Synergistic Activity of Ceragenins Against Carbapenem-Resistant *Acinetobacter baumannii* Strains in Both Checkerboard and Dynamic Time-Kill Assays

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Abstract

Acinetobacter baumannii is an emerging opportunistic pathogen that primarily infects critically ill patients in nosocomial settings and there is a need for identifying new alternative therapeutic agents against these organisms. Ceragenins are non-peptide, membrane-active agents that mimic the antimicrobial properties of antimicrobial peptides (AMPs) and affect the membrane permeability of microorganisms. The in vitro activities of CSA-8, CSA-13, CSA-44, CSA-131, CSA-138 either alone or in combination with colistin (sulphate) were determined against 25 carbapenem-resistant *A. baumannii* strains. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of selected ceragenins and colistin against these isolates were measured by in vitro microbroth dilution techniques. Checkerboard techniques and time-kill assays were performed to determine the activities of combinations. The MIC_{50} values (mg/L) of CSA-8, CSA-13, CSA-44, CSA-131, CSA-138 and colistin were 32, 4, 8, 2, 4 and 0.5, respectively. The MIC_{90} (mg/L) of CSA-8, CSA-13, CSA-44, CSA-131, CSA-138 and colistin were 128, 8, 16, 8, 16 and 16, respectively. At 6 h, 1×MIC and 2×MIC of CSA-13 were bactericidal. CSA-13 + colistin combination displayed synergistic interaction. Antagonism between antimicrobials was not observed. According to the results, CSA-13 and CSA-131 can be good alternatives for infections caused by carbapenem-resistant *A. baumannii*.

Introduction

Acinetobacter baumannii is a ubiquitous Gram-negative opportunistic pathogen that is commonly associated with aquatic environments [1]. *A. baumannii* has emerged as a serious cause of nosocomial infections, especially among critically ill patients in intensive care units, causing ventilator-associated pneumonia (VAP), septicemia, secondary meningitis, endocarditis, infections of the skin, soft tissues, urinary tract and those originating from prosthetic devices [2]. Due to the rapid development of resistance to various antibiotics, only a few antibiotics provide effective treatment

caused by this pathogen. For example, carbapenems have been one of the main antimicrobial classes used against *A. baumannii* infections, but the emergence and dissemination of carbapenemases have diminished the utility of this class of drugs from an already limited list of existing treatment options. In 2017, WHO published a list of antibiotic-resistant priority pathogens for which new antibiotics are urgently needed and the most critical group is carbapenem-resistant *A. baumannii* (CRAB). Among the alternatives for the treatment of infections due to CRAB, polymyxins and other non-β-lactam agents may be valuable options [3–6].

Endogenous AMPs have been the subjects of extensive studies, in part due to their breadth of activity and the proposition that bacterial activities are unlikely to readily develop resistance. However, AMPs have disadvantages such as having complex structures, difficult synthesis and purification mechanisms, and short half-lives due to proteases [7, 8]. Ceragenins have been developed as a result of studies to develop non-peptide derivatives of AMPs and thus reduce their disadvantages [8]. Ceragenins, which are membrane active molecules, combine with the cell surfaces of microorganisms and cause depolarization and death. Only a few

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studies have been reported in which the potential activities of ceragenins CSA-13 and CSA-131 have been evaluated against CRAB alone or in combination with other antibiotics [10–16]. However, there are no published studies describing the activities of ceragenins CSA-8, CSA-44 and CSA-138 against CRAB.

To better understand the antibacterial activity of ceragenins against CRAB, we evaluated the antibacterial activities of CSA-8, CSA-13, CSA-44, CSA-131 and CSA-138 against clinical isolates of CRAB. We compared ceragenin activity with that of colistin as well as potential synergy in combination using both checkerboard and time-kill curve experiments.

Material and Methods

Bacterial Strains

A total of 25 (Ab-1, Ab-2…Ab-25) non-repeat CRAB strains were obtained from the Department of Infectious Diseases, Faculty of Cerrahpasa Medicine, Istanbul University. API 20 NE System (bioMerieux Vitek, Marcy l'Etoile, France) was used to identify strains. Strains were determined as carbapenem-resistant according to disc diffusion and microdilution methods. For combination time-kill experiments, four strains were used and *Escherichia coli* ATCC 25,922 (Rockville, Md., USA) was quality control strain.

Antimicrobial Agents

CSA-8, CSA-13, CSA-44, CSA-131 and CSA-138 (Fig. 6) were synthesized and provided by one of the authors (P.B.S.) [8]. Colistin and meropenem were obtained from Sigma-Aldrich and Astra Zeneca (Turkey), respectively. Stock solutions of ceragenins and colistin from dry powders were prepared in distilled water, stored frozen at −80 °C and used within 6 months. Meropenem was prepared on the day of use.

