

Journal of Chemotherapy

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/yjoc20

Antibacterial and antibiofilm activities of ceragenins against *Achromobacter* species isolated from cystic fibrosis patients

Damla Damar-Çelik, Emel Mataracı-Kara, Paul B. Savage & Berna Özbek-Çelik

To cite this article: Damla Damar-Çelik, Emel Mataracı-Kara, Paul B. Savage & Berna Özbek-Çelik (2021) Antibacterial and antibiofilm activities of ceragenins against *Achromobacter* species isolated from cystic fibrosis patients, Journal of Chemotherapy, 33:4, 216-227, DOI: 10.1080/1120009X.2020.1819702

To link to this article: https://doi.org/10.1080/1120009X.2020.1819702

Published online: 26 Sep 2020.

Submit your article to this journal

Article views: 354

View related articles

View Crossmark data

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=yjoc20

Check for updates

Antimicrobial Original Research Paper

Antibacterial and antibiofilm activities of ceragenins against *Achromobacter* species isolated from cystic fibrosis patients

Damla Damar-Çelik[1], Emel Mataracı-Kara[1], Paul B. Savage[2], Berna Özbek-Çelik[1]

[1]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Istanbul University, Beyazit-Istanbul, Turkey; [2]Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT, USA

Achromobacter species, which are recognized as emerging pathogens isolated from patients with cystic fibrosis, are capable of forming biofilm in the respiratory tract in patients and innate multidrug resistance to antimicrobials. CSAs are cationic salt derivatives that mimic the activity of antimicrobial peptides and exhibit antimicrobial activity against bacteria. In this study, the in vitro activities of various ceragenins against Achromobacter-species biofilms were investigated comparatively with a conventional antibiotic (meropenem). Biofilm-formation inhibition and biofilm-adhesion inhibition were investigated on five strong biofilm-producing strains. The lowest MIC_{50} result was obtained with CSA-13. All of the tested CSAs showed significant biofilm inhibitory activity in the manner of a time- and concentration-dependent effect. To the best of our knowledge, this is the first article to evaluate the antibacterial and antibiofilm activities of tested CSAs against Achromobacter species.

Keywords: *Achromobacter* species, antibacterial, biofilm, ceragenins, cystic fibrosis

Introduction

Biofilms are aggregates of microorganisms embedded in an extracellular polymeric matrix that is self-produced, and they are commonly attached to living or nonliving surfaces and may be widespread in nature, hospital settings, and industry.[1–3] Biofilm formation performs an essential role in the development of infectious diseases because of the morbidity and mortality in patients and increased resistance to antimicrobial agents.[4–8] Notably, chronic lung infection and chronic inflammation-related biofilm promote mortality and morbidity in patients with cystic fibrosis (CF).[9] *Pseudomonas aeruginosa, Haemophilus influenzae, and Staphylococcus aureus* are known as typical CF pathogens. However, recently some pathogens infecting the lower airways have been identified using enhanced microbiologic techniques. One of them is *Achromobacter* spp.[10] The genus *Achromobacter* belongs to the class of non-fermentative bacilli, which are opportunistic pathogens in the CF patient. The prevalence of *Achromobacter* spp. infection/colonization varies between 5% and 29% in patients with CF and is rapidly increasing.[11–13] Although there is a limited number of studies examining the clinical impact of *Achromobacter* spp. infection in patients with CF, *Achromobacter xylosoxidans* is well-known in CF populations. Furthermore, this bacterium can cause various infections like bacteremia, endocarditis, and meningitis in non-CF or immunocompromised patients.

These bacteria are usually resistant to a wide range of antibiotics, including aminoglycosides, fluoroquinolones, and broad-spectrum beta-lactams. Antimicrobial treatment may be difficult due to increased antimicrobial resistance, and frequently, there is no correlation between susceptibility profiles and *Achromobacter* species. Thus, antimicrobial therapy should be conducted according to the susceptibility of each *Achromobacter* spp.[14,15]

Antimicrobial peptides (AMPs) have an essential role in innate immunity. Ceragenins or cationic steroid antibiotics (CSAs) are a family of bile acid derivatives that have been modified to yield an amphiphilic morphology similar to that of endogenous AMPs.[16,17] They were improved to mimic the activities of AMPs and destroy the cell membranes of bacteria, and they display antibacterial activity.[18]

Correspondence to: Berna Özbek Çelik, Istanbul University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, 34116 Beyazit, Istanbul, Turkiye. E-mail: bernaozbek@hotmail.com

© 2020 Edizioni Scientifi che per l'Informazione su Farmaci e Terapia
DOI 10.1080/1120009X.2020.1819702

They are not salt-sensitive or peptide-based and are relatively easy to prepare. CSAs can actively interact with anionic cell surfaces and cause transient pore formation in microbial membranes, leading to significant membrane depolarization and eventually resulting in cell death.

Moreover, the ceragenins have benefits over AMPs, such as their resistance to proteolysis and their predisposition to large-scale synthesis. While ceragenins possess bactericidal activity against both gram-negative and gram-positive bacteria, minimal inhibition concentrations (MICs) of them are typically lower against gram-positive organisms.[19,20]

Furthermore, only a few studies have investigated the antibiofilm activity of ceragenins against several microorganisms. For this reason, there are no published studies about the antimicrobial and antibiofilm activity of ceragenins against *Achromobacter* species biofilms. In this study, we aimed to determine the antimicrobial effects of ceragenins against *Achromobacter* species biofilms.

Materials and methods

Bacterial strains

For this study, 52 non-duplicate *Achromobacter* strains cultured from CF patients and the type strain (LMG 1863T) were obtained from the Department of Clinical Microbiology, Aarhus University Hospital, Aarhus, Denmark. The API 20 NE identification kits were used for the identification. Additionally, identification, *nrdA* allele, and PFGE profile were reported.[21] As reference strains, *P. aeruginosa* ATCC 27853 was used as quality control strains to verify the accuracy of the microdilution test procedure. Five *Achromobacter* strains that had the highest ability to form biofilms were selected for biofilm studies. PAO1 was used as a positive control to compare biofilm formation.[22,23]

Antimicrobial agents

CSA-13, CSA-44, CSA-131, CSA-138, CSA-142, and CSA-192 were synthesized from a cholic acid scaffold technique, as previously described.[24–26] Also, the CSA-131-P compound, which was formulated with a poloxamer (Pluronic® F127), was investigated for its antimicrobial activities. Stock solutions of CSAs were prepared in water and stored frozen at −80 °C to be used within six months. Meropenem solutions were prepared daily.

Media

Tryptic soy broth (TSB) supplemented with 1% glucose was used in studies for biofilm production and minimum biofilm eradication concentration (MBEC) determinations. Cation-adjusted Mueller-Hinton broth (CAMHB) was used for MIC determination. Tryptic soy agar (TSA) was used for colony counts and MBEC determinations.

MIC Determinations

MICs of CSAs and meropenem were determined by the broth microdilution technique that conformed with the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[27] Serial two-fold dilutions ranging from 64 to 0,125 mg/L were prepared in CAMHB for antimicrobial agents. The turbidity of pure broth cultures was adjusted to the 0.5 McFarland standard and diluted in CAMHB to give a final inoculum of 5×10^5 CFU/ml. Samples were allowed to incubate at 37 °C for 18–20 h. Each experiment was performed in duplicate.

