








Ceragenins exhibiting promising antimicrobial activity against various multidrug resistant Gram negative bacteria

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ABSTRACT

Ceragenins are novel promising agents for the treatment of infections caused by multi-drug resistant microorganisms. Since colistin resistance has become a worldwide problem, the need for new treatment agents has been increasing steadily. Therefore, this study aimed to investigate *in vitro* antimicrobial activities of ceragenins (Cationic Steroid Antibiotics) [CSA-8, CSA-13, CSA-142 and CSA-192] against multidrug resistant Gram negative isolates from Turkey. Experiments were performed by using broth microdilution method against *Klebsiella pneumoniae*, *Morganella morganii*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* isolates. All microorganisms except for three isolates were identified as multidrug resistant. Among tested ceragenins, CSA-13 showed the best results (MIC: 8-64 µg/ml). Nevertheless, the antimicrobial activity of CSA-8 was not significant. In conclusion, ceragenins appear to be a good candidate as antimicrobial therapy in the presence of multidrug (including colistin) resistant microorganisms.

Keywords: Ceragenins, colistin resistance, gram negative pathogens

INTRODUCTION

Antibiotic resistance is defined as the loss of activity of antibiotics against microorganisms. Antibiotic resistant bacteria, especially Gram negative bacteria, cause an increasing concern and antibiotic options used in the treatment of infectious diseases have been gradually decreasing (Zaman et al. 2017). For a long time now, colistin has been used by clinicians as only option to treat these infections. However, colistin resistance has been reported due to its widespread use in many countries. (Marchaim et al. 2011; Ah et al. 2014; Rojas et al. 2016; Sonnevend et al. 2016; Otter et al. 2017; Rossi et al. 2017). Therefore, discovering novel antimicrobials are essential in continuing to fight against antibiotic resistant bacteria.

Some of the new generation of promising agents are antimicrobial peptides (AMPs) (Döşler 2017) and ceragenins (Lai et al. 2008; Bolla et al. 2011). AMPs display broad-spectrum antimicrobial activities by interacting with the bacterial cell membrane. On the other hand, the clinical use of AMPs is problematic due to the difficulty of its synthesis and the lack of stability. Furthermore, AMP resistance may occur through some mechanisms such as secretion of proteases, release of AMP degrading enzymes, active efflux (Band and Weiss 2014; Döşler 2017). Ceragenins, Cationic Steroid Antibiotics (CSA), which are synthetic cholic acid derivatives, have the same mechanism of action as natural AMPs and mimic the activities of AMPs (Lai et al. 2008). Ceragenins can be prepared in large quantities because of their simple structures. Additionally, they are not degraded by proteases because they do not have peptide structure. Multiple studies have indicated that ceragenins display broad-spectrum activities against both Gram positive and Gram negative bacteria including drug-resistant bacteria, biofilms, fungi and viruses (Bozkurt-Guzel et al. 2014a; Durnas et al. 2016; Olekson et al. 2017). Besides, some studies have shown that the toxicity of the ceragenins is not remarkable (Saha et al. 2008; Leszczyńska et al. 2013).

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It has been shown that ceragenins have different resistance mechanisms than colistin and can be used in colistin-resistant *Klebsiella pneumoniae* isolates (Hashemi et al. 2017). Further studies are needed to determine the efficacy of ceragenins against multi-drug resistant bacteria. Thus, we aimed in this study to determine the effects of ceragenins against some multidrug resistant (including colistin) Gram negative bacteria obtained from Medical Microbiology Laboratories of the Faculty of Medicine, Çanakkale Onsekiz Mart University, Çanakkale, Turkey and Istanbul Faculty of Medicine, Istanbul, Turkey.

MATERIAL AND METHODS

Bacterial isolates

A total of 17 clinically isolates including *K. pneumoniae* (n=9), *Morganella morganii* (n=1), *Pseudomonas aeruginosa* (n=5) and *Stenotrophomonas maltophilia* (n=2) isolated from various specimens were obtained from the Medical Microbiology Laboratories of the Faculty of Medicine, Çanakkale Onsekiz Mart University, Çanakkale, Turkey and Istanbul Faculty of Medicine, Istanbul, Turkey between 2006-2016. All isolates were identified by Vitek 2 (BioMerieux, France) or API20 NE System (BioMerieux, France). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 are used as quality control strains.

