibitory Concentrations (MICs) of each Pelgipeptin isoforms have been studied previously (Ding et al., 2011; Wu et al., 2010), here we investigated the antimicrobial effects of combined isoforms, aiming to describe synergistic effects among them.

Materials and methods

Pelgipeptins were obtained from *Paenibacillus elgii* strain AC13, isolated from Brazilian Cerrado soil samples (Ortega et al., 2018), grown on chemically defined medium MMP (Patent BR102017018881-7). Purification was attained by high performance liquid chromatography (HPLC; Shimadzu) using a reverse phase column Shimadzu Shim-pack VP-ODS (4.6 μm, 150 × 4.6 mm). The mobile phase consisted by HPLC-grade water containing 0.1% of TFA (mobile phase A) and acetonitrile with 0.1% of TFA (mobile phase B). The molecular masses of the lipopeptides were confirmed by MALDI-ToF (Autoflex Speed; Bruker Daltonics) on reflected-positive mode (700–3500*m*/*z*).

The minimal inhibitory concentrations (MIC) for Pelgipeptins isoforms A–D were evaluated against *S. aureus* ATCC 14458, *E. coli* ATCC 11229, *K. pneumoniae* ATCC 13883; and two MDR strains obtained from Brasilia's *Laboratório Central* (LACEN), *K. pneumoniae* LACEN 3259271 and *E. coli* LACEN 3789319 isolated from hospitalized patients' blood samples in Brasilia, Brazil and all tested strains were used in a final suspension concentration of 5×10^5 CFU mL^{-1}. Test conditions followed the Clinical and Laboratory Standards Institute M07-A6 (Clinical and Laboratory Standards Institute – CLSI, 2004). Reference antibiotics were used as controls.

The Checkerboard testing method was used to evaluate synergism among Pelgipeptins A, B and C or combined with Chloramphenicol or Penicillin against the MDR K. pneumoniae (LACEN3259271). The Checkerboard method used was the broth microdilution assay performed in a 96-well plate with the final volume of 200 μl (Balouiri, Sadiki, & Ibnsouda, 2016; Doern, 2014). This assay applied the combination of two compounds in increasing concentrations to provide a final classification of the combined compounds based on a Fractional Inhibitory Concentration (FIC) Index (FICI) as follows: S, synergy (FIC ≤0.5); A, additive (FIC >0.50 and <1); I, indifferent (FIC >1 and ≤4) (Chin, Weitzman, & Della-Latta, 1997).

Results and discussion

All pelgipeptins were active against tested microorganisms. Pelgipeptin A showed lower MICs against both *K. pneumoniae* strains whereas Pelgipeptins C and D were the most active against multidrug-resistant *E. coli*. MICs for Pelgipeptins A, C and D were 8 μg mL^{-1} whereas Pelgipeptin B showed MICs varying from 64 to 16 μg mL^{-1} (Table 1). MICs for some commercial antibiotics tested against MDR *K. pneumoniae* strains were the same or higher (Huang & Yousef, 2014) than the ones observed for pelgipeptins.

Synergism among pelgipeptins was evaluated against two *K. pneumoniae* strains, by means of the Checkerboard testing method (Balouiri et al., 2016; Doern, 2014). Pelgipeptin D purification yield was the lowest and that isoform was not used to perform the synergy test. The combination Chloramphenicol + Pelgipeptin A and Pelgipeptins A + B resulted in indifferent effect over the growth inhibition of *K. pneumoniae* ATCC 13883 (FIC = 1.5 and 1.125, respectively). However, FIC values lower than 0.5 obtained for Pelgipeptin A + C; B + C; Penicillin + Chloramphenicol; and Pelgipeptin C + Penicillin or Chloramphenicol indicates synergism. When combined molecules were tested against MDR strain LACEN 3259271 the results were slightly different. Chloramphenicol + Pelgipeptin A, B or C; and Pelgipeptin C + Penicillin showed indifferent result (FIC = 1). The association of pelgipeptins A + B or A + C had an additive effect with FIC values varying from 0.5 and 0.75. Moreover, FIC values lower than 0.5 obtained on Pelgipeptin B + C or chloramphenicol associations indicates synergic effect (Table 2, Supplementary Tables 1 and 2).

