Susceptibility of Colistin-Resistant, Gram-Negative Bacteria to Antimicrobial Peptides and Ceragenins

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ABSTRACT The susceptibility of colistin-resistant clinical isolates of *Klebsiella pneumoniae* to ceragenins and antimicrobial peptides (AMPs) suggests that there is little to no cross-resistance between colistin and ceragenins/AMPs and that lipid A modifications are found in bacteria with modest changes in susceptibility to ceragenins and with high levels of resistance to colistin. These results suggest that there are differences in the resistance mechanisms to colistin and ceragenins/AMPs.

KEYWORDS colistin, resistant, antimicrobial peptides, ceragenin, Gram-negative bacteria

The continuous emergence of drug-resistant bacteria has led to dire predictions of a possible "postantibiotic" era in which common infections will not be treatable with the current arsenal of antibiotics (1, 2). Of particular concern are Gram-negative bacteria, because these organisms are inherently resistant to many antibiotics due to the permeability barrier provided by their outer membranes and the efflux pumps located therein (3, 4). To treat infections from Gram-negative bacteria, clinicians are increasingly using colistin, a member of the polymyxin family of antibiotics (5). Colistin is considered the antibiotic of last resort because, while it has side effects, including nephrotoxicity and ototoxicity, it is broadly active against Gram-negative bacteria (6, 7). Isolation of colistin-resistant bacteria in many countries underscores the need for development of novel strategies for targeting Gram-negative bacteria, including drug-resistant strains (8).

Endogenous antimicrobial peptides (AMPs) have played an important role in innate immunity for eons (9), and there have been efforts to use AMPs clinically. Challenges for clinical use of peptide therapeutics include the relatively high costs of large-scale production and the susceptibility of AMPs to degradation by proteases (10). We have developed a class of small molecules, termed ceragenins (Fig. 1), that circumvent these challenges while maintaining the same general mechanism of bactericidal activity of AMPs. Ceragenins can be prepared on a large scale, and because they are not peptide based, they are not substrates for proteases. As mimics of AMPs, ceragenins display broad-spectrum antibacterial activity, including potent antibiofilm activity (11–13). In *in vivo* studies involving medical devices and bone regrowth, ceragenins are effective in eliminating bacterial challenges, and local administration is well tolerated (14–16).

Consideration of the common structural features of colistin, AMPs, and ceragenins (multiple cationic groups, substantial hydrophobic character, and interaction with bacterial membranes) leads to three questions. (i) Are colistin-resistant bacteria also resistant to AMPs and ceragenins? (ii) Does generation of resistance to colistin occur at the same rate as potential generation of resistance to AMPs and ceragenins? (iii) Since the primary mechanism for bacterial resistance to AMPs and ceragenins is through modification of the lipid A portion of lipopolysaccharide (LPS) (17–19), how important
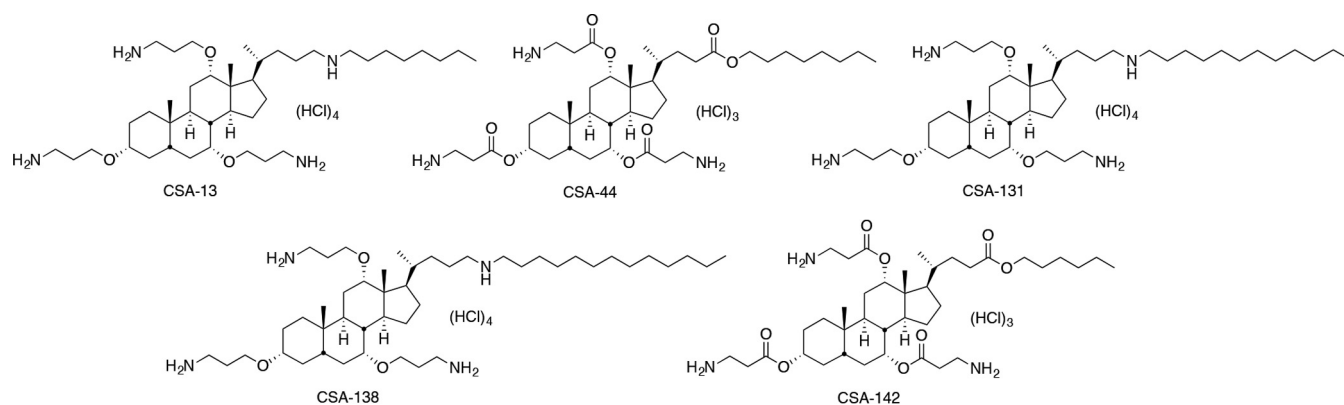
FIG 1 Structure of ceragenins CSA-13, CSA-44, CSA-131, CSA-138, and CSA-142.

are these modifications in the resistance of Gram-negative bacteria to colistin, AMPs, and ceragenins?

