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Activity of Cecropin A-Melittin Hybrid Peptides against Colistin-Resistant Clinical Strains of *Acinetobacter baumannii*: Molecular Basis for the Differential Mechanisms of Action

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***Acinetobacter baumannii* has successfully developed resistance against all common antibiotics, including colistin (polymyxin E), the last universally active drug against this pathogen. The possible widespread distribution of colistin-resistant *A. baumannii* strains may create an alarming clinical situation. In a previous work, we reported differences in lethal mechanisms between polymyxin B (PXB) and the cecropin A-melittin (CA-M) hybrid peptide CA(1-8)M(1-18) (KWKLFKKIGIGAVLKVLTTGLPALIS-NH_2) on colistin-susceptible strains (J. M. Saugar, T. Alarcón, S. López-Hernández, M. López-Brea, D. Andreu, and L. Rivas, Antimicrob. Agents Chemother. 46:875–878, 2002). We now demonstrate that CA(1-8)M(1-18) and three short analogues, namely CA(1-7)M(2-9) (KWKLFKKIGAVLKVL-NH_2), its N^{α}-octanoyl derivative (Oct-KWKLFKKIGAVLKVL-NH_2), and CA(1-7)M(5-9) (KWKLLKKIGAVLKVL-NH_2) are active against two colistin-resistant clinical strains. In vitro, resistance to colistin sulfate was targeted to the outer membrane, as spheroplasts were equally lysed by a given peptide, regardless of their respective level of colistin resistance. The CA-M hybrids were more efficient than colistin in displacing lipopolysaccharide-bound dansyl-polymyxin B from colistin-resistant but not from colistin-susceptible strains. Similar improved performance of the CA-M hybrids in permeation of the inner membrane was observed, regardless of the resistance pattern of the strain. These results argue in favor of a possible use of CA-M peptides, and by extension other antimicrobial peptides with similar features, as alternative chemotherapy in colistin-resistant *Acinetobacter* infections.**

The species belonging to the *Acinetobacter baumannii* complex act as causative agents for a wide variety of clinical conditions (7). It is particularly insidious in intensive care units (36), where the use of invasive devices, broad-spectrum antibiotics, and prolonged patient stay periods are associated with high morbidity and mortality rates (17, 31, 44, 45). *Acinetobacter* species have shown an outstanding capacity to develop resistance against common antibiotics such as carbapenems and other broad-spectrum ß-lactams, tetracyclines, fluoroquinolones, and aminoglycosides through a wide variety of mechanisms (35, 44, 45). This has led to a practical exhaustion in the repertoire of active antibiotics, including imipenem, which until recently was considered the gold standard for *Acinetobacter* treatment (21, 25). In view of this therapeutic problem different alternatives were implemented, one of which was the reappraisal of the administration of colistin (polymyxin E) against panresistant *Acinetobacter* infection (14), with a significant rate of success in a variety of clinical and experimental infections (22, 24, 38). This was further supported by new data showing milder nephrotoxic effects of polymyxins than previously reported (14, 27, 33).

Unfortunately, with the recent description of sporadic colistin-resistant isolates (20, 25, 34, 43, 50), the alarm signals have been reactivated. Partial solutions such as colistin combination therapy with other single antibiotics (azithromycin, rifampin, cotrimoxazole, or meropenem [25]) or even triple combination with rifampin plus imipenem (50), along with adequate prevention practices and disinfection protocols in hospitals (25), have been assayed. Nevertheless, due to the remarkable ability of *Acinetobacter* to develop resistance, increased numbers of outbreaks of panresistant *A. baumannii* are quite foreseeable and will be worsened by the paucity of new drugs developed against gram-negative bacteria.

