**In Vitro Activities of the 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 \textit{In Vitro} Aktivitesi

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**ABSTRACT**

**Objectives:** \textit{Pseudomonas aeruginosa} is an important opportunistic pathogen that is difficult to treat because of the antibiotic resistance that has developed in recent years. Increasing carbapenem resistance has led to a rise in hospital infections caused by this bacterium. As a result, researchers have begun to search for new molecules. Ceragenins are the general name for membrane-acting cationic steroid antimicrobial molecules that have activity similar to that of antimicrobial peptides. In this study, we investigated the \textit{in vitro} activities of the cationic steroid antibiotics (CSAs) CSA-13, CSA-131, CSA-138, CSA-142, CSA-192, and colistin on carbapenem-resistant \textit{Pseudomonas aeruginosa} (CRPA).

**Materials and Methods:** Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined by broth dilution method.

**Results:** The MIC\textsubscript{50} (µg/mL) values of CSA-13, CSA-131, CSA-138, CSA-142, CSA-192, colistin, and meropenem were 8, 4, 8, 16, 32, 1, and 16, respectively. The MBC values were equal to or twice the MIC values.

**Conclusion:** CSA-131 and CSA-138 appear to be good candidates for CRPA treatment. However, the lack of stability, efficacy, and pharmacokinetic properties of CSA requires further research in the future \textit{in vivo} and \textit{in vitro}.

**Key words:** Cationic steroid antibiotic, carbapenem-resistant \textit{Pseudomonas aeruginosa}, colistin, minimum inhibitory concentrations, minimum bactericidal concentrations

**ÖZ**

\textbf{Amaç:} Son yıllarda geliştirildiği antibiyotik direnci nedeniyle tedavisi zorlaşan önemli bakterilerden biri de \textit{Pseudomonas aeruginosa} (\textit{P. aeruginosa})\’dır. Önemli bir hastane enfeksiyon etkeni olan bu bakterinin karbapenem grubu antibiyotiklere karşı geliştirildiği direnç nedeniyle bu suşlarla meydana gelen enfeksiyonlarda önemli bir artış gözlüktür. Bu da araştırmacıları yeni moleküller üzerinde çalışmaya yönlendirmiştir. Cerageninler, antimikrobiyal peptidlere benzer şekilde aktivite gösteren, membrana etki eden kationik steroid antimikrobiyal moleküllerin genel ismidir. Bu çalışmada kationik steroid antibiyotiklerden kationik steroid antibiyotikler (CSA) olan CSA-13, CSA-131, CSA-138, CSA-142 ve CSA-192’nin ve kolistinin karbapenem dirençli \textit{P. aeruginosa} üzerine olan \textit{in vitro} etkilerini araştırılmıştır.

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Received: 02.04.2018, Accepted: 25.10.2018

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INTRODUCTION

Carbapenems are important frequently used antipseudomonal drugs, although high carbapenem resistance rates in Pseudomonas aeruginosa (P. aeruginosa) isolates have been increasingly reported worldwide.\(^\text{1,2}\) In 2017, the World Health Organization published a list of antibiotic-resistant priority pathogens for which new antibiotics are urgently needed. One of the most critical groups of all is carbapenem-resistant P. aeruginosa (CRPA).\(^\text{3}\) In such cases, colistin is the only option for treatment.\(^\text{4}\) Moreover, the lack of effective antibiotics against CRPA isolates has 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.\(^\text{5}\)

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.\(^\text{6}\) In addition to these properties of AMPs, there are problems in usage such as linear peptide structure, proteolysis, high cost of production in excess amount, short half-life in circulation, and less toxic effect against microorganisms in micromolar use.\(^\text{7}\) 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 they have been developed as nonpeptide 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 multiresistant strains, parasites, some viruses, and fungi.\(^\text{8,9}\)

CSA-13, the first generation prototype of CSA molecule, is highly effective against gram-positive and gram-negative bacteria.\(^\text{10,11}\) The low toxicity of CSA-13 in animal studies supports the possible application of this compound in human therapy.\(^\text{12}\) CSA-13, CSA-131, CSA-138, CSA-142, and CSA-192 molecules are in a later generation group. There are few published studies about these second generation molecules against resistant P. aeruginosa strains.\(^\text{12,13}\) Therefore, in the present study, we assessed the in vitro activities of CSA-13, CSA-131, CSA-138, CSA-142, and CSA-192 against CRPA strains and compared their activity to colistin.