Antimicrobial agents

CSA-8, CSA-13, CSA-142 and CSA-192 were synthesized from cholic acid as previously described (Guan et al. 2000). Chemical structures of ceragenins were shown in Figure 1. Meropenem, cefotaxime, colistin and levofloxacin were obtained from manufacturer (Sigma-Aldrich). Stock solutions from dry powders were prepared at a concentration of 5120 µg/mL and stored at -80°C and used within 6 months of preparation.

Antimicrobial susceptibility testing

Mueller-Hinton broth (MHB; Difco Laboratories, Detroit, Mich., USA) supplemented with divalent cations to a final

concentration of 25 mg of Mg²⁺ and 50 mg of Ca²⁺ per liter (CAMHB) was used for *in vitro* antimicrobial activity studies and Tryptic soy agar (TSA; Difco Laboratories) were used for colony counts. Minimum inhibitory concentrations (MICs) and Minimum bactericidal concentrations (MBCs) were determined by a microdilution method according to the International Organization for Standardization (ISO) (ISO 20776-1, 2006). Serial two-fold dilutions of antibiotics were prepared in CAMHB in the test tray. A final concentration of 5 × 10⁵ CFU/mL of inoculum was added. The trays were incubated at 37°C for 18-20 h. The MIC value was determined as the lowest concentration of antibiotics that led no visible bacterial growth. 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 plating onto TSA. The MBCs were defined as the lowest concentration of antibiotic giving at least 99.9 % (three-log) reduction of the initial inocula. All experiments were performed in duplicate.

RESULTS

According to the results, all isolates were evaluated considering EUCAST limits and most of them were found to be resistant to colistin, meropenem, cefotaxime and levofloxacin (Table 1) (EUCAST, 2018a). If an isolate is non-susceptible to at least one agent in three or more antimicrobial classes, it is defined as multidrug resistant (MDR) (Magiorakos et al. 2012). Accordingly, most of the tested isolates in this study (excluding two *K. pneumoniae* isolates and one *P. aeruginosa* isolate) were defined as MDR. Two *K. pneumoniae* isolates and one *P. aeruginosa* isolate were resistant to at least one agent in the two classes, but intermediate to meropenem (MIC: 8 µg/mL). CSAs, especially CSA-13 displayed considerable inhibitory effect (MIC: 8-64 µg/mL) against *K. pneumoniae*, *M. morganii*, *P. aeruginosa* and *S. maltophilia* isolates even though resistant to colistin (MIC: 16->256 mL) (Table 2). The least effective agent was CSA-8 (MIC: >128 µg/mL) against all microorganisms. CSA-142 (MIC: 16-64 µg/mL) showed moderate activity. Antibacterial activity of CSA-192 was determined from 32 µg/