Biofilm formation

52 *Achromobacter* strains isolated from CF patients were evaluated for biofilm-forming ability using the microtiter-plate method.[23] Briefly, to prepare bacterial inoculum, a loop of overnight *Achromobacter* strains was cultured in 5 ml TSB supplemented with 1% glucose in an orbital shaker (50 rpm) and then incubated at 37 °C for 24 h. Then, this overnight culture was diluted in fresh TSB supplemented with 1% glucose to provide a final concentration of approximately 1×10^7 CFU/200 µl. 200 µl of this suspension was added to the wells of 96-well tissue culture microtiter plates (Greiner) and allowed to incubate at 37 °C for 24 h. Control wells contained *Achromobacter* strains alone and medium alone. After the incubation, the waste medium was aspirated carefully. Each well was washed three times with 200 µl of sterile, physiological buffered saline to remove unattached bacteria from the wells and then air-dried. Next, the attached bacteria in the well were fixed with a 200 µl volume of 99% methanol for 15 min and aspirated; then the plates were left to dry. Plates were stained with 200 µl of 0.1% crystal violet (in water) for 5 min per well. The excess stain was quickly rinsed off with tap water, and the plates were left to dry. After the plates were dried, the stain was resolubilized using 200 µl of 95% ethanol with shaking on an orbital shaker for 30 min, and the OD 595 nm (OD 595) was measured.[22,23] All experiments were performed in two independent assays for each of the 52 *Achromobacter* strains.

Biofilm attachment assays

Biofilm attachment assays were done as described previously with the following modifications. An overnight culture of five strong, biofilm-producing *Achromobacter* strains was diluted 1/50 to obtain

Table 1 The distribution of 52 *Achromobacter* spp. and their biofilm-forming capabilities

Species	No. of strains tested.	No. of strains showing			
		No adherence (0)	Weak adherence (+)	Moderate adherence (+ +)	Strong adherence (+ + +)
A. xylosoxidans	22	12	7	–	3
A. insuavis	11	11	–	–	–
A. aegrifaciens	2	1	–	1	–
A. denitrificans	1	1	–	–	–
A. ruhlandii	9	8	1	–	–
A. dolens	1	–	1	–	–
A. genogroup	3	1	–	–	2
A. piechaudii	1	–	–	1	–
A. mucicolens	1	1	–	–	–
A spiritinus	1	1	–	–	–

1×10^7 CFU/200 µl in TSB supplement with 1% glucose. Then all strains were added to each well of 96-well tissue culture microtiter plates with 1/10× MIC of CSAs and meropenem, respectively. The plates were allowed to incubate for 1, 2, and 4 h at 37 °C. The positive control was *Achromobacter* strains in TSB supplemented with 1% glucose alone. After incubation, each well was washed with PBS solution three times and measured at OD 595 nm.[28]

Inhibition of biofilm formation

Each of the five tested *Achromobacter* strains were incubated in TSB supplemented with 1% glucose and antimicrobials (CSAs or meropenem at 1× and 1/10× in addition to 1/100× MIC) in 37 °C for 24 h in microtiter plates. Six wells were used for each CSA and antibiotic. The positive control was *Achromobacter* strains in TSB supplemented with glucose without antibiotics. After incubation, each well was washed with PBS solution three times and measured at OD 595 nm.

MBEC determinations

MBEC values for the ceragenins and meropenem against *Achromobacter* biofilms were performed according to a previously described procedure with the following modifications.[29] Five strong biofilm-producing *Achromobacter* strains were used for an MBEC assay for a range between 100× and 1000× MIC.[22,23] The biofilms (24 h) that were grown in 96-well tissue culture microtiter plates were gently washed with 200 µl PBS buffer three times and air-dried. Serial two-fold dilutions ranging from 16 to 0.025 mg/ml for CSAs and 2 to 0.025 mg/ml for antibiotics were prepared in CAMHB. A 200 µl solution of each concentration was added to a corresponding well, and the plates were incubated for 24 h at 37 °C. After the 24 h incubation at 37 °C, the plates were washed and sonicated to disrupt the biofilms, and samples were plated on TSA for colony counts. The MBEC was defined as the minimal concentration of meropenem or CSA that prevents bacterial regrowth in the medium.

Fluorescence microscopy

After the ceragenins and antibiotic treatments, the wells were immersed in SYTO 9 and propidium iodide (PI) to investigate live/dead cells in biofilms. SYTO 9 is a cell-permeable that causes green fluorescence at low concentration and interacts with DNA and RNA of viable cells. PI is impermeable to cellular membranes that cause orange fluorescence. However, it passes through the membranes of nonviable cells and interacts with DNA and RNA as Syto 9. Thus, under fluorescence microscopy, when SYTO 9 and PI are used, viable cells fluoresce green while dead cells are red/orange with damaged membranes.[30] After biofilm formation in 96 well plates and antimicrobial treatment, 1 µl SYTO 9 (5 mg/ml) and 1 µl PI (3 µg/ml) as dye solution were added in 1 ml PBS. Biofilms visualized by the Olympus BX51 fluorescence microscope digital photographs were taken by Olympus DP72 camera and DP2-TWAI software.[31]

Statistical analysis

Statistical analysis of the study was performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). Two way-ANOVA tests followed by Tukey's multiple comparison tests were used to determine the statistical significance among control and antimicrobial groups.

Results

Identification

The distribution of 52 *Achromobacter* spp. collected from CF patients and their biofilm-forming capabilities are given in Table 1. Of these strains, 22 were *A. xylosoxidans*, 11 were *Achromobacter insuavis,* two were *Achromobacter aegrifaciens,* one was *Achromobacter denitrificans,* nine were *Achromobacter ruhlandii,* one was *Achromobacter*

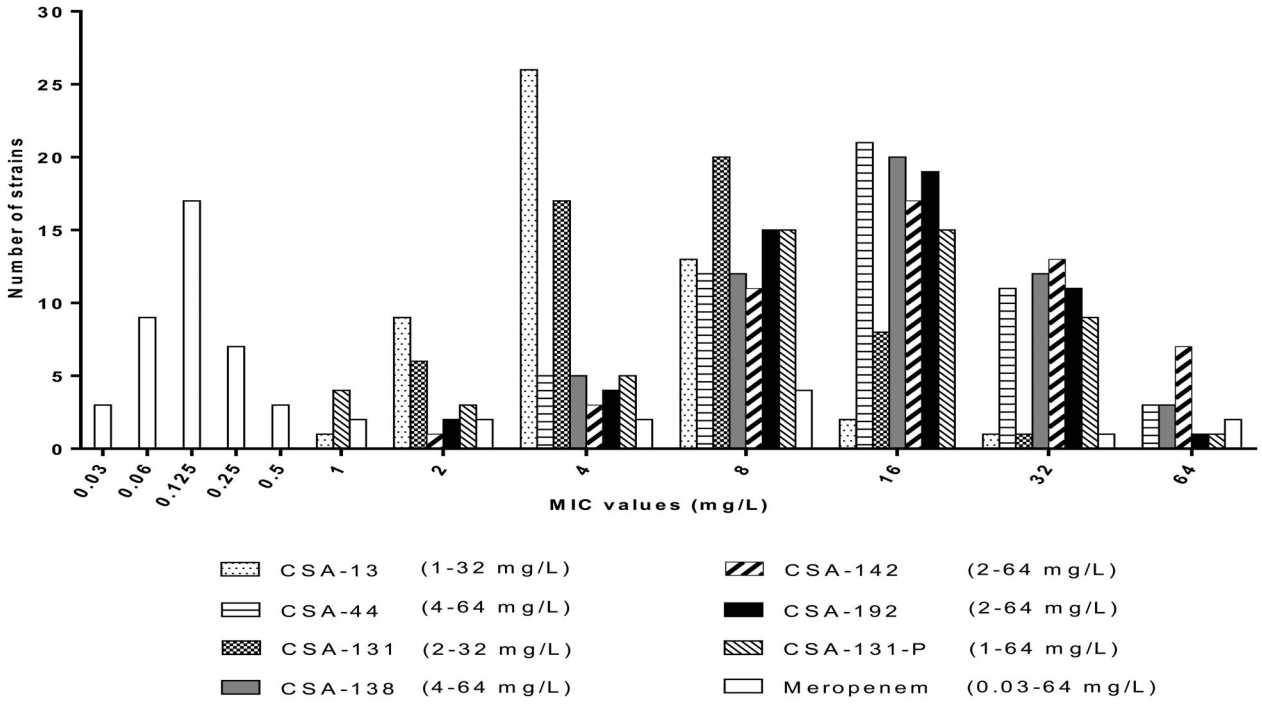


Figure 1 MIC distribution of 52 *Achromobacter* spp.

