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New antimicrobial cystatin C-based peptide active against gram-positive bacterial pathogens, including methicillin-resistant *Staphylococcus aureus* and multiresistant coagulase-negative staphylococci

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We describe the synthesis and antibacterial properties of a novel antimicrobial peptidyl derivative, (2*S*)-2-(*N*^α-benzyloxycarbonyl-arginyl-leucylamido-1-[(*E*)-cinnamoylamido]-3-methylbutane, structurally based upon the inhibitory centre of the human cysteine protease inhibitor, cystatin C. The derivative, here called Cystapep 1, displayed antibacterial activity against several clinically important gram-positive bacteria. It displayed minimal inhibitory and bactericidal concentrations of about 16 µg/ml for both *Staphylococcus aureus* and *Streptococcus pyogenes*. In radial agar diffusion assays, groups A, B, C and G streptococci as well as staphylococci were generally susceptible to the action of Cystapep 1, whereas pneumococci and enterococci were less susceptible. No activity against gram-negative bacteria was observed. Cystapep 1 also showed high activity against methicillin-resistant *S. aureus* (MRSA) and multiantibiotic-resistant coagulase-negative staphylococci (CNS), suggesting that its mechanism of action differs from those of most currently used antibiotics.

Key words: Cystatin C; antimicrobial peptide; gram-positive pathogens; *Staphylococcus aureus*; *Streptococcus pyogenes*.

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Resistance to antibiotics among bacteria is of great concern worldwide. During the last few decades, development of resistance has been noted in major human pathogens as exemplified by methicillin-resistant *Staphylococcus aureus* (MRSA) (1) and coagulase-negative staphylococci (CNS; MRSE), vancomycin-resistant en-

terococci (VRE) (2) and pneumococci with decreased susceptibility to penicillin (3). The increasing occurrence of resistance has been suggested to be caused by high levels of antibiotic consumption as well as by inadequate consumption patterns (4).

The resistance of bacterial pathogens to conventional antibiotics has resulted in efforts to develop antimicrobial compounds with new mechanisms of action. Antimicrobial peptides

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that are lethal to bacteria and fungi are ubiquitously produced in nature (5–7). Most of these peptides kill microorganisms by forming pores in their cell membranes (8). Due to the effect on microbial membranes, resistance acquisition by microorganisms may not occur easily, thus making these peptides very attractive for therapeutic use as antibiotics. Furthermore, some promising clinical data for novel peptides have already emerged. After solving the problems of large-scale synthesis, a number of new antimicrobial peptides, structurally based upon natural products, may be developed (6, 9, 10). Lantibiotics (8) and defensins (11), both groups comprising a high number of compounds, are among the most well-known antibacterial research agents. Lantibiotics are of microbial origin. In contrast, defensins are present in secretions as important constituents of the innate immune system. Although not clarified in detail, it appears that the main target of these agents is also the bacterial cell membrane (12–14).

A new group of potential antibacterial agents was described in 1989 when an oligopeptidyl derivative, N-benzyloxycarbonyl-leucyl-valyl-glycyl-diazomethane (Z-Leu-Val-Gly-DAM), structurally based upon the inhibitory centre of human cystatin C, was found to suppress the growth of *Streptococcus pyogenes* (15). The recently described cystatin superfamily of proteins, to which cystatin C belongs, comprises both eukaryotic and prokaryotic cysteine protease inhibitors (16). The human cystatins can be grouped into three subfamilies, out of which family 1 includes the mainly intracellular cystatins A and B, and family 2 the extracellular and transcellular cystatins C, D, E, F, G, S, SA and SN, while the intravascular proteins high- and low-molecular-weight kininogens constitute family 3. The ample occurrence of family 2 cystatins in all human secretions has led to the suggestion that these cystatins, in particular, might be involved in a nonimmune protective system by inhibiting extra- or intracellular cysteine proteases of bacterial, viral or fungal origin. Indeed, human cystatins C, D and S, rat cystatins A and S, chicken cystatin and oryzacystatin have been described to inhibit the replication of certain viruses and bacteria (17–22). The inhibitory centre of cystatin C, the most widely distributed family 2 cystatin, comprises three peptide segments, Arg⁸-Leu⁹-Val¹⁰-Gly¹¹, Gln⁵⁵-