Determinations of MICs and MBCs

MICs and MBCs were determined by using the microbroth dilution method according to CLSI [17]. Serial twofold dilutions ranging from 128 to 0.06 mg/L of antimicrobials were prepared in the 96-well U-bottom microplates. The inoculum was added with a final concentration of 5×10^5 cfu/mL. Microplates were incubated at 37 °C for 18–20 h. Experiments were done independently in duplicate. To determine MBCs, after incubation, 0.01 mL samples from each well where no growth was observed were taken and plated onto Tryptic soy agar (TSA). The lowest concentration of antibiotic giving at least a 99.9% killing of the initial inoculum was defined as the MBC [18].

Determination of Fractional Inhibitory Concentration Index (FICI)

The microbroth checkerboard technique was used to determine the effects of combinations [19]. A mixture of different concentrations of the antibiotics was added to the 96-well U-bottom microplates and inoculated with a final concentration of approximately 5×10^5 cfu/mL of inoculum. After incubation at 37 °C for 18–20 h, the fractional inhibitory concentration index (FICI) was determined as the sum of concentrations of the antimicrobials required for growth inhibition divided by the MICs of the antimicrobials. After incubation, FIC was determined and FIC of ≤ 0.5 was defined as synergism, FIC of > 0.5–4 as no interaction and FIC of 4.0 as antagonism [20].

Time-Kill Assays

Time-kill assays were performed on four CRAB strains (Ab-3, Ab-9, Ab-13, Ab-23). Tubes containing freshly prepared cation-adjusted Mueller–Hinton broth supplemented with the drug were inoculated with *A. baumannii* isolates to a density of 10^6 cfu/mL in a final volume of 20 mL and incubated in a shaking bath at 37 °C. Aliquots were removed at time 0, 1, 2, 4, 6 and 24 h post inoculation and serially diluted in saline for determination of viable counts. Diluted samples (10 μL) were plated on TSA plates, and bacterial counts were determined after 18 h of incubation at 37 °C. Concentrations of antimicrobial agents used were at 0.5×MIC, 1×MIC and 2×MIC alone, or in combination. The bactericidal activity was defined as a 3 log10 cfu/mL reduction in the colony count, relative to the initial inoculum. Synergy and indifference were, respectively, defined as a ≥ 2 log10 and < 2 log10 decrease in the cfu count when the combination was compared with the most active single drug after 24 h of incubation [18]. Experiments were performed independently in duplicate. The limit of detection (LOD) was ≤ 1.3 $\log_{10}$ cfu/mL. The mean cfu/mL values for the bacteria were also compared between groups for each antibiotic alone or in combination. Statistical analysis for the comparison of each combination was performed using one-way Anova test—the non-parametric analysis of variance—and Bonferroni correction for post hoc comparisons. Any value of P below 0.05 was considered as statistically significant.

Results and Discussion

Susceptibility

MICs and MBCs of CSA-8, CSA-13, CSA-44, CSA-131, CSA-138, colistin and meropenem were determined and

Table 1 MIC and MBC values (mg/L) of antimicrobial agents against 25 clinical CRAB strains

Antimicrobials	MICrange/MBC range	MIC50/MBC50	MIC90/MBC90
CSA-8	16–128/32–128	32/32	128/128
CSA-13	1–16/2–16	4/4	8/32
CSA-44	8–16/16–32	8/8	16/32
CSA-131	1–8/2–16	2/4	8/16
CSA-138	4–32/4–32	4/4	16/16
Colistin	0.125–4/0.5–4	0.5/1	4/4
Meropenem	8–128/8–256	16/32	32/64
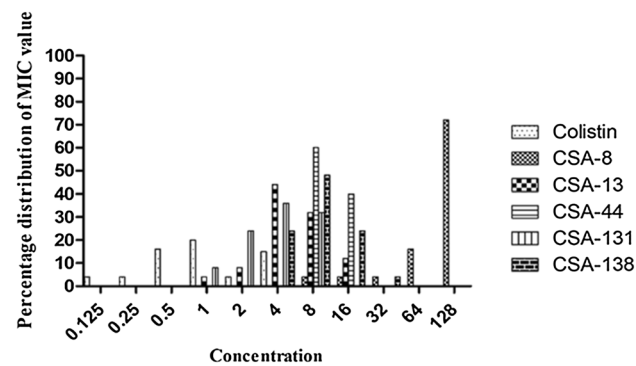
Fig. 1 Percentage distribution of MIC value of tested antimicrobials

summarized in Table 1 and Fig. 1. All the strains were resistant to meropenem.

