Histatin 5-Spermidine Conjugates Have Enhanced Fungicidal Activity and Efficacy as a Topical Therapeutic for Oral Candidiasis

Swetha Tati, Rui Li, Sumant Puri, Rohitashw Kumar, Peter Davidow, Mira Edgerton
Department of Oral Biology, University at Buffalo, The State University of New York, Buffalo, New York, USA

Oropharyngeal candidiasis (OPC) is caused by the opportunistic fungi *Candida albicans* and is prevalent in immunocompromised patients, individuals with dry mouth, or patients with prolonged antibiotic therapies that reduce oral commensal bacteria. Human salivary histatins, including histatin 5 (Hst 5), are small cationic proteins that are the major source of fungicidal activity of saliva. However, Hsts are rapidly degraded *in vivo*, limiting their usefulness as therapeutic agents despite their lack of toxicity. We constructed a conjugate peptide using spermidine (Spd) linked to the active fragment of Hst 5 (Hst 54–15), based upon our findings that *C. albicans* spermidine transporters are required for Hst 5 uptake and fungicidal activity. We found that Hst 54–15-Spd was significantly more effective in killing *C. albicans* and *Candida glabrata* than Hst 5 alone in both planktonic and biofilm growth and that Hst 54–15-Spd retained high activity in both serum and saliva. Hst 54–15-Spd was not bactericidal against streptococcal oral commensal bacteria and had no hemolytic activity. We tested the effectiveness of Hst 54–15-Spd *in vivo* by topical application to tongue surfaces of immunocompromised mice with OPC. Mice treated with Hst 54–15-Spd had significant clearance of candidal tongue lesions macroscopically, which was confirmed by a 3- to 5-log fold reduction of *C. albicans* colonies recovered from tongue tissues. Hst 54–15-Spd conjugates are a new class of peptide-based drugs with high selectivity for fungi and potential as topical therapeutic agents for oral candidiasis.

Oral candidiasis is prevalent in individuals who have compromised or suppressed Th17 immunity (1) and is a common sequela of antibiotic therapies that reduce commensal bacteria in the mouth (2). Failures in treatment of candidiasis and oral candidiasis are still encountered due to emergence of drug-resistant *Candida* species (3–5), thus emphasizing the need to develop alternate therapeutic agents.

Naturally occurring antimicrobial peptides are promising candidates for treatment of fungal infections because of their distinct mechanism of action fromazole- and polyene-based antifungal drugs (6). Several proteins with antifungal activities are produced by human salivary glands and contribute to inhibition of growth and viability of *Candida albicans* within the oral environment (7). Among these, histatins are a family of histidine-rich cationic peptides secreted by human parotid and submandibular-sublingual salivary glands (8) with selective antifungal activity and little or no bactericidal activity (9). Among at least 50 histatin peptides derived from post-translational proteolytic processing, histatin 5 (Hst 5), comprised of 24 amino acid residues, has the most potent fungicidal activity at physiological concentrations against *C. albicans* (10) and toward several medically important Candida species, such as *Candida kefyr*, *Candida krusei*, and *Candida parapsilosis*, as well as *Cryptococcus neoformans* and *Aspergillus fumigatus* (11–13). However, we found that many strains of *Candida glabrata* are significantly more resistant to Hst 5 due to their low uptake of Hst 5 (14).

