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ORIGINAL ARTICLE

Antibacterial and Antibiofilm Activities of Ceragenins against *Pseudomonas aeruginosa* Clinical Isolates

Cerageninlerin Klinik *Pseudomonas aeruginosa* Suşlarına Karşı Antibakteriyel ve Antibiyofilm Aktiviteleri

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ABSTRACT

Objectives: *Pseudomonas aeruginosa* can cause life-threatening infections that are difficult to treat due to its high resistance to antibiotics and its ability to form antibiotic tolerant biofilms. Ceragenins, designed to mimic the activities of antimicrobial peptides, represent a promising new group of antibacterial agents that display potent anti-*P. aeruginosa* activity. The aim of this study was to evaluate the antibacterial and antibiofilm activities of ceragenins in comparison to colistin and ciprofloxacin against *P. aeruginosa* strains.

Materials and Methods: Biofilm formation and determination of minimum inhibitory concentration (MIC) values of ceragenins (CSA-13, CSA-44, CSA-131, and CSA-138), ciprofloxacin, and colistin were evaluated against 25 *P. aeruginosa* isolates. Four good biofilm-producing strains were chosen for biofilm studies, and sessile MICs and inhibition of molecule adhesion and biofilm formation were evaluated.

Results: The MIC_{50} (μg/mL) values of CSA-13, CSA-44, CSA-131, CSA-138, ciprofloxacin, and colistin were 8, 8, 8, 16, 1, and 2, respectively. The sessile MICs for molecules were greater than planktonic MICs. CSA-13, CSA-44, and CSA-131 were more efficient after 4 h incubation while CSA-138, ciprofloxacin and colistin were more efficient after 1 h incubation. The most efficient agent for inhibition of adhesion was colistin (up to 45%). CSA-131, CSA-138, and colistin were the most efficient agents for inhibition of biofilm formation (up to 90%).

Conclusion: Our study highlights the potential of CSA-131 and CSA-138 as potential alternative agents to conventional antibiotics for the eradication of biofilms of *P. aeruginosa*.

Key words: *Pseudomonas aeruginosa*, biofilm, ceragenin

ÖZ

Amaç: *Pseudomonas aeruginosa*, antibiyotiklere oldukça dirençli ve biyofilm oluşturma yeteneği nedeniyle hayatı tehdit eden enfeksiyonlara neden olabilmektedir. Antimikrobiyal peptidlerin aktivitelerini taklit eden cerageninler, *P. aeruginosa*'ya karşı da güçlü etki gösteren yeni umut verici ajanlardır. Çalışmamızın amacı, cerageninlerin *P. aeruginosa* suşlarına karşı antibakteriyel ve antibiyofilm aktivitelerini değerlendirerek, kolistin ve siprofloksasinle karşılaştırmaktır.

Gereç ve Yöntemler: Yirmi beş *P. aeruginosa* suşunun biyofilm oluşturma özellikleri ve CSA-13, CSA-44, CSA-131, CSA-138, siprofloksasin ve kolistine karşı duyarlılıkları araştırılmış ve antimikrobiyal ajanların minimal inhibitör konsantrasyon (MİK) değerleri belirlenmiştir. Biyofilm çalışmaları için kuvvetli biyofilm oluşturan dört suş seçilerek, antimikrobiyal ajanların sesil MİK değerleri ve adezyon ve biyofilm oluşumuna etkileri araştırılmıştır.

Bulgular: CSA-13, CSA-44, CSA-131, CSA-138, siprofloksasin ve kolistinin $MİK_{50}$ (μg/mL) değerleri sırasıyla 8, 8, 8, 16, 1 ve 2, olarak bulunmuştur. Sesil MİK değerlerinin ise planktonik MİK değerlerinden daha büyük olduğu bulunmuştur. CSA-13, CSA-44 ve CSA-131'in, 4 saatlik inkübasyondan sonra, CSA-138, siprofloksasin ve kolistinin ise 1 saatlik inkübasyondan sonra biyofilme karşı daha etkili oldukları tespit edilmiştir. Adezyon inhibisyonu için kolistinin (%45'e kadar inhibisyon), biyofilm oluşumu inhibisyonu için ise yine kolistin, CSA-131 ve CSA-138'in (%90'a kadar inhibisyon) en etkili ajanlar oldukları tespit edilmiştir.

