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Shortened cecropin A–melittin hybrids

Significant size reduction retains potent antibiotic activity

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We have earlier reported two 26-residue antibacterial peptides made up from different segments of cecropin A (CA) and melittin (M). We now report a substantial reduction in size at the C-terminal section of the highly active hybrid CA(1–8)M(1–18), leading to a series of 20-, 18- and 15-residue analogs with antibiotic properties similar to the larger molecule. In particular, the 15-residue hybrids CA(1–7)M(2–9), CA(1–7)M(4–11) and CA(1–7)M(5–12) are the shortest cecropin-based peptide antibiotics described so far, with antibacterial activity and spectra similar or better than cecropin A and a 60% reduction in size. Their reduced size and highly α-helical structure require an alternative mechanism for their interaction with bacterial membranes.

Antibacterial peptide; Cecropin A; Melittin; Solid-phase peptide synthesis

1. INTRODUCTION

Antibacterial peptides have been known for over a decade now to play an important role in insect immunity [1,2]. In recent years, a more general view of their contribution to host defense mechanisms is gradually emerging, as new antimicrobial peptides are found in amphibians, higher mammals and humans [2–7]. In spite of their broad antibiotic spectrum and significant potency, attempts to develop some of these antibacterial peptides into useful, chemically synthesizable therapeutic agents have been somewhat impaired by the length and/or complexity of their structures. Thus cecropin A (CA), the first insect antibacterial peptide to be reported [8] and a rather potent antibiotic, has a linear but relatively long (37-residue) sequence which, though clearly accessible through chemical synthesis [9], is not an ideal candidate for drug development. PGL[a], PYL[a] [10,11] and other antibiotical peptides from amphibian skin such as the magainins [3] are smaller in size and thus less demanding synthetically [12], but somewhat less effective as antibiotics than the cecropins. Defensins [6], on the other hand, while showing interesting antibacterial, antifungal and antiviral properties, are rather complex, highly folded structures with three internal disulfide bonds and have so far proved rather difficult to prepare by chemical means [13].

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We have recently reported that certain hybrids of cecropin A and melittin (M) show powerful antibacterial activities. In particular, CA(1–13)M(1–13) and CA(1–8)M(1–18) [14,15] exhibit a wider spectrum and improved potency relative to cecropin A without the undesirable cytotoxic effects of melittin. Although, at 26 residues each, they represent a significant (30%) reduction in size from the parent cecropin A molecule, they are still relatively long and thus not yet quite appropriate for therapeutical consideration. We have therefore set as one of our goals to develop analogs of substantially reduced length that retain the antibacterial activity of the larger molecules. In this paper we report our results on several shortened cecropin–melittin hybrid peptides, leading to a group of 15-residue analogs with antibacterial spectra and potency essentially comparable to the larger-size peptides and which thus constitute a promising starting point for future development. Our findings also bear upon the current understanding of the structural features involved in the antibacterial action of this family of peptides.

2. MATERIALS AND METHODS

2.1. *Peptide synthesis*

All peptides were synthesized by the solid-phase method [16] as C-terminal carboxamides on *p*-methylbenzhydrylamine resin (0.81 mmol/g; Peptides International, Louisville, KY) using Boc-type chemistry and 2-chlorobenzyloxycarbonyl and formyl side protection for Lys and Trp, respectively. Chain assembly was done automatically on a 0.1-mmol scale in an Applied Biosystems 430A instrument running the opt21 run files provided by the manufacturer. The protected final peptide resins were sequentially treated with 40% (v/v) trifluoroacetic

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acid in dichloromethane (1 + 20 min, RT) and 20% (v/v) piperidine in *N,N*-dimethylformamide (2 × 1 min, RT) to respectively the N^{α}-Boc and formyl protections, then cleaved in HF-*p*-cresol (9:1 v/v; 0°C, 1 h). Following HF evaporation and diethyl ether extraction, the crude peptides were solubilized in 10% acetic acid, tested for purity on HPLC and lyophilized. They were purified in a single step by semi-preparative reverse-phase liquid chromatography on C_{18}-silica using linear 0–40% acetonitrile gradients in water, both containing 0.05% trifluoroacetic acid. Fractions judged to be of sufficient (>95%) purity by analytical HPLC were pooled and lyophilized. Peptide characterization was completed by amino acid analysis and plasma-desorption mass spectrometry.

2.2. *Circular dichroism*

Spectra were recorded on Aviv 62DS and Jasco 700 spectropolarimeters. Peptides were dissolved to 25 μM concentration in 2.5 mM sodium phosphate buffer, pH 7.4, containing 0–20% (v/v) hexafluoro-2-propanol (HFIP). Three consecutive 250–190 nm scans were made in a 1-mm cell at 10°C, averaged and fitted according to Chen and Yang [17] to a given α-helix, β-sheet, β-turn and random coil composition by means of the Prosec program.

