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# Fungicidal Activity of Cecropin A

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**Cecropin A (CA) fungicidal properties were explored. Nongerminated and germinated *Aspergillus* spp. and *Fusarium* spp. conidia were treated with CA. CA achieved complete lethality at ≤25 μM (99 μg/ml) for germinating, but not nongerminating, conidia of *Aspergillus* spp. CA achieved total lethality for nongerminated and germinating conidia of *Fusarium* spp at 1.5 μM (6 μg/ml). MIC and minimal lethal concentration assays in buffered RPMI medium gave similar results.**

*Aspergillus* spp. cause morbidity and mortality in immunocompromised hosts (6, 9, 15, 18, 21, 23, 27, 28, 34). *Fusarium* spp. are emerging pathogens resistant to amphotericin B in immunocompromised patients (1, 2, 24). This genus contains important toxigenic species causing several human and animal diseases (2, 16, 19, 29, 36).

The identification of naturally occurring fungicidal compounds without toxicity for mammalian cells would be beneficial. The cecropin family of lytic peptides may prove useful. Cecropins form large pores in bacterial cell membranes causing lysis but lack lethality for mammalian cells (4, 5, 14, 17, 30, 33). Cecropin A (CA), consisting of 37 amino acids, complexes with lipopolysaccharide (3, 8). Little is known, however, about the antifungal activity of CA. We, therefore, studied CA fungicidal activity and binding sites for five *Aspergillus* and *Fusarium* isolates.

**Antifungal activity against nongerminated and germinating conidia.** *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Fusarium moniliforme*, and *Fusarium oxysporum* were grown on potato dextrose agar (PDA; Difco, Detroit, Mich.) slants (7 days, 30°C). Conidia were harvested in 1% potato dextrose broth (PDB; Difco). Conidial suspensions ($10^4$/ml) were incubated for 8 h (30°C) to obtain germinating conidia (7). Nongerminated conidia ($10^4$ conidia/ml) were used immediately. Separate assays determined CA (Sigma Chemical Co., St. Louis, Mo.) fungicidal activity against nongerminated and germinating conidia. CA stock solutions (1 μg/μl) were freshly prepared with 1% PDB. Control samples consisted of 45 μl of conidia plus 405 μl of 1% PDB. Test samples composed of conidia (45 μl), the appropriate volume of stock CA, and 1% PDB (total volume, 450 μl) were mixed and incubated (30 min, 30°C). Sample aliquots (50 μl) were spread on each of 8 PDA plates and incubated (30°C, 24 h), and colonies were enumerated.

**MICs by microwell assays.** Fungal inhibition was studied (10, 11) by a standardized microdilution assay with MOPS (morpholinepropanesulfonic acid)-buffered (pH 7.0) RPMI medium plus glutamine (BioWhittaker, Inc., Walkersville, Md.). In addition, separate MIC assays in 1% PDB were performed to study the effect of PDB. Duplicate wells contained 20 μl of sample having $10^4$ conidia/ml with suitable amounts of stock CA and 1% PDB (final volume, 200 μl). Plates were shaken gently (5 min) and incubated (30°C, 96 h). MIC was defined as the lowest concentration having no visible growth in both wells (22). The minimal lethal concentration (MLC) was determined as described previously (35).

**Extraction and identification of conidial wall compounds.** All fungi were grown on PDA in Roux flasks (7 days at 30°C). Conidia were harvested with sterile 0.05% Triton X-100, passed through four layers of double cheesecloth, washed three times in sterile water, and freeze-dried. Germinated conidia were obtained by incubating (8 h, 30°C) freeze-dried conidia (0.3 g) in 20 ml of 1% PDB with occasional agitation; conidia were washed three times, freeze-dried, and stored at 0°C.

Nongerminated and germinating conidia (0.3 g) were extracted once (5 min) with 15 ml of high-pressure liquid chromatography (HPLC)-grade (J. T. Baker Chemical Co., Phillipsburg, N.J.) hexane or chloroform-methanol (2:1 [vol/vol]). After centrifugation, supernatants were passed through sintered glass filters and reduced to 100 μl with $N_2$. Samples (1 μl) were analyzed by gas chromatography-mass spectrometry in the electron ionization mode.

**Sedimentation analysis of ergosterol and cholesterol binding to CA.** Free ergosterol or cholesterol was mixed with CA (0.17 and 2.0 μM final concentrations, respectively) in phosphate-buffered saline with 0.05% peroxide-free Tween 20. Controls contained CA (2.0 μM). Triplicate samples were incubated (2 h, 37°C) and centrifuged (7,000 × *g*, 15 min, 4°C). Supernatants were analyzed in triplicate for unbound CA (32).

