In vitro Activities of Cationic Steroid Antibiotics, CSA-13, CSA-131, CSA-138, CSA-142 and CSA-192 against carbapenem-resistant *Pseudomonas aeruginosa*

Katyonik Steroid Antibiyotiklerden CSA-13, CSA-131, CSA-138, CSA-142 ve CSA-192'nin Karbapenem Dirençli *Pseudomonas aeruginosa* Suşlarına Karşı İn vitro Aktivitesi

Short title: Activities of CSAs against resistant *P. aeruginosa*

Kısa başlık: Dirençli *P. aeruginosa*'ya karşı CSA'ların aktiviteleri

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INTRODUCTION: *Pseudomonas aeruginosa* is one of the important opportunistic pathogen that is difficult to treat because of the antibiotic resistance developed in recent years.

Developing carbapenem-resistance led to an increase in hospital infections caused by this bacterium. As a result, researchers began to search for new molecules. Ceragenins are the general name of membrane-acting cationic steroid antimicrobial molecules that have activity similar to antimicrobial peptides. In this study, we investigated the in vitro activities of cationic steroid antibiotics (CSA's) CSA-13, CSA-131, CSA-138, CSA-142, CSA-192 and colistin on carbapenem-resistant *P. aeruginosa* (CRPA).

METHODS: MICs and MBCs were determined by broth dilution method.

RESULTS: MIC₅₀ (µg / ml) values of CSA-13, CSA-131, CSA-138, CSA-142, CSA-192, colistin and meropenem are 8, 4, 8, 16, 32, 1 and 16 respectively. The MBC values were equal to or twice the MIC values.

DISCUSSION AND CONCLUSION: In conclusion, we think CSA-131 and CSA-138 will be good candidates for CRPA treatment. However, the lack of stability, efficacy and pharmacokinetic properties of CSA requires further research in the future in vivo and in vitro.

Keywords: Cationic steroid antibiotic, carbapenem-resistant *Pseudomonas aeruginosa*, colistin, MIC, MBC

Introduction

Carbapenems are one of the frequently used important antipseudomonal drug, although high carbapenem resistance rates in *Pseudomonas aeruginosa* isolates has been increasingly reported worldwide.^{1,2,3} In 2017, WHO published the list of antibiotic resistant priority pathogens for which new antibiotics are urgently needed. One of the most critical group of all is carbapenem-resistant *P. aeruginosa* (CRPA).⁴ In such cases, colistin is the only option for treatment.⁵ Moreover, the lack of effective antibiotics against CRPA isolates led to the re-use of colistin. Colistin has shown good *in vitro* activity against Gram negative bacilli, including *P. aeruginosa*. However, resistance to colistin is emerging all across the globe.⁶

There is an urgent need to develop alternative compounds and approaches to combat CRPA. Antimicrobial peptides (AMPs) have recently been extensively researched as potential antimicrobial agents. AMPs have the advantages of selectivity, activity rate and the ability of bacteria not to develop resistance to these peptides.^{1,}

In addition to these properties of AMPs, there are problems in usage such as linear peptide structure, proteolysis by bacteria without, production in excess amount is expensive, short half-life in circulation and less toxic effect against microorganisms in micromolar use.⁸ Because of these reasons, researchers have sought new antimicrobial agents using peptide and peptide analogues. One of them, ceragenin, is a general name for membrane-acting cationic steroid antibiotics (CSAs) and have been developed as non-peptide forms of endogenous cationic AMPs. CSAs are cationic, amphiphilic molecules that mimic AMP but do not have the undesirable properties of AMPs. Due to their lipophilic nature they target pathogen membranes, causing morphological changes in membrane structure leading to cell death. Many CSAs display broad-spectrum antimicrobial activities against both Gram-positive and Gram-negative bacteria, including multi-resistant strains, parasites, some viruses and fungus.⁹⁻¹⁸

CSA-13, the first generation prototype of CSA molecule, is highly effective against Gram-positive and Gram-negative bacteria.^{12, 19} The low toxicity of CSA-13 in animal studies supports the possible application of this compound in human

therapy.²⁰ CSA-131, CSA-138, CSA-142 and CSA-192 molecules are in later generation group. There are few published studies about these second generation molecules against resistant *P. aeruginosa* strains.^{6, 19} Therefore, in the present study, we assess the in vitro activities of CSA-13, CSA-131, CSA-138, CSA-142 and CSA-192 against CRPA strains and compare their activity to the/with colistin.