2.3. *Antibacterial, hemolysis and antimalarial assays*

All three assays were performed as previously described [14]. Antibacterial and red blood cell lysis determinations were duplicated in independent sets of experiments (Stockholm, New York) using the same strains of bacteria. Lethal concentrations were calculated from inhibition zone diameters as described by Hultmark et al. [18].

3. RESULTS

The primary structures of the cecropin A–melittin hybrids reported here (one 20-mer, one 18-mer and six 15-mers) are depicted in Table I, which also includes CA(1–8)–M(1–18) [CA(1–8)M(1–18)] for comparison purposes. The melittin moiety of this parent structure was first decreased in length at its C-terminus to 12 and 10 residues (CA(1–8)M(1–12) and CA(1–8)M(1–10), respectively), then further reduced to 8 residues with simultaneous removal of the Ile^{8} residue of cecropin A to give the pentadecapeptide CA(1–7)M(1–8). Five other 15-mers were prepared in which the seven N-terminal residues of cecropin A are followed by 8-residue segments of melittin beginning at the N-terminus and shifting in one-residue steps towards the C-terminus. All peptides were prepared as C-terminal amides by automated solid phase synthesis using optimized Boc/benzyl chemistry, cleaved in HF and purified by reverse-phase chromatography in satisfactory yields (40–45%, combined cleavage and purification). They were found to be pure and of the correct structure on the basis of their amino acid analyses, HPLC homogeneity and mass spectra.

Table I

Primary structures of parent cecropin A(1–8)-melittin(1–18) and shortened analogs

Peptide name	No. of residues	Amino acid sequence	
		←cec A→	←melittin→
		1 8	1 18
CA(1–8)M(1–18)	26	KWKLFKKI	GIGAVLKVLTTGLPALIS
CA(1–8)M(1–12)	20	KWKLFKKI	GIGAVLKVLTTG
CA(1–8)M(1–10)	18	KWKLFKKI	GIGAVLKVLT
CA(1–7)M(1–8)	15	KWKLFKK	GIGAVLKV
CA(1–7)M(2–9)	15	KWKLFKK	IGAVLKVL
CA(1–7)M(3–10)	15	KWKLFKK	GAVLKVLT
CA(1–7)M(4–11)	15	KWKLFKK	AVLKVLTT
CA(1–7)M(5–12)	15	KWKLFKK	VLKVLTTG
CA(1–7)M(6–13)	15	KWKLFKK	LKVLTTGL

The solution conformations of the peptides were measured by circular dichroism at different concentrations of HFIP (0% to 20%, v/v, in 4% intervals). Two different types of conformational behavior could be broadly defined from these experiments (Tabel II). On the one hand, peptides CA(1–8)M(1–12), CA(1–8)M(1–10) and CA(1–7)M(1–8) in the absence of HFIP (5 mM sodium phosphate, pH 7.3) gave curves suggestive of significant levels of β-sheet structure which remained the prevalent conformation feature also in the 4–12% HFIP interval. Some incipient α-helix formation was observed even at 4% HFIP and, as expected, it rose to a plateau of ca. 50% helix at 16–20% HFIP, the highest concentrations of organic solvent studied. The other 15-residue peptides behaved somewhat differently. In the absence of organic solvent no defined structure was observed and, while moderate-to-medium levels of β structure were calculated for all five peptides at low (4–8%) HFIP concentrations, a much stronger tendency to adopt an α-helix structure became evident at 12% HFIP and higher, with no β-sheet present. Indeed, CA(1–7)M(2–9) was calculated by the Prosec program to be a 'perfect' α-helix at 16% HFIP and above (Fig. 1). The high helix contents of the peptides at and above 12% HFIP were found to be independent of the concentration in the 5–50 μM range (data not shown); at higher concentrations some loss of helicity was observed indicating possible aggregation. It should be noticed that the consistently high levels of α-helix found for all 15-residue hybrids except CA(1–7)M(1–8) (Table II) seem to be compatible with the presence of a single Gly residue in their central section, while in CA(1–7)M(1–8) and the larger hybrids the presence of the Gly-Ile-Gly-Ala tetrapeptide would appear to disrupt the helix continuity.

