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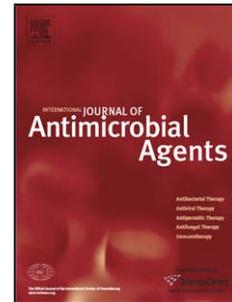
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The antimicrobial peptide Ci-MAM-A24 is highly active against multidrug-resistant and anaerobic bacteria pathogenic for humans

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ABSTRACT

Ci-MAM-A24, a synthetic antimicrobial peptide derived from a peptide precursor from immune cells of the marine invertebrate *Ciona intestinalis*, has been shown to be potently active against representatives of Gram-positive and Gram-negative bacteria by permeabilising their cytoplasmic membrane. In the present study, the activity of Ci-MAM-A24 against different bacterial pathogens frequently causing therapeutic problems was tested. In particular, the killing capacity of Ci-MAM-A24 against clinically important anaerobic bacteria as well as multiresistant aerobic strains such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci, extended-spectrum β -lactamase-producers and multiple-resistant *Pseudomonas aeruginosa* strains was monitored. Virtually all strains proved to be highly susceptible to Ci-MAM-A24 at low concentrations [minimum bactericidal concentration (MBC) < 10 μ g/mL].

1. Introduction

Antimicrobial peptides (AMPs) are key effector molecules of the innate immune system in the animal and plant kingdoms. In the search for new antimicrobial agents, these peptide antibiotics represent a promising class of substances that may be used as templates for the design of novel drugs [1].

Hundreds of AMPs have so far been identified from natural sources [2], some of which have already been evaluated in clinical trials [3]. In particular, marine organisms are an inexhaustible source of novel bioactive and antimicrobial compounds, including various peptides [4,5]. We recently described two new families of putative AMPs originating from the haemocytes of a marine invertebrate, the tunicate *Ciona intestinalis* [6,7]. A synthetic construct corresponding to the cationic amphipathic core region of one of these peptides (Ci-MAM-A24) efficiently killed a variety of different microbes including some human and marine pathogens [7]. Interestingly, human red blood cells were virtually unaffected by the peptide [7]. Moreover, it was demonstrated that the peptide kills bacteria by rapidly permeabilising their cytoplasmic membrane [7]. Although the killing efficiency of membrane-active peptides is often dramatically impaired by free ions in the surrounding media, Ci-MAM-A24 turned out to be exceptionally salt tolerant and remained highly active at human physiological conditions of 150 mM NaCl and pH 7.4 [7].

Consequently, the aforementioned characteristics of Ci-MAM-A24 suggested the idea that this peptide may be among the valuable candidates for the development of novel antibiotics.

However, one of the most important features of new antimicrobial drugs is their ability to kill efficiently microbes that cause serious therapeutic problems, in particular multidrug-resistant (MDR) bacteria whose growing emergence has long been a severe global health problem. The number of strains developing resistance against conventional antibiotics is constantly increasing [1,8]. Another prominent group of human pathogens regularly posing diagnostic and therapeutic challenges is represented by a multitude of anaerobic bacteria. Frequently overlooked, these strains often play a major role in a large variety of infectious diseases and processes such as those affecting the respiratory, gastrointestinal and female genital tracts as well as several soft tissues. Furthermore, anaerobes are primarily involved in various life-threatening systemic abscesses such as brain or intra-abdominal abscesses. Antibiotic treatment of these bacteria is often complicated by their slow growth, the low pH of the abscess environment and the increasing spread of resistance genes among these organisms [9]. Although the emergence of microbes posing severe therapeutic problems is constantly increasing, very few antibacterial therapeutic compounds of novel classes have been allowed admission to the market over the past 40 years [8,10]. Hence, there is still an urgent need for the development of new antibacterial drugs.

