0066-4804/99/$04.00+0
Copyright © 1999, American Society for Microbiology. All Rights Reserved.

Amphotericin B- and Fluconazole-Resistant Candida spp.,
Aspergillus fumigatus, and Other Newly Emerging Pathogenic Fungi Are Susceptible to Basic Antifungal Peptides

EVA J. HELMERHORST,1,* INGRID M. REIJNDERS,1 WIM VAN 'T HOF,1 INA SIMOONS-SMIT,2
ENNO C. I. VEERMAN,1 AND ARIE V. NIEUW AMERONGEN1

Academic Centre for Dentistry (ACTA), Department of Oral Biochemistry, Vrije Universiteit,1 and Department of
Clinical Microbiology, Academic Hospital Vrije Universiteit (AZVU),2 Amsterdam, The Netherlands

Received 20 August 1998/Returned for modification 3 November 1998/Accepted 17 December 1998

The present study shows that a number of basic antifungal peptides, including human salivary histatin 5, a
designed histatin analog designated dhvar4, and a peptide from frog skin, PGLa, are active against amphotericin B-resistant Candida albicans, Candida krusei, and Aspergillus fumigatus strains and against a fluconazole-resistant Candida glabrata isolate.

The AIDS epidemic, improved life-sustaining therapy, and aggressive anticancer therapy have contributed to the rise in the number of severely immunocompromised patients. This has led to an increase in mucosal and systemic fungal infections, and the concomitant increased usage of antifungal agents for prophylaxis is most likely the main cause of the development of antifungal drug resistance (21).

As most of the currently available drugs are directed against the ergosterol moiety in the fungal membrane (polyene antifungals) or against enzymes involved in the biosynthesis of ergosterol (azole antifungicals) (19) there is a threat of cross-resistance (7, 21) and a clear demand for a new class of antifungals that is not related to the ergosterol moiety in the fungal membrane. Peptide antibiotics, which are believed to interact with the microbial membrane leading to the disruption of cellular integrity and cell death, may be a promising new class of antifungal agents (3).

Histatins are natural human salivary peptides with strong fungicidal activities in vitro and have proven to be valuable design templates for the development of analogs with improved antifungal activities (5). The aim of the present study was to test the antifungal activities of a number of natural and designed basic antifungal peptides against yeasts and fungi that are isolated from clinical specimens with increasing frequency, such as Candida glabrata, Cryptococcus neoformans, and Aspergillus fumigatus (4, 8), and against fluconazole- or amphotericin B-resistant mutants. Fungicidal activities were compared to the activity of the amphipathic macrolide anticyotic drug amphoterin B, which is considered the “gold standard” in the treatment of disseminated candidosis (21).

Human salivary histatin 5 (sequence, DSHAKRHIGYKR KFHEKHSHRHYG) is an amphipathic analog of the C-14 terminus designated dhvar4 (KRLFKKLLFSLRKY), and a negative control peptide, dcysS, derived from cystatin S (SSSKEE NRIIPGGI) were chemically synthesized as described previously (5, 20). An antifungal peptide of amphibian origin, PGLa (15), was chemically synthesized and kindly provided by H. V. Westerhoff (Department of Microbial Physiology, Vrije Universiteit, Amsterdam, The Netherlands). The peptides were dissolved to a concentration of 2 mg/ml in 10 mM potassium phosphate buffer (PPB), pH 7.0, and stored at −20°C.

The fungicidal activities of these peptides were tested by incubating an inoculum of 2 × 106 yeast blastoconidia with a dilution series of peptide in 1 mM PPB, pH 7.0. After 1.5 h of incubation, 50-μl samples were diluted in 9 ml of phosphate-buffered saline, and viability was determined by plating 25 μl on Sabouraud dextrose agar (Oxoid, Hants, United Kingdom), as previously described (5). From the obtained killing curves, the concentrations of peptide giving 50% reduction in viable counts (IC50s) were determined (Table 1). Candida pseudotropicalis 311 (RIVM 4135), Candida albicans 315 (ATCC 10231), Candida krusei 355 (ATCC 6258), and Candida parapsilosis 356 (ATCC 90018) were all highly susceptible to histatin 5, dhvar4, and PGLa, with IC50s ranging from 0.3 to 2.4 μM. Also C. neoformans 316 (RIVM 39231), the causative agent of severe cryptococcosis in AIDS patients, was very susceptible to these peptides. For histatin 5, similar results have been reported for three clinically isolated C. neoformans strains (17). C. glabrata 359 was markedly less susceptible to histatin 5 and PGLa than the other strains tested; IC50s of 29 and 8.5 μM, respectively, were observed. The susceptibilities to amphotericin B differed among the strains tested. Most strikingly, C. kru sei 355 was resistant (IC50s > 70 μM). This and previous findings (10) might point to an intrinsically reduced susceptibility of C. krusei to amphotericin B.