The antibiotic and hemolytic activity of the peptides were measured by inhibition zone assays from which lethal concentrations were derived (Table III). Duplicate assays were performed independently in two of our laboratories and good correlation between them was found. Deviations in lethal concentration values from one assay to another were always within the ±50% experimental error range. All peptides were shown to be active against both Gram-negative and Gram-positive test microorganisms, in most cases at peptide concentrations similar to those of the parent analog CA(1–8)M(1–18). Thus, CA(1–8)M(1–12) and CA(1–8)M(1–10) were essentially comparable to CA(1–8)M(1–18), although the latter analog showed a significant reduc-

Table II

Calculated percentages[a] of α-helix, β-sheet and random coil in cecropin A–melittin hybrids

Peptide	0% HFIP			4% HFIP			8% HFIP			12% HFIP			16% HFIP			20% HFIP		
	α	β	r	α	β	r	α	β	r	α	β	r	α	β	r	α	β	r
CA(1–8)M(1–12)	0	35	58	9	52	37	17	52	31	35	37	28	52	18	30	56	6	38
CA(1–8)M(1–10)	0	33	62	6	41	43	5	56	33	23	46	31	48	21	31	52	12	36
CA(1–7)M(1–8)	0	21	60	0	16	50	0	52	35	13	52	35	41	18	41	44	25	31
CA(1–7)M(2–9)	0	0	69	0	8	62	28	63	9	91	0	9	100	0	0	100	0	0
CA(1–7)M(3–10)	0	0	65	0	11	59	0	44	45	63	0	37	91	0	9	100	0	0
CA(1–7)M(4–11)	0	0	69	0	19	67	0	33	53	75	0	25	88	0	12	90	0	10
CA(1–7)M(5–12)	0	0	69	0	22	56	35	33	32	78	0	22	82	0	18	88	0	12
CA(1–7)M(6–13)	0	4	62	0	13	57	0	53	36	75	0	25	88	0	12	90	0	10

[a]In cases where the sum of percentages is less than 100%, the remainder is predicted as a turn.

tion in activity against *S. aureus*. Of the six pentadecapeptides, CA(1–7)M(1–8) tested poorly against most organisms, but the other five showed antibacterial activity values within the same range as the parent structures. CA(1–7)M(2–9), CA(1–7)M(4–11) and CA(1–7)M(5–12), in particular, were essentially indistinguishable from the larger molecules in terms of molar antibiotic potency. In addition, all 15-residue peptides were also tested for antimalarial activy agains *P. falciparum*. In a reinvasion inhibition assay (Table IV), their activity was shown to be in most cases only slightly inferior to their parent structure, CA(1–8)M(1–18), and significantly better than a number of larger antibacterial peptides.

4. DISCUSSION

The aim of this study was to develop cecropin A–melittin hybrids of relatively simple structure that would be easily accessible by chemical synthesis and thus have increased therapeutic potential. For cecropin A it has been known for some time that the broad spectrum antibiotic activity decreased when the molecule was shortened by deletion of certain residues [8,19]. However, we recently reported several 26-residue cecropin A–melittin hybrids with improved antibacterial spectra and potencies relative to cecropin A [14,15]. CA(1–8)M(1–18) [CA(1–8)M(1–18)], one of the most active hybrids of this series, was chosen as a starting point for our present search. Since it is well known that the N-terminal section of cecropin A, while not sufficient in itself as an antibacterial agent, is nevertheless needed for optimal activity [8], we chose to explore the effect of deletions at the C-terminal melittin portion of CA(1–8)M(1–18). An encouraging initial finding was that removal of six or eight residues from the C-terminus of CA(1–8)M(1–18) did not significantly alter the biological activity of the resulting peptides. Thus,

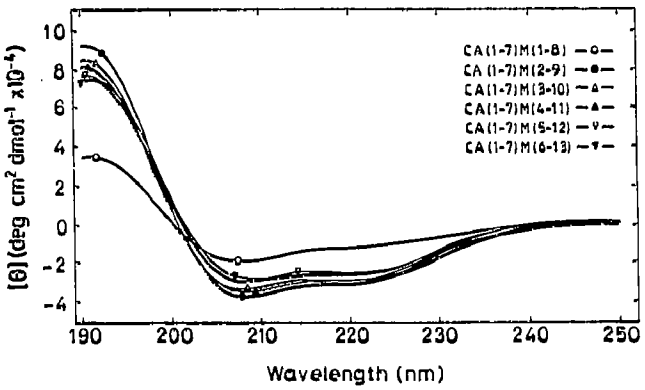

Fig. 1. Circular dichroism spectra of 15 residue cecropin A–melittin hybrids (25 μM) at 10°C in 2.5 mM sodium phospate, pH 7.4, containing 20% (v/v) hexafluoroisopropanol.

