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ORIGINAL ARTICLE

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 *İn Vitro* Aktivitesi

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ABSTRACT

Objectives: *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 *in vitro* activities of the cationic steroid antibiotics (CSAs) CSA-13, CSA-131, CSA-138, CSA-142, CSA-192, and colistin on carbapenem-resistant *Pseudomonas aeruginosa* (CRPA).

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

Results: The MIC_{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 *in vivo* and *in vitro*.

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

ÖZ

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

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Gereç ve Yöntemler: Minimum inhibitör konsantrasyonları (MİK) ve minimum bakterisidal konsantrasyonlar (MBK) değerleri mikrodilüsyon yöntemi ile belirlenmiştir.

Bulgular: $MİK_{50}$ (µg/mL) değerleri, CSA-13, CSA-131, CSA-138, CSA-142, CSA-192, kolistin ve meropenem için sırasıyla 16, 4, 8, 32, 32, 2 ve 32'dir. MBK değerleri, MİK değerlerine eşit ya da iki katı olarak saptanmıştır.

Sonuç: Sonuç olarak CSA-131 ve CSA-138'in karbepenem dirençli *P. aeruginosa* tedavisinde iyi birer aday olacağı düşüncesindeyiz. Ancak CSA'ların stabilite özellikleri, etkinlikleri ve farmakokinetik özelliklerindeki eksiklikler, gelecekte bu konuda daha fazla araştırma yapılmasını gerektirmektedir.

Anahtar kelimeler: Katyonik steroid antibiyotik, karbapenem dirençli *Pseudomonas aeruginosa*, kolistin, minimum inhibitör konsantrasyonları, minimum bakterisidal konsantrasyonlar

INTRODUCTION

Carbapenems are important frequently used antipseudomonal drugs, although high carbapenem resistance rates in *Pseudomonas aeruginosa* (*P. aeruginosa*) isolates have been increasingly reported worldwide.[1-3] 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).[4] In such cases, colistin is the only option for treatment.[5] 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.[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.[1,7] 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.[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 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.[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 a 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 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).[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 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.[22,23] 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 5×10^5 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