Following their secretion, all Hsts undergo proteolytic degradation by native enzymes found in whole saliva as well as enzymes of bacterial origin (9), so that their functional activity is reduced and must be continuously replenished by new secretion (15). This unique mixture of enzymes present in whole saliva represents a challenge for preventing degradation of added antifungal peptides to be used therapeutically. Several approaches have been tried to synthesize Hsts that have increased fungicidal activity and resist degradation. Hst variants (Dhvar) were designed by replacement of multiple amino acids within Hst 5 (residues 11 to 24) to increase helical structure and hydrophobicity (16, 17). Several of these variants displayed increased fungicidal activity but also had broad-spectrum antifungal and hemolytic activities (16), thus diminishing their utility as antifungal agents for oral candidiasis. Alternatively, several synthetic congeners of Hst 5 were designed based upon small regions within the Hst 5 parent protein (18, 19). A 12-amino-acid subunit of full-length Hst 5, here named Hst 54–15 (AKRHHGYKRKFH), was as active as the full-length protein in *Candida* candidacidal activity (20). Hst 54–15 has some toxicity towards skin and respiratory pathogenic microorganisms, such as streptococci, staphylococci, and *Pseudomonas aeruginosa* (19, 21, 22); however, it is inactive against most oral commensal bacteria (9). This selectivity for fungi among members of the oral microbiome makes Hst 54–15 an attractive potential drug for oral candidiasis as it spares and preserves beneficial microbiota.

The fungicidal mechanism of Hst 5 in *C. albicans* is not a result of cytolytic or membrane disruption (23). Instead, we found that all cytotoxic events are initiated once Hst 5 reaches the cytosol, so that the ability of this protein to be transported intracellularly is essential for its fungicidal activity (23). We found that Hst 5 utilizes the polyamine transporters Dur3 and Dur31 for its uptake in *C. albicans* (Dur3 is the major uptake transporter, while Dur31 only functions under high concentrations of Hst 5) (24), since it is likely recognized as a polyamine-like substrate. Polyamines (pu-
tein, and its smaller size facilitated chemical synthesis. Since we
it has slightly higher candidacidal activity than the full-length pro-
peptone-dextrose (YPD) medium with uridine added when required and
for murine oral infections. Cells were maintained in Difco yeast extract-
conjugate peptide having a polyamine added to the smaller active
Dur transporters. The objective of this study was to construct a
amine to either C or N termini in order to enhance its uptake by
porters (14), underscoring the importance of polyamine trans-
sitivity to Hst 5 in proportion to expressed levels of
C. albicans
Dur
transporters along with essential polyamines, so that
Hst 5, and are essential for cell growth (25). Hst 5 is taken up by C.
albicans
Dur
transporters that all carry a net positive charge (pKa values of 9 to 10), similar to
Hst 5 resistance (26). Heterologous expression of C.
albicans
Dur
expression levels are unlikely to be reduced in a manner
Hst 5::ura3::imm434::ura3::imm434
C. glabrata
93101
Wild type
90032
Wild type
90030
Wild type
Hst 5 Conjugates Have Enhanced Fungicidal Activity

Candida strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parent</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
</thead>
</table>
| C. albicans
CAF4-2 | CAF2-1 | Δura3::imm434/ura3::imm434 | 54 |
| CA4 | | Δura3::imm434/ura3::imm434 | 54 |
| flu1ΔΔ mutant | CAF4-2 | Δura3::imm434/ura3::imm434 Δflu1::FRT | 26 |
| dur3ΔΔ mutant | CAF4-2 | Δura3::imm434/ura3::imm434 Δdur3::FRT | 24 |
| dur3Δ31ΔΔ mutant | CAF4-2 | Δura3::imm434/ura3::imm434 Δdur3::FRT/Δdur31::FRT | 24 |
| C. glabrata
93101 | | | 55 |
| 90032 | | | ATCC |
| 90030 | | | ATCC |

trescine, spermidine [Spd], and spermine) are small molecules
that all carry a net positive charge (pKa values of 9 to 10), similar to
Hst 5, and are essential for cell growth (25). Hst 5 is taken up by C.
albicans
Dur transporters along with essential polyamines, so that
DUR expression levels are unlikely to be reduced in a manner
leading to Hst 5 resistance (26). Heterologous expression of C.
albicans
DUR3 and DUR31 genes in C. glabrata increased cell sen-
sitivity to Hst 5 in proportion to expressed levels of DUR
transporters (14), underscoring the importance of polyamine trans-
porters for Hst 5 toxicity. The critical role of polyamine transporters for Hst 5 uptake and toxicity suggested that the
potency of this peptide might be increased by addition of a poly-
amine to either C or N termini in order to enhance its uptake by
Dur transporters. The objective of this study was to construct a
conjugate peptide having a polyamine added to the smaller active
fragment of Hst 5 (Hst 54–15) and to examine its fungicidal activity.
Among naturally occurring polyamines (putrescine, spermi-
dine, and spermine), spermidine (Spd) was selected for conjuga-
tion. Among naturally occurring polyamines (putrescine, spermi-
dine, and spermine), spermidine (Spd) was selected for conjuga-
tion.