To address these questions, we compared the susceptibility of colistin-resistant clinical isolates of *Klebsiella pneumoniae* to colistin, representative AMPs (LL-37, cecropin A, and magainin 1), and the representative ceragenins shown in Fig. 1. Our initial focus on *K. pneumoniae* was due to its known pathogenicity and its ability to transfer resistance genes to other Gram-negative bacteria (20, 21). We and our collaborators previously found ceragenins to be active against other colistin-resistant bacteria (19, 22), and we have extended these observations to clinical isolates of *K. pneumoniae* using additional, later-generation ceragenins. MICs and minimum bactericidal concentrations (MBCs) were determined using a broth microdilution method (CLSI protocol, with Mueller-Hinton [MH] substituted for cation-adjusted MH) (23). Colistin-resistant clinical isolates of *K. pneumoniae* gave MICs of 16 to 200 μg/ml with colistin, while a susceptible strain (ATCC 13883) gave an MIC of 2 μg/ml (Table 1). MICs of LL-37 and magainin 1 were relatively high against the reference strain as well as the clinical isolates; MICs were lower and less varied with cecropin A. With the ceragenins, MICs with the susceptible strain were relatively low (1 to 3 μg/ml), and only small changes in MICs were observed with colistin-resistant isolates. MBCs with CSA-44 and CSA-131 were 2 to 10 μg/ml, demonstrating bactericidal rather than bacteriostatic activity, and colistin resistance did not significantly impact the MBCs of the ceragenins.

To quantify rates of bactericidal activity, time-kill assays were performed with CSA-44 and CSA-131 against the colistin-resistant strains ARLG-1127 and ARLG-1389 and compared to a susceptible strain of *K. pneumoniae* (Fig. 2). For these assays, the protocol for MIC measurement was used, and aliquots (10 μl) were removed at varied intervals, plated on nutrient agar, and incubated (24). At 2× MIC for both ceragenins, the inoculum was decreased by at least 3 logs within 2 h. At 4× MIC, the inoculum was decreased to the detection limit (2 logs) within the same time frame. These assays revealed that there are only minor differences in the kinetics of bactericidal activity

TABLE 1 MICs of colistin, AMPs, and CSAs against a standard strain of *K. pneumoniae* (ATCC 13883) and colistin-resistant clinical isolates

| *K. pneumoniae* strain	MICs (MBCs) (μg/ml) for:								
	Colistin	LL-37	Cecropin A	Magainin 1	CSA-13	CSA-44	CSA-131	CSA-138	CSA-142
ATCC 13883	2.0	32	2.0	64	2.0	1.0 (2.0)	1.0 (2.0)	3.0	3.0
ARLG-1127	32	64	2.0	64	2.0	1.0 (2.0)	1.0 (2.0)	2.0	2.0
ARLG-1340	100	100	NM[a]	NM	2.0	1.0 (2.0)	1.0 (6.0)	3.0	4.0
ARLG-1349	16	64	4.0	64	2.0	1.0 (2.0)	3.0 (4.0)	3.0	8.0
ARLG-1360	64	100	4.0	150	2.0	1.0 (2.0)	2.0 (6.0)	6.0	6.0
ARLG-1389	200	100	4.0	200	6.0	2.0 (2.0)	3.0 (10)	8.0	8.0
ARLG-1406	64	64	4.0	100	3.0	1.0 (3.0)	3.0 (8.0)	6.0	16