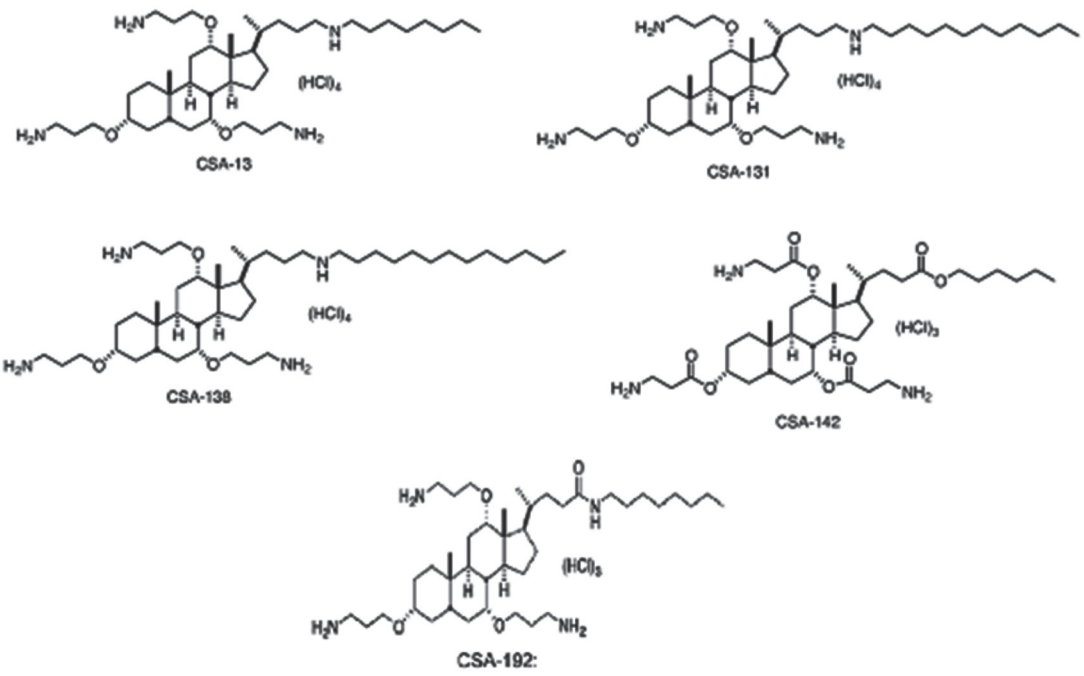

Figure 1. Structures of ceragenins CSA-13, CSA-131, CSA-138, CSA-142, and CSA-192

CSA: Cationic steroid antibiotics

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, 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.

Table 1. MIC and MBC values

Antimicrobials	MIC range	MIC_{50}	MIC_{90}	MBC range	MBC_{50}	MBC_{90}
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_{50}, MIC_{90}: Defined as the antimicrobial concentration that inhibited growth of 50% and 90% of the strains, MBC_{50}, MBC_{90}: Defined as the bactericidal concentration of 50% and 90% of the strains, MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, CSA: Cationic steroid antibiotics

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 MIC_{50} 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-131 is 4 μg/mL, while the MIC value of colistin is 1 μg/mL. The fact that CSA-13 and

Table 2. MIC, MIC_{50}, and MIC_{90} values of the tested antimicrobial agents (µg/mL)

Antimicrobial agents	MIC	Number of isolates	MIC_{50}	MIC_{90}
CSA-13	4 8 16	2 10 8	8	16
CSA-131	2 4 8	5 14 1	4	4
CSA-138	4 8 16	1 18 1	8	8
CSA-142	8 16 32	7 9 4	16	32
CSA-192	8 16 32	1 8 11	32	32
COL	0.5 1 2	2 8 10	1	2
MER	2 8 16 32 64 128	2 4 7 5 1 1	16	32

COL: Colistin, MER: Meropenem, MIC: Minimum inhibitory concentration, CSA: Cationic steroid antibiotic

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 3. MBC, MBC_{50}, and MBC_{90} values of the tested antimicrobial agents (µg/mL)

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

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.