Fungi are isolated from clinical specimens with increasing frequency, designed basic antifungal peptides against yeasts and fungi that proved antifungal activities (5). The aim of the present study designed templates for the development of analogs with improved antifungal activities in vitro and have proven to be valuable leading to the disruption of cellular integrity and cell death, which are believed to interact with the microbial membrane resulting in the number of severely immunocompromised patients. This has led to an increase in mucosal and systemic fungal infections, and the concomitant increased usage of antifungal agents for prophylaxis is most likely the main cause of the development of antifungal drug resistance (21).

Histatins are natural human salivary peptides with strong fungicidal activities in vitro and have proven to be valuable design templates for the development of analogs with improved antifungal activities (5). The aim of the present study was to test the antifungal activities of a number of natural and designed basic antifungal peptides against yeasts and fungi that are isolated from clinical specimens with increasing frequency, such as Candida glabrata, Cryptococcus neoformans, and Aspergillus fumigatus (4, 8), and against fluconazole- or amphotericin B-resistant mutants. Fungicidal activities were compared to the activity of the amphipathic macrolide anticyotic drug amphoterin B, which is considered the “gold standard” in the treatment of disseminated candidosis (21).

Human salivary histatin 5 (sequence, DSHAKRHIGYKR KFHEKHSHRHYG), an amphipathic analog of the C-14 terminus designated dhvar4 (KRLFKKLLFSLRKY), and a negative control peptide, dcysS, derived from cystatin S (SSSKEE NRIIPGGI) were chemically synthesized as described previously (5, 20). An antifungal peptide of amphibian origin, PGLa (15), was chemically synthesized and kindly provided by H. V. Westerhoff (Department of Microbial Physiology, Vrije Universiteit, Amsterdam, The Netherlands). The peptides were dissolved to a concentration of 2 mg/ml in 10 mM potassium phosphate buffer (PPB), pH 7.0, and stored at −20°C.

The fungicidal activities of these peptides were tested by incubating an inoculum of 2 × 106 yeast blastoconidia with a dilution series of peptide in 1 mM PPB, pH 7.0. After 1.5 h of incubation, 50-μl samples were diluted in 9 ml of phosphate-buffered saline, and viability was determined by plating 25 μl on Sabouraud dextrose agar (Oxoid, Hants, United Kingdom), as previously described (5). From the obtained killing curves, the concentrations of peptide giving 50% reduction in viable counts (IC50s) were determined (Table 1). Candida pseudotropicalis 311 (RIVM 4135), Candida albicans 315 (ATCC 10231), Candida krusei 355 (ATCC 6258), and Candida parapsilosis 356 (ATCC 90018) were all highly susceptible to histatin 5, dhvar4, and PGLa, with IC50s ranging from 0.3 to 2.4 μM. Also C. neoformans 316 (RIVM 39231), the causative agent of severe cryptococcosis in AIDS patients, was very susceptible to these peptides. For histatin 5, similar results have been reported for three clinically isolated C. neoformans strains (17). C. glabrata 359 was markedly less susceptible to histatin 5 and PGLa than the other strains tested; IC50s of 29 and 8.5 μM, respectively, were observed. The susceptibilities to amphotericin B differed among the strains tested. Most strikingly, C. kru sei 355 was resistant (IC50s > 70 μM). This and previous findings (10) might point to an intrinsically reduced susceptibility of C. krusei to amphotericin B.

Aspergillus fumigatus is a mold which causes life-threatening infections in granulocytopenic patients. A clinical isolate from sputum, A. fumigatus X 807219, was grown on Sabouraud dextrose agar for 4 days at 30°C. To separate the conidia from the conidiophores, the agar was covered with 20 ml of sterile 1 mM PPB, pH 7.0, followed by filtration of the mycelial suspension over a sterile cotton plug fitted as described previously (11). The filtrate, containing 2 × 105 colony-forming conidia/ml, was used in a killing assay. Conidia of A. fumigatus were resistant to the killing activity of amphotericin B (Fig. 1). In contrast, the IC50s of histatin 5, dhvar4, and PGLa for A. fumigatus conidia could be determined (IC50s, 18, 18, and 9 μM, respectively).