Table III

Antibiotic and lytic activity of cecropin A–melittin hybrids and parent compounds

Peptide	Lethal concentration[a] (μM)						
	D21	OT97	Bs11	Bm11	Staph.	Strep.	SRC
cecropin A[b]	0.3	2	4	0.5	>200	4	>400
melittin[c]	0.8	3	0.2	0.6	0.2	0.5	4–8
CA(1–13)M(1–13)[c]	0.5	1	0.7		2	1	>500
CA(1–8)M(1–18)[c]	0.3	0.7	0.4	0.4	1	2	>600
CA(1–8)M(1–12)	0.4	0.9	0.3	0.2	2	0.1	>600
CA(1–8)M(1–10)	0.5	3	0.3	0.5	7	0.8	>600
CA(1–7)M(1–8)	4	15	1.4	0.8	29	0.7	>300
CA(1–7)M(2–9)	1	4	0.5	0.7	2	0.5	>300
CA(1–7)M(3–10)	3	15	3	0.9	>200	1	>600
CA(1–7)M(4–11)	0.6	4	0.6	0.5	8	0.7	>600
CA(1–7)M(5–12)	0.5	2	0.3	0.5	8	0.4	>500
CA(1–7)M(6–13)	2	8	1	0.5	51	0.4	>500

[a] Calculated from inhibition zones on thin agarose plates seeded with the respective organisms. Test strains are: D21 = *E. coli* D21; OT97 = *P. aeruginosa* OT97; Bs11 = *B. subtilis;* Bm11 = *B. megaterium;* Staph. = *S. aureus* Cowan 1; Strep. = *S. pyogenes;* SRC = sheep red cells.

[b] Data from [8].

[c] Data from [13].

Table IV

Antimalarial (*P. falciparum*) activity of natural antibacterial peptides and cecropin A–melittin hybrids

Peptide	50% reinvasion inhibition (μM)
Cecropin A	n.a.*
Cecropin B	110
Cecropin P-1	90
Magainin	80
PGL[a]	40
CA(1–13)M(1–13)	17
CA(1–8)M(1–18)	4
CA(1–7)M(1–8)	7.0
CA(1–7)M(2–9)	7.5
CA(1–7)M(3–10)	8.0
CA(1–7)M(4–11)	8.0
CA(1–7)M(5–12)	9.0
CA(1–7)M(6–13)	9.0

*n.a. = no activity.

Table III shows that activity against *S pyogenes* increased by a factor of 20 when the hybrid was shortened from 26 to 20 residues. A further decrease in size [CA(1–8)M(1–10) did have only a small but probably significant effect. In addition, both peptides were found to be non-cytotoxic (i.e. non-hemolytic), a desirable property in any antibacterial agent. These results stimulated a search for structures of even smaller size and comparable activity that resulted in six 15-residue hybrids (Table I) in which seven N-terminal residues of cecropin A are followed by a segment of eight consecutive residues from melittin. While the first member of this series, CA(1–7)M(1–8), was not a very effective antibacterial agent, the other five retained significant potency, in some cases [CA(1–7)M(2–9) and CA(1–7)M(5–12)] comparable to the larger structures. The latter peptides are the shortest (60% reduction in size relative to cecropin A), most active cecropin-based antibiotics described to date and show interesting antimalarial properties as well. Work at our laboratories is currently aimed at a wider exploration of their antimicrobial spectrum and therapeutical possibilities. We believe their structures should provide helpful leads in the search for even smaller antibacterial peptide molecules.

The present finding that pentadecapeptides derived from cecropin A are powerful antibiotics must be reconciled with our previous ion-channel model for the membrane activity of the cecropins [15]. Up until now we had examined cecropin-based peptides of such a length (26–37 residues) that could be assumed to span the ca. 30 Å-thick membrane of a bacterial cell. Most of these peptides could be viewed as two α-helical segments separated by a Gly-Pro-containing 'hinge' region providing the necessary conformational flexibility required for membrane channel formation [15,19,20]. In the previously described CA(1–13)M(1–13) and CA(1–8)M(1–18) hybrids, the melittin-derived Gly-Ile-Gly-Ala tetrapeptide located at the middle of the sequence might play a similar role. In agreement with this model, the antibacterial activity of the larger (20- and 18-residue) peptides in the present series could still be explained by making allowance for some flexibility in the membrane-spanning ability of the peptides. Our present findings, however, showing several 15-residue peptides to be powerful membrane-active antibiotics while achieving remarkably high levels of α-helix (and thus total spans of ca. 22.5 Å, clearly shorter than the bacterial membrane thickness), seem to point towards a slightly different mechanism for antibacterial action. The possibility that the peptides adopt a 3_{10} helix conformation (total length 30 Å) instead of an α-helix is an attractive model that needs to be investigated. Another alternative is that the peptides associate in non-covalent form to give dimeric or multimeric structures spanning the entire membrane. Until further experimental testing, however, caution should be exercised in the interpretation of these results. Recent solid phase NMR work [21] has shown magainin, a membrane channel-forming peptide [22], to interact with membranes in a parallel fashion while another membrane-active peptide did interact perpendicular to the membrane. It may therefore be too early to suggest a general mechanism of action for the present short cecropin A–melittin hybrids, if indeed a single mechanism is involved.

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