MATERIALS AND METHODS

Strains and peptides. All Candida strains used in this study are listed in
Table 1. Candida albicans CAF4-2 and Candida glabrata 931010, 90032,
and 90030 were used as wild-type (WT) strains. C. albicans CA4 was used for
murine oral infections. Cells were maintained in Difco yeast extract-
peptone-dextrose (YPD) medium with uridine added when required and
stored at ~78°C. Streptococcus gordoni D1L1 was kindly provided by S.
Ruhl (University at Buffalo). Streptococcus sanguinis SK36 and Streptococ-
cus parasanguis FW213 were gifts from Hui Wu (University of Alabama).
Spermidine was obtained from Sigma. Hst 54–15 Peptides conjugated with spermidine (Spd-Hst 54–15 and Hst 54–15-Spd) were synthesized by Ge-
netmed Synthesis (San Antonio, TX) using 9-fluorenylmethoxy carbonyl
(Fmoc) chemistry and N,N-di-cyclohexylcarbodiimide coupling reagent.
Conjugates were designed with either a GGG linker region between

Candidacidal and bactericidal assays. Candidacidal assays were per-
formed using the microdilution plate method, as we have previously de-
scribed (27). Of specific note, the activity of Hsts is quenched by the salts
in RPMI 1640 medium, which is the test medium recommended to be
used in CLSI and EUCAST reference microdilution assays for antifungal
susceptibility testing. Therefore, a modified microdilution method out-
lined in reference 27 was used in this study. Briefly, single colonies of each
strain were inoculated into 10 ml of YPD medium with uridine (50 μg/ml)
and grown overnight at room temperature. Overnight-grown cells were
diluted to an A600 of 0.3 to 0.4 and were incubated at 30°C with shaking
at 250 rpm until an A600 of ~1.0 was attained. Cells were washed twice
with 10 mM pH 7.4 sodium phosphate buffer (NaPB) and grown overnight
at room temperature. Overnight-grown cells were diluted to an A600 of 0.3 to 0.4 and were incubated at 30°C with shaking until they reached an A600 of ~0.7 to 0.8. Fetal bovine serum (FBS) (Life
Technologies, Grand Island, NY) was added to sterilized YPD medium
(9:1) to a final concentration of 10% FBS. Cells were incubated in YPD
plus 10% FBS medium at 37°C with shaking for 1 h so that ~90% of cells formed
germ tubes that were not more than 2× the length of the mother
cell in order to allow colony counting of single cells. Cells were washed
twice with 10 mM pH 7.4 NaPB, and then 1 × 106 cells were mixed with
serial dilutions of different peptides (0, 30, 60, 90, and 120 μM) at 30°C for
30 min. Surviving cells were calculated as described above. For can-
didacidal assays in the presence of salvia, assays were performed as described
above, except whole saliva was added to phosphate buffer (1:1 by volume)
with or without 1× proteinase inhibitor cocktail (Complete Mini EDTA-
free; Roche, Mannheim, GmbH).