Checkerboard

In vitro activities of CSAs combined with colistin or CSAs against CRAB are shown in Tables 2, 3. CSA-13 + colistin, CSA-13 + CSA-131, CSA-13 + CSA-138 and CSA-131 + CSA-138 combinations were determined as synergistic because their FIC indexes were less than 0.5. However, additive effects were detected in all combinations. Any combination did not show any antagonistic interaction.

Time-Kill Assays

Time-kill assays were performed on four isolates with colistin, CSA-13 and CSA-131 to observe their behaviours alone and in combinations and the results are given in Table 4 and Figs. 2, 3, 4, 5. Concentrations of 1×MIC and 2×MIC of CSA-13 were bactericidal along the curve at 6 h, with re-growth being observed at 24 h. In addition to this, earlier bactericidal effects were documented at the end of 2 h and 4 h with colistin, CSA-13 and CSA-131. In combination

Table 2 In vitro activity of CSA-13, CSA-131, CSA-138 combined with colistin against CRAB by using the microbroth checkerboard technique

Antimicrobials	N	Synergistic effects FIC 0.5	Additive effects FIC > 0.5–4.0
Colistin + CSA-13	7	1	6
Colistin + CSA-131	2	0	2
Colistin + CSA-138	2	0	2

Table 3 In vitro activity of some CSA molecules combined with CSA molecules against CRAB by using the microbroth checkerboard technique

Antimicrobials	N	Synergistic effects FIC 0.5	Additive effects FIC > 0.5–4.0
CSA-13 + CSA-131	7	1	6
CSA-13 + CSA-138	2	1	1
CSA-131 + CSA-138	5	1	4

time-kill studies, CSA-13 + colistin combination demonstrated synergistic activity against two of four strains at 0.5×MIC and 1×MIC and against one of four strains at 2×MIC. CSA-131 + colistin combination demonstrated synergistic activity against two of four strains at 1×MIC and against one of four strains at 2×MIC. CSA-13 + CSA-131 combination demonstrated synergistic activity against one of four strains at 1×MIC and against two of four strains at 2×MIC. Earlier synergistic effects were noted at 2, 4 and 6 h.

Recently, outbreaks caused by resistant *A. baumannii* have been reported in Turkey as well as in all parts of the World [21–23]. In the past decade, there has been a dramatic increase in CRAB [22]. Therefore, in order to take the microorganism under control, selection of the antimicrobial agents is extremely important. Ceragenins are a group of cholic acid derivatives that have potent activities against various microorganisms. Recently, it is documented that CSA-13 and CSA-131 are active both against colistin-resistant and carbapenem-resistant *A. baumannii* strains [9–16]. Villa-Farres et al. showed that CSA-13 and CSA-131 showed good activity against resistant-*A. baumannii*, with a 2 mg/L of MIC$_{50}$ value [16]. In this study, the MIC$_{50}$ values of the studied antibiotics were ranked as follows: colistin > CSA-131 > CSA-13 = CSA-138 > CSA-44 > CSA-8. It is thought that CSA-131 has the best activity because it has long hydrophobic chain at C12 carbon (Fig. 6). The MICs and MBCs of the CSA-13 and CSA-131 are closer to those of colistin. The current results are similar to those reported by Bozkurt-Guzel et al. [9] and Villa-Farres et al. [16] Regarding all tested CSAs, the results obtained reaffirm the good activity

Table 4 Extent of bacterial killing exerted by antibiotics alone and in combination (time-kill curve) over time against CRAB clinical strains