Eukaryotic antimicrobial peptides (EAPs) are ubiquitous elements of the innate defense systems of all higher organisms (4). Reasonable expectations have been raised about their pharmacological exploitation as potent antibacterial agents based on (i) the wide range of pathogens susceptible to them; (ii) their extremely low rate of resistance compared with classical antibiotics, resulting from a distinctive mechanism of membrane perturbation based on stoichiometric interaction of the EAP with exposed anionic lipids; and (iii) their anti-endotoxic properties, which result from their ability to interact with lipopolysaccharide (LPS). A number of EAPs have been tested, both in vitro against different species of the genus *Acinetobacter* (18, 39, 40), including multiresistant strains (1, 18, 40), and in vivo on animal models of bacteremia by *Acinetobacter baumannii* (3, 12).

Though polymyxins share major structural features (i.e., a cationic peptide nature) and self-promoted uptake mecha-

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nisms with most EAPs, we earlier demonstrated that polymyxin B (PXB) and the cecropin A-melittin (CA-M) hybrid CA(1-8)M(1-18), an EAP, differed in the last steps of their lethal activity on *A. baumannii* (40), warranting exploitation of the latter against colistin-resistant strains. Similar pioneering work by Urban et al. established the activity of the EAPs $rBPI_{21}$ (the recombinant 21-kDa amino-terminal fragment of the bactericidal/permeability-increasing protein) and cecropin P_1 on a single polymyxin B-resistant *A. baumannii* isolate (43). In the present work, we provide further evidence of the activity of four selected CA-M analogues on two clinical strains of *A. baumannii* with different degrees of colistin resistance. We show that the CA-M hybrids have higher affinity than colistin towards LPS isolated from colistin-resistant *Acinetobacter baumannii* strains, a plausible ground for their superior activity. These results are a first step towards clinical use of these peptides as a feasible alternative to polymyxin-resistant *A. baumannii*.

MATERIALS AND METHODS

Reagents. SYTOX green was obtained from Molecular Probes (Leiden, The Netherlands). Protected 9-fluorenylmethoxy carbonyl (Fmoc) amino acids, resins, and other reagents for peptide synthesis were from Neosystem (Strasbourg, France), Bachem (Bubendorf, Switzerland), or Novabiochem-Calbiochem (Läufelfingen, Switzerland). The following four CA-M sequences (CA and M stand for cecropin A and melittin, respectively; the positional stretch of each parental sequence included in the hybrid is within parentheses) were prepared as C-terminal carboxamides by optimized Fmoc-based methods of solid-phase synthesis (16): the 26-mer CA(1-8)M(1-18) (KWKLFKKIGIGAVLKVLTTGLPALIS-NH_2; molecular weight, 2793.8), the 15-mer CA(1-7)M(2-9) (KWKLFKKIGAVLKVL-NH_2; molecular weight, 1770.2) and its N^{α}-octanoyl derivative (C_7H_{13}CO-KWKLFKKIGAVLKVL-NH_2; molecular weight, 1898.5) (6, 40), and the short 12-mer CA(1-7)M(5-9) (KWKLLKKVLKVL-NH_2; molecular weight, 1544.1). After chain assembly and acid deprotection cleavage, the synthetic products were purified to near homogeneity (>95%) by reverse-phase high-performance liquid chromatography and were further characterized by matrix-assisted laser desorption ionization-time of flight mass spectrometry and amino acid analysis (6).

Standard laboratory powder of colistin sulfate salt (Sigma, St. Louis, MO) was prepared according to guidelines from the National Committee for Clinical Laboratory Standards (8). This was the only form of colistin used in this study.

Bacterial strains. *A. baumannii* clinical panresistant strains 208628 and 201630, resistant to cefotaxime, ceftazidime, imipenem, amoxicillin, amikacin, piperacillin-tazobactam, doxycycline, and colistin sulfate (9), were selected from 13 panresistant strains because their colistin sulfate MICs were different (64 and 8 mg/liter, respectively). The strains were from the collection of clinical bacterial strains at the "Virgen del Rocío" University Hospital, Seville, Spain. *A. baumannii* was identified by Gram stain appearance, colonial morphology, motility, cytochrome oxidase reaction, and growth at 44°C, as well as by the semiautomated MicroScan Walk Away method (Dade-Behring). The strains were confirmed as *A. baumannii* by amplified ribosomal rRNA gene restriction analysis (46). Strains were stored at −70°C between experiments.