dolens, three were *Achromobacter genogroup,* one was *Achromobacter piechaudii,* one was *Achromobacter mucicolens,* and one was *Achromobacter spiritinus.*

Susceptibility results against planktonic cells (MIC)

The in vitro activities of ceragenins and meropenem against 52 *Achromobacter* strains are summarized in Figure 1. The MICs of the meropenem against the quality control strain *P. aeruginosa* ATCC 27853 were within the accuracy range described by EUCAST throughout the study. Susceptibility testing showed that the MIC ranges for CSA-13, CSA-44, CSA-131, CSA-138, CSA-142, CSA-192, CSA-131-P and meropenem were 1–32 mg/L, 4–64 mg/L, 2–32 mg/L, 4–64 mg/L, 2–64 mg/L, 2–64 mg/L, 1–64 mg/L, 0.03–64 mg/L, respectively. The lowest MIC_{50} results were obtained with CSA-13. CSA-131 and CSA-131-P displayed the second most effective results with a MIC_{50} value of 8 mg/L. CSA-44, CSA-138, CSA-142, and CSA-192 showed the same MIC_{50} results.

Minimum biofilm eradication concentration (MBEC)

The antibiofilm activities of ceragenins and meropenem against five strong biofilm-producing *Achromobacter* strains are summarized in Table 2. However, MBECs were greater than MICs; ceragenins' MBEC values ranged from 1 to 4 mg/ml. Susceptibility results demonstrated that the MBEC (mg/ml) values of CSA-13, CSA-131, and CSA-131-P were 1 to 4 mg/ml, 2 to 4 mg/ml, and 1.6 mg/ml, respectively. Meropenem showed similar MBEC values, which were over 1000 times the MICs (0.03–0.125 mg/ml) (Table 2). Among the tested ceragenins, CSA-13 was the most effective agent against mature biofilms.

Inhibition of adhesion

When the 1/10 × MICs of tested ceragenins and meropenem were investigated for 1, 2, or 4 hours at 37 °C for adherence to the wells of tissue culture microtiter plates, all of the tested agents inhibited biofilm attachment processes. Inhibition rates of adhesion showed a time- and concentration-dependent effect. All of the tested agents, CSA-13, CSA-131, CSA-131-P, and meropenem, were found to be more efficient after four hours of incubation. The most potent agent for inhibition of adhesion was CSA-131-P (up to 84%) (Figure 2).

Inhibition of biofilm formation

All of the tested CSAs and meropenem showed significant inhibitory activity against biofilm formation of *Achromobacter* strains at 24 hours. The rates of biofilm formation inhibition were dependent on concentration; the highest inhibition rates were shown at 1 × MICs for all agents, as expected. Although CSA-131-P was the most efficient agent for inhibition of biofilm formation (up to > 90%) (Figure 3), CSA-13 and CSA-131 had potent inhibitory effects on tested *Achromobacter* biofilm formations. All tested CSAs showed a better inhibitory effect on biofilms than meropenem did.

Table 2 In vitro antibacterial and antibiofilm activities of ceragenins comparatively to meropenem against five strong biofilm producing *Achromobacter* isolates

	Strains				
Antimicrobial agents	*A. xylosoxidans*-1	*A. genogroup*-1	*A. xylosoxidans*-2	*A. xylosoxidans*-3	*A. genogroup*-2
	Antimicrobial susceptibility, MIC (mg/L)				
CSA-13	4	4	8	4	4
CSA-131	8	8	16	4	4
CSA-131-P	16	16	16	16	4
Meropenem	0.125	0.125	2	0.06	0.03
	Minimum biofilm eradication concentration, MBEC (mg/ml)				
CSA-13	2	4	1	4	4
CSA-131	4	2	2	2	2
CSA-131-P	1.6	1.6	1.6	1.6	1.6
Meropenem	>0.125	>0.125	>2	>0.06	>0.03

Fluorescence microscopy

A. xylosoxidans-1 biofilm was formed for 24 hours in the wells of a microtiter plate. After treatment, the cells were stained with the PI and Syto 9 stains and examined by fluorescence microscopy. The cells formed a uniform and smooth biofilm (Figure 4). All the cells of the field were stained with Syto9 and appeared green (Figure 4A, E, I, M). Increasing concentrations of CSAs provoked the appearance of damaged cells (small red spots) homogeneously dispersed in the biofilm (Figure 4B, F, J). All studied CSAs had more potent antibiofilm effects than meropenem (Figure 4).

Discussion

Recently, the rising proportion of drug-resistant bacterial infections in clinics has drawn attention to novel approaches for limiting and treating these infections. One of these efforts has involved studies of AMPs as antibacterial agents. Ceragenins, also known as CSAs, are cholic acid derivatives that are similar in antibacterial activity to condensed amino acid (derivatives of cholic acid marked with L-arginine), which was first synthesized in 1979.[32,33] Like antibacterial peptides, ceragenins demonstrate positive charges arranged on one face and hydrophobic residues on the other.[34,35] Ceragenins can be separated into two categories: polymyxin mimics and squalamine mimics. Ceragenins are unique, cationic steroid compounds, low-molecular-mass, and produced to copy the activities of natural AMPs. Because ceragenins target cell membranes based in part on electrostatic interactions, they efficiently kill many microorganisms that are extraordinary to induce resistance.[36] Various ceragenins have been developed and studied until today.

CSA-1 to CSA-50 as first-generation ceragenins; CSA-50 and over numbers as second-generation ceragenins have been developed.[16,37] Previous reports have been demonstrated, with the potent in vitro activities of ceragenins against various bacteria, viruses, and parasites.[38–44] In the present research, we first showed the susceptibility and antibiofilm activity of CSAs against the *Achromobacter* species.