**Fluorescent microscopy.** Conidia also were studied (13) by using CA tagged with fluorescein isothiocyanate (CA-FL; Sigma). Nongerminating conidia and hyphae of *F. moniliforme* and germinating conidia of *A. flavus* were observed for complexing with CA-FL. Conidial controls were observed in parallel.

**CA degradation.** Suspensions (250 μl) of washed and unwashed nongerminated or germinated conidia of *A. flavus* ($10^6$/ml in 1% PDB) were added to CA (100 μg), and the mixtures were mixed and incubated (30°C, 30 min). Controls (100 μg of CA in 250 μl of 1% PDB), nongerminated conidia ($10^6$/ml in 1% PDB), and germinating conidia ($10^6$/ml in 1% PDB) were incubated similarly. Slant culture supernatants of *A. flavus* and unwashed nongerminated and germinating conidia of *F. moniliforme* were also incubated with CA. To timed samples was added 10% trifluoroacetic acid (1:10 [vol/vol]). CA and its breakdown products were analyzed by HPLC.

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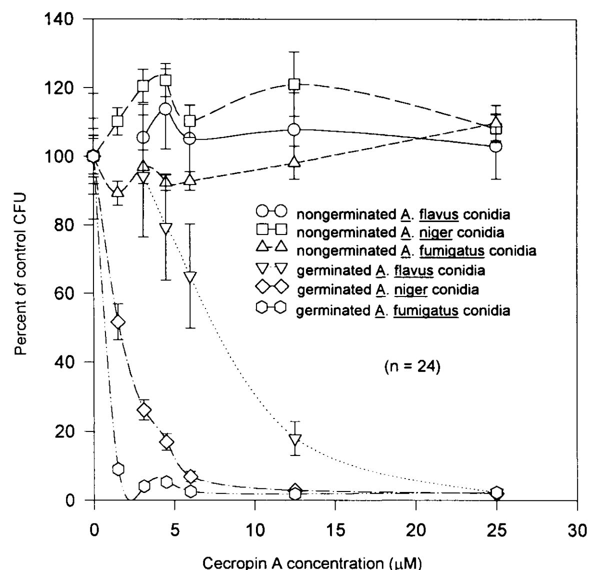


FIG. 1. CA lethality for nongerminated and germinating *Aspergillus* species conidia. Values are means ± standard errors.

**Statistical analysis.** Viable count bioassays were performed on three occasions. Analysis of variance and the least significant difference test were performed on the data. Data were pooled by sample type ($n = 24$). A 95% level of significance was employed.

**Determination of antifungal activity by viable count bioassay.** CA had fungicidal activity against germinating *Aspergillus* species conidia (Fig. 1). The susceptibility of *Aspergillus* spp. to CA was in the following order (least susceptible to most susceptible): *A. flavus*, *A. niger*, *A. fumigatus*. CA was not lethal to nongerminated conidia of *Aspergillus*. CA was lethal at concentrations as low as 0.5 μM for both nongerminated and germinating conidia of both *Fusarium* species (Fig. 2), with *F. moniliforme* slightly more susceptible than *F. oxysporum.*

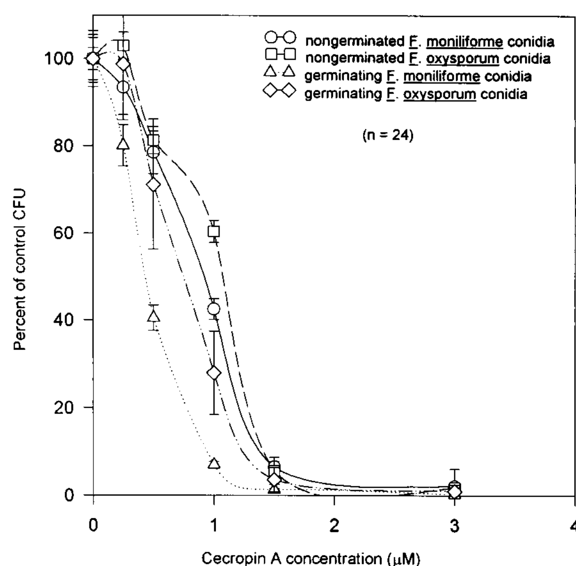


FIG. 2. CA lethality for nongerminating and germinating *Fusarium* species conidia. Values are means ± standard errors.