Antibiotics and concentrations (mg/L)	No. of isolates showing the following log10 cfu/ml decrease at the designated incubation time														
	1.h			2.h			4. h			6.h			24.h		
	−1	−2	−3	−1	−2	−3	−1	−2	−3	−1	−2	−3	−1	−2	−3
CSA-13[a]															
0.5×MIC	2	0	0	3	1	0	0	3	1	1	0	0	0	0	0
1×MIC	4	0	0	1	2	1	0	1	3	0	3	1	0	0	0
2×MIC	3	1	0	0	2	2	0	0	4	0	3	1	0	0	0
CSA-131[a]															
0.5×MIC	3	0	0	1	3	0	0	4	0	4	0	0	0	0	0
1×MIC	4	0	0	0	4	0	0	4	0	3	1	0	0	0	0
2×MIC	3	1	0	0	0	4	0	0	3	0	4	0	0	0	0
Colistin[a]															
0.5×MIC	1	0	0	3	1	0	0	4	0	3	1	0	0	0	0
1×MIC	4	0	0	0	3	1	0	1	2	0	4	0	0	0	0
2×MIC	3	0	0	0	3	1	0	1	1	0	4	0	0	0	0
CSA-13 + Colistin[b]															
0.5××MIC	1	0	0	2	0	0	1	1	0	3	0	0	2	1	1
1×MIC	1	2	0	4	0	0	3	1	0	3	1	0	2	2	0
2×MIC	3	1	0	2	2	0	4	0	0	4	0	0	3	1	0
CSA-131 + Colistin[b]															
0.5×MIC	0	0	0	2	1	0	3	1	0	4	0	0	4	0	0
1×MIC	1	0	0	1	0	0	3	0	0	4	0	0	2	2	0
2×MIC	1	0	0	1	0	0	4	0	0	3	1	0	3	1	0
CSA-13 + CSA 131[b]															
0.5×MIC	0	0	0	2	0	0	3	1	0	4	0	0	3	1	0
1×MIC	1	0	0	1	0	0	1	1	1	4	0	0	4	0	0
2×MIC	1	0	0	3	0	0	3	0	0	4	0	0	2	2	0

[a]Bactericidal activity was defined as a ≥ 3-log10 cfu/ml decrease from the initial inoculum

[b]Synergy was defined as a 100-fold increase in colony count at 24 h by the combination compared with the most active agent alone

of these compounds except CSA-8 against CRAB, highlighting CSA-13 and CSA-131 as the best compounds, with lower MICs against all of the strains (Table 1). We found the highest MIC results with CSA-8. MIC ranges of CSA-44 and CSA-138 are almost the same. Our study also shows that CSA-13, CSA-44 and CSA-138 have an MIC_{50}/MBC_{50} ratio of 1, suggesting that the bactericidal activity is close to the inhibitory concentration.

Furthermore, according to time-kill assay, concentrations of 1×MIC and 2×MIC of CSA-13 were bactericidal along the curve at 6 h, with re-growth being observed at 24 h. In addition to this, earlier bactericidal effects were documented at the end of 2 h and 4 h with colistin, CSA-13 and CSA-131.

In checkerboard, different effects were observed with additive effect being the most frequent interaction between

colistin + CSA-13 and CSA-13 + CSA-131 combinations (Tables 2, 3). Colistin + CSA-13 was synergistic with one of the seven strains and showed an additive effect with the remaining strains. A synergistic effect was observed for CSA-13 + CSA-131, CSA-13 + CSA-138 and CSA-131 + CSA-138 combinations. Notably, an antagonistic effect was never detected. In the literature, combinations of CSA-13-colistin have previously been reported to be synergistic against CRAB [9], which is in line with our results.

The results of combination time-kill assay showed that not only, 2×MIC combinations of CSA-13 + colistin are synergistic, but also 0.5×MIC combinations are synergistic. In CSA-131 + colistin combination, synergistic activities are determined when the agents are used at 1×MIC or 2×MIC. CSA-13 + CSA-131 combination demonstrated synergistic

