JOURNAL OF INTEGRATED OMICS
A METHODOLOGICAL JOURNAL
HTTP://WWW.JIOMICS.COM

III INTERNATIONAL CAPARICA CONFERENCE IN ANTIBIOTIC RESISTANCE 2019 PRELIMINARY COMMUNICATION | DOI: 10.5584/JIOMICS.V9I2.296

One Enterocin AP-7121: combination with colistin against human multi-drug resistant Gram-negative pathogens

G. Delpech[1], S. Lissarrague[1], M. Ceci[2], L. García Allende[2], A. Lallee[1], B. Baldaccini[1], M. Sparo[1*]

[1] Microbiología Clínica, Medicina, Universidad Nacional del Centro de la Provincia de Buenos Aires, Olavarría (7400), Argentina;[2] Centro de Estudios Bioquímicos, Tandil (7000), Argentina.

Received: 15 August 2019 **Accepted:** 28 October 2019 **Available Online:** 15 November 2019

PRELIMINARY COMMUNICATION

The significant prevalence of Gram-negative bacteria as health-care associated pathogens and their increased antimicrobial multi-drug resistance highlight the need for new therapeutic options. Colistin is a conventional antimicrobial currently employed for the treatment of nosocomial infections caused by multi-drug resistant Gram negative bacteria such as Pseudomonas aeruginosa and Acinetobacter baumannii complex with a main drawback, its toxicity. Doses of this drug, and its toxic effects, can be potentially reduced by using it combined with bacteriocins. AP-7121 is an enterocin produced by the probiotic strain Enterococcus faecalis CECT7121. The aim of this study was to investigate the synergistic activity of AP-7121 combined with colistin against multi-drug resistant Gram-negative pathogens. P. aeruginosa (n: 3) only susceptible to colistin and A. baumannii complex (n: 3) only susceptible to colistin and tigecycline were included. These human isolates were recovered from blood cultures (hemoculture) of patients with catheter-related bloodstream infections at the Intensive Care Unit (Hospital Ramon Santamarina de Tandil Argentina). Minimum Inhibitory Concentration (MIC) for AP, colistin, and colistin/AP-7121 combination against Gram-negative bacteria was assayed (micro-dilution method, CLSI 2018). In vitro bactericidal activity of AP alone or combined with colistin (MIC/4), for assessing a synergistic effect, was studied carrying out time-kill curves. Samples were obtained for viable cell counts (0, 4, 8 and 24 h). MIC and time-kill curves were carried out three times, in duplicate. Results were expressed as their average values. All isolates were resistant to AP (MICAP-7121 > 128 mg/L). Colistin showed anti-P. aeruginosa (MICcolistin 0.5 mg/L) and anti-A. baumannii complex (MICcolistin 0.5-1.0 mg/L) activity in each isolate. Colistin/AP-7121 Combination showed bactericidal activity against P. aeruginosa (MICcolistin/AP-7121 ≤ 0.06/11-0.12/16 mg/L) and A. baumannii (MICcolistin/AP-7121 ≤ 0.12-0.20/16 mg/L). A synergistic effect (colistin/AP-7121) was observed at 4-8 and 24 h for P. aeruginosa (-1.8 to -3.8 Δlog10 CFU/mL) and for A. baumannii complex isolates (-2.0 to -3.8 Δlog10 CFU/mL). AP-7121 is a candidate as an alternative option for the combination with colistin, against human P. aeruginosa and A. baumannii complex isolates producers of bloodstream infections. Their synergistic activity against these bacteria, leads to a bactericidal activity of AP, with lower MIC values and a potential reduction of colistin toxicity, to be thoroughly investigated.

Keywords: enterocin, AP-7121, colistin, Gram positive, human, multi-drug resistant, pathogens

1. Introduction

The significant prevalence of Gram-negative bacteria as health-care associated pathogens and their increased antimicrobial multi-drug resistance highlight the need for new therapeutic options [1-3].

Among health-care associated infectious diseases, a significant problem for Public health are catheter-associated bloodstream infections, where Pseudomonas aeruginosa and Acinetobacter baumannii complex are reported as part of the commonest bacterial agents of these infections [4].

Previous studies showed that multi-drug resistant P. aeruginosa was considered as a risk factor for increased in-hospital mortality and 30-day mortality after infection, as well as the presence of catheters was considered a risk factor for colonization with this species. In the case of A. baumannii, the fatal outcome of infections has increased due to carbapenems resistance, with a mortality rate approaching to 60%, including bloodstream infections [2, 5].

