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Communication

Hematological and antifungal properties of temporin A and a cecropin A-temporin A hybrid★✪

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Temporin A (TA) and a cecropin A-temporin A hybrid peptide (CATA) were synthesized and assayed for their hemolytic, anticoagulant, and antifungal properties. CATA retains significant antifungal activity, is less hemolytic than TA, and inhibits blood coagulation. These results recommend further studies of the biological activities of CATA.

Temporin A (TA; Table 1) is an antibiotic peptide that was originally isolated from the skin of the European red frog, *Rana temporaria* [1]. It is among the shortest naturally occurring, gene encoded, antibiotic peptides to be isolated and exhibits antibiotic activities primarily against Gram-positive organisms, but also against some Gram-negatives, the

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Abbreviations: APTT, activated thromboplastin time; CA, cecropin A; CAMel, CA(1-7)Mel(2-9)NH_2; CATA, cecropin A(1-7)-temporin A(2-9)NH_2; Fmoc, 9-fluorenylmethoxycarbonyl; Mel, melittin; PT, prothrombin time; TA, temporin A.

fungus *Candida albicans*, and human erythrocytes, with the latter result depending upon the assay system used [1, 2]. Cecropin A (CA; Table 1) is a gene encoded antibiotic peptide that was originally isolated from the hemolymph of larva of the silkmoth *Hyalophora cecropia* [3]. A synthetic hybrid peptide, CA(1-7)Mel(2–9)NH_2 (CAMel; Table 1), containing portions of the amino-acid sequences of CA and melittin (Mel; Table 1), the latter an antimicrobial and hemolytic peptide that is the major toxic component of the venom of the honeybee (*Apis mellifera*), has been found to exhibit excellent antibiotic activities with minimal hemolytic activity [4, 5]. The sequence of the amino terminal portion of TA is somewhat similar to that of the Mel(2-9) portion of the CAMel hybrid, and it was hypothesized that a hybrid peptide CA(1-7)TA-(2-9)NH_2 (CATA; Table 1) might also have useful biological properties. The sequences of the CATA and CAMel hybrids are 69% identical, and there is an additional 23% sequence homology (I ~ L ~ V; K ~ R) between the two peptides. The CATA hybrid was synthesized and preliminary biological assay data indicate that it has different hematological and antifungal properties than TA.

MATERIALS AND METHODS

Peptide synthesis, purification, and characterization. TA and the CATA hybrid were synthesized by solid phase peptide synthesis techniques, using Fmoc chemistries as described [6]. The peptides were purified by reverse phase (RP) HPLC and characterized by amino acid analysis and electrospray ionization mass spectrometry as described [6, 7].

Hemolysis assay. Hemolytic activity was monitored in isotonic saline using human erythrocytes, essentially as described [2, 8].

Blood coagulation assay. Anticoagulant activity of the peptides dissolved in isotonic saline was tested by determination of prothrombin time (PT) and activated thromboplastin time (APTT) of a Coagulation Reference Plasma (Baxter AG), with reagents from Instrumentation Laboratory.

Antifungal assay. To examine peptide inhibition of growth of *Batrachochytrium dendrobatidis* [9], 5×10^4 mature cells or 5×10^5 zoospores in 50 μl H-broth were plated in replicates in a 96-well microtiter plate with or without addition of 50 μl serial dilutions of each peptide in broth. Positive control wells received 50 μl broth without peptide, and negative control wells (on a separate plate) received 50 μl broth containing 0.4% paraformaldehyde. Growth at 96 h (23°C) was measured as increased absorbance at 492 nm with an ELISA plate reader.

RESULTS

Synthetic peptide characterization

Synthetic TA and CATA preparations were pure as determined by analytical RP-HPLC and had the correct amino-acid compositions and masses.

Hemolysis assay

CATA was relatively nonhemolytic compared with TA, but the hemolytic activity of both increased with increasing peptide concentration (Table 2). For comparison, the hemolytic activities are expressed as the concentration of peptide yielding 50% hemolysis, and this value was > 130 μM for both peptides. Previous studies found that the 50% hemolysis value for TA was >120 μM in agarose [1], and 30 μM in isotonic saline [2].