Here we report the antimicrobial activity of Ci-MAM-A24 against a large panel of medically important bacterial strains. Minimal bactericidal concentrations (MBCs) and

90% lethal doses (LD₉₀) were determined against different strains of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), multiresistant *Pseudomonas aeruginosa* and extended-spectrum β -lactamase (ESBL)-producing strains of *Escherichia coli* and *Klebsiella pneumoniae* as well as against a variety of anaerobic human pathogens.

2. Materials and methods

2.1. Bacteria

A total of 52 clinically relevant bacterial strains were used in this study. The following reference strains were obtained from the American Type Culture Collection (ATCC) and the German Collection of Microorganisms and Cell Cultures (DSMZ), respectively: MRSA (ATCC 33593 and ATCC 43300); VRE (*Enterococcus faecalis* ATCC 51299 and *Enterococcus faecium* DSM 17050); ESBL-producing enterobacteria (*K. pneumoniae* ATCC 700603); and 12 strains of anaerobic bacteria (*Clostridium perfringens* ATCC 13124, *Bacteroides fragilis* ATCC 25285, *Bacteroides ovatus* ATCC 8483, *Bacteroides thetaiotaomicron* ATCC 29148, *Prevotella oralis* ATCC 33321, *Prevotella intermedia* ATCC 25611, *Fusobacterium nucleatum* ATCC 10953, *Veillonella parvula* ATCC 10790, *Peptostreptococcus anaerobius* ATCC 27337, *Propionibacterium acnes* ATCC 6919, *Propionibacterium avidum* ATCC 25577 and *Eubacterium lentum* ATCC 43055). All other strains, including several multiresistant pseudomonads, were isolates from human clinical specimens. *Escherichia coli* K-12 D31, an ampicillin- and streptomycin-resistant strain, containing

a lipopolysaccharide (LPS) core that has lost part of the glucose, galactose and rhamnose [11], served as a quantitative positive control in each experiment.

2.2. Peptide

The peptide Ci-MAM-A24 was synthesised with an amidated C-terminus (WRSLGRTLLRLSHALKPLARRSGW-NH₂) and was obtained from Biosynthan (Berlin, Germany) at a purity grade of >95%. Homogeneity and the molecular identity of the synthetic peptide were verified by mass spectrometry. Ci-MAM-A24 was dissolved in 1 mM HCl to prepare a stock solution of final peptide at a concentration of 1 mg/mL.

2.3. Antimicrobial assays

To test the antimicrobial activity of Ci-MAM-A24, a microdilution assay was performed as described previously [12]. Briefly, bacteria (10^4 – 10^5 cells/mL) were incubated at 37 °C with different concentrations of Ci-MAM-A24 in 10 mM sodium phosphate buffer (pH 7.4) supplemented with 1% tryptic soy broth. After an incubation period of 2 h, the antimicrobial activity of Ci-MAM-A24 was analysed by plating serial dilutions of the incubation mixture onto brain–heart infusion agar plates and determining the number of colony-forming units the following day. The Ci-MAM-A24 solvent, 1 mM HCl, served as negative control in each experiment. None of the bacterial strains was affected by the solvent. To monitor and to demonstrate the high reproducibility of the method and the activity of the Ci-MAM-A24 peptide, the reference strain *E. coli* K-12 D31 was used in each test as a positive control, ensuring high quantitative interassay reproducibility. Results are given as MBC and

LD₉₀, i.e. the concentrations necessary to kill ≥99.9% and ≥90% of the microorganisms, respectively.

For susceptibility testing of anaerobic bacteria, the strains were incubated under anaerobic conditions using pre-reduced media. *Escherichia coli* K-12 D31 subjected to these conditions served as control for unimpaired Ci-MAM-A24 activity under anaerobic conditions.