Fig. 2 Time-kill assays for CSAs and colistin alone and in combination against Acb-3. **a** 0.5×MIC, **b** 1×MIC **c** 2×MIC. The X-axis represents the killing time, and the Y-axis represents the logarithmic bacterial survival. Error bars indicate standard deviation. cfu: colony-forming units
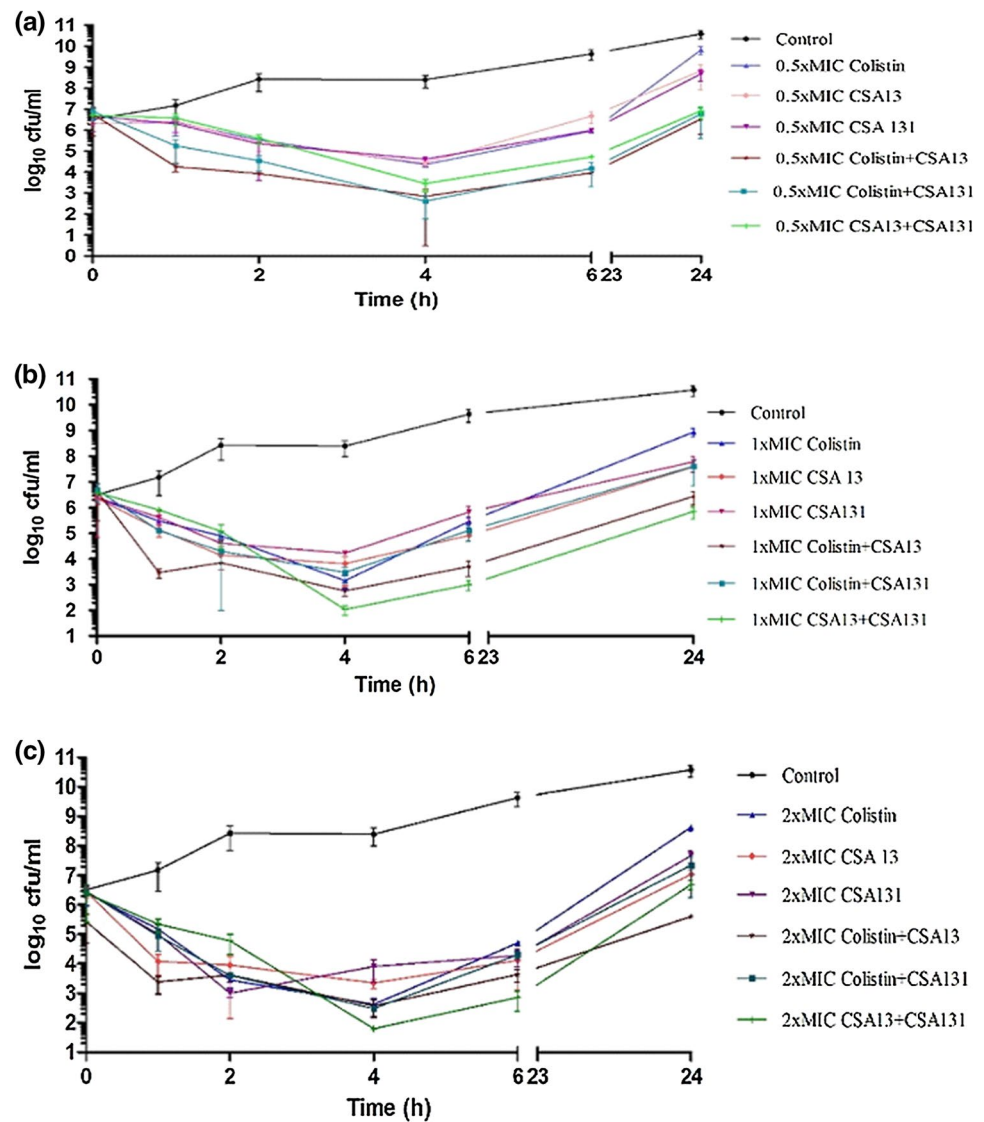

activity against one of four strains at 1×MIC and against two of four strains at 2×MIC (p < 0.05). Earlier synergistic effects were noted at 2, 4 and 6 h.

According to these combination results, it seems to be promising to combine CSA-13, CSA-131 and colistin where antibacterial agents provide a bactericidal effect in a concentration-dependent manner. Ceragenins can increase susceptibility to antimicrobials by affecting the permeability of both the outer membrane and cytoplasmic membrane of Gram-negative bacteria [12]. The findings of the study extend current knowledge on synergistic activity of CSA-13 and CSA-131 in combination with colistin and support the potential role of CSAs as a novel therapeutic agent against CRAB.