Ile⁵⁶-Val⁵⁷-Ala⁵⁸-Gly⁵⁹ and Pro¹⁰⁵-Trp¹⁰⁶ (23, 24). The above-mentioned peptidyl derivative Z-Leu-Val-Gly-DAM is based upon the aminoterminal segment of the inhibitory centre. While retaining the capacity to inhibit cysteine proteases, it shows a narrow antibacterial spectrum by selectively inhibiting the growth of *S. pyogenes* a bacterial species that produces a cysteine protease (25). Subsequent analysis of a number of related compounds, however, showed that their antimicrobial effect was probably not ascribable to any protease inhibition (25). In addition, the antimicrobial spectrum of both linear and cyclic substances within this class of compounds was found to differ from that of Z-Leu-Val-Gly-DAM against several gram-positive pathogens. For one of the most promising linear derivatives, here named Cystapep 1, comparatively low MIC and MBC values for both *S. pyogenes* and *Staphylococcus aureus* were recorded (25), and a strong mouse protective capacity of some of the compounds against lethal streptococcal infections was noted (25). In the present work we studied the antimicrobial effect of Cystapep 1 against a larger collection of clinically important pathogens, including several strains resistant to a number of currently used antibiotics. Our results indicate that Cystapep 1 is active mainly against streptococcal and staphylococcal species and that its mechanism of action may differ from those of most antibiotics currently in clinical use.

MATERIALS AND METHODS

Synthesis of Cystapep 1

(2S)-1-amino-2-[N-(*tert*-butyloxycarbonyl)-amino]-3-methylbutane hydrochloride was prepared from Boc-*L*-valinol following standard procedures (25). The free amino function of mono-Boc-diamine was protected with the Fmoc group and the resulting product was used for synthesis of Cystapep 1. Elongation of the peptide chain was performed using Boc-Leu and Z-Arg and the DCC/HOBt method for peptide bond formation. The Boc protecting group was removed with 4N HCl in dioxane. Finally, the Fmoc group was removed with 20% piperidine in DMF and the resulting product was isolated by ion-exchange chromatography using S-Sepharose FF. The obtained (2S)-1-amino-2-(N^α-benzyloxycarbonyl-arginyl-leucylamido)-3-methylbutane was finally purified using RP-HPLC and then acylated with (*E*)-cinnamic acid, using the DCC/HOBt method. The

product (Cystapep 1) was isolated using ion-exchange chromatography on a column of S-Sepharose FF and finally purified by RP – HPLC. The structure of Cystapep 1 (Fig. 1) was confirmed by FAB-MS and ¹H-NMR, including COSY and NOESY techniques.

Test of concentration of Cystapep 1

Different solutions of Cystapep 1 were centrifuged at 300 g for 15 min. Aliquots of the clear supernatants were used for quantitative amino acid analysis after evaporation followed by hydrolysis at 110°C for 20 h in 6 M HCl. An automated system, Beckman High Performance Analyzer, model 6300, was used for the amino acid analysis. The amounts of amino acids released were then used to calculate the concentration of Cystapep 1 from its known structure.

Bacterial strains

The following bacterial reference strains were used: *Streptococcus pyogenes* type M1 (40/58), *Streptococcus agalactiae* (NCTC 8181), *Streptococcus equisimilis* (ATCC 12388) and *Streptococcus anginosus* (CCUG 27298), *Streptococcus pneumoniae* (ATCC 49619), *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 14990). In addition, a variable number of clinical isolates of groups A, B, C and G streptococci (GAS, GBS, GCS, GGS, respectively), *Streptococcus aureus*, coagulase-negative staphylococci (CNS), *Enterococcus faecalis*, *Enterococcus faecium*, viridans streptococci, *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were obtained from our Clinical Microbiology Department.