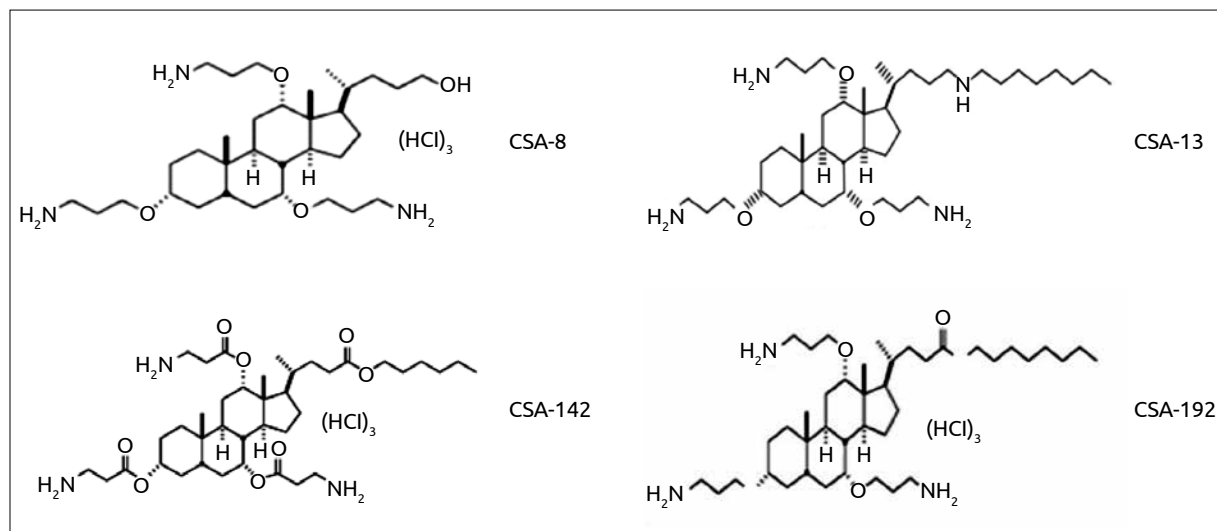


Figure 1. Chemical structures of ceragenins

mL to >128 µg/mL. Among microorganisms, all CSAs showed the lowest activity against *M. morgani* (one isolate). The MBCs were generally equal to or two fold greater than those

of the MICs. All quality control results were within acceptable ranges reported in the EUCAST quality control tables (EUCAST, 2018b).

Table 1. *In vitro* antibacterial activities of antibiotics against various Gram negative bacteria

Microorganisms	Colistin		Meropenem		Cefotaxime		Levofloxacin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	µg/mL							
<i>K. pneumoniae</i> 1	256	256	32	128	>128	>128	8	8
<i>K. pneumoniae</i> 2	>256	>256	8	8	>128	>128	8	8
<i>K. pneumoniae</i> 3	32	32	32	32	>128	>128	16	16
<i>K. pneumoniae</i> 4	32	32	32	64	>128	>128	32	32
<i>K. pneumoniae</i> 5	32	32	16	16	>128	>128	32	32
<i>K. pneumoniae</i> 6	>256	>256	64	64	>128	>128	32	64
<i>K. pneumoniae</i> 7	32	32	8	8	>128	>128	32	32
<i>K. pneumoniae</i> 8	32	64	16	16	>128	>128	16	16
<i>K. pneumoniae</i> 9	64	64	64	128	>128	>128	32	32
<i>M. morgani</i>	>256	>256	16	16	128	128	16	32
<i>P. aeruginosa</i> 1	32	32	32	32	128	128	128	128
<i>P. aeruginosa</i> 2	16	16	32	32	>128	>128	8	8
<i>P. aeruginosa</i> 3	32	32	16	32	>128	>128	32	64
<i>P. aeruginosa</i> 4	32	32	16	32	>128	>128	>128	>128
<i>P. aeruginosa</i> 5	32	32	8	16	>128	>128	64	64
<i>S. maltophilia</i> 1	128	>128	>128	>128	>128	>128	16	64
<i>S. maltophilia</i> 2	32	32	128	128	>128	>128	16	32

MIC: Minimum inhibitory concentrations, MBC: Minimum bactericidal concentrations

Table 2. *In vitro* antibacterial activities of ceragenins against various Gram negative bacteria

Microorganisms	CSA-8		CSA-13		CSA-142		CSA-192	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	µg/mL							
<i>K. pneumoniae</i> 1	>128	>128	8	16	64	64	64	64
<i>K. pneumoniae</i> 2	>128	>128	16	16	64	64	64	64
<i>K. pneumoniae</i> 3	>128	>128	8	8	64	64	64	64
<i>K. pneumoniae</i> 4	>128	>128	16	32	32	32	128	128
<i>K. pneumoniae</i> 5	>128	>128	16	16	32	32	64	64
<i>K. pneumoniae</i> 6	>128	>128	16	16	32	32	128	128
<i>K. pneumoniae</i> 7	>128	>128	8	8	64	64	64	64
<i>K. pneumoniae</i> 8	>128	>128	16	16	16	16	64	64
<i>K. pneumoniae</i> 9	>128	>128	16	16	32	64	128	128
<i>M. morgani</i>	>128	>128	64	64	64	64	128	128
<i>P. aeruginosa</i> 1	>128	>128	16	16	32	32	128	>128
<i>P. aeruginosa</i> 2	>128	>128	8	8	32	32	32	32
<i>P. aeruginosa</i> 3	>128	>128	16	16	32	32	128	128
<i>P. aeruginosa</i> 4	>128	>128	16	32	32	32	128	128
<i>P. aeruginosa</i> 5	>128	>128	16	32	32	64	128	128
<i>S. maltophilia</i> 1	>128	>128	16	16	64	64	64	64
<i>S. maltophilia</i> 2	>128	>128	64	64	64	128	64	128