MATERIALS AND METHODS

Bacterial isolates

Twenty clinical strains of unrelated CRPA were isolated from various samples from Çanakkale Onsekiz Mart University, Health Practice and Research Center Microbiology Laboratory, Turkey, in 2016-2017. The isolates were defined as carbapenem-resistant strains using disc diffusion and microdilution. 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).\(^\text{14}\) 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 a 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

Minimum inhibitory concentrations (MICs) were determined by the microbroth dilution technique as described by the CLSI.\(^\text{15,16}\) Serial twofold dilutions ranging from 128 to 0.06 µg/mL were prepared in CSMHB. The inoculum was prepared with 4- to 6-h broth culture that gives a final concentration of 5×10^6 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. The experiments were performed in duplicate. Minimum bactericidal concentrations (MBCs) 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 overnight incubation at 37°C. The MBC was defined as the lowest concentration of antibiotic giving at least 99.9% killing of the initial inocula.24

RESULTS

MIC ranges for CSA-13, CSA-131, CSA-138, CSA-142, CSA-192, colistin, and meropenem against 20 clinical CRPA strains were 4-16, 2-8, 4-32, 8-32, 0.5-2, and 8-128 (µg/mL), respectively (Table 1, Table 2). The MBCs were generally equal to or twofold greater than those of the MICs (Table 1, Table 3). 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 the MIC results against quality control strains were as expected (Table 4).

DISCUSSION

Today, the treatment of P. aeruginosa infections is becoming increasingly difficult due to growing antibiotic resistance. CRPA is the second microorganism, after carbapenem-resistant A. baumannii, in the list of bacteria for which new antibiotics are urgently needed published by the WHO in 2017.4 This has led researchers to search for new therapeutic agents. One of them is CSA molecules. In the present 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.6 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.

In the present study, the MIC50 values (µg/mL) of 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 a long hydrophobic chain at C12 (Figure 1). It is promising that the MIC value of CSA-13 is 4 µg/mL, while the MIC value of colistin is 1 µg/mL. The fact that CSA-13 and

![Figure 1. Structures of ceragenins CSA-13, CSA-131, CSA-138, CSA-142, and CSA-192](image)

CSA: Cationic steroid antibiotics

| 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    |

MIC<sub>50</sub>, MIC<sub>90</sub>: Defined as the antimicrobial concentration that inhibited growth of 50% and 90% of the strains, MBC<sub>50</sub>, MBC<sub>90</sub>: Defined as the bactericidal concentration of 50% and 90% of the strains, MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, CSA: Cationic steroid antibiotics.
CSA-138 have the best activity after CSA-131 is considered to be because of the long hydrophobic chain at 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 MIC 50 value was 1 µg/mL and the MIC 90 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 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 increasing antibiotic resistance. The MBC values of the antibiotics tested in our study were equal to or twice the MIC values. This suggests that these antibiotics may produce the desired bactericidal effect in the treatment of CRPA infections.

### Table 2. MIC, MIC<sub>50</sub>, and MIC<sub>90</sub> values of the tested antimicrobial agents (µg/mL)

| Antimicrobial agents | MIC | Number of isolates | MIC<sub>50</sub> | MIC<sub>90</sub> |
|----------------------|-----|--------------------|----------------|----------------|
| 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                  |                |                |
|                      | 16  | 8                  | 32             | 32             |
|                      | 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, MIC: Minimum inhibitory concentration, CSA: Cationic steroid antibiotic

### Table 3. MBC, MBC<sub>50</sub>, and MBC<sub>90</sub> values of the tested antimicrobial agents (µg/mL)

| Antimicrobial agents | MBC | Number of isolates | MBC<sub>50</sub> | MBC<sub>90</sub> |
|----------------------|-----|--------------------|----------------|----------------|
| CSA-13               | 4   | 2                  | 16             | 32             |
|                      | 8   | 4                  |                |                |
|                      | 16  | 8                  |                |                |
|                      | 32  | 6                  |                |                |
| CSA-131              | 2   | 1                  |                |                |
|                      | 4   | 14                 | 4              | 8              |
|                      | 8   | 5                  |                |                |
| CSA-138              | 4   | 1                  | 8              | 16             |
|                      | 16  | 5                  |                |                |
|                      | 32  | 1                  |                |                |
| CSA-142              | 8   | 1                  | 16             | 32             |
|                      | 32  | 7                  |                |                |
|                      | 64  | 4                  |                |                |
| CSA-192              | 8   | 1                  |                |                |
|                      | 16  | 3                  | 32             | 32             |
|                      | 32  | 15                 |                |                |
|                      | 64  | 1                  |                |                |
| COL                  | 0.5 | 2                  | 2              | 4              |
|                      | 1   | 4                  |                |                |
|                      | 2   | 11                 |                |                |
|                      | 4   | 3                  |                |                |
| MER                  | 2   | 1                  |                |                |
|                      | 4   | 1                  |                |                |
|                      | 8   | 2                  | 32             | 64             |
|                      | 16  | 3                  |                |                |
|                      | 32  | 7                  |                |                |
|                      | 64  | 5                  |                |                |
|                      | 256 | 1                  |                |                |

COL: Colistin, MER: Meropenem, MBC: Minimum bactericidal concentration, CSA: Cationic steroid antibiotic

### Table 4. MIC values (µg/mL) of antimicrobial agents against Pseudomonas 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          |

CSA: Cationic steroid antibiotic MIC: Minimum inhibitory concentration

### CONCLUSION

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

ACKNOWLEDGEMENTS

This work was supported by the Research Fund of İstanbul University under project no: 23843.

Conflicts of interest: No conflict of interest was declared by the authors.

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