The observed synergic effect against *K. pneumoniae* ATCC13883 of Pelgipeptin isoforms and the control antimicrobials was expected, due to the differences in the targeted cell structure of each antimicrobial. Lipopeptides from the *Paenibacillus* spp. present in its structure both, D and L amino acids, resulting in higher stability over proteolytic enzymes (Cochrane & Vederas, 2016; Huang & Yousef, 2014). The presence of the nonproteinogenic amino acid Dab results an overall cationic charge of pelgipeptins, suggesting that its primary mechanism of action may be the membrane disruption of targeted organisms (Cochrane & Vederas, 2016). Penicillin is a bactericidal molecule that inhibits trans peptidase, enzymes that catalyzes reactions between peptidoglycan chains of the bacterial cell wall (Tipper & Strominger, 1965); leading to the cell wall stiffness suppression and turning bacterial cells susceptible to osmotic variations. Chloramphenicol binds to the ribosome 50S subunit and inhibits protein synthesis (Cushnie, O'Driscoll, & Lamb, 2016; Nussbaum, Brands, Hinzen, Weigand, & Habich, 2006). Furthermore, no synergic effect against the MDR strain was observed between Penicillin G and Pelgipeptin isoforms since the antimicrobial effect is solely due to the Pelgipeptins mechanism of action.

Despite the different cell target of each drug tested, only some combinations with Pelgipeptins were synergic (Table 2). This fact may be a function of the pelgipeptins structural difference on the fatty acid chains,

Table 1 Minimum inhibitory concentration (MIC): Pelgipeptin A–D and reference antibiotics against tested strains ($\mu g.mL^{-1}$).

	Pelgipeptin A	Pelgipeptin B	Pelgipeptin C	Pelgipeptin D	Chloramphenicol	Penicillin G
Escherichia coli ATCC 11229	8	32	32	8	NT[a]	NT[a]
Escherichia coli LACEN 3789319	16	16	8	8	NT[a]	NT[a]
Staphylococcus aureus ATCC 14458	128	16	32	16	8	NT[a]
Klebsiella pneumoniae ATCC 13883	8	64	64	8	16	0.5
Klebsiella pneumoniae LACEN 3259271	8	32	16	16	32	>1024

[a] NT, not tested.

Table 2 Fractional Inhibitory Concentration (FIC) Index: Pelgipeptins A–C and other antibiotics against *Klebsiella pneumoniae* ATCC13883 and the MDR strain LACEN 3259271.

Combinations		*K. pneumoniae* ATCC13883		*K. pneumoniae* LACEN 3259271	
Molecule A	Molecule B	FIC	Interpretation[a]	FIC	Interpretation[a]
Pelgipeptin A	Chloramphenicol	1.5	I	2	I
	Pelgipeptin B	1.125	I	0.75	A
	Pelgipeptin C	0.375	S	0.562	A
Pelgipeptin B	Chloramphenicol	0.25	S	0.312	S
	Pelgipeptin C	0.265	S	0.5	S
	Penicillin G	0.5	S	1.5	I
Pelgipeptin C	Chloramphenicol	0.265	S	1.25	I
	Penicillin G	0.5	S	1.5	I

[a] S, synergy (FIC $\leq$ 0.5); A, additive (FIC >0.50 and <1); I, indifferent (FIC >1 and $\leq$4).

since its length and structural formation may change the hydrophobicity level, directly interfering over bacterial cell membrane interaction (Wu et al., 2010); and pelgipeptin B seem to be the best synergic agent.

Conclusions

The synergic effect of combinations with Pelgipeptins was demonstrated by means of Checkerboard testing method. The synergic effect against *K. pneumoniae* ATCC13883 of Pelgipeptins and the standard antimicrobials was expected, since they target distinct cell structures. However, the synergic effect of Pelgipeptin B and C against multidrug resistant *K. pneumoniae* may be due to their longer fatty acid chain resulting in a higher hydrophobicity.

After validation by in vivo experiments, synergism could be explored for developing novel therapeutic options, since this approach could be used to reduce the needed dosage of a compound, and consequently reduce toxicity (e.g. chloramphenicol), or even rescue compounds that lost activity in the past (e.g. penicillin). Despite an ongoing discussion if combined antibiotic administration is an efficient therapeutic method, it is important to express that the results of this study should be considered to improve medical practices in Brazil.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biori.2018.12.001.

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