[a]NM, not measured.
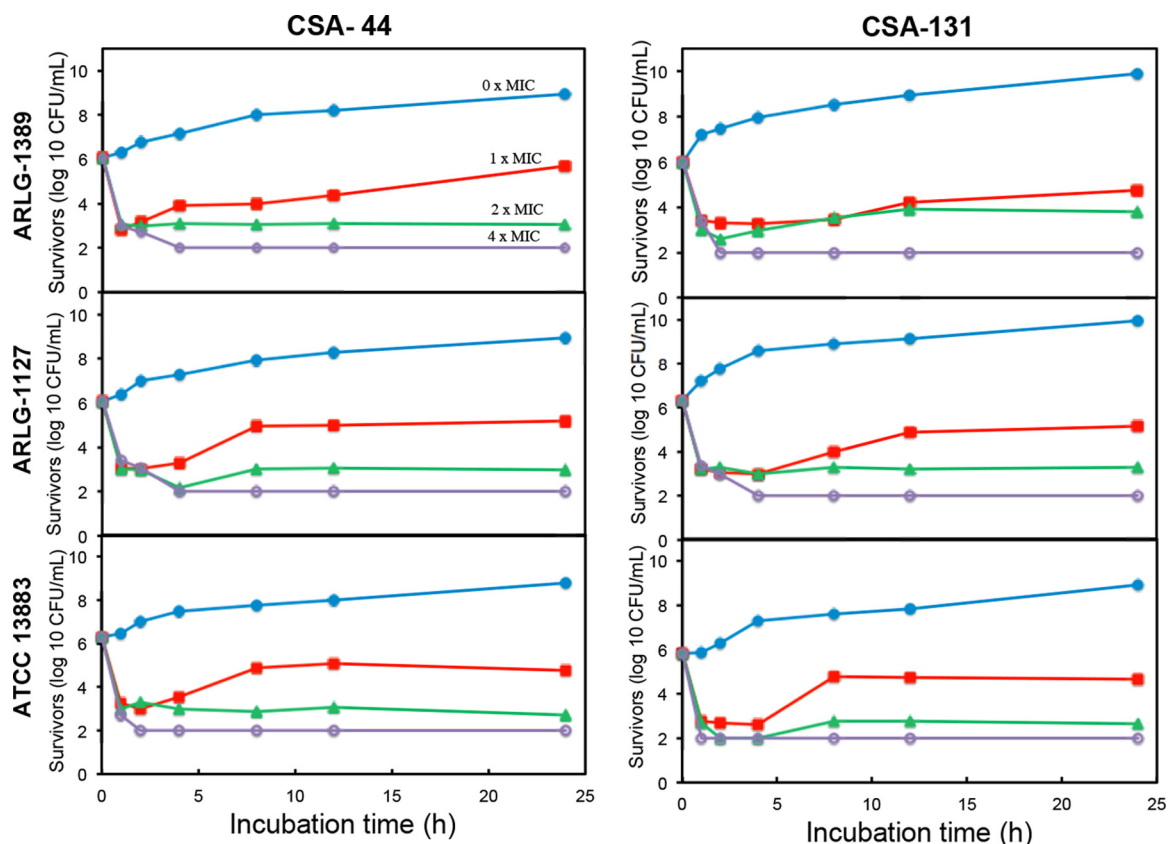
FIG 2 Rates of bactericidal activity of ceragenins are similar among colistin-resistant and colistin-susceptible strains. Time-kill curves with CSA-44 and CSA-131 against colistin-resistant *K. pneumoniae* strains (ARLG-1127 and ARLG-1389) and colistin-susceptible strain ATCC 13883. Detection limit is 2 logs (CFU/ml).

among the colistin-resistant and colistin-susceptible strains, again suggesting that colistin resistance does not significantly influence susceptibility to ceragenins.

We compared the relative rates at which *K. pneumoniae* (ATCC 13883) and other Gram-negative bacteria (*Pseudomonas aeruginosa* [ATCC 27853] and *Acinetobacter baumannii* [ATCC 19606]) become resistant to colistin and ceragenin CSA-131 by serially exposing these organisms to both antimicrobials and monitoring susceptibility. MICs (at 24 h) for the strains were determined, and bacterial populations growing at the highest concentrations of the antimicrobials were used to inoculate fresh medium. This process was repeated every 18 to 24 h. Concentrations of the antimicrobials were adjusted to allow determination of MICs (19). This process was repeated for 10 periods (of 24 h each) with colistin, with MICs rising from 1 to 2 μg/ml to $\geq$350 μg/ml, and for 30 days with CSA-131. Resulting MICs are shown in Table 2, along with the susceptibility of the resulting bacteria to colistin, CSA-131, and representative AMPs. Serial exposure to colistin, resulting in the generation of highly resistant organisms, caused little or no change in MICs with CSA-131. Some changes in MICs were observed with the AMPs against colistin-resistant organisms (1.3- to 8-fold increases). Serial exposure to CSA-131 resulted in increases of MICs from 1 to 2 μg/ml to 2 to 8 μg/ml and resulted in increased MICs with AMPs (2.3- to 12-fold increases).