Previous researches have explained that ceragenins are efficient antimicrobials against many microorganisms, including multidrug-resistant microorganisms. Chin et al. evaluated the efficacy of CSA-13 against 50 clinical isolates of *P. aeruginosa* and compared them with several antibiotics.[17] CSA-13 showed a MIC range of 1–32 mg/L, with an MIC_{50} of 16 mg/L. Hashemi et al. found that CSA-13 (MIC: 2–6 mg/L) and CSA-142 (MIC: 2–16 mg/L) had significant antimicrobial effects, and CSA-44 and CSA-131 were bactericidal against colistin-resistant *Klebsiella pneumoniae* strains.[45] Also, Bozkurt-Guzel et al. demonstrated the antibacterial activities of CSA-13 against clinical *P. aeruginosa* isolates with an 8–16 mg/L MIC range and 8 mg/L MIC_{50}.[46] Another study by Vila-Farrés et al. examined the MIC_{50} (mg/L) values of CSA-13, CSA-44, CSA-131, and CSA-138 against *P. aeruginosa* strains, which were 4 mg/L, 4 mg/L, 1 mg/L, and 2 mg/L, respectively.[47] Wnorowska et al. demonstrated with cell line studies that combinations of LL-37 with ceragenins (CSA-13 and CSA-131) possess the potential to be used as a novel strategy to treat urinary tract infections caused by antibiotic-resistant *Escherichia coli* strains.[48] Here we report that the MIC_{50} (mg/L) values of CSA-13, CSA-44, CSA-131, CSA-138, CSA-142, CSA-192, CSA-131-P, and meropenem were 4 mg/L, 16 mg/L, 8 mg/L, 16 mg/L, 16 mg/L, 16 mg/L, 8 mg/L and 0,125 mg/L, respectively. These findings are similar to those of other previous studies.[17,45–47] In our research, the lowest MIC_{50} and MIC_{90} results were achieved with CSA-13 against tested *Achromobacter* strains. Furthermore, CSA-131 and CSA-131-P showed good MIC_{50} results as 8 mg/L against tested strains.

Biofilms are unique and organized populations of bacteria and are regularly controlled by signaling

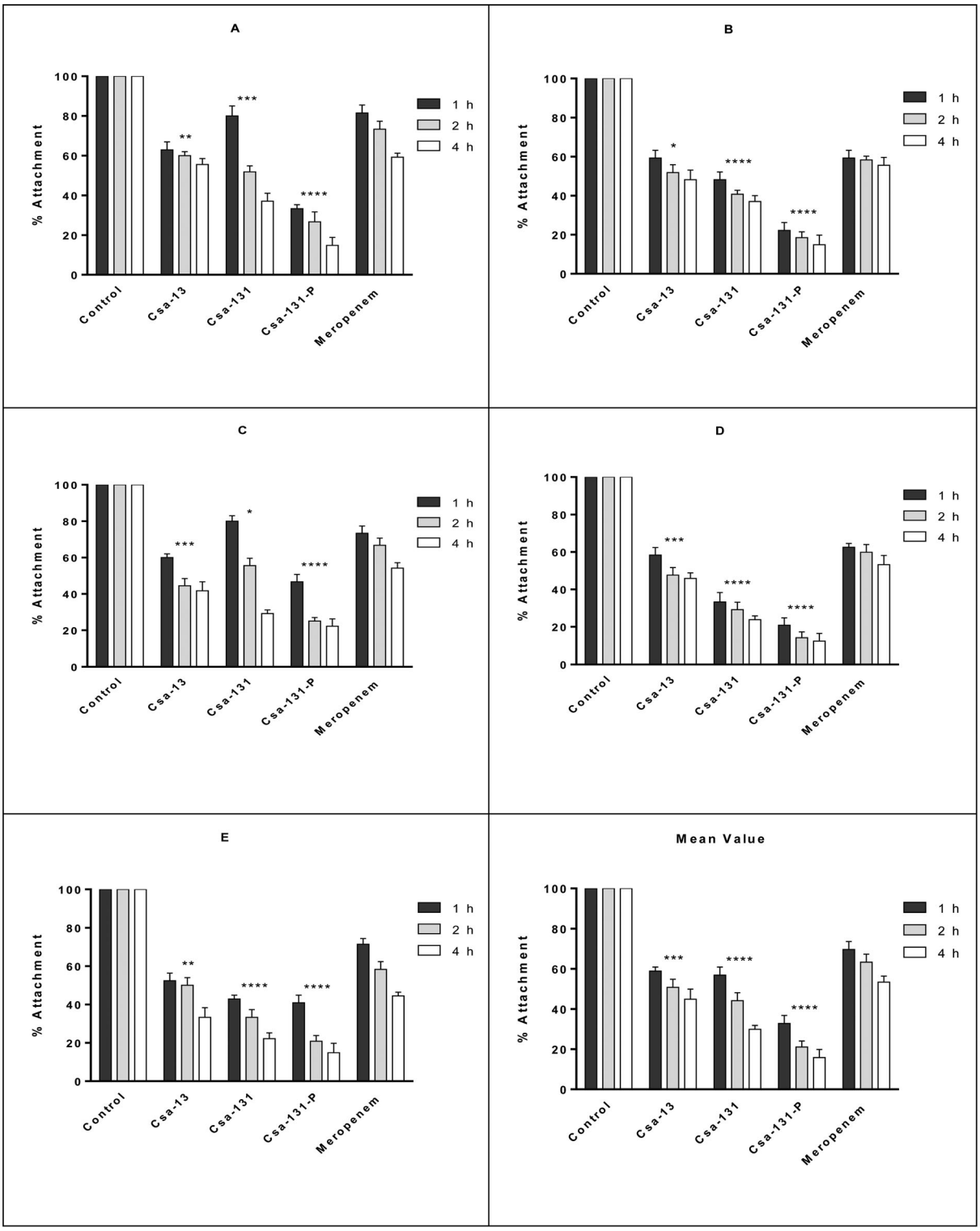


Figure 2 Attachment of five strong biofilm producing *Achromobacter* isolates biofilm by the studied antimicrobials. (A) *A. xylosoxidans*-1; (B) *A. genogroup*-1; (C) *A. xylosoxidans*-2; (D) *A. xylosoxidans*-3; (E) *A. genogroup*-2; and mean value of antimicrobials. Control bars indicate *Achromobacter* strains without antimicrobial agent and accepted as 100%. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (CSAs vs. meropenem).

molecules rather than random accumulations of cells following from cell division. It is well recognized that biofilm-embedded bacterial cells are resistant to both host immune responses and antimicrobial agents when compared to their planktonic forms.[49] Novel approaches to treat biofilms might take one of three primary forms: noticeable reduction of planktonic cells before biofilm formation, inhibition of bacterial adhesion and biofilm formation, or elimination of established biofilm.[39,50] A new field of research has consequently focused on preventing biofilm formation and bacterial adherence. For this purpose, in the present study, we evaluated the inhibition of bacterial attachment to the surfaces as well as the inhibition