3. Results and discussion

Ci-MAM-A24 exhibited a broad spectrum of potent antimicrobial activity against various bacterial pathogens (Tables 1 and 2). The MBC of the peptide against multiresistant strains was almost consistently 3.125 µg/mL, with very few exceptions of 1.56 µg/mL and 6.25 µg/mL corresponding to one dilution step in either direction in the assay (Table 1). The MBC of Ci-MAM-A24 against different anaerobic bacteria was in the range 0.39–6.25 µg/mL, with the only exception being the two species of the genus *Prevotella* that were more or less resistant to the peptide (MBC ≥ 100 µg/mL) (Table 2). Moreover, the LD₉₀ was always one or two dilution steps lower than the respective MBC, except for *E. faecium* clinical isolate no. G 70 and the two anaerobic strains *B. thetaiotaomicron* and *P. avidum* where a difference of three dilution steps was observed (Tables 1 and 2). In accordance with previous findings [7], Ci-MAM-A24 did not show any preference in killing for either Gram-positive or Gram-negative bacteria. The MBCs determined against Gram-positive MRSA and VRE strains were exactly in the same range of those against the Gram-negative ESBL-producing strains and multiresistant *P. aeruginosa* isolates (Table 1). Likewise,

no clear difference could be observed between the susceptibility of Gram-positive and Gram-negative anaerobic bacteria (Table 2). This is particularly remarkable as all representatives of novel classes of antibiotics that have reached the market in recent years, such as daptomycin and the oxazolidinones, are primarily effective against Gram-positive bacteria [8]. Exhibiting a high positive charge and an amphipathic potential, Ci-MAM-A24 represents a typical member of the class of cationic AMPs. It is believed that such positively charged peptides bind to negatively charged surface components of bacteria such as the LPS of Gram-negative bacteria and lipoteichoic acid of Gram-positive bacteria [3]. Subsequently, most cationic AMPs kill bacteria by disruption of their cytoplasmic membrane integrity, which has also been shown for Ci-MAM-A24 [7]. In the present study, we have shown that the activity of Ci-MAM-A24 was not diminished by the diverse resistance mechanisms that different bacteria have developed against conventional antibiotics. Consequently, membrane permeabilisation by Ci-MAM-A24 appears to be a highly effective killing mechanism to fight against MDR strains.

Although some cases of a modest decrease in susceptibility for cationic AMPs have been described [13], complete resistance has been proposed to be unlikely to occur [10]. In this respect, peptide antibiotics constitute an attractive alternative to conventional antimicrobial drugs. Moreover, we have demonstrated that the peptide Ci-MAM-A24 kills a large variety of anaerobic bacteria at very low concentrations. Despite the fact that anaerobic infections frequently cause serious health problems, anaerobes are in general underrepresented in studies determining the activity spectrum of novel antibiotic compounds. This is probably due to the more difficult and time-consuming techniques that are required for culturing of such strains. Hence, few

AMPs have been investigated to date regarding their activity against anaerobic bacteria [14,15].

In conclusion, the synthetic peptide Ci-MAM-A24 represents a promising template for alternative antimicrobial drug design according to its overall advantageous features such as salt tolerance, low cytotoxicity, broad-spectrum antimicrobial properties and, most notably, potent activity against various multiresistant as well as anaerobic pathogenic bacterial strains.

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Competing interests

None declared.

Ethical approval

Not required.

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Table 1

Activity of the antimicrobial peptide Ci-MAM-A24 against multidrug-resistant bacteria