<table>
<thead>
<tr>
<th>NAME</th>
<th>Mol wt</th>
<th>Sequence</th>
<th>Net change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hst 5 Spd-Hst 54–15</td>
<td>3,035.8</td>
<td>DHSHARNQHYGKMRKFFEKHRHSGHRG</td>
<td>+7</td>
</tr>
<tr>
<td>Hst 54–15-Spd</td>
<td>1,978.3</td>
<td>Sperrimidine-succinic-GGG-AKRHHGYRKRFH</td>
<td>+8</td>
</tr>
<tr>
<td>Hst 54–15-Spd</td>
<td>1,863.2</td>
<td>AKRRHHGYRKRFH-GGSPerrimidine</td>
<td>+8</td>
</tr>
</tbody>
</table>
The antimicrobial activity of peptides against S. gordonii, S. sanguinis, and S. parasanguinis was tested by the broth microdilution method using Todd-Hewett (TH) broth (Becton, Dickinson), based on the CLSI approved standard (28). Initial inoculums of 5 × 10⁴ CFU/ml were incubated with and without Hst 5 and Hst 5₄₋₁₅-Spd in polystyrene 96-well plates (Becton, Dickinson) for 24 h at 37°C with shaking at 150 rpm. The MIC was taken as the lowest concentration of each peptide that resulted in more than 99.9% reduction of growth. Experiments were performed in triplicate.

**Hst 5₄₋₁₅-Spd competition assays.** Competition assays were performed as described previously (24). Briefly, overnight-grown wild-type cells were prepared as for candidicidal assays. Cells (1 × 10⁶) were suspended in 50 μl of NaPB, and then Hst 5₄₋₁₅-Spd (15, 30 and 60 μM) and fluorescently labeled spermidine (BODIPY-X-Spd; 100 μM) were added to the cells. Fluorescent counts were recorded at 30°C using a Bio-Tek multilabel plate reader and Gen5 software (provided by the University at Buffalo Confocal Microscopy and Flow Cytometry Core Facility). Cellular uptake of BODIPY-X-Spd resulted in a decrease in total fluorescence counts that was normalized with control wells, and uptake values were determined using a standard curve of fluorescence counts for known amounts (3.5 to 10 nmol) of BODIPY-X-Spd.

**Biofilm formation with antifungal peptides.** Overnight-grown cells (C. albicans CAF4-2 and C. glabrata 90030) were diluted to an A₆₀₀ of 0.3 to 0.4 and were incubated at 30°C with shaking at 250 rpm for 4 h. Cells were harvested, washed, and resuspended to an A₆₀₀ of ~1 in NaPB (pH 7.4). One milliliter of cells was added to each well of a 12-well tissue culture plate (Becton, Dickinson, Franklin Lakes, NJ). Plates were incubated at 37°C for 3 h, and then nonadherent cells were removed by gently washing the wells with NaPB, 1 ml of fresh yeast nitrogen base (YNB) medium was added, and the plates were incubated for 24 h at 37°C. After 24 h, all group samples were washed twice with 1 ml NaPB. For control groups, 1 ml of YNB was added to each well after washing, and then the plates were incubated at 37°C for another 36 h. For peptide-treated groups, 500 μl of antimicrobial peptide (Hst 5 or Hst 5₄₋₁₅-Spd at a final concentration of 60 μM) was added to each well, and the mixture was incubated at 37°C for 1 h, and then 500 μl of YNB medium was added to quench the activity of antifungal peptides and biofilms were incubated at 37°C for another 36 h. Initially, we performed XTT assays to assess biofilm mass, but found that this assay did not give colorimetric readings with Candida glabrata.

**Antifungal activity assay of Hst 5₄₋₁₅-Spd.** BODIPY-X-Spd (3.5 to 10 nmol) was added to the respective groups, and the plates were incubated for 24 h at 37°C. After 24 h, all group samples were washed twice with 1 ml NaPB. For control groups, 1 ml of YNB was added to each well after washing, and then the plates were incubated at 37°C for another 36 h. For peptide-treated groups, 500 μl of antimicrobial peptide (Hst 5 or Hst 5₄₋₁₅-Spd at a final concentration of 60 μM) was added to each well, and the mixture was incubated at 37°C for 1 h, and then 500 μl of YNB medium was added to quench the activity of antifungal peptides and biofilms were incubated at 37°C for another 36 h. We performed XTT assays to assess biofilm mass, but found that this assay did not give colorimetric readings with Candida glabrata.

