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Antibacterial and antimalarial properties of peptides that are cecropin-melittin hybrids

H.G. Boman, D. Wade*, I.A. Boman, B. Wåhlin† and R.B. Merrifield*

Departments of Microbiology and †Immunology, University of Stockholm, S-10691 Stockholm, Sweden and **Rockefeller University, New York, NY 10021-6399, USA*

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Solid phase synthesis was used to produce 5 hybrid peptides containing sequences from the antibacterial peptide, cecropin A, and from the bee venom toxin, melittin. Four of these chimeric peptides showed good antibacterial activity against representative Gram-negative and Gram-positive bacterial species. The best hybrid, cecropin A(1–13)-melittin(1–13) was 100-fold more active than cecropin A against *Staphylococcus aureus*. It was also a 10-fold better antimalarial agent than cecropin B or magainin 2. Sheep red cells were lysed by melittin at low concentrations, but not by the hybrid molecules, even at 50 times higher concentrations.

Cecropin A; Melittin; (*Staphylococcus aureus, Plasmodium falciparium*)

1. INTRODUCTION

Cecropins are a family of broad spectrum antibacterial peptides that constitute a main part of the humoral immune system in insects (review [1]). However, cecropins may also be widely distributed in the aminal kingdom because a cecropin was recently isolated from pig intestine [2]. Cecropins are composed of two different domains linked by a hinge region containing Gly or Pro or both. The N-terminal domain is strongly basic and forms a nearly perfect amphipathic α-helix, while the C-terminus is a more hydrophobic helix [3–6]. Cecropins act on a wide variety of Gram-positive and Gram-negative bacteria but as far as tested they do not lyse eucaryotic cells [1,3].

The bee venom toxin, melittin, has a principal design that resembles the cecropins but the polarity is reversed: here the N-terminus is hydrophobic and the C-terminus is basic [7,8]. Like the cecropins, melittin has a hinge region and an amidated C-terminus. In both of the peptides there is also a conserved tryptophan residue on the N-terminal side of the basic residues. As far as known cecropin is not toxic, while melittin has a variety of toxic properties (review [9]). The most recorded function of melittin is the ability to lyse red cells. Melittin is also antibacterial [10] although this is not widely recognized.

The ideal immune substance should act against a very wide range of foreign cells without causing any destruction of the host and the same would in many respects apply also to toxins. It was therefore interesting to make chimeric peptides that are cecropin-melittin hybrids and assay them for antibacterial activity and ability to lyse red cells. This was a natural extension of our earlier work on cecropin analogs with single residue substitutions [11,12], on cecropin model peptides [13], and on a hybrid between cecropins A and D [14] with an improved ability to form ion channels [15]. Two analogs of cecropin B have been reported to inhibit the blood stream forms of the malaria parasite [16], and recently magainin 2 and cecropin B were found to inhibit the mosquito forms of the parasite [17]. We have therefore also tested our most potent hybrid peptide for ability to inhibit the growth of the blood stream forms of the malaria parasite.

Correspondence address: H.G. Boman, Department of Microbiology, University of Stockholm, S-10691 Stockholm, Sweden

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2. MATERIAL AND METHODS

2.1. *Solid phase synthesis*

The new peptides and also melittin were synthesized by a combination of manual and automated (Applied Biosystem 320A) solid phase techniques as described before in detail [11–14]. The purification was first by gel filtration on Sephadex G-25 in 1 M HOAc. The next step was low pressure, reverse phase, preparative liquid chromatography on a Vydac C18 column (218TPB10) and elution with a linear gradient of 25–65% acetonitrile. The molecular weight of the peptides was determined by fission-fragment mass spectrometry. Synthetic magainin 2 with a C-terminal amide and PGLa were kindly obtained from M. Zasloff.

2.2. *Antibacterial assays*

For each peptide and for each test bacterium 3 μl of a series of dilutions were placed in small wells in thin agarose plates seeded with the respective test bacteria. We earlier used agar plates [18] but many basic peptides are adsorbed to agar, and agarose gives LC values lower by a factor of 2–5 (Zasloff, personal communication). Plates

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were incubated overnight at 30°C, the inhibition zones were recorded and the lethal concentration for each peptide (LC, the lowest concentration that inhibits growth) was calculated as described [18]. Since large inhibition zones give low LC values, such values are more accurate than high LC values which come from small zones.

2.3. *Assay of red cell lysis*

This was recorded by an adaptation of the antibacterial inhibition zone assay [18] to plates with sheep red cells (SRC). Sterile agarose plates contained 6 ml of medium with 1% agarose, 0.9% NaCl and 10% SRC suspended in Alsevers solution. A dilution series of peptide was applied in 3 mm wells each loaded with 3 μl of the respective samples. The plates were incubated at 30°C for 24 h, the zones were recorded and LC values calculated as described [18]. Clear zones were recorded after a few hours of incubation and the use of such data gave an LC value of the same order as readings after 24 h. This plate assay is faster and more convenient than the lysis assay normally used for melittin.