Materials and Methods

Bacterial isolates:

Twenty clinical strains of unrelated CRPA recently isolated from various samples from Canakkale Onsekiz Mart University, Health Practice and Research Center Microbiology Laboratory, Turkey, in 2016-2017. Isolates were defined as carbapenem-resistant strains using the disc diffusion and microdilution method. All strains were identified by the Vitek2 Compact System. *P. aeruginosa* ATCC 27853 (Rockville, Md., USA) was used as a quality control strain.

Antimicrobial agents:

CSA-13, CSA-131, CSA-138, CSA-142 and CSA-192 were synthesized from a cholic acid scaffold technique as previously described (Figure 1).²¹ Colistin and meropenem were obtained from AstraZeneca (Turkey). Ceragenins were prepared at a concentration of 2560 µg/ml and stored frozen at -80°C. Colistin and meropenem were prepared at a concentration of 5120 µg/ml. Meropenem was prepared on the day of use. Frozen solutions of antibiotics were used within 6 months.

Media:

Mueller-Hinton Broth (MHB, Difco Laboratories, Detroit, MI, USA) supplemented with divalent cations to final concentration of 25 mg of Mg⁺² and 50 mg of Ca⁺² per liter (CSMHB) was used for all the experiments. Pour plates of tryptic soy agar (TSA, Difco Laboratories, Detroit, MI, USA) were used for colony counts.

Determination of MICs and MBCs:

MICs (Minimum inhibitory concentrations) were determined by the microbroth dilution technique as described by the CLSI.^{22, 23} Serial two-fold dilutions ranging from 128 to 0.06 µg/ml were prepared in CSMHB. The inoculum was prepared with 4- to 6- hour broth culture that gives final concentration of 5×10^5 cfu/ml in the test tray. Viable cell counts were performed with each test to verify the number of colony-forming units in

each inoculum. The trays were covered and placed in plastic bags to prevent evaporation and incubated at 37°C for 18-20 h. The MIC was defined as the lowest concentration of antibiotic giving complete inhibition of visible growth. Experiments were performed duplicate. MBCs (minimum bactericidal concentrations) were determined at the conclusion of the incubation period by removing two 0.01 ml samples from each well demonstrating no visible growth and plated onto TSA. Resultant colonies were counted after an overnight incubation at 37°C. The MBC was defined as the lowest concentration of antibiotic giving at least a %99.9 killing of the initial inoculums.²⁴

Results

MIC ranges for CSA-13, CSA-131, CSA-138, CSA-142, CSA-192, colistin and meropenem against 20 clinical CRPA were 4-16, 2-8, 4-32, 8-32, 8-32, 0.5-2 and 8-128 (µg/ml), respectively (Table I, Table III). The MBCs were generally equal to or two fold greater than those of the MICs (Table I, Table IV). MIC and MBC studies were conducted with three replicates. CSA-131 and CSA-138 followed by CSA-13 may be good candidates for infections caused by these bacteria. CSA-142 and CSA-192 are less effective. *P. aeruginosa* ATCC 27853 strain was used as a standard strain and MIC results against quality control strains are as expected (Table II).

Discussion

Today, the treatment of *P. aeruginosa* infections is becoming increasingly difficult due to developing antibiotic resistance. CRPA is in the second microorganism after carbapenem resistant *A. baumannii*, in the list of bacteria for which new antibiotics are urgently needed published by WHO in 2017.⁴ This led researchers to search for new therapeutic agents. One of them is CSA molecule. In this study, we investigated the in vitro effects of CSA-13, CSA-131, CSA-138, CSA-142 and CSA-192 against 20 clinical unrelated CRPA strains.

There are several studies in the literature investigating the effect of CSA molecules on *P. aeruginosa*. Vila-Farrés et al. investigated the in vitro activities of CSA-13, CSA-44, CSA-131 and CSA-138 against colistin-susceptible and resistant strains of *A. baumannii* and *P. aeruginosa* in 2015. They showed that, CSA-131 is a good candidate for the treatment of *P. aeruginosa* and *A. baumannii* infections.⁶