**Hemolysis assays.** Serial dilutions of 100 μl Hst 5 or Hst 5₄₋₁₅-Spd (0, 7.5, 15, 30, 60, 120, and 250 μM) were mixed with equal volumes of 4% murine red blood cells (RBC) suspensions (100 μl) in normal saline prepared from freshly collected blood (30). The mixtures were incubated at 37°C for h and centrifuged at 1,000 × g for 5 min. Aliquots (100 μl) of supernatant were transferred to 96-well plates. The effect of Hst 5 or Hst 5₄₋₁₅-Spd on erythrocyte membranes was estimated by calculating the amount of hemoglobin released from disrupted erythrocytes, which was determined spectrophotometrically at A₅₄₀. Zero lysis and 100% hemolysis were determined in Tris buffer (pH 8.10, 20 mM Tris, 0.1 M NaCl) and 0.05% Triton X-100, respectively. Percentage hemolysis was calculated as [(A₅₄₀ in the peptide solution – A₅₄₀ in Tris buffer)/(A₅₄₀ in 0.05% Triton X-100 – A₅₄₀ in Tris buffer) × 100%].

**Efficacy of antifungal peptides in murine oral candidiasis.** Murine oral candidiasis infection was performed as previously described (26) using a protocol approved by the University of Buffalo Institutional Animal Care and Use Committee (project no. ORB06042Y) with slight modifications (31, 32). All infections were performed under anesthesia using ketamine (10 mg/ml) and xylazine (1 mg/ml) to a final concentration of 110 mg/kg body weight, and all topical treatment procedures were performed under anesthesia using isoflurane gas. Every experimental replicate had a total of 15 C57BL/6 mice (6- to 8-week-old, female mice) (Jackson Labs, Bar Harbor, ME), and the mice were grouped as untreated or phosphate-buffered saline (PBS)-treated controls (group 1; n = 5), Hst 5 treated (group 2; n = 5), and Hst 5₁₅₋₅-Spd treated (group 3; n = 5). Experiments were repeated independently at least three times. Immunomodulation was performed by subcutaneous injection of 225 mg/kg body weight cortisone 21-acetate (Sigma-Aldrich; C3130-5G) on day 1 prior to the infection and days 1 + 1 and 3 + 3 after infection. All three groups of mice were infected with C. albicans strain CAH (URA³ × 1 × 10⁷ cells/ml) impregnated in a cotton swab for 2 h under the tongue. In the control group, phosphate-buffered saline (PBS) solution was used as a treatment compound. Treatment was started on second day after infection (because mice start developing visible lesions around day 2) with 50 μl of 250 μM (781 μg/ml) Hst 5 or Hst 5₁₅₋₅-Spd in respective groups. Mice were anesthetized using isoflurane gas (XG-8 gas anesthesia System; Xenogen USA) for 2 min prior to topical treatment. Solutions consisting of 50 μl of PBS, Hst 5 (250 μM), or Hst 5₁₅₋₅-Spd (250 μM) were applied onto the anterior dorsal of the tongue for 60 s. Mice fully recovered within 5 min and were returned to cages without restrictions on eating or drinking. Topical treatment was repeated on days 3 and 4. After 5 days of infection, mice were euthanized by cervical dislocation under anesthesia (ketamine-xylazine), and the tongue and adjacent hypoglossal tissues were excised and cut into halves laterally. One-half was weighed and homogenized for quantification of infection levels by CFU/tg tongue tissue, and the other half was processed for histopathological analysis following fixation in zinc-buffered formalin followed by 70% ethanol and then embedded in paraffin. Thin sections were cut and stained with periodic acid-Schiff stain.