2.4. *Antiparasitic assay*

The blood stream forms of the malaria parasite, *Plasmodium falciparium*, primarily late trophozoites and early schizonts, were assayed by recording the inhibition of the reinvasion of human red cells [19]. Quadruplicate microcultures of strain F32 (Tanzania) were incubated for 20 h at 37°C in a complete tissue culture medium, with or without different concentrations of the peptides to be assayed. After acridine orange staining, the percentage of newly infected red cells was scored in a fluorescence microscope. For each culture 40 000 red cells were analysed for parasitic infection.

3. RESULTS

The sequences of the parent peptides, cecropin A (CA) and melittin (M), are given in fig.1. Assuming that the region with a Trp followed by a number of basic residues can be termed 'head' and that the hydrophobic region could be referred to as a 'tail', 5 hybrid peptides were made based on the following considerations:

(1) CA(1-24)M(1-13)	Long, cecropin-like but with melittin 'tail'
(2) CA(1-13)M(1-13)	Short, cecropin-like but with melittin 'tail'
(3) CA(25-37)CA(1-13)	Shortened and inverted cecropin
(4) M(1-13)CA(1-13)	Short, melittin-like with cecropin 'head'
(5) M(16-26)M(1-13)	Shortened and inverted melittin

The term 'like' only refers to whether the basic 'head' is in the C-terminus as in melittin or in the N-terminus as for all cecropins. A further attempt to study the effects of the polarity was the design of the two inverted molecules.

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            1           13              24 25          37
Cecropin A: KWKLFKKIEKVGQ--NIRDGIIKAGP--AVAVVGQATQIAK

            1           13      16        26
Melittin:   GIGAVLKVLTTGL--PA--LISWIKRKRQQ
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Fig.1. Amino acid sequences of the two parental peptides cecropin A and melittin. The break points used in the construction of hybrids are indicated by the respective numbers and by two hyphens. The basic residues are in bold type. The C-terminals in both parents and all hybrid peptides are amidated.

All peptides were found to be homogeneous by analytical HPLC, of correct amino acid composition and with expected molecular weights by mass spectroscopy. In addition the parental compounds and 3 of the hybrids were investigated with acidic polyacrylamide gel electrophoresis by staining of one gel and with overlay of bacteria on another gel [20]. All compounds gave sharp bands coinciding with the bactericidal activity as scored by the bacterial overlay.

The peptides were then assayed quantitatively for antibacterial activity against 2 Gram-negative and 3 Gram-positive bacteria as well as for lysis of sheep red cells (SRC). The results in table 1 show that under the present assay condition (with agarose instead of agar in the plates) melittin is an even better broad-spectrum antibiotic than cecropin A. However, melittin is hemolytic and caused lysis of SRC at 4–8 μM. When the C-terminus in cecropin A was replaced with the sequence M(1–13), (the melittin 'tail' in hybrids 1 and 2) the bactericidal activity was increased by a factor of 30–100 in case of *S. aureus* and by a factor of 6–8 in the case of *B. subtilis*. Equally important, these peptides caused no lysis of SRC up to 200 μM.

It was not possible to make a shortened and inverted cecropin (hybrid 3) with activity. However, activity against 4 organisms, *E. coli*, *P. aeruginosa*, *B. subtilis*, and *S. pyogenes* was restored in hybrid 4 in which the melittin 'tail', M(1–13), replaced CA(25–37) in hybrid 3. This peptide gave doubtful results in two cases: with *S. aureus* the concentration dependence of the inhibi-

Table 1

Lethal and lysis concentrations (μM) for parental compounds and cecropin A-melittin hybrids

Compound	Plate					
	D21	OT97	Bs11	Sa CI	Sp 1	SRC
Parental peptides:						
CA(1-37)	0.2	2	4	>200	4	>200
M(1-26)	0.8	3	0.2	0.2	0.5	4-8
Hybrid peptides:						
(1) CA(1-24)M(1-13)	0.3	1	0.5	6	2	>200
(2) CA(1-13)M(1-13)	0.5	1	0.7	2	1	>200
(3) CA(25-37)CA(1-13)	200	300	20	>300	20	>300
(4) M(1-13)CA(1-13)	1	5	0.3	5*	1	80?
(5) M(16-26)M(1-13)	0.7	8	0.7	10	1	>200

Lethal concentrations calculated [18] from inhibition zones on thin agarose plates seeded with the respective organisms. Abbreviations for the organisms used in the assay plates: D21, *Escherichia coli* K12; OT97, *Pseudomonas aeruginosa*; Bs11, *Bacillus subtilis*; Sa CI, *Staphylococcus aureus* Cowan I; Sp 1, *Streptococcus pyogenes*; SRC, sheep red cells