MIC determinations. MICs of colistin and the four CA-Ms were determined in duplicate against the *A. baumannii* strains by the broth microdilution method. Mueller-Hinton II Broth Cation Adjusted (Becton Dickinson, Cockeysville, Md.) was used as growth medium, and an initial inoculum of 5×10^5 CFU/ml at exponential growth phase was used (8). The MIC was defined as the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the microdilution wells measured by turbidimetry at 24 h at 450 nm in a Bio-Rad 680 Microplate enzyme-linked immunosorbent assay reader. Initial inoculum and bacterial growth were determined by serial dilutions in physiologic serum that were subcultured in Columbia III agar with 5% sheep agar plates and incubated at 37°C for 20 h. The concentrations of antimicrobials ranged from 0.06 to 128 mg/liter. *A. baumannii* ATCC 19606, a reference strain that is susceptible to colistin sulfate, was used as a control.

Permeation of *Acinetobacter baumannii* to SYTOX green. The permeation of the bacterial inner membrane induced by peptides was assessed by the increase of fluorescence of the vital dye SYTOX green (molecular weight, 600). This cationic dye is excluded from cells with intact membranes but enters the cytoplasm of those with compromised structures, binding their nucleic acids and promoting an increase in fluorescence. We adapted previously described protocols to a 100-μl total volume microscale (40). Briefly, bacteria were harvested at exponential growth phase, washed twice in Hanks medium supplemented with 10 mM D-glucose (Hanks plus glucose), and resuspended in the same medium at an optical density at 495 nm (OD_{450}) of 0.05 (approximately 10^7 CFU/ml) in Hanks plus glucose containing a final concentration of 1 μM SYTOX green. One-hundred-microliter aliquots of this suspension were transferred into polypropylene 96-well microplates. Fluorescence (excitation wavelength [λ_{exc}], 485 nm; emission wavelength [λ_{em}], 520 nm) was measured in a Polarstar Galaxy plate reader (BMG Labotechnologies, Offenburg, Germany) at 37°C; once basal fluorescence was stabilized, peptides were added at the corresponding concentration, and the increase in fluorescence was monitored for 60 min. Triton X-100 (final concentration, 0.1% [wt/vol]) was then added in order to observe full permeation.

Isolation of lipopolysaccharide. Lipopolysaccharide (LPS) from *A. baumannii* strains 208628, 201630, and ATCC 19606 was extracted from bacteria harvested at late exponential growth phase according to the hot phenol-water method, dialyzed extensively against distilled water, and quantified for 3-deoxy-D-manno-2-octulosonic acid content by the thiobarbituric acid method, assuming three molecules for each *A. baumannii* unit, as described previously (40).

Displacement of dansyl-polymyxin bound to LPS. Dansyl-polymyxin B (DPXB) was synthesized and quantified by dinitrophenylation. LPS was solubilized in 5 mM HEPES, pH 7.0, at a final concentration of 3 μM, and then DPXB was added (5 μM final concentration). The increase in fluorescence due to the binding between these two molecules was monitored in a Hitachi F2000 spectrofluorometer (λ_{exc} = 340 nm; λ_{em} = 460 nm). Once fluorescence was stabilized, addition of a given peptide displaced LPS-bound DPXB, with consequent decrease in DPXB fluorescence. Results were expressed as I_{50} (peptide concentration causing 50% reduction of maximal binding) and I_{max} (percentage of maximal displacement) (28).