**RESULTS**

**Candidacidal activity of Hst 5₁₅₋₅-Conjugated with Spd.** Susceptibility of C. albicans to both conjugates was tested, along with that to Hst 5 and Spd as controls (Fig. 1A). Spd alone had no toxicity toward cells at 7 to 15 μM and only 3 ± 0.4 to 10 ± 0.15% killing at 30 μM to 60 μM (data not shown). Both Hst-Spd conjugates showed at least twice the candidacidal activity at all concentrations of Hst 5 tested in C. albicans (Fig. 1A). Both conjugates had equivalent killing activity at higher concentrations; however, Hst 5₁₅₋₅-Spd had significantly (P < 0.05) higher candidacidal activity than Spd-Hst 5₁₅₋₅ at lower (7.5 μM) concentrations. Since the Hst 5₁₅₋₅-Spd conjugate had slightly better activity, we focused subsequent experiments on examination of this construct. Also, we assessed the bactericidal activity of Hst 5₁₅₋₅-Spd against oral commensal bacteria (Streptococcus gordoni, Streptococcus sanguinis, and Streptococcus parasanguinis) and found that its MIC was >781 μg/ml (>250 μM), thus showing the selectivity of this peptide conjugate for C. albicans.

Candidacidal activity of Hst 5₁₅₋₅-Spd against three strains of C. glabrata (931010, 90030, and 90032) was at least 2 times higher than that of Hst 5 (Fig. 1B). Interestingly, although there were significant differences among the three strains in sensitivity to-
wards Hst 5 due to differences in Hst 5 uptake (14), all three C. glabrata strains were equally sensitive to Hst 54–15-Spd, suggesting that the conjugate was equally well transported by all strains.

**Dur3 is the preferential uptake transporter for Hst 54–15-Spd in C. albicans.** We previously found that Hst 5 competes for C. albicans intracellular uptake with Spd (24); so to confirm that Hst 54–15-Spd also utilizes Spd (Dur) transporters for its uptake, competition assays using BODIPY-X-Spd were performed. A dose-dependent reduction in Spd uptake in the presence of different concentrations (15, 30, and 60 μM) of Hst 54–15-Spd was found (Fig. 2A) that was similar to that of Hst 5 in our previous study, thus showing that the Hst 54–15-Spd conjugate utilizes the same transporters as Spd and Hst 5 for its entry into cells. Since C. albicans Dur3 and Dur31 polyamine transporters are involved in Hst 5 uptake (24), we expected similar use of these transporters for Hst 54–15-Spd translocation.

**FIG 1.** Hst 5 and Spd conjugates have higher candidacidal activities towards both C. albicans and C. glabrata. Susceptibility of C. albicans and C. glabrata was tested using candidacidal assays. (A) C. albicans cells (CAF4-2) were exposed to Spd, Hst 5, Hst 54–15, Spd-Hst 54–15, and Hst 54–15-Spd (7.5 to 31 μM), and the percentage of killing was calculated. Both Spd-Hst 54–15 and Hst 54–15-Spd showed significantly ($P < 0.05$) higher candidacidal activity in C. albicans than Hst 5. (B) For C. glabrata, three wild-type strains (Cg 931010, 90032, and 90030) were exposed to Hst 54–15-Spd and Hst 5. Candidacidal activity of Hst 54–15-Spd against three strains of C. glabrata was increased by at least 2 times compared with Hst 5.

Previously, we found C. albicans Flu1 is able to transport Hst 5 out of cells and thus reduce its toxicity (26). To determine whether Flu1 also plays a role in efflux of Hst 54–15-Spd, we tested antifungal activity of Hst 54–15-Spd in the C. albicans wild type and flu1 mutant strain. There was no significant difference in Hst 54–15-Spd toxicity between these two strains (data not shown), showing that Flu1 does not contribute to Hst 54–15-Spd export from the cells.

**Hst 54–15-Spd inhibits growth of C. albicans and C. glabrata biofilms.** Biofilms formed by yeast cells often have reduced susceptibility to antifungal agents compared with planktonic cells; therefore, we examined the sensitivities of biofilms formed by C. albicans and C. glabrata to Hst 5 and Hst 54–15-Spd (Fig. 3). C. albicans biofilm mass was reduced by only 24% following incubation with Hst 5, while Hst 54–15-Spd reduced biofilm formation by 43%. In contrast, Hst 5 had no ability to reduce C. glabrata biofilm mass, while incubation with Hst 54–15-Spd significantly reduced C. glabrata biofilm production by 41%.

