Fungal Lethality, Binding, and Cytotoxicity of Syringomycin-E

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Syringomycin-E (SE) was significantly lethal to Aspergillus and Fusarium species at between 1.9 and 7.8 µg/ml. SE complexed with the following fungal wall constituents (in order of binding): β-1,3-glucan > chitin > mannan > ergosterol = cholesterol. Cytotoxicity in HeLa cells was proportional to the SE concentration, while the amount required for cytotoxicity was 3 to 20 times that needed to kill 95% of the fungi tested.

Fungi are important medical pathogens that are increasingly resistant to current control strategies. This situation has prompted increased efforts to find novel antimicrobial agents. Peptides from widely divergent species have been studied for their antimicrobial properties. For example, cecropins, present in insects and pigs, have potent antimicrobial activity (7, 8, 11–13). Syringomycins, which are produced by Pseudomonas syringae, are a new class of potent antimicrobial peptides. They are small, cyclic lipodepsinonapeptides (approximately 1,200 kDa), of which syringomycin-E (SE) is the major form (17, 21). SE (molecular weight, 1,240) and related P. syringae cyclic lipodepsinonapeptides have been considered putative virulence factors against plants. However, some strains produce these peptides and are saprophytic, indicating that SE is not the primary cause of plant disease (1). SE was evaluated in vitro with standard broth microdilution assays and found to inhibit the medically important yeasts Aspergillus fumigatus, Mucor sp., and Trichophyton sp. (18). SE action in yeast is influenced by sterols and may involve formation of voltage-sensitive ion channels of weak anion selectivity (10, 20).

Aspergillosis and fusariosis are life-threatening mycoses in immunocompromised hosts. The most prevalent species causing aspergillosis are A. flavus, A. fumigatus, and A. niger (9, 15, 23). Fusarium species are emerging pathogens that are resistant to amphotericin B in immunocompromised patients (2, 3, 16). Little is known about the activity of SE against these species of medically important filamentous fungi, nor is the physiological interaction between SE and such fungi understood. We therefore studied the potential lethality of SE for A. flavus, A. niger, A. fumigatus, Fusarium moniliforme, and Fusarium oxysporum. We also explored the physicochemical interactions between SE and fungal wall components, as well as the cytotoxicity of SE for HeLa cells.

Bioassays to determine SE lethality against fungi. Fungi were grown on potato dextrose agar (Difco, Detroit, Mich.) slants for 7 days (30°C). Conidia were harvested in 1% potato dextrose broth (PDB), pH 5.0 (Difco). Conidial suspensions (10^4 conidia/ml) were incubated for 8 h (30°C) to obtain germinating conidia (6). Viable conidial numbers were consistent with the high percentage observed earlier (7). Nongerminating conidia (10^6 conidia/ml) were used immediately. Control samples consisted of 45 µl of conidia plus 405 µl of 1% PDB. Test samples were composed of 45 µl of conidia and the appropriate volumes of stock SE and 1% PDB to give the desired peptide concentration, ranging from 0.05 to 6.4 µM, in a volume of 450 µl. SE was produced as previously described (4). SE was purified to homogeneity based on high-performance liquid chromatography profiles and fast atom bombardment mass spectrometry analysis. Samples were mixed, incubated, and plated out as previously described (7, 8).

Determination of peptide binding to fungal conidial walls and mammalian components. We determined the ability of SE to complex with fungal conidial constituents, which included ergosterol and cholesterol (Sigma Chemical Co., St. Louis, Mo.), mannan (Sigma Chemical Co.), chitin (a gift from B. Triplett, Southern Regional Research Center, U.S. Department of Agriculture), and β-1,3-glucan (Wako Pure Chemical Co., Osaka, Japan). Polymers and sterols were finely dispersed with ground-glass hand homogenizers. The final SE concentration in the SE control and all test mixtures was 23.8 µg/ml. Ergosterol and cholesterol (final concentrations, 1.8 ± 10^{-3} M) were separately mixed with SE, as were β-1,3-glucan, chitin, and mannan (final concentrations, 200 µg/ml). Binding assays were performed three separate times, each time in triplicate. After gentle mixing, test samples and SE controls were incubated for 30 min at 37°C. Samples were centrifuged at 8,000 × g (10 min) in a swinging bucket. Degrees of dispersion before and after centrifugation were not determined. After centrifugation, lipids formed floating fat pads and polymers formed pellets. This force easily removed insoluble sterols and other fungal wall constituents, producing a clear supernatant for peptide analysis. Unbound peptide concentrations in solution were determined by the Waddell method (22), which estimates contents of peptide bonds independent of aromatic residues. For our purposes, this method was preferred to other estimations for protein content, according to criteria published earlier (24).