Strain	MBC ($\mu\text{g/mL}$)	LD ₉₀ ($\mu\text{g/mL}$)
MRSA strains		
ATCC 33593	3.125	0.78
ATCC 43300	3.125	0.78
Clinical isolate no. 344	3.125	1.56
Clinical isolate no. 355	3.125	1.56
Clinical isolate no. 358	3.125	1.56
Clinical isolate no. 595	3.125	0.78
Clinical isolate no. 596	3.125	1.56
Clinical isolate no. 597	3.125	1.56
Clinical isolate no. 598	3.125	1.56
Clinical isolate no. 599	6.25	3.125
VRE strains		
<i>Enterococcus faecalis</i> ATCC 51299	3.125	1.56
<i>Enterococcus faecium</i> DSM 17050	3.125	1.56
<i>E. faecium</i> clinical isolate no. 354	3.125	1.56
<i>E. faecium</i> clinical isolate no. 356	3.125	1.56
<i>E. faecium</i> clinical isolate no. G 56	3.125	0.78
<i>E. faecium</i> clinical isolate no. G 57	3.125	1.56
<i>E. faecium</i> clinical isolate no. G 58	3.125	1.56
<i>E. faecium</i> clinical isolate no. G 59	6.25	3.125
<i>E. faecium</i> clinical isolate no. G 70	6.25	0.78
<i>E. faecium</i> clinical isolate no. G 71	3.125	1.56
ESBL-producing enterobacteria		
<i>Klebsiella pneumoniae</i> ATCC 700603	3.125	0.78
<i>K. pneumoniae</i> clinical isolate no. CF 1	3.125	1.56
<i>K. pneumoniae</i> clinical isolate no. CF 7	3.125	1.56
<i>K. pneumoniae</i> clinical isolate Obels ESBL	3.125	0.78
<i>K. pneumoniae</i> clinical isolate no. ESBL 8	3.125	1.56
<i>K. pneumoniae</i> clinical isolate no. ESBL 23	1.56	0.39

<i>Escherichia coli</i> clinical isolate no. E 4	6.25	1.56
<i>E. coli</i> clinical isolate no. E 9	3.125	1.56
<i>E. coli</i> clinical isolate no. E 85	3.125	0.78
<i>E. coli</i> clinical isolate no. E 86	3.125	0.78
Multiresistant <i>Pseudomonas aeruginosa</i> strains		
Clinical isolate no. CF 453 mr	3.125	1.56
Clinical isolate no. CF 479 mr	1.56	0.78
Clinical isolate no. CF 509 mr	3.125	1.56
Clinical isolate no. CF 629 mr	3.125	1.56
Clinical isolate no. CF 640 mr	3.125	1.56
Clinical isolate no. CF 601 mr mukoid	3.125	1.56
Clinical isolate no. CF 602 mr mukoid	3.125	1.56
Clinical isolate no. CF 643 mr mukoid	3.125	0.78
Clinical isolate no. CF 644 mr mukoid	3.125	1.56
Clinical isolate no. CF 646 mr mukoid	3.125	3.125
Control strain		
<i>Escherichia coli</i> K-12 D31	1.56/3.125	0.78/1.56

MBC, minimal bactericidal concentration; LD₉₀, 90% lethal dose; MRSA, meticillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant enterococci; ESBL, extended-spectrum β -lactamase.

Table 2

Activity of the antimicrobial peptide Ci-MAM-A24 against anaerobic bacteria

Strain	MBC ($\mu\text{g}/\text{mL}$)	LD ₉₀ ($\mu\text{g}/\text{mL}$)
Gram-positive anaerobes		
<i>Clostridium perfringens</i> ATCC 13124	0.78	0.39
<i>Eubacterium lentum</i> ATCC 43055	3.125	0.78
<i>Peptostreptococcus anaerobius</i> ATCC 27337	0.78	0.20
<i>Propionibacterium acnes</i> ATCC 6919	6.25	1.56
<i>Propionibacterium avidum</i> ATCC 25577	3.125	0.39
Gram-negative anaerobes		
<i>Bacteroides fragilis</i> ATCC 25285	1.56	0.39
<i>Bacteroides ovatus</i> ATCC 8483	0.39	0.20
<i>Bacteroides thetaiotaomicron</i> ATCC 29148	3.125	0.39
<i>Prevotella oralis</i> ATCC 33321	100	50
<i>Prevotella intermedia</i> ATCC 25611	>100	>100
<i>Fusobacterium nucleatum</i> ATCC 10953	1.56	0.39
<i>Veillonella parvula</i> ATCC 10790	3.125	0.78
Control strain		
<i>Escherichia coli</i> K-12 D31	1.56	0.39/0.20

MBC, minimal bactericidal concentration; LD₉₀, 90% lethal dose.