In the present study, the MIC₅₀ values of (µg/ml) CSA-13, CSA-131, CSA-138, CSA-142, CSA-192 and colistin against 20 CRPA strains isolated from various samples were 8, 4, 8, 16, 32 and 1, respectively. It is thought that CSA-131 has the best activity because it has long hydrophobic chain at C12 (Figure 1). It is promising that the MIC value of CSA-131 is 4 µg/ml, while the MIC value of colistin is 1 µg/ml. The fact that CSA-13 and CSA-138 have the best activity after CSA-131 is considered to have the long hydrophobic chain in the C8. As a result, the long hydrophobic chain structure that CSA possesses causes these molecules to move strongly through the bacterial membrane, leading to their strong activity. CSA-142 and CSA-192 were found to be less effective than the others. In our study, the MIC ranges of colistin against 20 CRPA strains were 0.5-2 µg/ml, and the MIC₅₀ value was 1 µg/ml and the MIC₉₀ value was 2 µg/ml. No colistin resistance was detected against any of the strains.

Sensitivity tests are of great importance for the selection of antibiotics to be used in the treatment. One of them is, the MIC test. MICs are predictive values of clinical outcome and estimate the bacteriostatic or inhibitory activity of antimicrobial agents. However, it is important to know bactericidal activity in the treatment of serious infections that may result in death due to inadequate treatment. Therefore, it is also important to determine the MBC values of *P. aeruginosa*, which is difficult to treat due to the developing antibiotic resistance. The MBC values of the antibiotics tested in our study were found to be equal to or twice the MIC values. This suggests that these antibiotics may produce the desired bactericidal effect in the treatment of CRPA infections.

In conclusion, according to our results, ceragenins especially CSA-131 and CSA-138 appear to be good candidates in the treatment of CRPA infections. But, future in vivo and in vitro studies should be performed to correlate the safety, efficacy, and pharmacokinetic parameters of these molecules.

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The authors report no declarations of interest.

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Figures:

Figure I: Structures of ceragenins CSA-13, CSA-131, CSA-138, CSA-142 and CSA-192.

Tables:

Table I: MIC and MBC values

Antimicrobials	MIC range	MIC₅₀	MIC₉₀	MBC range	MBC₅₀	MBC₉₀
CSA-13	4-16	8	16	4-32	16	32
CSA-131	2-8	4	4	2-8	4	8
CSA-138	4-32	8	8	4-32	8	16
CSA-142	8-32	16	32	8-64	32	64
CSA-192	8-32	32	32	8-64	32	32
Colistin	0.5-2	1	2	0.5-4	2	4
Meropenem	8-128	16	32	8-256	32	64

MIC₅₀, MIC₉₀: Defined as the antimicrobial concentration that inhibited growth of 50% and 90% of the strains.

MBC₅₀, MBC₉₀: Defined as the bactericidal concentration of 50% and 90% of the strains.

Table II: MIC values ($\mu\text{g/ml}$) of antimicrobial agents against *P. aeruginosa* ATCC 27853 strain

Antibiotics	MIC values
CSA-13	16
CSA-131	8
CSA-138	32
CSA-142	16
CSA-192	32
Colistin	2
Meropenem	1

Table III: MICs, MIC₅₀s and MIC₉₀s of the tested antimicrobial agents (µg/ml).

Antimicrobial agents	MIC	Number of isolates	MIC₅₀	MIC₉₀
CSA-13	4	2	8	16
	8	10		
	16	8		
CSA-131	2	5	4	4
	4	14		
	8	1		
CSA-138	4	1	8	8
	8	18		
	16	1		
CSA-142	8	7	16	32
	16	9		
	32	4		
CSA-192	8	1	32	32
	16	8		
	32	11		
COL	0.5	2	1	2
	1	8		
	2	10		
MER	2	2	16	32
	8	4		
	16	7		
	32	5		

	64	1
	128	1

COL:	Colistin,	MER:	Meropenem
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Table IV: MBCs, MBC₅₀s and MBC₉₀s of the tested antimicrobial agents (µg/ml).

Antimicrobial agents	MBC	Number of isolates	MBC ₅₀	MBC ₉₀
CSA-13	4	2	16	32
	8	4		
	16	8		
	32	6		
CSA-131	2	1	4	8
	4	14		
	8	5		
CSA-138	4	1	8	16
	8	13		
	16	5		
	32	1		
CSA-142	8	1	32	64
	16	8		
	32	7		
	64	4		
CSA-192	8	1	32	32
	16	3		
	32	15		
	64	1		
COL	0.5	2	2	4
	1	4		
	2	11		
	4	3		

MER		32	
	2	1	64
	4	1	
	8	2	
	16	3	
	32	7	
	64	5	
	256	1	
COL:	Colistin,	MER:	Merop