In 2017, in Argentina, a nation-wide surveillance reported

Corresponding Author: M. Sparo, Microbiología Clínica, Medicina, UNCPBA. Av. Pringles 4375, CP 7400, Olavarría, Argentina. +54 249 4428797, E-mail: monicasparo@gmail.com

resistance to fluorquinolones (25%) and carbapenems (29%) in P. aeruginosa, while 76% of Acinetobacter spp. isolates showed carbapenems resistance [6].

Colistin is a conventional antimicrobial currently employed for the treatment of nosocomial infections caused by multi-drug resistant Gram negative bacteria such as P. aeruginosa and A. baumannii complex with a main drawback, its toxicity [7].

A strategy for the treatment and control of Gram-negative pathogens has been testing the synergistic effect between a conventional antimicrobial together with natural compounds as bacterial antimicrobial peptides [8]. Therefore, doses of colistin and its toxic effects could be potentially reduced by using this drug combined with a bacteriocin such as AP-7121.

AP-7121 (formerly MR99) is an enterocin produced by the probiotic strain Enterococcus faecalis CECT7121, a non-hemolytic, gelatinase negative strain recovered from a natural corn silage in Tandil District, Argentina, which recently has been sequenced. Moreover, it does not show antimicrobial multi-resistance, with Minimum Inhibitory Concentration (MIC)vancomycin 0.12 μg/mL, MICampicillin 0.5 μg/mL, MICgentamicin < 500 μg/mL, and MICstreptomycin < 1000 μg/mL. Physicochemical studies showed that AP-7121 presents heat-stability (1 h, 75 °C). Also, it is sensitive to proteolytic enzymes, detergents, and chelants; in addition, it is stable against the activity of enzymes such as DNase, RNase, amilase, glucuronidase, and lipase. Also, presents inhibitory activity among a wide range of pH values (4.0–8.0). The mechanism of action is mediated by formation of pores in bacterial membranes, followed by osmotic shock and cell lysis [9-11].

The aim of this study was to investigate the synergistic activity of AP-7121 combined with colistin against multi-drug resistant Gram-negative pathogens.

2. Material and Methods

During the period July-December 2018, human isolates were recovered from blood cultures (hemoculture) of patients with catheter-related bloodstream infections at the Intensive Care Unit (Hospital Ramon Santamarina, Tandil Argentina). Phenotypic characterization was performed with conventional tests. For A. baumannii characterization was carried out to the complex level [12]. Disk diffusion susceptibility tests were done following Clinical and Laboratory Standards Institute`s guidelines [13].

P. aeruginosa (n: 3) only susceptible to colistin and A. baumannii complex (n: 3) only susceptible to colistin and tigecycline were included. Each bacterium was recovered from different patients, and one representative isolate was considered from each patient.

Phenotypic characterization and antimicrobial susceptibility to colistin (P. aeruginosa, A. baumannii complex) and tigecycline (A. baumannii complex) were validated with the Vitek 2 CompactTM automated system (bioMérieux, Buenos Aires, Argentina).

Isolation of AP-7121 was carried out according to a previously standardized protocol [14]. Probiotic strain E. faecalis CECT7121 (deposited at the Spanish Collection of Type Cultures, CECT, Burjasot, Valencia, Spain) was incubated in brain-heart infusión (BHI) broth (Laboratorio Britania, Buenos Aires, Argentina) at 35 ± 2 °C for 18 h. This culture was inoculated in 4 L of BHI broth and incubated at 35 ± 2 °C for 9 h.

Then, it was centrifuged at 15,000g, 4 °C, for 20 min. Supernatant was adjusted to pH: 7.0 and precipitated. After centrifugation at 20,000g, 4 °C, for 20 min, the pellet was re-suspended in 40 mL of phosphate buffer saline (PBS), pH: 7.0 (50 mM). AP-CECT7121 was isolated by physicochemical extraction employing Sep-Pak™ C18 cartridges (Waters, Milford, MS, USA). E. faecalis extract (5.0 mL) was loaded into a cartridge, previously washed with acetonitrile in trifluoroacetic acid (TFA, 0.1%), and it was eluted with acetonitrile (60%)-TFA (0.1%).

Eluate was concentrated to dryness using a vacuum centrifuge (Thermo Savant Instuments, Hollbrook, NY, USA). The obtained pellet was re-suspended in PBS (250 μL). Aliquotes (20 μL) of the suspensión were injected in a reverse-phase HPLC system (Shimadzu, Kyoto, Japan) and separated in a Nucleosil C18 (5 μm, Pharmacia, Uppsala, Sweden) column. Mobile phase: buffer A (TFA 0.1%) and buffer B (acetonitrile 95% in TFA 0.1%). AP-7121 was eluted using a linear gradient (95% A/5% B to 15% A/85% B), with a flow rate of 0.2 mL/min, controlling elution with a UV detector.