Susceptibility testing

The antibacterial activity of Cystapep 1 was tested by agar well diffusion. Strains were grown aerobically at 37°C for 18 h on blood agar base (LabM) with 4% defibrinated horse blood. However, *H. influenzae* was grown on Blood agar base No. 2 (Oxoid) containing 7% haematinized horse blood in 5% CO₂ atmosphere.

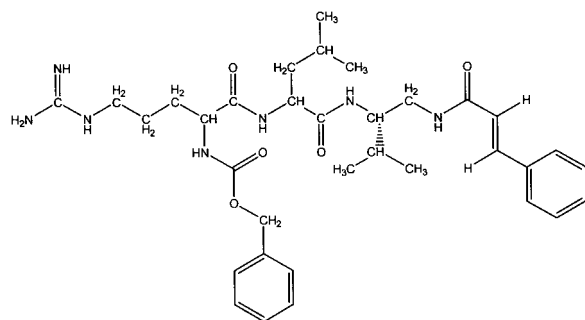


Fig. 1. Chemical structure of (2*S*)-2-(*N*^α-benzyloxy-carbonyl-arginyl-leucylamido)-1-[(*E*)-cinnamoylamido]-3-methylbutane, here named Cystapep 1.

From each strain, 5–10 colonies were suspended in 10 ml saline to an optical density of approximately 0.5 McFarland units, vortexed rigorously and inoculated onto IsoSensitest agar (Oxoid), or onto haematin agar as indicated above, using a cotton-tipped swab. The thickness of the solid media was 5 mm. Wells of 5 mm diameter were punched in the agar and 50 μl of a solution of Cystapep 1 (1.0 mg/ml) in dimethylsulfoxide (DMSO) was applied in each hole. In pilot experiments, phosphate-buffered saline (PBS) or PBS containing either 5% or 50% DMSO as solvents was also tested. After prediffusion at room temperature for 0.5 h, the plate was incubated at 37°C for 14 h aerobically or in 5% CO₂ as described above. The antibacterial effect was classified according to inhibition zone diameters into four groups: <7 mm, 8–11 mm, 12–15 mm, and more than 16 mm.

For determination of minimal bactericidal (MBC) and inhibitory (MIC) concentrations, a broth dilution method was used (26). Strains to be examined were grown overnight on blood agar. Two-fold dilutions of the test substance in 2 ml Todd Hewitt broth containing 5% DMSO were inoculated with a loopful of bacteria to final concentrations of approximately 10⁵ CFU/ml and the suspensions were dispensed in microtitre plates (Nunclon). Following incubation at 37°C for 20 h, MIC was defined as the lowest concentration yielding no visible growth. From each dilution, 10 μl was inoculated onto horse blood agar and, following 18 h incubation, MBC was defined as the lowest concentration yielding growth of no more than 10 colonies, corresponding to at least 99.9% killing.

RESULTS

Solubility of Cystapep 1 and stability of its solutions

Cystapep 1 was found to be soluble to at least 1 mg/ml in both PBS and DMSO. However, in PBS a visible precipitate formed after some hours both at room temperature and at 4°C. Cystapep 1 precipitated also from PBS solutions containing 5% or 50% DMSO, although after a prolonged storage period of 5–24 h. The antibacterial activity of the solutions was the same in all the fresh preparations but decreased as a function of precipitation of Cystapep 1 from the PBS solutions. However, in 100% DMSO, no precipitation and no decrease in the antibacterial capacity occurred even after storage for at least 1 month at room temperature or at 4°C. Since DMSO did not influence the growth of any of the bacterial strains tested, a

TABLE 1. Susceptibility of selected gram-positive bacterial species to Cystapep 1^a

Species (no. of strains tested)	Zone diameter (mm)			
	≤7	8–11	12–15	≥16
MSSA (57)	0	0	29	28
MRSA (97)	0	0	1	96
CNS (56)	0	0	16	40
<i>S. pyogenes</i> (63)	0	0	0	63
<i>S. agalactiae</i> (33)	0	0	11	22
Group C streptococci (25)	0	0	21	4
Group G streptococci (25)	0	0	22	3
<i>S. pneumoniae</i> (39)	0	33	6	0
Viridans streptococci (64)	15	4	33	12
<i>Listeria monocytogenes</i> (11)	0	0	11	0
Total	15	37	150	268

^a Tested by agar well diffusion (See Materials and Methods for details).