Fig. 3 Time-kill assays for CSAs and colistin alone and in combination against Acb-9. **a** 0.5×MIC, **b** 1×MIC **c** 2×MIC. The X-axis represents the killing time, and the Y-axis represents the logarithmic bacterial survival. Error bars indicate standard deviation. cfu: colony-forming units
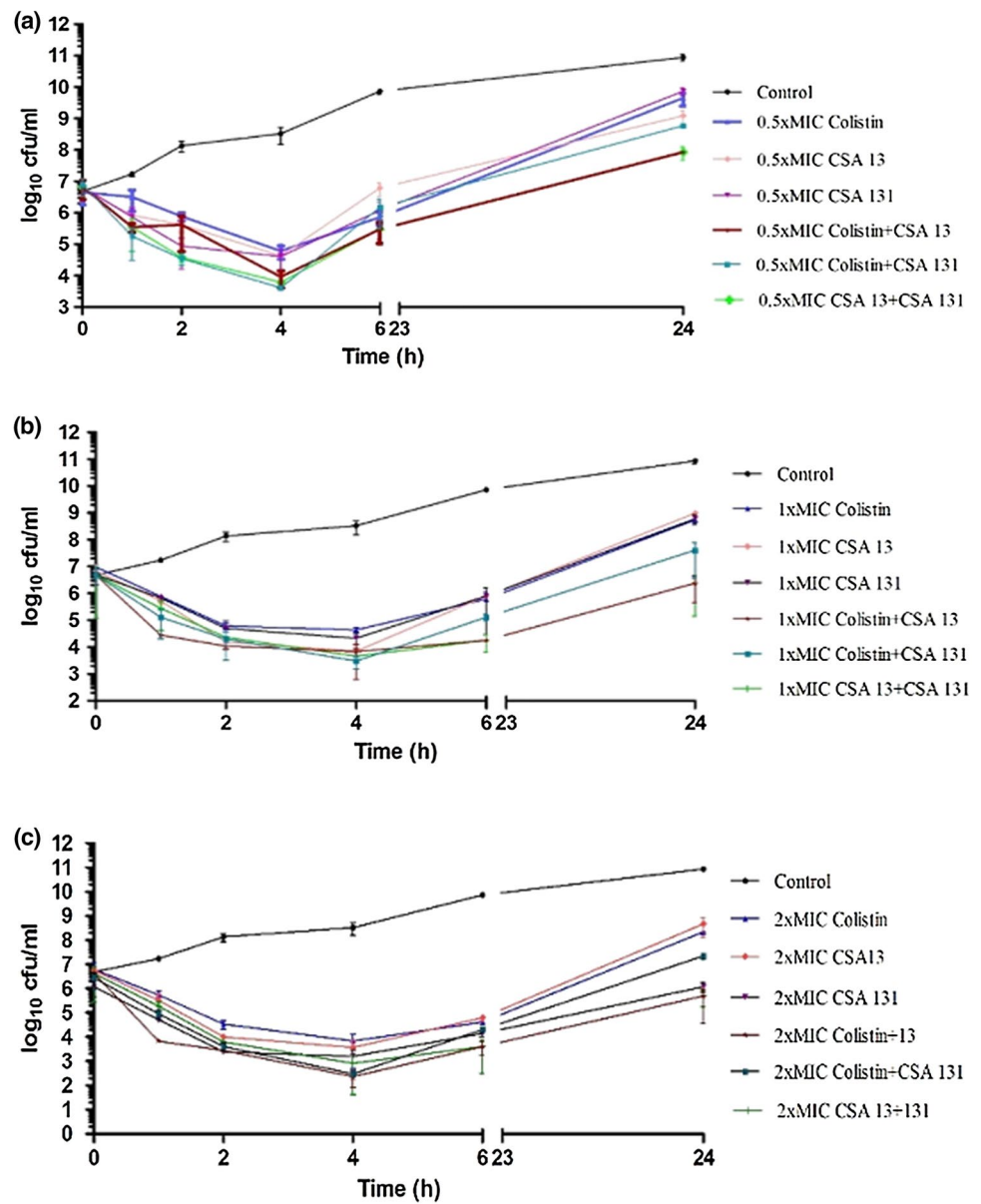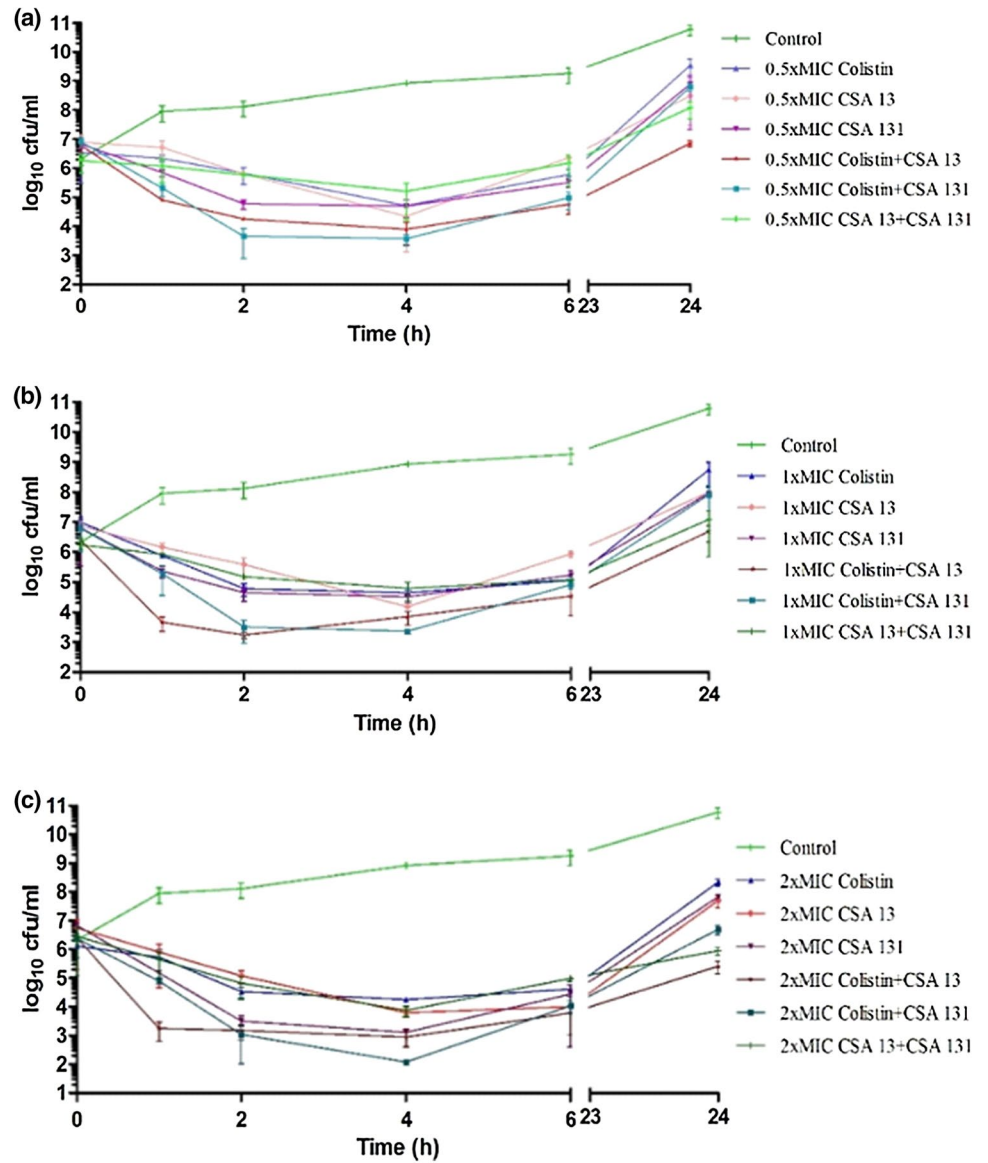