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Sonuç: Çalışmamızda CSA-131 ve CSA-138'in *P. aeruginosa*'nın biyofilmlerine karşı konvansiyonel antibiyotiklere alternatif olarak kullanılabileceği vurgulanmıştır.

Anahtar kelimeler: *Pseudomonas aeruginosa*, biyofilm, ceragenin

INTRODUCTION

Biofilms are communities of bacterial cells surrounded by an extracellular matrix.[1] According to the National Institutes of Health, biofilms are estimated to account for over 80% of all nosocomial infections and are particularly common with device implants, such as contact lenses, ventricular assist devices, vascular and urinary catheters, and endotracheal tubes.[2-4] *Pseudomonas aeruginosa* forms a highly virulent biofilm that has been associated with higher mortality rates compared with other bacterial pathogens, and there are growing concerns over its increased antimicrobial and multidrug resistance. Because the use of conventional antimicrobial compounds in many cases cannot eradicate biofilms, there is an urgent need to develop alternative compounds and approaches to combat biofilm-based infections.[5-9] One frequently studied target is the bacterial membrane. Most antimicrobial peptides display broad-spectrum antibacterial activities and target the bacterial membrane. However, many antimicrobial peptides are difficult to synthesize and purify due to their complexity and size. In addition, antimicrobial peptides can be substrates for proteases, which limit their *in vivo* half-lives.[10] Recently, a series of cationic derivatives of cholic acid have been synthesized and have been found to have properties that may make them useful antimicrobial agents. The ceragenins, designed to mimic the activities of antimicrobial peptides, are a new class of antimicrobial agent. Ceragenins are not peptide based, are not salt sensitive, and are relatively simple to prepare and purify on a large scale.[11] CSAs, which stands for cationic steroidal antimicrobials, are strongly associated with anionic cell surfaces and in the formation of transient pores in the membrane, resulting in membrane depolarization and cell death. As well as their antibacterial activities against resistant strains of *P. aeruginosa*, *Acinetobacter baumannii*, and *Staphylococcus aureus*, these molecules can also display candidacidal, antiviral, antiparasitic, and anticancer effects.[12-18] Additionally, previous studies have demonstrated the efficacy of ceragenins, especially the leading ceragenin CSA-13, on sessile cells including *P. aeruginosa*.[11-13] However, there has been no study in the literature describing the efficacy of CSA-44 and CSA-131 against *P. aeruginosa* biofilms. The present work examines the *in vitro* antibiofilm activities of CSA-13, CSA-44, CSA-131, and CSA-138 in comparison to colistin and ciprofloxacin against *P. aeruginosa* strains.

MATERIALS AND METHODS

Bacterial isolates

A total of 25 *P. aeruginosa* isolates from various sources including blood, catheters, urine, endotracheal aspirate, esophageal aspirate, ears, sputum, abscesses, and bronchoalveolar lavage fluid submitted to the Clinical Microbiology Laboratories of Cerrahpaşa Faculty of Medicine Hospitals in Turkey (2011) were used. All the strains were identified using API 20 NE (BioMérieux, France) systems. *P. aeruginosa* ATCC 27853 strain was used as the quality control strain.

Antimicrobial agents

The CSAs CSA-13, CSA-44, CSA-131, and CSA-138 were synthesized from a cholic acid scaffold technique as previously described (Figure 1).[19] Colistin and ciprofloxacin were kindly provided by their respective manufacturers. Stock solutions of CSAs from dry powders were prepared in water and the antibiotics were prepared according to the manufacturers' recommendation and stored frozen at -80°C for up to 6 months. Final concentrations of antimicrobials were prepared in cation-adjusted Mueller-Hinton broth (CAMHB, Difco Laboratories, Franklin Lakes, NJ, USA) prior to use.

Media

Tryptic soy broth supplemented with 1% glucose (TSB-glucose, Difco Laboratories) was used for biofilm production and to determine minimum biofilm eradication concentration (MBEC) values, CAMHB was used to determine the minimum inhibitory concentration (MIC), and tryptic soy agar (TSA, Difco Laboratories) was used for culturing bacteria.

MIC determinations

MICs of CSA-13, CSA-44, CSA-131, CSA-138, ciprofloxacin, and colistin were determined against *P. aeruginosa* (planktonic forms) by the microbroth dilution technique as described by CLSI.[20] MIC_{50} is the MIC that inhibits 50% of isolates.