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

References

1. Bassetti M, Poulakou G, Ruppe E, Bouza E, Van Hal SJ, Brink A. Antimicrobial resistance in the next 30 years, humankind, bugs and drugs: a visionary approach. Intensive Care Med. 2017;43:1464-1475.
2. Davies TA, Marie Queenan A, Morrow BJ, Shang W, Amsler K, He W, Lynch AS, Pillar C, Flamm RK. Longitudinal survey of carbapenem resistance and resistance mechanisms in Enterobacteriaceae and non-fermenters from the USA in 2007-09. J Antimicrob Chemother. 2011:66:2298-2307.
3. El-Mahdy TS. Expression of ampC, oprD, and mexA, outer membrane protein analysis and carbapenemases in multidrug resistant clinical isolates of Pseudomonas aeruginosa from Egypt. J Chemother. 2014:26:379-381.
4. World Health Organization. WHO publishes list of bacteria for which new antibiotics are urgently needed (2017). Accessed: 14 March. 2017. http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/
5. Falagas ME, Bliziotis IA. Pandrug-resistant Gram-negative bacteria: the dawn of the postantibiotic era? Int J Antimicrob Agents. 2007;29:630-636.
6. Vila-Farrés X, Callarisa AE, Gu X, Savage PB, Giralt E, Vila J. CSA-131, a ceragenin active against colistin-resistant Acinetobacter baumannii and Pseudomonas aeruginosa clinical isolates. Int J Antimicrob Agents. 2015:46:568-571.
7. Smeianov V, Scott K, Reid G. Activity of cecropin P1 and FA-LL-37 against urogenital microflora. Microbes Infect. 2000;2:773-777.
8. Epand RM, Epand RF, Savage PB. Ceragenins (Cationic Steroid Compounds), a novel class of antimicrobial agents. Drug News Perspect. 2008:21:307-311.
9. Chin JN, Jones RN, Sader HS, Savage PB, Rybak MJ. Potential synergy activity of the novel ceragenin, CSA-13, against clinical isolates of Pseudomonas aeruginosa, including multidrug-resistant P. aeruginosa. J Antimicrob Chemother. 2008:61:365-370.
10. Chin JN, Rybak MJ, Cheung CM, Savage PB. Antimicrobial activities of ceragenins against clinical isolates of resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2007:51:1268-1273.
11. Durnaś B, Wnorowska U, Pogoda K, Deptuła P, Wątek M, Piktel E, Głuszek S, Gu X, Savage PB, Niemirowicz K, Bucki R. Candidacidal Activity of Selected Ceragenins and Human Cathelicidin LL-37 in Experimental Settings Mimicking Infection Sites. PLoS One. 2016;11:e0157242.
12. Epand RF, Pollard JE, Wright JO, Savage PB, Epand RM. Depolarization, bacterial membrane composition, and the antimicrobial action of ceragenins. Antimicrob Agents Chemother. 2010:54:3708-3713.
13. Epand RF, Savage PB, Epand RM. Bacterial lipid composition and the antimicrobial efficacy of cationic steroid compounds (Ceragenins). Biochim Biophys Acta. 2007;1768:2500-2509.
14. Howell MD, Streib JE, Kim BE, Lesley LJ, Dunlap AP, Geng D, Feng Y, Savage PB, Leung DY. Ceragenins: a class of antiviral compounds to treat orthopox infections. J Invest Dermatol. 2009:129:2668-2675.
15. Lara D, Feng Y, Bader J, Savage PB, Maldonado, RA. Anti-trypanosomatid activity of ceragenins. J Parasitol. 2010;96:638-642.
16. Leszczynska K, Namiot D, Byfield FJ, Cruz K, Zendzian-Piotrowska M, Fein DE, Savage PB, Diamond S, McCulloch CA, Janmey PA, Bucki R. Antibacterial activity of the human host defence peptide LL-37 and selected synthetic cationic lipids against bacteria associated with oral and upper respiratory tract infections. J Antimicrob Chemother. 2013;68:610-618.
17. Polat ZA, Savage PB, Genberg C. *In vitro* amoebicidal activity of a ceragenin, cationic steroid antibiotic-13, against Acanthamoeba castellanii and its cytotoxic potential. J Ocur Pharmacol Ther. 2011;27:1-5.
18. Savage PB, Li C, Taotafa U, Ding B, Guan Q. Antibacterial properties of cationic steroid antibiotics. FEMS Microbiol Lett. 2002;217:1-7.
19. Bozkurt-Guzel C, Savage PB, Gerceker AA. In vitro activities of novel ceragenin, CSA-13, alone or combination with colistin, tobramycin and ciprofloxacin against Pseudomonas aeruginosa strains isolated from cystic fibrosis patients. Chemotherapy. 2011;57:505-510.
20. Saha S, Savage PB, Bal M. Enhancement of the efficacy of erythromycin in multiple antibiotic resistant gram-negative bacterial pathogens. J Appl Microbiol. 2008;105:822-828.
21. Guan Q, Li C, Schmidt EJ, Boswell JS, Walsh JP, Allman GW, Savage PB. Preparation and characterization of cholic acid-derived antimicrobial agents with controlled stabilities. Org Lett. 2000;2:2837-2840.
22. Clinical and Laboratory Standards Institute.Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard-(10 th ed). CLSI document. USA; 2015:7-10.
23. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. CLSI document (27th ed). USA; 2016:100-127.
24. National Committee for Clinical Laboratory Standarts. Methods for Determining Bactericidal Activity of Antimicrobial Agents-Approved Guideline, USA; 2005:26.