Lysis of *A. baumannii* spheroplasts by colistin and CA(1-8)M(1-18). Spheroplasts from *A. baumannii* colistin-susceptible strain ATCC 19606 and the colistin-resistant strains 208628 and 201630 were prepared according to Dathe et al. (10). Briefly, bacteria were harvested at an OD_{450} of 0.8 and washed extensively with Hanks plus glucose, and spheroplasts were obtained in isosmotic media (10% sucrose, 10 mM $MgCl_2$ in 50 mM Tris, pH 8.0) by incubation first with 0.8 mg/ml lysozyme (5 min, 4°C) and afterwards with 6.3 mM EDTA (5 min, 4°C, gentle shaking). Spheroplasts were centrifuged and resuspended in 10 mM HEPES, 150 mM NaCl, 50 mM sucrose, pH 7.0, and adjusted to an OD_{450} of 0.5. Aliquots (100 μl) were transferred into a 96-well microplate thermostatized at 37°C; the peptides at the corresponding concentration were then added, and the decrease in light scattering due to spheroplast lysis was monitored for 30 min at 450 nm.

RESULTS

Activity of CA-M peptides on *A. baumannii* colistin-resistant strains. In a preliminary step, the microbicidal activity of CA-M peptides was assayed on both susceptible and resistant strains of *A. baumannii* and compared with that of colistin. Since all experiments in this work were done in vitro, a commercial preparation of colistin sulfate was used rather than the noncationic methanesulfonate prodrug used clinically for parenteral administration (14). The four CA-M peptides had MICs in the 1.1 to 2.5 μM range against all tested strains (Table 1), with less than twofold differences among all peptides and strains assayed and with no evident correlation to colistin resistance patterns. In contrast, for colistin the MICs were 1.1 μM for the susceptible ATCC 19606 strain and 4.6 and 36.5 μM for the resistant strains 201630 and 208628, respectively (Table 1).

Permeation to the vital dye SYTOX induced by peptides. The microbicidal activity of most CA-Ms assayed to date relies on the permeation of the plasma membrane of target cells (37). A substantial difference between these peptides and PXB in the ability to permeate the inner membrane was found earlier in the colistin-susceptible strain ATCC 19606 (40). Aiming to extend those observations to colistin-resistant strains, we as-

TABLE 1. MICs of colistin sulfate and CA-M peptides for *Acinetobacter baumannii* strains

Peptide	Peptide MIC[a] for strain:		
	ATCC 19606	208628	201630
Colistin	1.1 (2)	36.5 (64)	4.6 (8)
CA(1-8)M(1-18)	1.4 (4)	1.4 (4)	1.4 (4)
CA(1-7)M(2-9)	1.1 (2)	1.1 (2)	1.1 (2)
Oct-CA(1-7)M(2-9)	1.1 (2)	1.1 (2)	2.2 (4)
CA(1-7)M(5-9)	2.5 (4)	2.5 (4)	2.5 (4)

[a] Values are reported in micromolar concentrations; values in parentheses are in milligrams/liter.

sayed the four CA-M peptides at equipotent concentration, namely, their respective MICs. Under such conditions, and regardless of the level of colistin resistance, the fluorescence obtained for the CA-M peptides reached maximal value very close to that obtained with a final addition of Triton X-100, which was conventionally assumed to entail full permeation of the bacterial inner membrane when the outer membrane was leaky (Fig. 1). In contrast, when colistin sulfate was assayed at its respective MICs, important differences among strains were observed. The highest permeation was observed on the susceptible ATCC 19606 strain, whereas the resistant 208628 strain was hardly permeated. Notably, the MIC of colistin sulfate for this strain is 32-fold higher than that for ATCC 19606. For strain 201630, with a low-resistance profile, permeation to colistin was intermediate between the previous two strains. A common feature for all three strains after colistin treatment was sensitization to lysis by Triton X-100, suggestive of outer membrane damage. Interestingly, the 201630 strain showed an intrinsic sensitivity (albeit only partial) to Triton X-100 (Fig. 1) even without colistin pretreatment, which is not observed in the other two strains.