Biofilm formation

Biofilm formation by *P. aeruginosa* strains was evaluated using a crystal violet staining method.[21,22] Briefly, populations of suspensions of bacteria were adjusted with TSB-glucose. Biofilms were formed by pipetting cell suspensions into wells of microtiter plates (Greiner Bio-One, Kremsmuenster, Austria) followed by incubation for 24 h at 37°C. After incubation, the remaining medium was aspirated gently, nonadherent cells were removed, and the wells were stained with 0.1% crystal violet, followed by measurement of optical density (OD) at 600 nm. For each isolate, biofilm production was measured in triplicate and *P. aeruginosa* ATCC 27853 was used as a standard strain. The extent of biofilm production (weak, moderate, and strong) was calculated. Four strong biofilm-producing clinical isolates of *P. aeruginosa* (PA-1, PA-2, PA-3, PA-4) and control strain biofilms were selected. Biofilms were formed in the wells of microtiter plates as previously described by Dosler and Karaaslan[22] with some modifications. An overnight culture of isolates from 24 h growth in TSA was inoculated in TSB-glucose in an orbital shaker at 37°C overnight. Cultures were

centrifuged (about 3000 rpm, 5-10 min) and washed twice with sterile phosphate-buffered saline (PBS) and resuspended in TSB-glucose to a cellular density equivalent to 1×10^6 cells/mL. Biofilms were formed by pipetting 200 µL of the standardized cell suspension into selected wells of sterilized polystyrene flat-bottomed 96-well tissue culture microtiter plates and incubated for 24 h at 37°C. After incubation, the waste medium was aspirated gently, and nonadherent cells were removed by washing the biofilms three times with sterile PBS.

Biofilm attachment assay

Biofilm attachment assays were performed using a previously described method with some modifications.[22,23] The overnight cultures of isolates were prepared to cellular density equivalent to 1×10^6 cells/mL, as described above. Four strong biofilm producing clinical isolates of *P. aeruginosa* were added to each well of 96-well tissue culture microtiter plates with 1X MIC of CSAs and antibiotics. A positive control without antimicrobial agent and a negative control without cells were also prepared. The plates were incubated for 1, 2, and 4 h at 37°C. After incubation, the wells were washed twice with PBS and were measured spectrophotometrically at OD 450 nm on a microplate reader (BioRad Novapath).

Inhibition of biofilm formation

Four strong biofilm producing clinical isolates of *P. aeruginosa* strains (1×10^6 cells/mL) were added to each well of 96-well tissue culture microtiter plates with 1X MIC, 1/10X MIC, and 1/100X MIC of ceragenins and antibiotics.[22,23] A positive control without antimicrobial agent and a negative control without cells were prepared. The plates were incubated for 24 at 37°C. After incubation, the wells were washed twice with PBS and were measured spectrophotometrically at OD 450 nm on a microplate reader (BioRad Novapath).

MBEC determinations

To evaluate the activities of antimicrobial agents on 24-h-old mature *P. aeruginosa* biofilms, biofilms were formed as described above and metabolic activities of the biofilms were assessed using the standardized static microtiter plate model and measured by 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[8phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT, Sigma-Aldrich, St. Louis, MO, USA) reduction assay.[24] The 24-h biofilms in 96-well tissue culture microtiter plates were washed three times with 200 µL of PBS solution and air-dried. Doubling concentrations of antimicrobials were added to the pre-formed biofilms. Aliquots of 200 µL of each concentration were added to each corresponding well and the plates were incubated for 24 h at 37°C. Drug-free biofilm wells containing only TSB-glucose were used as controls. After incubation, the medium was aspirated and washed with PBS three times. An XTT solution was prepared as previously published and added to each well. The microtiter plates were incubated in the dark for 6 h at 37°C. Biofilm growth was measured spectrophotometrically at 480 nm on a microplate reader (BioRad Novapath). MBECs were determined as the minimum antibacterial drug concentration that caused 50% reduction of biofilm compared to drug-free untreated biofilm controls. Each experiment was performed in four wells and was repeated twice.