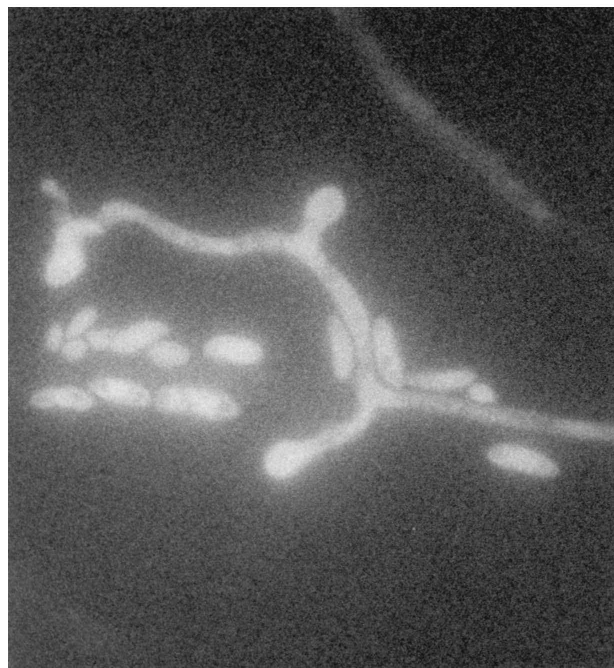

FIG. 3. CA-FL binding to *F. moniliforme* conidia and hyphae. Controls without peptides showed no fluorescence. Magnification, ×1,000.

**MIC and MLCs.** MICs and MLCs in buffered RPMI broth for both *Fusarium* spp. were ≤3.0 μM; the corresponding MICs and MLCs for *Aspergillus* spp. were as follows: *A. flavus*, ≤25.0 μM; *A. fumigatus*, 12.5 μM; and *A. niger*, 12.5 μM. The MICs for nongerminated and germinating conidia of *A. fumigatus* in 1% PDB were 12.0 and 6.25 μM, respectively, and those for *A. niger* were 12.5 and 25.0 μM, respectively. In this menstruum *A. flavus* was not affected by the tested concentrations. The MICs in 1% PDB for nongerminated and germinating conidia of *F. moniliforme* and *F. oxysporum* were 1.5 μM.

**Hexane and chloroform-methanol extracts of fungal conidia.** Nongerminated and germinated conidia of *Aspergillus* spp. and *F. oxysporum* contained ergosterol. Cholesterol was observed in nongerminated conidia of *F. moniliforme*. Germinated conidia of *F. moniliforme* contained only ergosterol.

**Ergosterol and cholesterol binding to CA.** Soluble CA in the CA-ergosterol and CA-cholesterol mixtures was reduced by 43.4 and 41.8%, respectively, as compared to the level in the CA control. The results indicated that binding occurred between CA and these sterols.

**Fluorescent microscopy.** Nongerminated *F. moniliforme* conidia and hyphae were observed to brightly fluoresce when treated with CA-FL (Fig. 3). Nongerminated *A. flavus* conidia treated with CA-FL did not fluoresce. Germinating conidia of *A. flavus* incubated with CA-FL fluoresced brightly. Controls did not fluoresce.

**CA degradation.** Nongerminated conidial supernatant and unwashed nongerminated conidia of *A. flavus* degraded CA. Unwashed germinating conidia of *A. flavus* mixed with CA only weakly degraded CA. Washed nongerminated or germinating conidia of *A. flavus* did not degrade CA. Neither nongerminated nor germinating conidia suspensions of *F. moniliforme* degraded CA.

**Discussion.** CA was lethal against germinating *Aspergillus* spp. and nongerminating and germinating *Fusarium* spp. at concentrations as low as 0.5 μM (2 μg/ml) within 30 min.

Lethality was dependent on genus, species, and conidial stage. CA-FL bound to germinating but not to nongerminating *A. flavus* conidia, suggesting that the conidial wall was changing during hyphal formation, thus offering binding sites to CA unavailable on nongerminating conidia. Ergosterol, present in conidia of *A. flavus*, complexed with CA. By comparison, CA complexed with ergosterol in both conidial and hyphal elements of *Fusarium* spp., which is consistent with the antifungal activity of CA against nongerminated and germinating fusarial conidia.

Freshly harvested conidial suspensions and culture supernatant samples of *A. flavus* degraded CA. Unwashed, germinating conidial samples of *A. flavus* only weakly degraded CA, suggesting that protease activity was lost during incubation. Washed nongerminated and germinating conidia in *A. flavus* samples had no proteolytic activity, indicating the presence of an extracellular protease. *F. moniliforme* did not degrade CA.

The fungicidal activity of CA against filamentous fungi was validated in two systems. Unlike antifungal azoles, which are fungistatic, CA is fungicidal under standard in vitro conditions. CA fungicidal activity is more analogous to that of amphotericin B against *Aspergillus* spp. CA was effective against nongerminated and germinated conidia of the *Fusarium* spp. The identification of fungicidal agents against *Fusarium* spp. is especially important, as there are no commercially available agents other than amphotericin B with in vitro antifungal activity against *Fusarium* spp. (26). In addition to its potential as a systemic agent, CA could be useful as a topical agent for treatment of *Aspergillus* and *Fusarium* keratitis (31). Since its fungicidal activity is rapid, CA may prevent irreparable corneal tissue damage. Research has demonstrated that cecropins can be safely administered to animals to combat cancer and bacterial infection (12, 20, 25).

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