Lysis of *A. baumannii* spheroplasts by colistin and CA(1-8)M(1-18). In order to gain insight into whether the bacterial components involved in colistin resistance were restricted to differences in the outer membrane or whether others located at the inner membrane took part as well, we assayed the lysis capacity of both colistin sulfate and CA(1-8)M(1-18) on spheroplasts obtained from the *A. baumannii* colistin-susceptible ATCC 19606 and colistin-resistant 208628 and 201630 strains. Lysis was monitored by a decrease in light scattering. As shown in Fig. 2, the extent of lysis achieved by either colistin or CA(1-8)M(1-18) showed scarce differences among strain types. Again, and in agreement with the aforementioned results on inner membrane permeation, colistin induced smaller effects than those of CA(1-8)M(1-18). These results ruled out a major role for inner membrane components in *Acinetobacter* resistance to colistin.

Displacement of DPXB bound to LPS by colistin and CA-M peptides. The previous results suggested that strain differences for colistin susceptibility were due to outer membrane components, with LPS as the most likely candidate. In order to investigate this hypothesis, we looked for differences in LPS affinity between colistin and CA-M peptides by monitoring the displacement of previously bound DPXB. Results are shown on Table 2. Interestingly, for the two colistin-resistant strains, the displacement by colistin of DPXB bound to LPS, as measured by either I_{50} or I_{max} parameters, was significantly smaller ($P < 0.05$, paired t test) than those obtained with the four CA-M peptides. This is in sharp contrast to the data corresponding to LPS from colistin-susceptible *A. baumannii* strain ATCC 19606, for which all peptides, including colistin, showed very similar affinity ($P > 0.1$).

DISCUSSION

The emergence of multiresistant *Acinetobacter baumannii* has singled out polymyxin as the last universally active drug against this pathogen, despite its controversial toxicity and lower success rate compared with other antimicrobials (14).

Like most EAPs, polymyxins display strong cationic character and amphipathicity and enter bacteria by a self-promoted uptake mechanism, disrupting the tight packing of LPS molecules. Nevertheless, they differ from EAPs in the essential step of their respective lethal mechanisms. Thus, for polymyxins a detergent-like effect on the inner membrane was initially proposed on the basis of their amphipathic nature, their high affinity for acidic phospholipids exposed at the inner membrane (23), and their ability to permeate model membranes