RESULTS

Susceptibility results against planktonic cells (MIC)

The *in vitro* activities of the studied antimicrobials against 25 *P. aeruginosa* isolates are summarized in Table 1. Susceptibility testing demonstrated that the MIC_{50} (µg/mL) values of CSA-13, CSA-44, CSA-131, CSA-138, ciprofloxacin, and colistin were 8, 8, 8, 16, 1, and 2, respectively (Table 1). CSA-13, CSA-44, and CSA-131 showed similar MIC_{50} results. The highest MIC_{50} results were obtained with CSA-138.

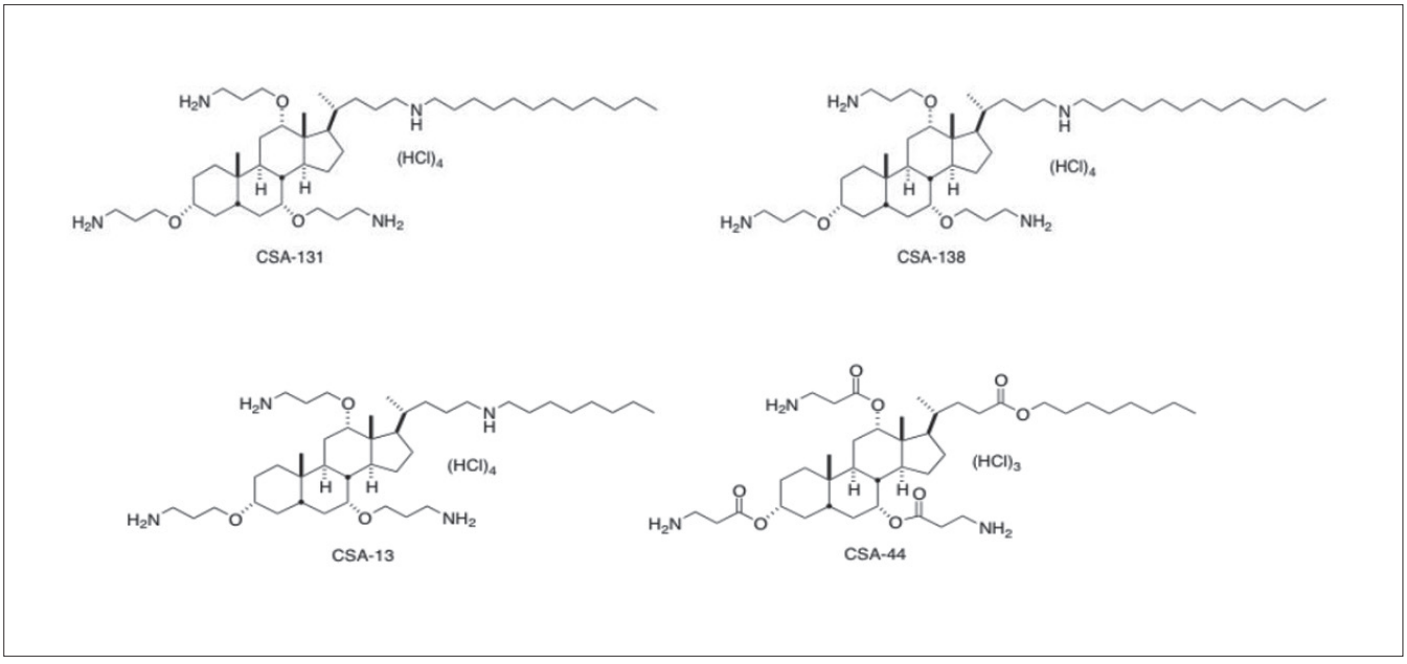

Figure 1. Chemical structures of CSA-13, CSA-44, CSA-131, and CSA-138

Inhibition of adhesion

Inhibition of adhesion rates depended on time. CSA-13, CSA-44, and CSA-131 were more efficient after 4 h incubation, while CSA-138, ciprofloxacin, and colistin were more efficient after 1 h incubation. The most efficient agent for inhibition of adhesion was colistin (up to 45%) (Figure 2).

Inhibition of biofilm formation

Inhibition of biofilm formation rates depended on concentration; the highest inhibition rates were shown at MICs for all agents, as expected. CSA-131, CSA-138, and colistin were the most efficient agents for inhibition of biofilm formation (up to 90%) (Figure 3).