In a previous study, we found that serial exposure of Gram-negative bacteria to ceragenin CSA-13 resulted in increased MICs and modifications to the lipid A portion of lipopolysaccharide (19). Lipid A is a primary target of colistin (25), ceragenins (11), and AMPs (26), and lipid A modifications were observed as mechanisms for the generation of resistance to these antimicrobials (26–30). To determine if colistin resistance and serial exposure to CSA-131 result in comparable lipid A modifications (phosphate ester formation with 4-aminoarabinose and/or ethanolamine), lipid A was isolated from three

TABLE 2 MICs of colistin, CSA-131, LL-37, magainin 1, and cecropin A with susceptible standard strains of *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* and with strains serially exposed to colistin or CSA-131

Strain	MICs (μg/ml) for:				
	Colistin	CSA-131	LL-37	Magainin 1	Cecropin A
K. pneumoniae ATCC 13883	2.0	1.0	32	64	2.0
Serially exposed to colistin[a]	350	1.5	64	82	16
Serially exposed to CSA-131[b]	32	8.0	100	150	24
A. baumannii ATCC 19606	1.0	2.0	16	32	4.0
Serially exposed to colistin[a]	400	2.0	64	100	16
Serially exposed to CSA-131[b]	32	2.0	128	150	32
P. aeruginosa ATCC 27853	1.0	2.0	32	64	4.0
Serially exposed to colistin[a]	350	2.0	64	100	8.0
Serially exposed to CSA-131[b]	32	4.0	100	150	16

[a]Ten days of exposure.
[b]Thirty days of exposure.

colistin-resistant clinical isolates of *K. pneumoniae*, and bacteria were serially exposed to colistin or CSA-131. Lipid A was isolated using the TRI reagent method (31) and analyzed via mass spectrometry (electrospray ionization, negative-ion mode, Agilent 6230 series time-of-flight spectrometer). Lipid A from the parent strain (*K. pneumoniae* [ATCC 13883]) showed the expected masses lacking 4-aminoarabinose and ethanolamine, while lipid A from each of the clinical isolates and from bacteria serially exposed to colistin or CSA-131 showed these modifications (Table 3 [mass spectra are shown in the supplemental material]), as expected from activation of two-component systems (e.g., PhoP/PhoQ and PmrA/PmrB) (26–30). Additions of fatty acids were also observed. Comparable masses were observed for parent and modified lipid A (27, 32, 33). Susceptibility of these strains to colistin or CSA-131 varies dramatically, and yet there are modifications to lipid A common to these organisms. These modifications to lipid A may impact the activities of colistin and CSA-131 differently; alternatively, there may be other mechanisms of resistance with these organisms (e.g., loss of lipopolysaccharide) (34) that provide high levels of colistin resistance without influencing susceptibility to CSA-131.

TABLE 3 Masses of isolated lipid A from colistin-susceptible (ATCC 13883) and colistin-resistant strains of *K. pneumoniae* and bacteria serially exposed to colistin and CSA-131

K. pneumoniae strain	Observed lipid A mass (*m/z*)	Mass of parent lipid A structures (*m/z*)	Additions to parent lipid A structures
ATCC 13883[c]	1,795		
	1,840		
	1,853		
	1,910		
ARLG-1389	2,023	1,840	Lauric acid
	2,209	1,840	Aminoarabinose and palmitic acid
ARLG-1349	2,021	1,840	Lauric acid
	2,209	1,840	Aminoarabinose and palmitic acid
ARLG-1360	2,034	1,910	Ethanolamine
ATCC 13883 serially exposed to colistin[a]	1,795	1,795	
	1,987	1,840	Aminoarabinose and hydroxyl group
ATCC 13883 serially exposed to CSA-131[b]	1,840	1,840	
	2,021	1,840	Lauric acid
	2,152	1,840	Aminoarabinose and lauric acid

[a]Ten days of exposure.
[b]Thirty days of exposure.
[c]ATCC 13883 is the fully susceptible strain, so no modifications to lipid A are identified (this is the source of the parent lipid A structures).

Considering the common structural features of colistin, AMPs, and ceragenins, the issue of cross-resistance arises. Among colistin-resistant isolates and strains generated by serial exposure to colistin, MICs increase up to several hundredfold compared to susceptible strains, while up to 5-fold increases in MICs were observed with the AMPs and ceragenins tested. However, in some cases, MICs of ceragenins were the same with both colistin-susceptible and -resistant bacteria. While specific mechanisms for resistance of Gram-negative bacteria to colistin have been identified, multiple mechanisms likely influence resistance (35). At least one of these mechanisms causes very high resistance to colistin but does not appear to impact, to the same extent, susceptibility to AMPs and ceragenins. These observations are consistent with the reported susceptibility of colistin-resistant bacteria to a variety of AMPs (36, 37). The fact that lead ceragenins (CSA-44 and CSA-131) retain bactericidal activity against highly colistin-resistant bacteria provides further support for development of these compounds as broad-spectrum antibacterial agents in multiple potential clinical applications.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC .00292-17.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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