Abbreviations: MSSA=methicillin-sensitive *S. aureus*; MRSA=methicillin-resistant *S. aureus*; CNS=coagulase-negative staphylococci.

The tests were generally performed once; however, for the reference strains of *S. aureus*, *S. epidermidis*, *S. pyogenes*, *S. pneumoniae* and *E. faecalis*, tests repeated five times showed closely similar results with zone diameters varying no more than 2 mm.

solution of Cystapep 1 in 100% DMSO was used in all tests of antibacterial activity.

Screening of various pathogens for susceptibility to Cystapep 1

Gram-negative strains obtained at our diagnostic department were tested for susceptibility to Cystapep 1. All isolates of *H. influenzae*, *M. catarrhalis*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, 25 of each, were resistant to the action of Cystapep 1. In contrast, most of the examined Gram-positive bacteria were susceptible, as specified below and in Table 1.

Staphylococci. A total of 154 strains of *S. aureus* and 56 strains of CNS was included in testing. A high proportion of the *S. aureus* strains (97/154) and of the CNS strains (26/56) were methicillin-resistant. Though all the methicillin resistant strains were susceptible to vancomycin, many of the strains were also resistant to commonly used drugs, such as erythromycin, clind-

amycin, fusidic acid, netilmicin, and levofloxacin. With no exception, Cystapep 1 produced inhibitory zones with diameters greater than 14 mm for these multiresistant strains, which were thus regarded as susceptible to Cystapep 1. There was no apparent difference in degree of susceptibility between the methicillin-resistant and methicillin-sensitive *S. aureus* or CNS strains; furthermore, all *S. aureus* and CNS strains, including the reference strains ATCC 29213 and ATCC 14990, showed an equal level of susceptibility to Cystapep 1.

Beta-haemolytic streptococci. One hundred and forty-six isolates of beta-haemolytic streptococci were tested, viz. 63 *S. pyogenes*, 33 *S. agalactiae*, 25 group C, and 25 group G strains, among those the reference strains mentioned in Materials and Methods. All strains were clearly inhibited by Cystapep 1, showing zones of inhibition of at least 10 mm in diameter. The inhibitory zones of GAS were larger than those of GBS, GCS and GGS.

Pneumococci. All the 38 pneumococcal isolates were inhibited by Cystapep 1 though to a moderate degree, most showing inhibitory zones of 8–11 mm. A zone of 10 mm was observed for the reference strain ATCC 49619.

Viridans streptococci. The majority, 45 out of 64 strains of the viridans group of streptococci tested, showed good susceptibility to Cystapep 1; however, 15 of the strains were resistant.

Enterococci. None of 64 *E. faecalis* and 10 *E. faecium* strains was susceptible to Cystapep 1.

L. monocytogenes. All 11 strains examined were susceptible to Cystapep 1, showing inhibitory zones of 12–15 mm.

Inhibitory and bactericidal concentrations of Cystapep 1 for selected strains

The reference strains of *S. pyogenes*, *S. agalactiae*, *S. equisimilis*, *S. anginosus* and *S. aureus* were tested by broth dilution to determine MBC and MIC of Cystapep 1. The *S. pyogenes* and *S. aureus* strains showed MBC and MIC of 16 µg/ml as compared to a slightly higher level, 32 µg/ml, shown by the other three strains (Table 2).

TABLE 2. MIC/MBC determinations of some reference strains^a

Strain	MIC ($\mu\text{g/ml}$)	MBC ($\mu\text{g/ml}$)
<i>S. pyogenes</i>	16	16
<i>S. agalactiae</i>	32	32
<i>S. equisimilis</i>	32	32
<i>S. anginosus</i>	32	32
<i>S. aureus</i>	16	16

^a See Materials and Methods for details.

Strains were grown on solid media, and then tested by broth dilution at a concentration of approximately 10^5 CFU/ml for susceptibility to Cystapep 1. The tests were performed five times with similar outcomes.