MIC: Minimum inhibitory concentrations, MBC: Minimum bactericidal concentrations

DISCUSSION

The emergence of MDR microorganisms, which are not affected by most of the drugs, is a growing health threat all over the world. The World Health Organization has published a list of priority pathogens resistant to antimicrobials in order to assist in the research and development of new and effective antibacterial agents. One of the most important outputs of the report is that researchers should be focused on the discovery and development of active new antibiotics against multi-drug resistant Gram negative bacterial strains. According to this report, carbapenem-resistant *P. aeruginosa*, *K. pneumoniae*, *M. morgani* are among the pathogens against which urgent precautions should be taken (WHO, 2017). Therefore, our study involves multi-drug resistant Gram negative microorganisms, including carbapenem-resistant *P. aeruginosa*, *K. pneumoniae*, *M. morgani*.

As reported previously, ceragenins have potent antimicrobial activity (Bozkurt-Güzel et al. 2014a; Bozkurt-Guzel et al. 2014b; 2014b; Durnas et al. 2016; Olekson et al. 2017). Hashemi et al. (2017) found that CSA-13 (MIC: 2-6 µg/mL) and CSA-142 (MIC: 2-16 µg/mL) had significant antimicrobial effects and CSA-44 and CSA-131 were bactericidal against colistin resistant *K. pneumoniae* strains. Furthermore, it was also reported that ceragenins (CSA-44 and CSA-131) retained bactericidal activity against colistin-resistant bacteria. Similarly, the data shown here established that CSA-13 and CSA-142 had higher activity than other ceragenins against colistin resistant *K. pneumoniae* strains and CSA-13 (MIC: 8-16 µg/mL) had lower MIC value than CSA-142 (MIC: 16-64 µg/mL).

In the study performed by Vila-Farrés et al. (2015) regarding the activity of ceragenins against *P. aeruginosa*, it was determined that CSA-13 had the same MIC values against both colistin resistant and non-resistant strains. Chin et al. (2008) showed that CSA-13 MIC₅₀ was 16 µg/mL and had potential synergistic activity against multi-drug resistant *P. aeruginosa*. In this study, CSA-13 showed similar activity (MIC: 8-16 µg/mL) against colistin resistant *P. aeruginosa* strains. According to these results, it was concluded that colistin resistance did not alter the effect of ceragenins. In another study, erythromycin-ceragenin combination against multi-drug resistant pathogens was investigated and it was shown that CSA-13 increased the activity of erythromycin by depolarizing the bacterial outer membrane. Moreover, the toxicity of CSA-13 was determined to be insignificant (Saha et al. 2008). Our study also demonstrated high antimicrobial activity of CSA-13 against multi-drug resistant *P. aeruginosa*.

To our knowledge, there is no study evaluating the activities of ceragenins against *M. morgani* and *S. maltophilia*. The study mentioned herein showed that the highest MIC value of the tested ceragenins was against *M. morgani* strain (MIC: 64->128 µg/mL). Outer membranes of these microorganisms can be a barrier for ceragenins and therefore they do not have low MIC values (Pollard et al. 2012).

According the data in Table 2, CSA-8 was found to be the least active agent. Previous studies also have shown that CSA-8 was

more effective against Gram positive bacteria than Gram negative bacteria and fungi (Bozkurt-Güzel et al. 2014a; Bozkurt-Guzel et al. 2014b). The low activity of CSA-8 may be due to the lack of the hydrophobic side chain and being more hydrophilic than the other compounds.

Consequently, in the present study, multiple drug resistance was detected in most of the tested bacteria. Apparently, new agents are needed to treat the diseases caused by these bacteria. Our study showed that ceragenins, especially CSA-13, are promising agents against colistin-resistant Gram negative strains. However, additional studies are also needed to determine activity and safety of ceragenins.

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