Fractions were collected at regular time period. Then, the active fraction was evaporated to dryness and re-suspended in phosphate buffer (50 mM, pH: 7.0). Biological activity of AP-7121 was detected in the eluate fractions after 30 min of the sample injection, when it was ca. 40% of buffer B.

Minimum Inhibitory Concentration (MIC) for AP-7121, colistin and the colistin/AP-7121 combination against P. aeruginosa and A. baumannii complex isolates was assayed with the broth micro-dilution method, according to the Clinical and Laboratory Standards Institute`s recommendations [13]. P. aeruginosa ATCC 27853 and Escherichia coli ATCC 25922 were employed as quality control strains.

In vitro bactericidal activity of AP-7121 alone or combined with colistin (MIC/4), for assessing a synergistic effect, was studied carrying out time-kill curves. Fresh cultured bacterial cells were washed, suspended, and diluted in PBS, 50 mM, pH: 7.0, for reaching a 106 CFU inoculum. Samples (100 μL) of each bacterial suspension were obtained at 0, 4, 8, and 24 h of incubation (35 ± 2 °C). Viable colony counts were performed in BHI agar, after incubation at 35 ± 2 °C for 24 h. A viable cell count in the same experimental conditions, with PBS, was performed as quality control [14].

MIC and time-kill curves were carried out three times, in duplicate. Results were expressed as their average values, in mg/L for MIC and Δlog10 CFU/mL for viable counts.

3. Results

3.1. Bactericidal activity of colistin, AP-7121 and colistin/AP-7121 combination against human P. aeurginosa and A. baumannii complex isolates

All the studied P. aeruginosa and A. baumannii complex isolates were resistant to AP-7121, with MICAP-7121 > 128 mg/L (Table 1).

Anti-P. aeruginosa and anti-A. baumannii complex activity in each isolate, MIC: 0.5 mg/L and MIC: 0.5-1.0 mg/L, was detected respectively, for colistin (Table 1).

Combination of colistin and AP-7121 showed bactericidal activity against P. aeruginosa (MICcolistin/AP-7121 ≤ 0.06/11-0.12/16 mg/L) and A. baumannii complex (MICcolistin/AP-7121 ≤ 0.12-0.20/16 mg/L).

Table 1: Location Minimum Inhibitory Concentrations for AP-7121, colistin and colistin/AP-7121 against Pseudomonas aeruginosa and Acinetobacter baumannii complex from blood of patients with catheter-related infections.

Pseudomonas aeruginosa			
MIC	AP-7121	Colistin	Colistin/AP-7121
CEBUTI428	> 128	0.5	0.12/16
CEBUTI571	> 128	0.5	0.12/16
CEBUTI783	> 128	0.5	≤ 0.06/11
***Acinetobacter baumannii* complex**			
CEBUTI463	> 128	0.5	0.12/16
CEBUTI656	> 128	0.5	0.12/16
CEBUTI802	> 128	1.0	0.20/16

3.2 Assessment of early and late synerfystic effect of colistin/AP-7121 combination

A synergistic effect when colistin and AP-7121 (Table 2) were combined, it was observed at 4-8 h (early synergy) and 24 h for P. aeruginosa (-1.8 to -3.8 Δlog10 CFU/mL) and for A. baumannii complex (-2.0 to -3.8 Δlog10 CFU/mL).

4. Discussion

In this study, the synergistic activity of AP-7121 combined with colistin against human multi-drug resistant P. aeruginosa and A. baumannii complex isolates was investigated.

Worldwide, catheters-related infections are considered as one of the main causes of bloodstream infections associated with significant patient morbidity and mortality and increased health care costs [15]. Multi-drug resistance bacteria have become a critical risk factor for patients with bloodstream infections due to the limited therapeutic options. A French multi-center study showed that optimizing bloodstream infections management by increasing rapidity of appropriate treatment initiation may decrease short-term mortality, when patients received at least one active antibiotic within the first 48 hours [16].

In this sense, P. aeruginosa and A. baumannii complex constitute representative bacterial agents of catheter-related bloodstream infections due to their prevalence and their tendency to express multi-drug resistance and can be considered as bacterial models for the study of new alternatives for the treatment of these infections, namely bacteriocins such as enterocin AP-7121.