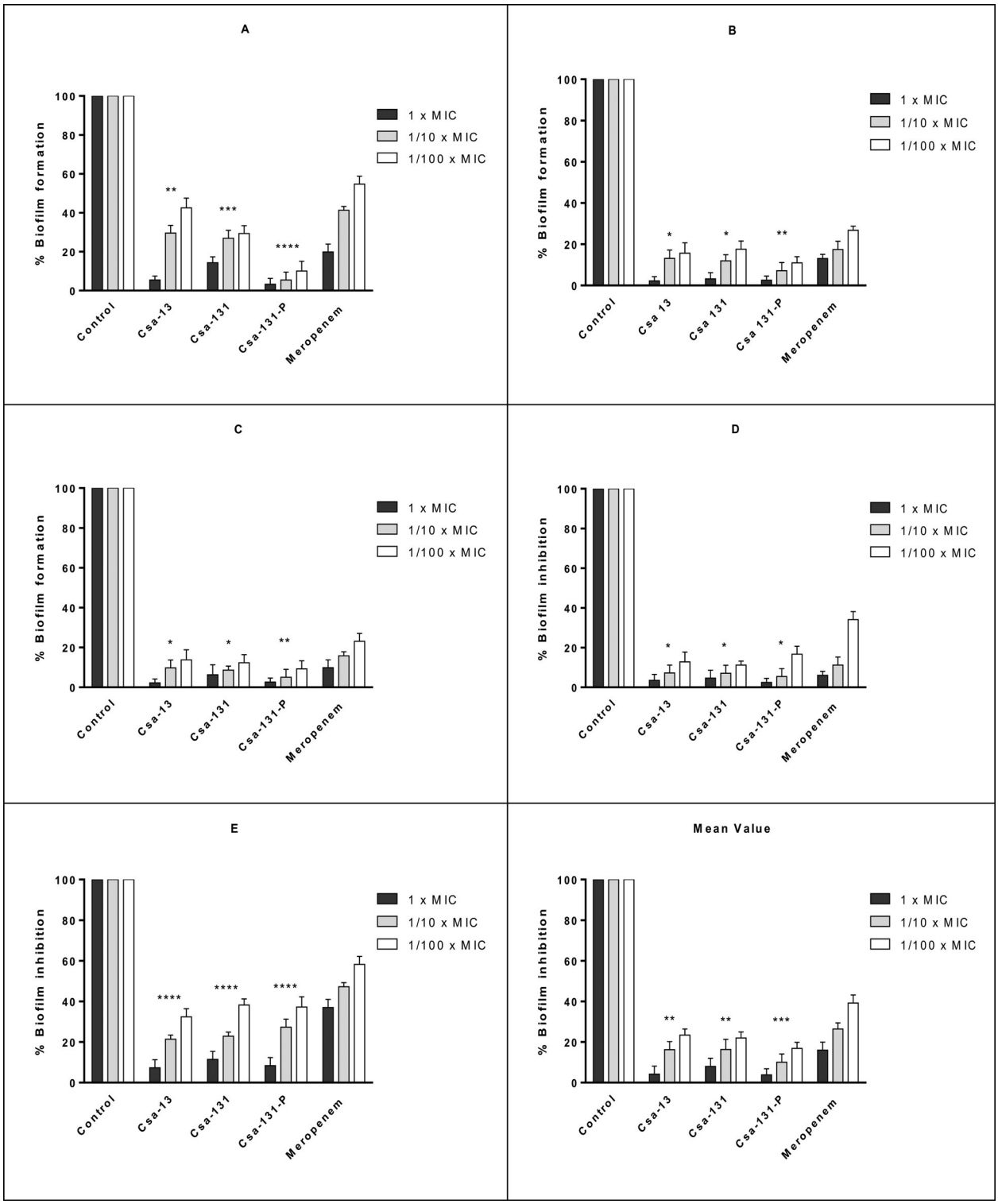


Figure 3 Prevention of biofilm of *Achromobacter* strains by the studied antimicrobials. (A) *A. xylosoxidans*-1; (B) *A. genogroup*-1; (C) *A. xylosoxidans*-2; (D) *A. xylosoxidans*-3; (E) *A. genogroup*-2; and mean value of antimicrobials. Control bars indicate *Achromobacter* strains without antimicrobial agent and accepted as 100%. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (CSAs vs. meropenem).

of biofilm production by the MIC or subMIC values of ceragenins and meropenem. In the present work, in addition to a susceptibility test, the antibiofilm effect (inhibition of biofilm formation or inhibition of biofilm adhesion) of CSA-13, CSA-131, and CSA-131-P was investigated against five potent biofilm-producing *Achromobacter* strains using the crystal violet staining method. Additionally, meropenem, a conventional antibiotic, was used for comparison. According to the results of the present study, although MBECs of ceragenins were higher than MICs, as expected, meropenem MBECs were over a thousandfold greater than MICs of this antibiotic.

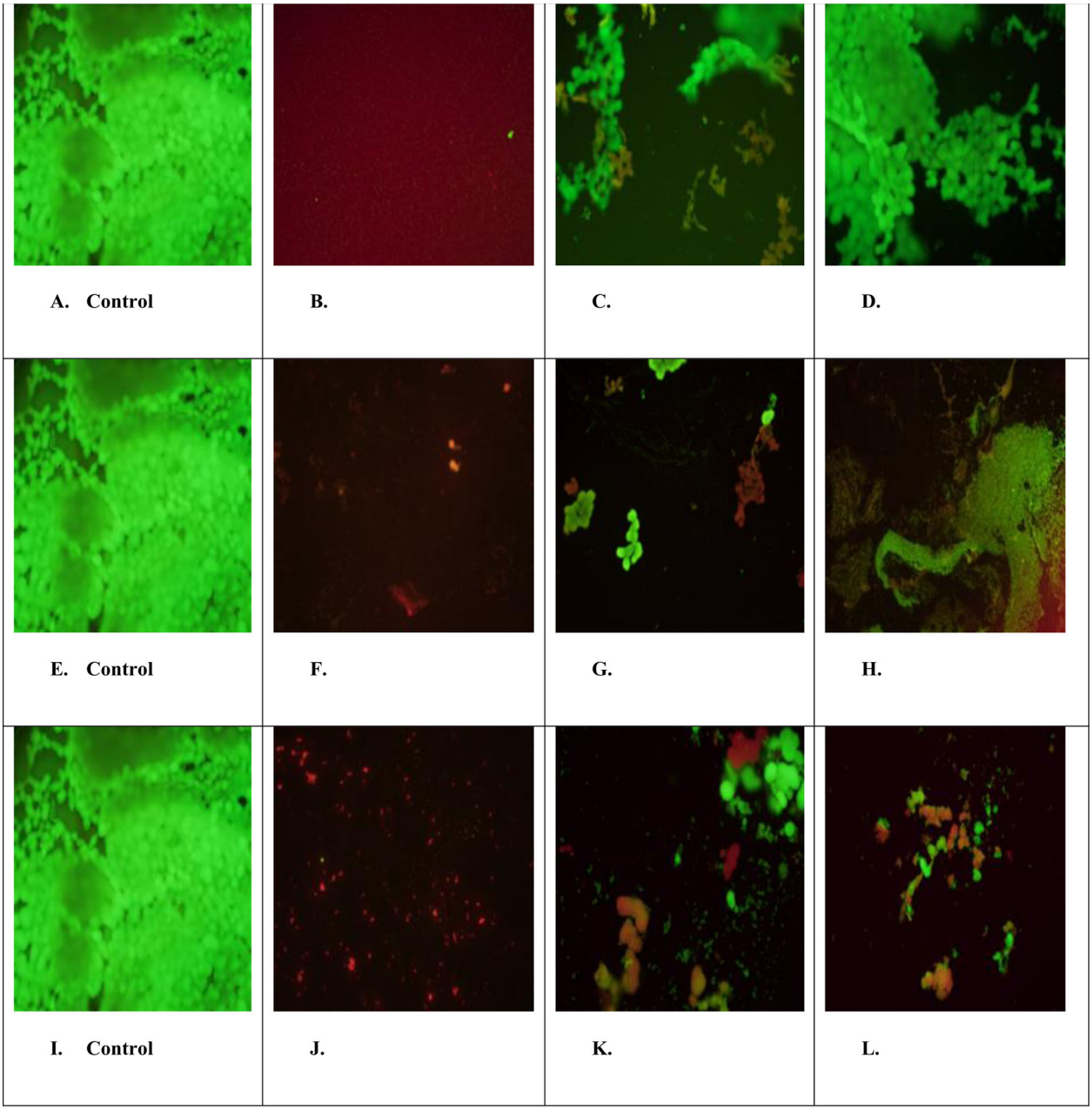


Figure 4 Fluorescence images of *A. xylosoxidans*-1 biofilms with or without CSAs and antibiotic treatment. A. E. İ. M. Control; B., C., and D. images of *A. xylosoxidans*-1 treated with 1× MIC, 1/10× MIC, and 1/100× MIC CSA-13, respectively. F., G., and H. images of *A. xylosoxidans*-1 treated with 1× MIC, 1/10× MIC, and 1/100× MIC CSA-131, respectively. J., K., and L. images of *A. xylosoxidans*-1 treated with 1× MIC, 1/10× MIC, and 1/100× MIC CSA-131-P, respectively. N., O., and P. images of *A. xylosoxidans*-1 treated with 1× MIC, 1/10× MIC, and 1/100× MIC meropenem, respectively.