Coagulation assay

The results of the coagulation assay are shown in Table 3. Normal values for PT and APTT times are 11–14.5 s and 25–37 s, respectively [10]. In the PT assay, these values were not exceeded at any concentration of TA,

Table 1. Amino-acid sequences of peptides

Peptide (abbreviation)	Amino-acid sequence[a]
Temporin A (TA)	FLPLIGRVLSGIL-NH_2
Cecropin A (CA)	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH_2
Melittin (Mel)	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2
CA(1-7)Mel(2-9)NH_2 (CAMel)	KWKLFKKIGAVLKVL-NH_2
CA(1-7)TA(2-9)NH_2 (CATA)	KWKLFKKLPLIGRVL-NH_2

[a]-NH_2 indicates amide (i.e. -$CONH_2$).

Table 2. Percent hemolysis of human erythrocytes by TA and CATA

Peptide conc. (μM)[a]	14	29	43	58	72	86	101	115	130
TA	0	1	4	6	14	20	28	33	44
CATA	0	1	1	1	1	1	2	3	3

[a]100% hemolysis occurred at 225 μM TA. CATA was not tested above 130 μM.

Table 3. Prothrombin (PT) and activated partial thromboplastin (APTT)

Time with TA or CATA				
Peptide conc. (μM)	PT (s)		APTT (s)	
	TA	CATA	TA	CATA
Control[a]	12.6	12.4	36.1*	36.1*
26	12.9	–	36.6	–
40	–	14.1*	–	39.6*
52	12.9	–	37.9*	–
80	–	15.4*	–	51.1*
130	13.1	–	38.0*	–
261	13.2	–	39.2*	–
400	–	19.5*	–	81.5*

[a]Control is saline added to the Reference Plasma. Values out of range are marked with an asterisk. Control for APTT is out of the normal range because the substrate normal plasma was diluted with saline. This effect was not seen in the control for the PT test.

but were exceeded by a concentration of 40 μM CATA. In the APTT assay, the normal values were exceeded by 52 μM TA and 40 μM CATA. In comparison, the range of concentrations at which TA is antibacterial for Gram-positive and some Gram-negative organisms and the fungus *Candida albicans*, is generally less than 12 μM [1, 6].

Antifungal assay

The results of assays with the fungus *Batrachochytrium dendrobatidis* are shown in Table 4. TA was more active than CATA against both the zoospore form of the fungus (lacking cell walls) and the mature cell form, but CATA retained significant inhibitory activity. In comparison, the lethal concentration of TA

Table 4. Results of assay with the fungus *Batrachochytrium dendrobatidis*

	50% inhibition conc. (μM) with:	
Peptide	Zoospores	Mature cells
TA	23	48
CATA	> 47	> 47

against the fungus *Candida albicans* was 3.4–4.0 μM [1].

DISCUSSION

During the past two decades, a new class of antibiotic peptides has been discovered, the gene encoded, antibiotic peptides obtained from animal, plant and bacterial sources [11]. A great deal of attention has been focused on these peptides due to the development of a public health crisis as a consequence of the appearance of drug resistant microorganisms, and the hope is that these new antibiotic peptides will provide a partial solution to this crisis. Their small sizes and relatively simple structures make them ideal candidates for modification by solid phase peptide synthesis technologies. Many synthetic analogs of the naturally occurring structures have been developed, including hybrids containing portions of the sequences of two or more antibiotic peptides [12]. Several have been found to have improved antibiotic properties with respect to the parent peptides, and some of the new synthetic antibiotic peptides are either in, or have successfully completed, clinical trials. Previous successes in the development of hybrid peptides from cecropin A and melittin with improved antimicrobial activities in relation to the parent peptides but with no hemolytic activity indicated that it might be possible to do the same starting with cecropin A and TA. The preliminary data reported here indicate that: 1) both TA and CATA are not hemolytic at concentrations at which TA yields antimicrobial effects (i.e. generally less than 12 μM [1, 6]); 2) TA does not affect coagulation times at these concentrations; 3) CATA inhibits coagulation at all concentrations tested; and 4) CATA has a somewhat weaker but still significant activity against the chytrid fungus *Batrachochytrium dendrobatidis* (which has been linked to recent declines in amphibian populations [13]) and *Candida albicans* [1] in comparison with TA. These results support the concept that TA may be a potentially useful antibiotic, whereas CATA requires further study. Additional antifungal and antibacterial studies are under way.

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