Beside the selection of fungi with lower intrinsic susceptibility during therapy, resistant mutants may also arise as a result of selection during treatment. A fluconazole-resistant C. glabrata strain (B 57149), isolated from a patient receiving a combination therapy of fluconazole and ciprofloxacin (12), was kindly provided by M. Marichal (Janssen Research Founda-
tion, Beerse, Belgium) and included in our study. The molecular characterization of this strain has revealed that the acquired resistance was due to an increased copy number of the CYP51 (ERG11) gene encoding 14α-lanosterol demethylase, which is involved in the ergosterol biosynthesis route (12). In a killing assay it was found that this strain was very susceptible to basic peptides and even more susceptible than fluconazole-sensitive *C. glabrata* 359 (IC₅₀s, 1.5 and 29 mM, respectively; Table 1). Interestingly, for recombinant histatin 5, a similar result has been reported: an azole-resistant *C. glabrata* strain overexpressing 14α-lanosterol demethylase was more susceptible to histatin 5 than an azole-sensitive *C. glabrata* strain (18).

With increasing frequency case studies report on azole-resistant *Candida* spp. that are cross resistant to amphotericin B (1, 2, 9, 13, 16). Kelly et al. (6, 7) recently described two clinical fluconazole-resistant *C. albicans* isolates defective in sterol Δ⁵,⁶-desaturation. As a result, these mutants synthesized 14α-methyllecoesterol instead of ergosterol and in consequence were cross resistant to amphotericin B. The emergence of this kind of cross-resistant strain is a threatening development and underlines the requirement for new antifungals with a different target. We tested whether a mutant of *C. albicans* (ATCC 32354) lacking ergosterol was susceptible to basic peptides. This strain was a generous gift from J. Brajtburg (Washington University School of Medicine, St. Louis, Mo.) and was raised by random mutagenesis and selected by subculturing on amphotericin B-containing plates (14). In Fig. 2A and B it is shown that the ergosterol-deficient mutant, in contrast to the wild-type strain, was resistant to amphotericin B (IC₅₀ > 70 μM) but susceptible to histatin 5, dhvar4, and PGLa (IC₅₀s, 2.5, 0.9, and 3.5 μM, respectively). These results indicate that the antifungal target of the antibiotic peptides tested is not the ergosterol moiety in the yeast membrane.

In conclusion, this study demonstrates that newly emerging strains that are untreatable with currently available antifungotics are susceptible to natural and designed basic antimicrobial peptides. These peptides could possibly provide an attractive alternative for or a supplement to classic antifungotics in the treatment of persistent fungal infections.

![FIG. 1. Killing activities of histatin 5, dhvar4, PGLa, and amphotericin B against *A. fumigatus* conidia. Error bars indicate standard deviations.](image1)

![FIG. 2. Killing activities of histatin 5, dhvar4, PGLa, and amphotericin B against *C. albicans* B311 (A) and an ergosterol-deficient laboratory mutant of *C. albicans* B311 (B). Error bars indicate standard deviations.](image2)

### TABLE 1. Killing activities of basic antifungal peptides and amphotericin B against pathogenic yeasts

<table>
<thead>
<tr>
<th>Strain</th>
<th>Histatin 5</th>
<th>dhvar4</th>
<th>PGLa</th>
<th>dcysS</th>
<th>Amphotericin B</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. pseudotropicalis</em> 311</td>
<td>1.0 ± 0.5</td>
<td>2.0 ± 0.0</td>
<td>1.3 ± 0.7</td>
<td>&gt;125</td>
<td>7.2 ± 1.9</td>
</tr>
<tr>
<td><em>C. albicans</em> 315</td>
<td>2.4 ± 0.4</td>
<td>0.9 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>&gt;125</td>
<td>2.2 ± 0.8</td>
</tr>
<tr>
<td><em>C. neoformans</em> 316</td>
<td>0.7 ± 0.2</td>
<td>0.8 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>&gt;125</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td><em>C. krusei</em> 355</td>
<td>1.2 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>0.8 ± 0.3</td>
<td>&gt;123</td>
<td>&gt;70</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> 356</td>
<td>2.0 ± 1.0</td>
<td>1.2 ± 0.8</td>
<td>0.8 ± 0.4</td>
<td>&gt;123</td>
<td>6.0 ± 1.0</td>
</tr>
<tr>
<td><em>C. glabrata</em> 359</td>
<td>29 ± 11</td>
<td>2.0 ± 0.1</td>
<td>8.5 ± 1.0</td>
<td>&gt;123</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td><em>C. glabrata</em> B57149</td>
<td>1.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>&gt;123</td>
<td>4.6 ± 0.1</td>
</tr>
</tbody>
</table>

* Data are means ± standard deviations from two independent experiments.

* Fluconazole-resistant clinical isolate.
This project was financially supported by the Dutch Technology Foundation (STW project VTH44.3302) and by Unilever Research, Oral Care, Bebington, United Kingdom.

The generous gifts of the mutant \textit{Candida} strains from J. Brajtburg and P. Marichal are gratefully acknowledged.

\textbf{REFERENCES}


14. Sokol-Anderson, M., J. E. Sligh, Jr., S. Elberg, J. Brajtburg, G. S. Koba-