In contrast to the antimicrobial effects against fungi and bacteria, the antibiofilm activity of ceragenins is not well defined against biofilms. A few studies demonstrated that ceragenins, especially CSA-13, have antibiofilm effects against *P. aeruginosa*.[50–52] The findings of the study also showed that among the ceragenins, CSA-131-P, and CSA-13 were the most effective agents against mature biofilms. Although the inhibition of biofilm adhesion and biofilm formation rates depended on time and concentration, it was also shown that CSA-131-P at 1 × MIC was the most efficient agent with robust inhibition rates of biofilm adhesion of 84.17% and biofilm formation of 96.14%. Our results clearly showed that ceragenins are much more active in the inhibition of mature biofilm formation than the inhibition of the biofilm attachment process (Figure 4). These results meant that biofilm formation might be blocked by inhibiting not only attachments but also additional mechanisms. Previously, it was revealed by Olekson et al. that many ceragenins, including CSA-13, CSA-44, CSA-131, CSA-138, CSA-142, and CSA-192, had antibiofilm actions against mixed-species biofilms produced by *P. aeruginosa* and *S. aureus*.[39] Furthermore, Gu et al. confirmed that CSA-138

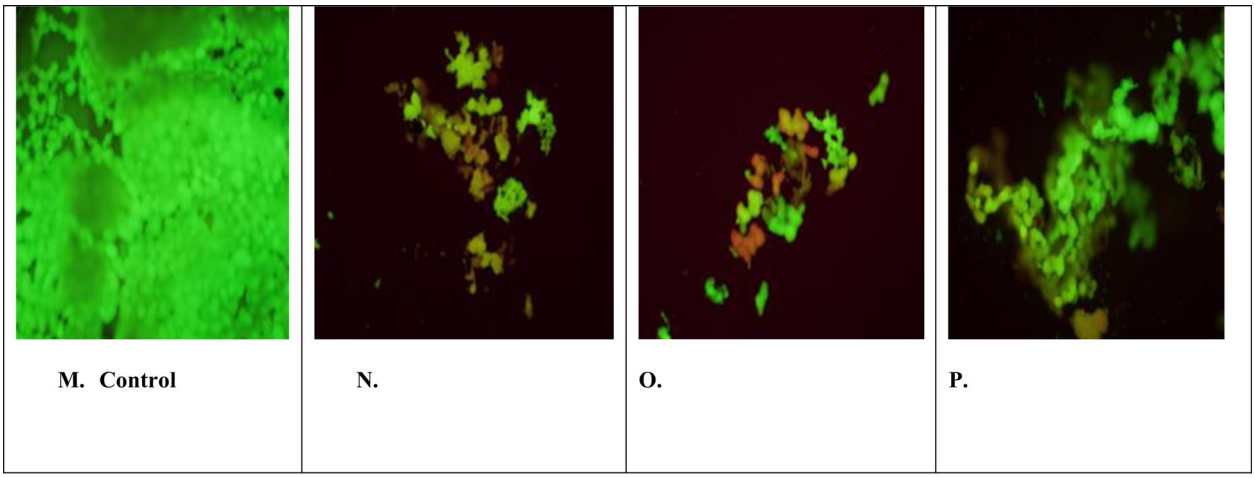


Figure 4 Continued.

also influenced biofilm formation of *P. aeruginosa* on lenses, and Nagant et al. pointed out that CSA-13 inhibited biofilm formation, the surface of biofilms, and the inside of built biofilms of *P. aeruginosa*.[50,51] Present research had similar results with the previous investigations.

On the other hand, up to this time, different poloxamers have been produced for use as drug carriers. The poloxamer Pluronic® F127 (hereafter referred to as pluronic) has been particularly well-studied due to its capabilities to create well-defined micelles and appropriate drugs for transfer to specific tissues.[53] In two previous investigations, it was recognized that combinations of this non-ionic surfactant and ceragenins diminished host cell cytotoxicity without substantially reducing antibacterial activities.[54,55] The mechanism of reduced toxicity is presumably due to the transient encapsulation of ceragenins in micelles formed by the pluronic.

While ensconced in micelles, few ceragenins are predisposed to associate with host membranes, yet the higher attraction of ceragenins for microbial membranes—and the dynamic nature of micelles—provide ceragenins to achieve antimicrobial activities.[56] As a result of this, pluronic micelles may work to increase the membrane selectivity of ceragenins. Via formulating CSA-131 in poloxamer micelles, cilia injury was prevented, and antimicrobial activity was unaltered. The ability of CSA-131, formulated with a poloxamer, to decrease the communities of fungal pathogens in tracheal and lung tissue, was also examined in ex vivo investigations by Hashemi et al.[45] These findings indicate that CSA-131, formulated in micelles, may serve as a possible therapeutic for polymicrobial and biofilm-related infections in the lung and trachea. Similarly, in this study, it was revealed for the first time for *Achromobacter* strains that CSA-131-P was not different from CSA-13 on antimicrobial and antibiofilm activities.

To the best of our knowledge, our study is the first record to evaluate the antibacterial and antibiofilm activities of tested ceragenins against *Achromobacter* species. According to our findings, ceragenins, especially CSA-13, CSA-131, and CSA-131-P, seem to be possible candidates for the drug development process for *Achromobacter* infections as well as biofilm-related ones.

Consequently, CSAs could be suitable candidates for further analysis as antibacterial drugs, and future investigations should be carried out to correlate the potency, safety, and pharmacokinetic parameters of ceragenins.

Acknowledgments

We thank Dr. Niels Norskov Lauritsen for providing us *Achromobacter* species from the Department of Clinical Microbiology, Aarhus University Hospital, Aarhus, Denmark. Also, we thank Dr. Çağla Bozkurt Güzel for providing us CSA-131-P from the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Istanbul University, Beyazit-Istanbul, Turkey.

Disclosure statement

The authors declare that they have no competing interests.

Funding

This work was supported by a grant from the Research Fund of The University of Istanbul (Istanbul, Turkey) Project no. 52726.

Notes on contributors

Damla Damar-Çelik was born in İstanbul, Turkey on 27 February 1992. She graduated from Istanbul University Department of Biology in June 2014. She earned her Master of Science degree in January 2018 at the University of Istanbul

Department of Pharmaceutical Microbiology. Her thesis is "Investigation of the Postantibiotic effects of various antibiotics alone and in combination on Achromobacter xylosoxidans strain." During her master of science studies, she received Erasmus grant and studied in Aarhus University Hospital Department of Clinical Microbiology as part of the Erasmus Internship Programme for 2.5 months. She studied the antibiotic resistance mechanism in Achromobacter xylosoxidans in Denmark. She is currently in her third year of her PhD in Pharmaceutical Microbiology at Istanbul University, Faculty of Pharmacy under the supervision of Prof. Dr. Berna Özbek Çelik. She studies bacterial biofilm formation, antimicrobial resistance and bacteriophages. She published some articles in academic journals and attented some conferences like the European 42nd Cystic Fibrosis Conference in Liverpool-UK and VI Congress of Macedonian Microbiologists with International participation in Ohrid-Macedonia. She has been working as a research assistant at Marmara University Faculty of Pharmacy Department of Pharmaceutical Microbiology since 2017.