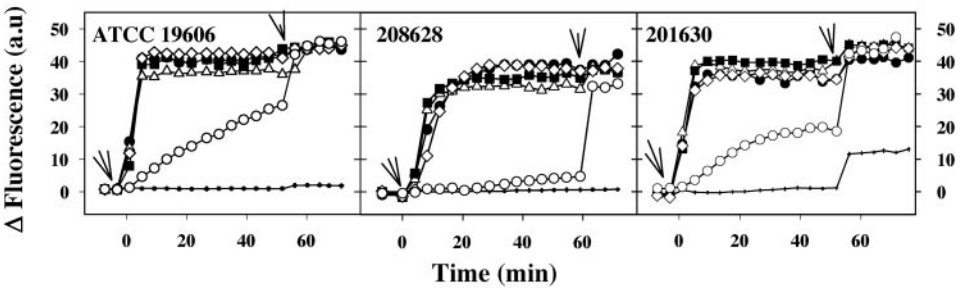

FIG. 1. Inner membrane permeation of *A. baumannii* strains by colistin and four CA-M peptides. Permeation was measured as the increase of fluorescence of SYTOX green (λ_{exc} = 485 nm; λ_{em} = 520 nm) after peptide addition (first arrow). Peptides were assayed at their corresponding MICs, as expressed in Table 1: 1.4 μM for CA(1-8)M(1-18); 1.1 μM for CA(1-7)M(2-9); 1.1 μM (ATCC 19606 and 208628) and 2.2 μM (201630) for Oct-CA(1-7)M(2-9); 2.5 μM for CA(1-7)M(2-5); 1.1 (ATCC 19606), 36.5 (208628), and 4.6 μM (201630) for colistin sulfate. In order to obtain complete permeation, 0.1% Triton X-100 was added after 1 h of incubation (second arrow). Symbols: control, crosshair; CA(1-8)M(1-18), solid circle; CA(1-7)M(2-9), empty triangle; Oct-CA(1-7)M(2-9), solid square; CA(1-7)M(5-9), empty diamond; colistin sulfate, empty circle. ATCC 19606 (left panel) was susceptible to colistin, whereas 208628 (middle panel) and 201630 (right panel) showed high and low resistance to colistin, respectively. a.u, arbitrary units.

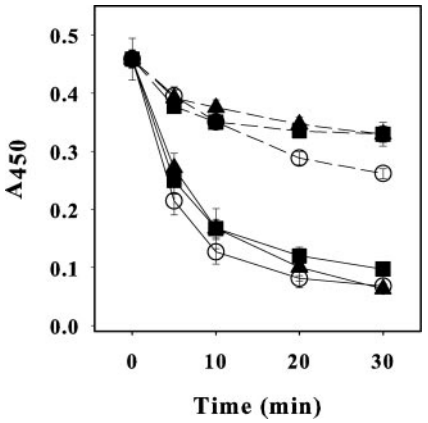

FIG. 2. Lysis of *A. baumannii* spheroplasts by colistin and CA(1-8)M(1-18). Spheroplasts from colistin-susceptible *A. baumannii* ATCC strain 19606 (empty circles) and clinical isolates 208628 (solid square) and 201630 (solid triangle), representative of high and low levels of colistin resistance, respectively, were resuspended in isotonic medium and, once absorbance at 495 nm became stabilized, wre treated with either CA(1-8)M(1-18) (solid lines) or colistin sulfate (dashed lines) at 8 or 16 μM final concentration, respectively. Spheroplast lysis was monitored as a decrease in light scattering at 450 nm.

(49), though this latter effect was achieved only at concentrations higher than the MIC (11). Another proposed mechanism, not exclusive of the above one, is the induction of a bacterial stasis by means of a hyperosmotic stress caused by phospholipid intermixing between the periplasmic leaflets of inner and outer membranes (32), similar to the process observed for vesicles in vitro (5). For EAPs, on the other hand, permeation of the inner membrane by disruption of phospholipid packing and subsequent loss of intracellular homeostasis appears to be the critical final step. In a previous work (40), we demonstrated that the bactericidal activities of cecropin A-melittin hybrid peptide CA(1-8)M(1-18) and PXB on a colistin-susceptible multiresistant *A. baumannii* strain mirrored such differences. Whether or not these features justify the use of CA-M peptides as a potential alternative to the rising polymyxin resistance in *Acinetobacter* was the purpose of this work.

The four CA-M peptides here considered were active at micromolar concentrations on the *A. baumannii* strains tested, with a maximal twofold variation in their respective MICs, in agreement with similar reports for the EAPs $rBPI_{121}$ and cecropin P1 on another polymyxin B-resistant *A. baumannii* strain (43).

We have shown that the outer membrane is responsible for colistin sulfate resistance, as the drug lyses in a similar fashion the spheroplasts of different *A. baumannii* strains regardless of their respective levels of resistance. CA(1-8)M(1-18) follows a similar trend but with substantially higher efficacy. In gram-negative bacteria, resistance to polymyxins has been consistently linked to the presence of LPS with a less anionic lipid A, to acylation with an additional fatty acid, or to the presence of an O antigen (reviewed in reference 13). Such polymyxin resistance often runs parallel to that for EAPs, although different LPS modifications may introduce substantial differences between the two types of antibiotics (30, 42). Thus, the DPXB displacement results in the present work evidenced a strong link between LPS affinity and antimicrobial activity, which was also observed for other CA-M peptides on *Pseudomonas aeruginosa* (41). Remarkably, the concentration needed to achieve 50% displacement of LPS-bound DPXB from colistin-resistant strains was significantly lower for CA-M peptides than for colistin sulfate, whereas in the ATCC 19606 reference strain, susceptible to colistin, such difference was blurred. While an LPS structure for ATCC 19606 is available (47), such is not the case for the colistin-resistant strains in this work, so one can only hypothesize on some still unknown modifications in their LPS being responsible for their resistance.