Table 1. MICs and MIC_{50}s of the tested antimicrobial agents (µg/mL)

Antimicrobial agents	MIC	Number of isolates	MIC_{50}
CSA-13	4 8 16 32	4 12 7 2	8
CSA-44	8 16 32	17 5 3	8
CSA-131	4 8 16	8 15 2	8
CSA-138	4 8 16 32	1 9 8 7	16
CIP	0.5 1 2 4	4 10 5 6	1
COL	0.5 1 2 4	1 2 17 5	2

CIP: Ciprofloxacin, COL: Colistin, MIC: Minimum inhibitory concentration

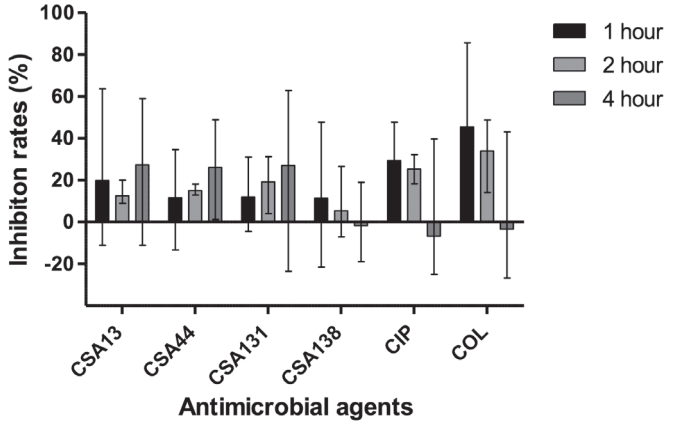

Figure 2. Inhibition of adhesion rates of antimicrobials after 1, 2, and 4 h incubation

Susceptibility results against biofilm cells (MBEC)

We showed that ceragenins are also active against four biofilm producing clinical isolates of *P. aeruginosa* and ATCC 27853 strains. However, MBECs were greater than MICs; ceragenins' MBEC values differ from 16 to 512 µg/mL. Susceptibility results demonstrated that the MBECs (µg/mL) of CSA-13, CSA-44, CSA-131, and CSA-138 were 32-64, 32-256, 16-128, and 128-512, respectively. Ciprofloxacin and colistin showed similar MBEC values (8-64 µg/mL) (Table 2). Among the tested ceragenins, CSA-13 was the most effective agent against mature biofilms.

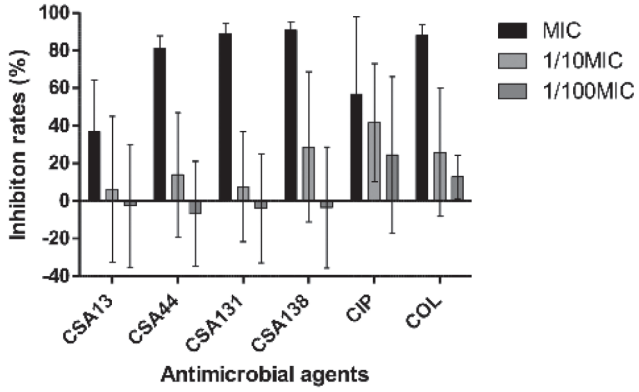

Figure 3. Inhibition of biofilm formation rates of antimicrobials at different concentrations

Table 2. MBECs of the tested antimicrobial agents (µg/mL)

	CSA-13	CSA-44	CSA-131	CSA-138	CIP	COL
PA-1	64	256	128	256	16	16
PA-2	64	512	16	128	32	8
PA-3	32	32	64	256	8	8
PA-4	64	64	128	128	64	64
P. aeruginosa ATCC 27853	64	64	128	512	32	32

CIP: Ciprofloxacin, COL: Colistin, MBEC: Minimum biofilm eradication concentration

DISCUSSION

Ceragenins are unique, low molecular mass, cationic steroid compounds. These compounds mimic the activity of naturally occurring antimicrobial peptides. Previous studies have shown that ceragenins, especially CSA-13, are potent antimicrobials against various microorganisms including multidrug-resistant *P. aeruginosa*.[11-13] In the present work, in addition to CSA-13, we investigated the effect of CSA-44, CSA-131, and CSA-138 on the formation and adhesion of a biofilm by five good biofilm forming strains of *P. aeruginosa* using the crystal violet staining method. Ciprofloxacin and colistin were used as comparative conventional antibiotics. Here we report that the MIC_{50} (µg/mL) values of CSA-13, CSA-44, CSA-131, CSA-138, ciprofloxacin,

and colistin were 8, 8, 8, 16, 1, and 2, respectively, for 25 *P. aeruginosa* strains and these results are similar to those of other published studies.