* indicates a very flat concentration dependence
? indicates turbid zones

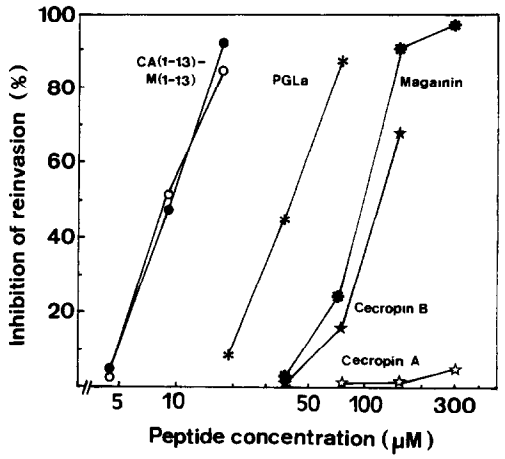

Fig.2. Inhibition of the growth of the blood stream forms of *P. falciparium* by different amounts of hybrid 2, CA(1-13)M(1-13), and four other peptides used for comparison. Details of the assay are given in section 2 and in [19]. Duplicate assays were run for hybrid 2.

tion zones was abnormally flat and with SRC turbid zones were obtained. Finally, in the inverted melittin (hybrid 5), where the basic 'head' was moved from the C-terminus to the N-terminus, there was a significant antibacterial activity against 3 test species, but still no lysis of SRC, two rather surprising results.

Cecropins A and B and our most potent hybrid, CA(1-13)M(1-13), were then assayed for activity against the blood stream form of *Plasmodium falciparium*. For comparison we also included the frog skin peptides magainin 2 and PGLa, but melittin could not be tested because it is strongly hemolytic. Fig.2 shows that cecropin A had only negligible activity while cecropin B was about as potent as magainin 2. The hybrid CA(1-13)M(1-13) was an order of magnitude more potent than cecropin B while PGLa had an intermediate activity. Only a trace of hemolytic activity was observed with the hybrid.

4. DISCUSSION

Both cecropins and melittin are composed of a hydrophilic and a hydrophobic domain that are separated by a flexible hinge region. However, the polarity of the two peptides is reversed, with the hydrophilic and basic region of melittin in the C-terminus while in all cecropins the strongly basic region is at the N-terminus. In addition, it should be noted that cecropin A contains 37 residues while melittin is composed of only 26. The size of an antibiotic peptide is relevant because a large peptide is more likely to be immunogenic than a small one. It is also more difficult and expensive to synthesize a larger molecule. The difference in size between the two parental peptides is therefore quite relevant from a therapeutic aspect.

We have earlier made truncated cecropins and analogs with single residue replacements in the hydrophilic part [11,12] but in every case obtained peptides with more or less decreased antibacterial potency. We have also made model peptides containing the first 11 residues of cecropin A linked to two theoretically constructed fragments that were either amphipathic, flexible or hydrophobic [13]. One of the model peptides showed moderate activities and lends support to the concept that active cecropins will have to be composed of a hydrophilic and a hydrophobic domain linked by a hinge region.

The hybrid (chimeric) concept was first tested on a peptide that contained residues 1-11 from cecropin A and 12-37 from cecropin D [14]. This compound was the first case where we succeeded in improving the overall activity of a cecropin-like molecule. The results in table 1 and fig.2 clearly show that the hybrid concept is fruitful and that it can give antimicrobial peptides with better properties than those of the parental compounds. In particular, the hybrid CA(1-13)M(1-13) has interesting properties. It has melittin size without melittin toxicity to red cells. In the plate assay 200 μM gave no lysis of normal sheep red cells while with parasite-infected human red cells some lysis was observed with hybrid 2 at 20 μM, the peptide concentration that gave maximum inhibition (fig.2). However, parasite growth was already 50% inhibited at 10 μM. Hybrid 2, CA(1-13)M(1-13), was more active than cecropin A agsinst all of the 3 Gram-positive organisms tested (table 1). However, the practical use of this compound cannot be judged without an extensive toxicity test.

The ability of melittin to lyse red cells has previously been shown to depend on the C-terminal hexapeptide [21], and a melittin analog with an intact basic 'head' and an idealized repeating hydrophobic 'tail' structure [22] showed a somewhat improved hemolytic activity. However, none of the melittin-like hybrid peptides described here were effective hemolytic agents. Our prediction was that the melittin-like hybrid 4 should lyse red cells but this peptide only produced small turbid zones on the SRC plate, and did not give clear evidence of lysis. Thus, since neither the two melittin-like hybrids 3 and 4, nor the inverted melittin (hybrid 5) gave convincing lysis of SRC we conclude that both the design of the basic 'head' and the location in the C-terminus seem to be critical properties for the ability to lyse red cells. The inverted melittin (hybrid 5) showed a good antibacterial activity against 3 of the 5 test bacteria. Thus, a potent antibacterial activity seesm to be a less sequence-dependent property than the lysis of red cells.

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