CA-M hybrid peptides have been extensively reviewed as bactericidal agents (37), and some of them have been tested on *Acinetobacter* (1, 18, 40). In the present work, the four CA-M peptides displayed very similar antimicrobial activity on *A. baumannii*, particularly so when activities were expressed in molar terms. In contrast to other peptides (2, 48), N-terminal acylation of CA(1-7)M(2-9) did not provide a significant advantage over the unmodified analogue, as it does on other pathogens with accessible membranes, such as *Leishmania* spp. (6).

From a clinical perspective, the percentage of colistin-resistant *Acinetobacter* sp. strains is not yet very significant, despite the consistent evidence for the acquisition of foreign genetic elements involved in antibiotic resistance by *Acinetobacter* (15, 35, 51) that may include genetic elements underlying colistin resistance. Nevertheless, colistin resistance is not a trivial issue for the pathogen: replication times are much higher for resistant than for wild-type strains (data not shown), and in the 201630 strain a partially leaky outer membrane was observed, suggesting defective LPS packing. As this feature appears only in one of the resistant strains, coexistence of more than a single LPS modification or, alternatively, more than one mechanism of resistance against colistin must be expected. In fact, the

TABLE 2. Displacement of dansyl-polymyxin bound to lipopolysaccharides from ATCC 19606, 208628, and 201630 *Acinetobacter baumannii* strains by cecropin A-melittin analogues and colistin sulfate

Peptide	LPS from *Acinetobacter baumannii* strain:					
	ATCC 19606		208628		201630	
	I_{max} (%)	I_{50} (μM)	I_{max} (%)	I_{50} (μM)	I_{max} (%)	I_{50} (μM)
CA(1-8)M(1-18)	27.2	1.6	96.5	0.6	71.5	0.5
CA(1-7)M(2-9)	33.5	1.8	96.9	0.5	78.1	0.6
Oct-CA(1-7)M(2-9)	29.9	1.5	92.2	0.5	76.3	0.5
CA(1-7)M(5-9)	23.2	1.9	83.9	0.4	70.6	0.4
Colistin	40.2	1.3	50.5	35.4	26.3	13.4

maintenance of the resistance to colistin implies a severe burden to the bacterium; for *Pseudomonas aeruginosa* strains with high-level resistance to polymyxin, instability in absence of the antibiotics was reported (29).

Whether the CA-M peptides will deserve an opportunity as clinical alternatives against the rising threat of widespread dissemination of panresistant (including colistin) *A. baumannii* is still a matter for further studies. The potential of these and other EAPs as antimicrobial leads may partly rely on the fairly broad range of structural modulations with activity-improving effects they can accommodate. For instance, a diastereomer form of a designed antimicrobial peptide was recently tested successfully in a murine model of *A. baumannii* bacteremia (3). In addition, CA-M peptides have shown remarkably low records of induction of resistance, including those mechanisms triggered by the peptide itself (26). Moreover, the possibility of combination therapy, such as their demonstrated synergy with ß-lactams (18), may reduce even further the risk of inducing resistance against these peptides, an admittedly unlikely occurrence which nevertheless has been described and discussed by some authors (19, 26). As a further exploratory step towards the potential therapeutic application of CA-M peptides, we are currently testing their antimicrobial activity in an animal model of colistin-resistant *Acinetobacter* sepsis.

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