All the assayed isolates showed resistance to AP-7121 when it was not combined with colistin. Previously, enterocin AP-7121 presented homogeneous bactericidal activity against phylogenetically related bacterial species, i.e. Gram positive pathogens, from different origin, such as Enterococcus spp., Streptococcus spp., Staphylococcus aureus and Listeria monocytogenes but it was bacteriostatic against Gram negative bacteria, when it was assayed alone [9, 10].

Similar results were obtained by other authors when a bacteriocin was assayed against different bacteria. Garvicin KS, a broad-spectrum bacteriocin produced by Lactococcus garvieae, is effective against Gram positive and Gram negative bacteria. Nevertheless, this bacteriocin showed no bactericidal activity against P. aeruginosa isolates when it was tested alone. In addition, a lack of inhibition against A. baumannii isolates was observed for the lantibiotic nisin when it was not combined with other antimicrobial or bacteriocin [8].

When AP-7121 and colistin were assayed together against the bacteria, the determined MICs values were lower for the antimicrobial and significantly lower for the bacteriocin, compared with the obtained MICs for colistin and AP-7121

Table 2: Location Minimum Inhibitory Concentrations for AP-7121, colistin and colistin/AP-7121 against Pseudomonas aeruginosa and Acinetobacter baumannii complex from blood of patients with catheter-related infections.

Isolate	**Δlog_{10} CFU/mL**			
P. aeruginosa	0 h	4 h	8 h	24 h
Control	0	1.3	2.4	3.5
AP-7121	0	1.4	2.3	3.2
Colistin	0	-0.4	-1.2	-3.1
Colistin/AP-7121	0	**-1.8**	**-2.6**	**-3.8**
***A. baumannii* complex**				
Control	0	1.1	2.2	3.1
AP-7121	0	1.0	2.4	3.3
Colistin	0	-0.5	-1.4	-3.0
Colistin/AP-7121	0	**-2.0**	**-2.9**	**-3.8**

alone, showing a bactericidal effect achieved with the combination antimicrobial/bacteriocin for the studied isolates.

A traditional strategy for the treatment of infections with multi-resistant P. aeruginosa or A. baumannii is based on the combination of conventional antimicrobials in order to decrease the MIC values and be effective. A previous study assessed the efficacy colistin in combination with three different antimicrobials. Even though a drop of MICs was observed when compared to the ones for the individual antimicrobials, combinations were effective only in 13-20% of the resistant isolates while in most cases there was an additive/indifferent effect [17].

In addition, there was a lower fold decrease of MIC values for the combinations of colistin with other antimicrobials than the obtained with the combination colistin/AP-7121.

Combination of two or more conventional antimicrobials for the treatment of multi-drug resistant infections has another disadvantages compared to the use of a bacteriocin-antimicrobial combination. Resistance to new antimicrobials already emerged and together with an inadequate and non-controlled use of these drugs might contribute with a higher increase in resistance. The emergence and widespread of resistance of new antimicrobials is a significant drawback for therapy of these infections since might delay active treatment in patients with severe infections [7].

Decreased MIC values of the combination colistin-AP-7121 in the assayed human bloodstream P. aeruginosa and A. baumannii complex needs to be highlighted not only for contributing with less selective pressure for the emergence and spread of colistin resistance. Also, these results reinforce the possibility that a colistin-bacteriocin combination could potentially lead to a reduction of the toxic effects of colistin, as it was reported when this antimicrobial was assayed together with nisin [18].

The observed MICs reduction needs to be highlighted from the microbiological and therapeutical points of view, considering that nowadays colistin is still considered as one of the first-line treatment options for multi-drug resistant isolates of these bacterial species [7, 19].

Also, an early (4-8 h) and late (24 h) synergistic effect of the combination between colistin and AP-7121 was detected for P. aeruginosa and A. baumannii complex isolates in this study.

Recently, the synergistic effect of AP-7121 combined with conventional antimicrobials such as gentamicin and vancomycin, against Gram positive pathogenic bacteria was achieved [14]. Furthermore, when AP-7121 was assayed together with colistin for Gram negative bacteria of food origin (ground-beef Escherichia coli), bactericidal activity and a synergy were proven. These results suggested that the detergent effect of colistin against the outer membrane of Gram negative bacteria allowed AP-7121 to form pores that would lead to an osmotic shock followed by cell death, as it was previously observed for the enterocin against Gram positive bacteria [10, 20].