DISCUSSION

We have previously reported on antibiotic properties of some synthetic peptidyl derivatives, structurally based upon the aminoterminal segment of the inhibitory centre of human cystatin C (16, 24). The peptidyl derivative named Cystapep 1, described in this discourse, was selected for further study since it showed both good in vitro inhibitory activity for staphylococci and streptococci as well as a high protective capacity against invasive *S. pyogenes* infection in the mouse. In the present work, the antimicrobial spectrum of Cystapep 1 was further characterized by testing its activity against several species including methicillin-resistant staphylococcal isolates.

Cystapep 1 was found equally effective against antibiotic-resistant staphylococci and streptococci as against antibiotic-susceptible strains of these species. In a large collection of MRSA isolates comprising many strains with additional resistance to several non-beta-lactam antibiotics, the in vitro susceptibility to Cystapep 1 proved invariably high. Similarly, a substantial number of clinical CNS isolates, many found multiresistant to commonly used antimicrobials, also showed high susceptibility to Cystapep 1. At present these staphylococci represent leading agents in nosocomial and bio-material-associated infections posing significant therapeutic problems due to shortage of effective antibacterial agents. In addition, the susceptibility of beta-haemolytic streptococci to Cystapep 1 may prove important because of problems when treating both invasive and superficially located infections. In contrast, en-

terococci appeared to be poorly sensitive to Cystapep 1.

Though several hundred isolates have been studied, we have not so far observed any strains of staphylococci or beta-haemolytic streptococci resistant to Cystapep 1. The possibility of selecting resistant mutants by repeated passages in media containing Cystapep 1 is currently being investigated. So far no resistant mutants have been obtained (own unpublished observations).

The stability of Cystapep 1 in different types of solutions was also investigated. Cystapep 1 proved to be soluble and stable in 100% DMSO. It was also soluble in several other solvents, e.g. PBS, but precipitated slowly from the other solvents tested. Since DMSO did not influence the growth of any bacterial species investigated in preliminary testing, DMSO solutions of Cystapep 1 could be used in in vitro susceptibility testing. However, as DMSO displays some toxicity for mammalian cells, alternative non-toxic solvents should preferably be used in further studies of Cystapep 1. Although Cystapep 1 has been successfully used to protect animals from lethal streptococcal infections (25) the intrinsic toxicity of Cystapep 1 also needs to be further investigated in order for a clinically useful drug to be developed. For optimal use, derivatives of Cystapep 1 with higher antibacterial activity would also have to be designed.

It is notable that the activity of Cystapep 1 appears to be limited to Gram-positive bacteria and that the effect is bactericidal. The structure of Cystapep 1 bears little resemblance to those of previously reported naturally occurring antimicrobial peptides, e.g. defensins. Cystapep 1 is much smaller than these peptides, contains extensively modified amino acid residues and mimics the active centre of a human major protease inhibitor, cystatin C, although it does not retain any protease inhibitory properties (25). The mode of action of Cystapep 1 therefore probably differs from those of known membrane pore-forming peptides. Furthermore, the lack of action on gram-negative bacteria noted for Cystapep 1, with a molecular size that would permit its transport across the outer membrane pores, may argue against its target of action being the bacterial cytoplasmic membrane. Indeed, the observation that Cystapep 1 was as active against multiresistant staphylococci and

other antibiotic-resistant strains as against corresponding antibiotic-susceptible strains might suggest its action to be distinct from those of most currently used antibiotics.

In conclusion, our previous and present results suggest that various synthetic peptidyl derivatives structurally based upon the inhibitory centre of human cystatin C may be promising candidates for the development of clinically useful antibacterial drugs. As previously reported, these compounds do not require chemically reactive groups for their action. The presently described compound, Cystapep 1, showed pronounced activity against important gram-positive pathogens, including multiresistant staphylococci, whereas no activity for gram-negative organisms was found. Obviously, such selectivity in its antibacterial spectrum may be advantageous from an ecological point of view. Further studies will be required to establish the mechanism of action of Cystapep 1, supposedly distinct from those of most, or all, known antibiotics, and whether resistance development among key organisms may be anticipated.

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