Emel Mataracı-Kara received the M.Sc. and PhD. degrees from the Istanbul University Faculty of Pharmacy, Istanbul, Turkey, in 2010 and 2016, respectively. She is still working as an Assistant Professor at the Department of Pharmaceutical Microbiology Faculty of Pharmacy Istanbul University, Istanbul, Turkey. She is the author or co-author of numerous papers in both international refereed journals and conference contributions. Her work focuses on indwelling catheter infections, bloodstream infections, biofilms, in vitro pharmacokinetics and pharmacodynamics parameters of antimicrobial cationic peptides and antibiotics alone or combinations. She has received several important recognitions to her research career, including the Drug Research Prize, and Successful Researcher Awards given by Istanbul University for her academic papers.

Paul B. Savage received his undergraduate degree in chemistry from Brigham Young University in 1988. He completed his PhD in organic chemistry with Samuel Gellman at the University of Wisconsin in 1993. He completed NIH-funded postdoctoral training with Leo Paquette at the Ohio State University in 1995. His tenure at BYU began in 1995. He has risen through the academic ranks and is now the Reed M. Izatt Professor of Chemistry and Biochemistry. His research interests are tied to innate immune function, specifically, the discovery of structural requirements of glycolipids for stimulation of natural killer T cells and the development of non-peptide mimics of endogenous antimicrobial peptides. His research has led to over 200 papers and more than 50 issued US patents. His research has been funded by the National Institutes of Health, the National Science Foundation and by corporate sponsors.

Berna Özbek-Çelik received her undergraduate degree in biology from İstanbul University in 1996. She completed her Master Degree in Pharmaceutical Microbiology with Prof. A. Alev Gerçeker at the University of Istanbul in 2000. She completed her Ph.D. degree in Medical Microbiology in Istanbul Universitesi Medical Faculty. Prof.Berna Özbek Çelik achieved FEMS funded postdoctoral training with Prof. Patrice Nordmann at the Paris Sud University in 2007. She has risen through the academic ranks and is now the Professor of Pharmaceutic Microbiology. Her research interests are antimicrobial compounds, antibiotic combinations effects, antibiotic resistance mechanisms, bacterial biofilms specifically, the investigation of antimicrobial catheter lock solutions, and bacteriophage therapies. Her research has led to over 60 papers. His research has been funded by the İstanbul University Scientific Research Corporation Unit and the Scientific and Technological Research Council of Turkey (TUBITAK).

References

1 Lappin-Scott HM and JW Costerton, editors. Microbial biofilms. Cambridge University Press; 1995.
2 Kolenbrander PE. Coaggregation of human oral bacteria: potential role in the accretion of dental plaque. J Appl Microbiol. 1993;74:79S–86S.
3 Ghiorse WC, Wilson JT. Microbial ecology of the terrestrial subsurface. Adv Appl Microbiol. 1988;33:107–73.
4 Lawrence JR, Korber DR, Wolfaardt GM, Caldwell DE. Bacterial behavioral strategies at interfaces. Adv Microb Ecol. 1995;14:1–75.
5 McLean RJC, Fortin D, Brown DA. Microbial metal-binding mechanisms and their relationship to nuclear waste disposal. Can J Microbiol. 1996;42(4):392–400.
6 Costerton JW, Lappin-Scott HM. Behaviour of bacteria in biofilms. ASM News. 1989;55:650–4.
7 Marshall KC. Biofilms: an overview of bacterial adhesion, activity, and control at surfaces. ASM News. 1992;58:202–7.
8 Durham-Colleran MW, Verhoeven AB, Van Hoek ML. *Francisella novicida* forms in vitro biofilms mediated by an orphan response regulator. Microb Ecol. 2010;59(3):457–65.
9 Stoltz DA, Meyerholz DK, Welsh MJ. Origins of cystic fibrosis lung disease. N Engl J Med. 2015;372(4):351–62.
10 Lambiase A, Catania MR, Del Pezzo M, Rossano F, Terlizzi V, Sepe A, Raia V. *Achromobacter xylosoxidans* respiratory tract infection in cystic fibrosis patients. Eur J Clin Microbiol Infect Dis. 2011;30(8):973–80.
11 Parkins MD, Floto RA. Emerging bacterial pathogens and changing concepts of bacterial pathogenesis in cystic fibrosis. J Cyst Fibros. 2015;14(3):293–304.
12 Trancassini M, Iebba V, Citera N, Tuccio V, Magni A, Varesi P, et al. Outbreak of *Achromobacter xylosoxidans* in an Italian cystic fibrosis center: genome variability, biofilm production, antibiotic resistance, and motility in isolated strains. Front Microbiol. 2014;5:138.
13 De Baets F, Schelstraete P, Van Daele S, Haerynck F, Vaneechoutte M. Achromobacter xylosoxidans in cystic