Cytotoxicity assay. Human epithelioid cervical carcinoma cells (HeLa cells; ATCC 2.2CCL) were grown in minimal essential medium (Mediatech) supplemented with fetal bovine sera and penicillin. The cells were rinsed with phosphate-buffered saline and harvested with trypsin. Cell counts were determined manually with trypan blue, and cells were diluted to a final concentration of 2.5 × 10^5 CFU/ml. Aliquots of 100 µl
per well were dispensed in a 96-well plate. Cells were incubated overnight (18 to 24 h) at 37°C in a humidified 5% CO₂ environment. Neutral red dye solution was then added to each well, and the mixtures were reincubated for 3 h. Twofold serial dilutions of SE were prepared to final concentrations of 30, 15, 7.5, and 3.5 µM. Negative controls (without peptide) were included. Cells were exposed to the peptide for 1 h. These experiments consisted of three wells per concentration in each of three experiments. Supernatants from each well were transferred into another 96-well plate. The remaining cells were lysed with a 2% Triton solution. Glacial acetic acid was then added to supernatants and lysates and read at A540.

**Statistical analysis.** Viable count bioassays with SE were performed on three separate occasions per fungus. 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 used. Gompertz plot analysis was employed to determine the significance of the fungicidal properties of SE for the tested fungi relative to those of cecropins A and B and dermaseptin, as published previously by our laboratory (7, 8).

**SE lethality for fungi.** SE proved to be significantly lethal for the germinated conidia of the test aspergilli (Fig. 1A). Nearly all of the germinated *A. niger* and *A. fumigatus* conidia were killed at an SE concentration of 1.9 µg/ml. The number of CFU was reduced by 95% at 7.8 µg of SE per ml for the germinated conidia of *A. flavus*. SE did not affect the nongerminated conidial viability of the tested *Aspergillus* species. SE was rapidly and significantly effective against *Fusarium* species (Fig. 1B). At 1.9 µg/ml, CFU viabilities of the nongerminated and germinated conidia of *F. moniliforme* and *F. oxysporum* were reduced by 95 and 99%, respectively.

**Binding of SE to cell wall and membrane constituents.** Less than 1% of the ergosterol or cholesterol bound with SE. The fungal wall constituents chitin, mannan, and β-1,3-glucan bound 3.3, 4.2, and 10.7%, respectively, of the SE. Variabilities of binding were less than 0.1%.

**Cytotoxicity assay.** Percent cytotoxicity of SE against HeLa cells was directly related to the concentration of the peptide (Fig. 2). The threshold for cytotoxicity appeared to be ≥12 µg/ml. At concentrations of less than 12 µg/ml, cell viability appeared to be slightly enhanced, while at concentrations of ≥12 µg/ml, cytotoxicity increased in direct relation to the increased levels of SE. These findings indicate that SE has minimum to no cytotoxicity at concentrations which have ≥95% lethality against germinating conidia of *A. flavus*, *A. fumigatus*, and *A. niger*, as well as nongerminated and germinating conidia of *F. moniliforme* and *F. oxysporum*.

Sorensen et al. (18) showed that SE causes lysis of sheep erythrocytes. However, comparison of our bioassay data with their erythrocyte cytotoxicity data shows that SE reduces fungal viability by 90 to 98% at concentrations producing less than 20% release of hemoglobin from erythrocytes. Lethality for erythrocytes does not necessarily mean that a compound cannot be used as an antibiotic. Amphotericin, by comparison, also causes hemolysis (5, 14) but does so at concentrations above those used therapeutically. Structural modification of SE may improve the therapeutic index of this potent peptide. It should be noted that the HeLa cytotoxicity assay cannot predict toxicity of SE in humans and animals.

We determined the lethality of SE for fungi relative to the lytic peptides cecropin A, cecropin B, and dermaseptin, which we studied previously (7, 8). Statistical analysis with Gompertz
plots showed SE to be significantly more fungicidal than these peptides against the tested germinated conidia of *Aspergillus* spp. and to be the second most lethal against the tested Fusarium spp. and to be the second most lethal against the tested *Fusarium* conidia of *Aspergillus*. Formulations (19). This finding and the cytotoxicity and bioassay results reported here suggest that SE has potential for development as a novel agent against invasive fungal infections.

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