Other authors studied the possible combinations of an antimicrobial, polimixin B, with two bacteriocins, nisin and garvicin KS, with different results for each scheme. When there were assayed against A. baumannii, synergy was observed for garvicin KS-polimixin B but not for nisin-polimixin B mixtures, showed by the prevention or failing of bacteria regrowth. When the antimicrobial was tested together with both bacteriocins, they showed, as AP7121 did, an early synergistic effect after 4 h. However, there was no synergistic effect detected against P. aeruginosa when combinations of polimixin B with the two bacteriocins [8].

5. Concluding Remarks

According to the in-vitro obtained results, AP-7121 could be a candidate as an alternative option for the combination with colistin against human P. aeruginosa and A. baumannii complex isolates producers of bloodstream infections.

Their synergistic activity against these bacteria, leads to a bactericidal activity of AP7121, with lower MIC values and a potential reduction of colistin toxicity.

Further in vitro and in vivo studies need to be conducted in order to achieve a more comprehensive and thorough knowledge about the toxicity reduction effect over colistin, as well as the potential future availability and application of AP7121 in combination with this antimicrobial as a complementary tool for the treatment of these severe infectious diseases caused by multi-resistant Gram negative bacteria, and consider it as basic step for extending its usefulness for the prevention or treatment of other kinds of human infections.

Acknowledgements

Authors thank to the Centro de Estudios Bioquímicos (Tandil, Argentina) for supporting and funding this study.

References

[1} B. Isler, Y. Doi, R.A. Bonomo, D.L. Paterson, Antimicrob Agents Chemother 63 (2018) e01110–e01118.
[2] L. Nguyen, J. Garcia, K. Gruenberg, C. MacDougall, Curr Infect Dis Rep 20 (2018) 23.
[3] R. Gahlot, C. Nigam, V. Kumar, G. Yadav, S. Anupurba, Int J Crit Illn Inj Sci 4 (2014) 162-167.
[4] Red WhoNet Argentina (2018). http://antimicrobianos.com.ar/2018/?cat=10.
[5] M. Bassetti, A. Vena, A. Croxatto, E. Righi, B. Guery, Drugs Context 7 (2018) 212527.
[6] M. Bassetti, P. Peghin, M. Vena, D.R. Giacobbe. Front Med (Lausanne) 6 (2019) 74.
[7] H. Chi, H. Holo, Curr Microbiol 75 (2018) 272-277.
[8] G. Delpech, N. García Allende, S. Lissarrague, M. Sparo, Open Infect Dis J 10 (2018a) 79-87.
[9] G. Delpech, M. Bistoletti, M. Ceci, S. Lissarrague, S.S. Bruni,

M. Sparo, Probiotics Antimicrob Proteins 9 (2017) 355-362.
[10] G. Delpech, E.M. Hebert, M. Sparo, L. Saavedra, Microbiol Resour Announc 8 (2019) e00245-19.
[11] M. Sparo, (2006a) Ph.D. thesis. University of Buenos Aires, Argentina.
[12] M.D. Sparo, M.S. Castro, P.J. Andino, M.V. Lavigne, C. Ceriani, G.L. Gutiérrez, M.M. Fernández, M.C. De Marzi, E.L. Malchiodi, M.A. Manghi, J Appl Microbiol 100 (2006b) 123-134.
[13] A.M. Leber, ed., Clinical Microbiology Procedures Handbook, fourth ed., ASM Press, Washington DC, 2016..
[14] Clinical and Laboratory Standards Institute. CLSI (2018) M100-S28.
[15] National Nosocomial Infections Surveillance System. National Nosocomial infections surveillance (NNIS) system report, data summary from January 1992 through June 2004. Am J Infect Control. 2004;32:470–485.
[16] O. Robineau, J. Robert, C. Rabaud, J.P. Bedos, E. Varon, Y. Péan, R. Gauzit, S. Alfandari, Infect Drug Resist 11 (2018) 1259-1368.
[17] H. Lee, K.H Roh, S.G. Hong, H.B. Shin, S.H. Jeong, W. Song, Y. Uh, D. Yong, K. Lee, Ann Lab Med 36 (2016) 138-144.
[18] G. Delpech, M, Ceci, L. García Allende, M. Sparo, J Vet Pharm Therap 41, Suppl 1 (2018b) 126.
[19] K. Naghmouchi, J. Baah, D. Hober, E. Jouy, C. Rubrecht, S. Famara, D. Drider, Antimicrob Agents Chemother 57 (2013) 2719-2725.
[20] N. Martis, S. Leroy, V. Blanc, J Infect 69 (2014) 1-12.