Fig. 4 Time-kill assays for CSAs and colistin alone and in combination against Acb-13. **a** 0.5×MIC, **b** 1×MIC **c** 2×MIC. The X-axis represents the killing time, and the Y-axis represents the logarithmic bacterial survival. Error bars indicate standard deviation. cfu: colony-forming units
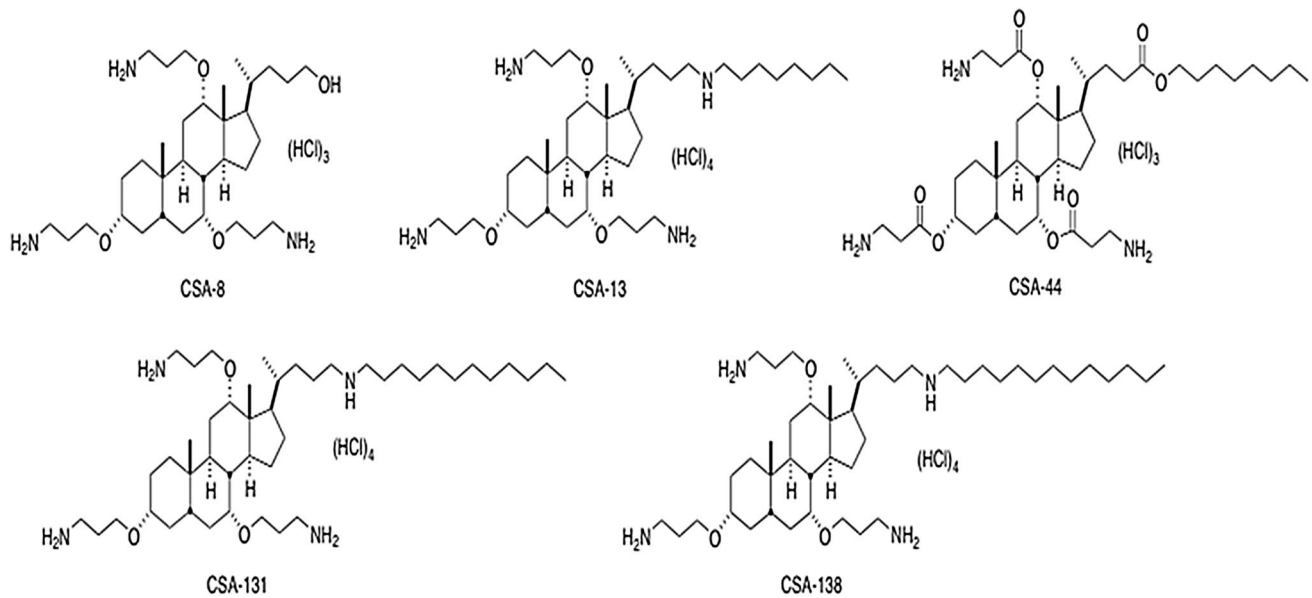
Fig. 5 Time-kill assays for CSAs and colistin alone and in combination against to Acb-23. **a** 0.5×MIC, **b** 1×MIC **c** 2×MIC. The X-axis represents the killing time, and the Y-axis represents the logarithmic bacterial survival. Error bars indicate standard deviation. cfu: colony-forming units
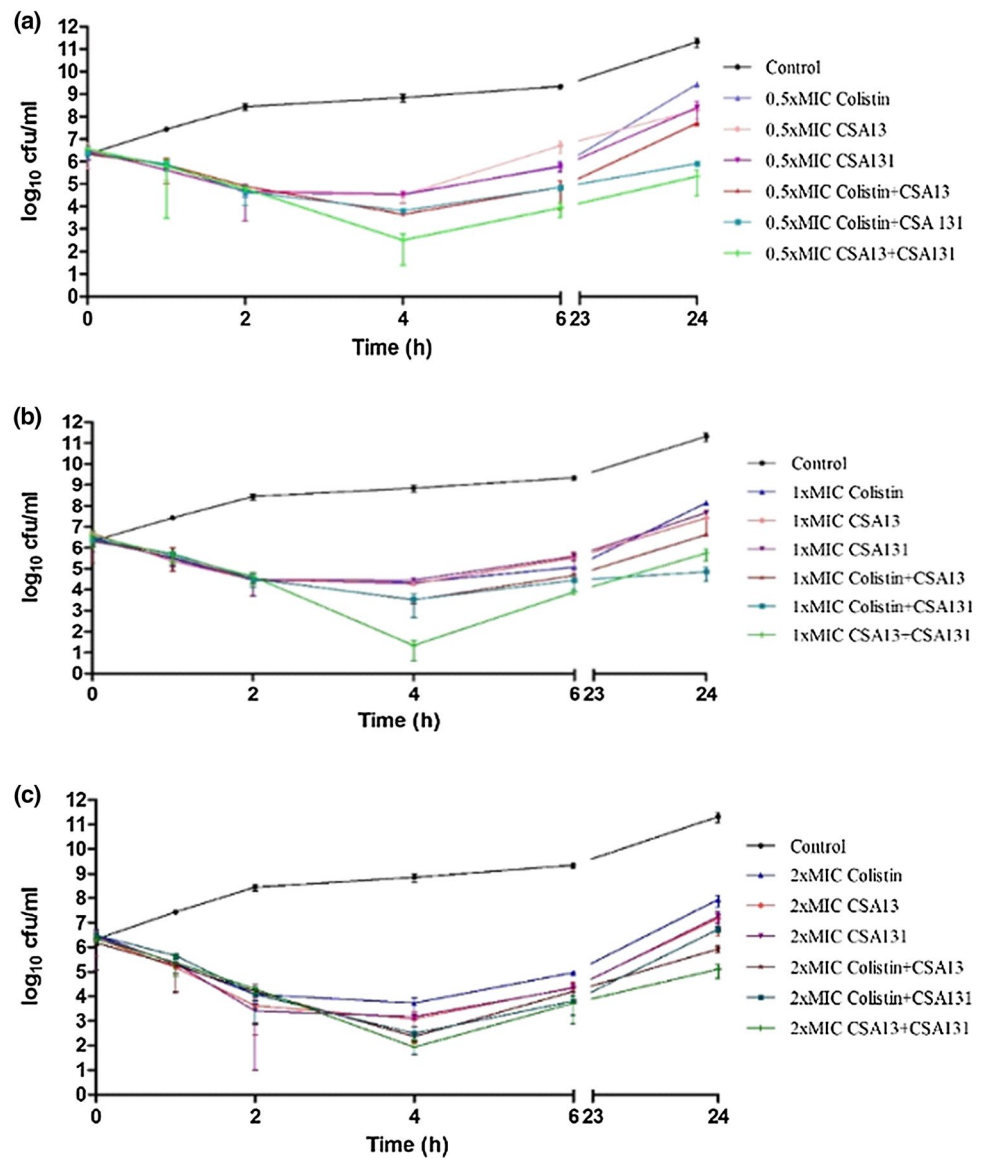
Fig. 6 The structure of ceragenins used in the present study

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest for this study.

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