Chin et al.[11] investigated the efficacy of CSA-13 against 50 clinical isolates of *P. aeruginosa*, and compared them with various antibiotics. CSA-13 was shown to have a 1-32 µg/mL MIC range and 16 µg/mL MIC_{50}. Bozkurt Guzel et al.[25] showed the antibacterial activities of CSA-13 against clinical isolates of *P. aeruginosa* with 8-16 µg/mL MIC range and 8 µg/mL MIC_{50} (Poster presentation, ECCMID, 2017). In another study, Vila-Farrés et al.[26] determined the MIC_{50} (µg/mL) values of CSA-13, CSA-44, CSA-131, and CSA-138 against *P. aeruginosa* strains as 4, 4, 1, and 2, respectively. As shown in previous studies and our study, CSA-13, CSA-44, and CSA-131 showed similar MIC_{50} results. In our study the highest MIC and MIC_{50} results were obtained with CSA-138 against *P. aeruginosa*.

Biofilms are specific and organized communities of cells under the control of signaling molecules, rather than random accumulations of cells resulting from cell division. It is well known that biofilm embedded microorganisms possess resistance to both antimicrobial agents and host immune responses when compared to their planktonic forms.[1] In the present study, MBECs of ceragenins and antibiotics were higher than MICs, as expected. Ciprofloxacin's and colistin's MBECs were less than those of ceragenins, but among the ceragenins CSA-13 was the most effective agent against mature biofilms. In contrast to the antimicrobial effects against bacteria and fungi, the antibiofilm activity of ceragenins is not well defined against biofilms. Only a few studies showed that ceragenins, especially CSA-13, have antibiofilm effects against *P. aeruginosa*.[27-29]

Novel approaches to biofilm control might take one of three main forms: effective reduction of planktonic cells before biofilm formation, inhibition of cell adhesion and biofilm formation, or removal of established biofilm.[22,30] A novel field of research has accordingly focused on preventing biofilm development and adherence. For this purpose in the present study, we investigated the inhibition of bacterial attachment to the surfaces, as well as the inhibition of biofilm production by MIC or subMIC values of ceragenins, ciprofloxacin, and colistin. Although inhibition of adhesion and biofilm formation rates depended on time and concentration, it was shown that CSA-131 and CSA-138 were the most efficient agents with inhibition rates of adhesion of 11.38 and 11.97 and biofilm formation of 90.7 and 88.69 (%), respectively, at MIC. Our results clearly showed that ceragenins are much more effective on biofilm formation rather than attachment. These results indicated that biofilm formation can be prevented by inhibiting not only attachment but also other mechanisms.

In one of our studies the effects of CSA-13, CSA-8, CSA-44, CSA-131, and CSA-138 were evaluated on adhesion and biofilm formation of *C. albicans* and it was shown that all of the studied CSAs inhibited *C. albicans* biofilm formation in a concentration-dependent manner.[15]

Recently, Olekson et al.[30] also showed antibiofilm activities of various ceragenins including CSA-13, CSA-44, CSA-131, CSA-138, CSA-142, and CSA-192 against preformed mixed-species biofilms of *P. aeruginosa* and *S. aureus*. Nagant et al.[27,29] demonstrated that CSA-13 affected biofilm formation, the surface of biofilms, and the inside of established biofilms of *P. aeruginosa*. Gu et al.[28] showed that CSA-138 also inhibited biofilm formation of *P. aeruginosa* on lenses. Our results were similar to those of these studies.

CONCLUSION

To the best of our knowledge this is the first report to evaluate the antibiofilm activities of CSA-44 and CSA-131 against *P. aeruginosa*. According to our results, ceragenins, especially CSA-13, CSA-131, and CSA-138, appear to be good candidates in the treatment of *Pseudomonas* infections as well as biofilm-related ones. Therefore, CSAs are promising candidates for further research as antibacterial drugs and as agents for treatment of biofilm infections. Future studies should be performed to correlate the safety, efficacy, and pharmacokinetic parameters of these molecules.

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Conflict of Interest: No conflict of interest was declared by the authors.

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