fibrosis: prevalence and clinical relevance. J Cyst Fibros. 2007;6(1):75–8.
14 Amoureux L, Bador J, Fardeheb S, Mabille C, Couchot C, Massip C, Salignon A-L, Berlie G, Varin V, Neuwirth C, et al. Detection of *Achromobacter xylosoxidans* in hospital, domestic, and outdoor environmental samples and comparison with human clinical isolates. Appl Environ Microbiol. 2013;79(23):7142–9.
15 Papalia M, Steffanowski C, Traglia G, Almuzara M, Martina P, Galanternik L, Vay C, Gutkind G, Ramírez MS, Radice M, et al. Diversity of Achromobacter species recovered from patients with cystic fibrosis, in Argentina. Rev Argent Microbiol. 2020;52(1):13–8.
16 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(18):2837–40.
17 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(2):365–70.
18 Surel U, Niemirowicz K, Marzec M, Savage PB, Bucki R. Ceragenins – a new weapon to fight multidrug resistant bacterial infections. Med Stud. 2014;3 (3):207–13.
19 Ozbek-Celik B, Damar-Celik D, Mataraci-Kara E, Bozkurt-Guzel C, Savage PB. Comparative in vitro activities of first and second-generation ceragenins alone and in combination with antibiotics against multidrug-resistant *Klebsiella pneumoniae* strains. Antibiotics. 2019;8(3):130.
20 Isogai E, Isogai H, Takahashi K, Okumura K, Savage PB. Ceragenin CSA-13 exhibits antimicrobial activity against cariogenic and periodontopathic bacteria. Oral Microbiol Immunol. 2009;24(2):170–2.
21 Gade SS, Nørskov-Lauritsen N, Ridderberg W. Prevalence and species distribution of *Achromobacter* sp. cultured from cystic fibrosis patients attending the Aarhus centre in Denmark. J Med Microbiol. 2017;66(5):686–9.
22 O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. Annu Rev Microbiol. 2000;54(1):49–79.
23 Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods. 2000;40(2):175–9.
24 Lai X-Z, Feng Y, Pollard J, Chin JN, Rybak MJ, Bucki R, Epand RF, Epand RM, Savage PB. Ceragenins: cholic acid-based mimics of antimicrobial peptides. Acc Chem Res. 2008;41(10):1233–40.
25 Durnaś B, Wnorowska U, Pogoda K, Deptuła P, Wątek M, Piktel E, Głuszek S, Gu X, Savage PB, Niemirowicz K, et al. Candidacidal activity of selected ceragenins and human cathelicidin LL-37 in experimental settings mimicking infection sites. PLoS One. 2016;11(6):e0157242.
26 Butt AM, Amin MCIM, Katas H, Sarisuta N, Witoonsaridsilp W, Benjakul R. In vitro characterization of pluronic F127 and D–tocopheryl polyethylene glycol 1000 succinate mixed micelles as nanocarriers for targeted anticancer-drug delivery. J Nanomater. 2012;2012:1–11.
27 The European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters. Version 10.0, 2020. http://www.eucast.org.
28 Overhage J, Campisano A, Bains M, Torfs EC, Rehm BH, Hancock RE. Human host defense peptide LL-37 prevents bacterial biofilm formation. Infect Immun. 2008;76(9):4176–82.
29 Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. The Calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol. 1999;37(6):1771–6.
30 Li CS, Chia WC, Chen PS. Fluorochrome and flow cytometry to monitor microorganisms in treated hospital wastewater. J Environ Sci Health A Tox Hazard Subst Environ Eng. 2007;42(2):195–203.
31 Bektaş M, Hacıosmanoğlu E, Ozerman B, Varol B, Nurten R, Bermek E. On diphtheria toxin fragment A release into the cytosol-cytochalasin D effect and involvement of actin filaments and eukaryotic elongation factor 2. Int J Biochem Cell Biol. 2011;43(9):1365–72.
32 Ding B, Taotofa U, Orsak T, Chadwell M, Savage PB. Synthesis and characterization of peptide-cationic steroid antibiotic conjugates. Org Lett. 2004;6(20):3433–6.
33 Bellini AM, Vertuani G, Quaglio MP, Cavazzini G. Bile acid derivatives with antimicrobial activity. Farmaco Sci. 1979;34(11):967–78.
34 Zanetti M, Gennaro R, Scocchi M, Skerlavaj B. Structure and biology of cathelicidins. Adv Exp Med Biol. 2000;479:203–18.
35 Bucki R, Pastore JJ, Randhawa P, Vegners R, Weiner DJ, Janmey PA. Antibacterial activities of rhodamine B-conjugated gelsolin-derived peptides compared to those of the antimicrobial peptides cathelicidin LL37, magainin II, and melittin. Antimicrob Agents Chemother. 2004;48(5):1526–33.
36 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 Ocul Pharmacol Ther. 2011;27(1):1–5.
37 Savage PB, Li C, Taotafa U, Ding B, Guan Q. Antibacterial properties of cationic steroid antibiotics. FEMS Microbiol Lett. 2002;217(1):1–7.
38 Chin JN, Rybak MJ, Cheung CM, Savage PB. Antimicrobial activities of ceragenins against clinical isolates of resistant *Staphylococcus aureus*. AAC. 2007;51(4):1268–73.
39 Olekson MA, You T, Savage PB, Leung KP. Antimicrobial ceragenins inhibit biofilms and affect mammalian cell viability and migration in vitro. FEBS Open Bio. 2017;7(7):953–67.
40 Piktel E, Pogoda K, Roman M, Niemirowicz K, Tokajuk G, Wróblewska M, Szynaka B, Kwiatek WM, Savage PB, Bucki R. Sporicidal activity of ceragenin CSA-13 against *Bacillus subtilis*. Sci Rep. 2017;7:44452.
41 Hacioglu M, Bozkurt-Guzel C, Savage PB, Tan ASB. Antifungal susceptibilities, in vitro production of virulence factors and activities of ceragenins against *Candida* spp. isolated from vulvovaginal candidiasis. Med Mycol. 2019;57(3):291–9.
42 Howell MD, Streib JE, Kim BE, Lesley LJ, Dunlap AP, Geng D, Feng Y, Savage PB, Leung DYM. Ceragenins: a class of antiviral compounds to treat orthopox infections. J Invest Dermatol. 2009;129(11):2668–75.
43 Lara D, Feng Y, Bader J, Savage PB, Maldonado RA. Antitrypanosomatid activity of ceragenins. J Parasitol. 2010;96(3):638–42.
44 Bozkurt-Guzel C, Savage PB, Akcali A, Ozbek-Celik B. Potential synergy activity of the novel ceragenin, CSA-13, against carbapenem-resistant *Acinetobacter baumannii* strains isolated from bacteremiapatients. Biomed Res Int. 2014;2014:710273.
45 Hashemi MM, Rovig J, Weber S, Hilton B, Forouzan MM, Savage PB. Susceptibility of colistin-resistant, Gram-negative bacteria to antimicrobial peptides and ceragenins. Antimicrob Agents Chemother. 2017;61(8):e00292–17.
46 Bozkurt-Guzel C, Oyardi O, Savage PB. Comparative in vitro antimicrobial activities of CSA-142 and CSA-192, second-generation ceragenins, with CSA-13 against various microorganisms. J Chemother. 2018;30(6–8):332–7.
47 Vila-Farrés X, Callarisa AE, Gu X, Savage PB, Giralt E, Vila J. CSA131, a ceragenin active against colistin-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* clinical isolates. Int J Antimicrob Agents. 2015;46(5):568–71.
48 Wnorowska U, Piktel E, Durnaś B, Fiedoruk K, Savage PB, Bucki R. Use of ceragenins as a potential treatment for urinary tract infections. BMC Infect Dis. 2019;19(1):369.
49 Branda SS, Vik S, Friedman L, Kolter R. Biofilms: the matrix revisited. Trends Microbiol. 2005;13(1):20–6.
50 Gu X, Jennings JD, Snarr J, Chaudhary V, Pollard JE, Savage PB. Optimization of ceragenins for prevention of bacterial colonization of hydrogel contact lenses. Invest Ophthalmol Vis Sci. 2013;54(9):6217–23.
51 Nagant C, Pitts B, Stewart PS, Feng Y, Savage PB, Dehaye JP. Study of the effect of antimicrobial peptide mimic, CSA-13, on an established biofilm formed by *Pseudomonas aeruginosa*. Microbiologyopen. 2013;2(2):318–25.
52 Bozkurt-Guzel C, Hacioglu M, Savage PB. Investigation of the in vitro antifungal and antibiofilm activities of ceragenins CSA-8, CSA-13, CSA-44, CSA-131, and CSA-138 against Candida species. Diagn Microbiol Infect Dis. 2018;91(4):324–30.
53 Pitto-Barry A, Barry NPE. Pluronic block-copolymers in medicine: From chemical and biological versatility to rationalization and clinical advances. Polym Chem. 2014;5(10):3291–7.

54 Nagant C, Savage PB, Dehaye JP. Effect of pluronic acid F-127 on the toxicity towards eukaryotic cells of CSA-13, a cationic steroid analogue of antimicrobial peptides. J Appl Microbiol. 2012;112(6):1173–83.
55 Leszczyńska K, Namiot A, Cruz K, Byfield FJ, Won E, Mendez G, Sokołowski W, Savage PB, Bucki R, Janmey PA. Potential of ceragenin CSA-13 and its mixture with pluronic F-127 as treatment of topical bacterial infections. J Appl Microbiol. 2011;110(1):229–36.
56 Ding B, Yin N, Liu Y, Cardenas-Garcia J, Evanson R, Orsak T, Fan M, Turin G, Savage PB. Origins of cell selectivity of cationic steroid antibiotics. J Am Chem Soc. 2004;126(42):13642–8.