The translocation rate of Hst 54–15-Spd into C. albicans cells is higher than that of Hst 5. Since we were unable to synthesize an N-terminally labeled Hst 54–15-Spd as we did for Hst 5, we could...
not measure uptake of Hst 54–15-Spd directly. As propidium iodide (PI) uptake is a direct functional consequence of ionic efflux and vacuole expansion induced only after uptake of Hst 5 (23), we used intracellular PI as an indirect measurement for Hst 54–15-Spd uptake visualized by time-lapse confocal microscopy. C. albicans cells treated with Hst 54–15-Spd showed rapid PI uptake within 5 min of treatment: 37% PI positive, compared with only 16% PI-positive cells treated with Hst 5 (Fig. 4A and B). Ninety percent of C. albicans cells were PI positive within 10 min of treatment with Hst 54–15-Spd. In contrast, only 50% of Hst 5 treated cells were PI positive within 15 min, while over 30 min was required to achieve 80% PI-positive Hst 5-treated cells (data not shown). C. albicans dur3Δ/Δ knockouts had significantly fewer PI-positive cells when treated with Hst 54–15-Spd, supporting the role of these transporters in Hst 54–15-Spd uptake (Fig. 4B).

Hst 54–15-Spd is more stable than its parent peptide, Hst 5. In order to determine the relative susceptibility of Hst 54–15-Spd to proteolytic degradation in whole saliva, we examined the candidacidal activity of peptides added to fresh whole human saliva with and without protease inhibitors. Since Hst 5 degradation in saliva is increased with physiological temperatures (33), we tested peptides at both 30°C and 37°C (Fig. 5), using whole saliva (WS) alone as a control. WS alone had low candidacidal activity that was increased by addition of protease inhibitors at 30°C. Addition of an equimolar concentration of spermidine (31 mM) to WS did not alter its fungicidal activity, irrespective of the presence or absence

FIG 2 Hst 54–15-Spd competes for uptake with spermidine and uses Dur3 transporters in C. albicans. (A) Spermidine (Spd) uptake was calculated for 5 min after addition of BODIPY-X-Spd (100 μM). Hst 54–15-Spd showed significant (P < 0.01) competition for Spd uptake in C. albicans cells in a dose-dependent manner. (B) The C. albicans wild-type (CAF4-2) and Hst 5 uptake transporter-deficient (dur3Δ/Δ, dur31Δ/Δ, and dur3/31Δ/Δ) strains were exposed to Hst 54–15-Spd, and the percentage of killing was calculated. Both the dur3Δ/Δ and the double gene deletion dur3/31Δ/Δ strains were found to be significantly (P < 0.05) less sensitive to Hst 54–15-Spd.

FIG 3 C. albicans and C. glabrata biofilms were more sensitive to Hst 54–15-Spd than Hst 5. Biofilms were formed for 24 h in tissue culture plates, and then Hst 54–15-Spd or Hst 5 (60 μM) was added for 1 h, and the cells were grown further for 24 h. (A) Reduction of biofilm formation in C. albicans was detected in the presence of both Hst 54–15-Spd and Hst 5. (B) Significant (P < 0.005) reduction in C. glabrata biofilms was detected only in the presence of Hst 54–15-Spd (**, P < 0.005; ***, P < 0.0001).
of protease inhibitors (data not shown). Hst 5 4–15-Spd (31 μM) added to whole saliva showed significantly (P < 0.001) higher (3-fold) candidacidal activity (67% ± 3.8% and 62% ± 0.7% killing at 30 and 37°C, respectively) than that of Hst 5 added to whole saliva (21% ± 0.84% and 13% ± 2.1% killing at 30 and 37°C, respectively). Hst 5 candidacidal activity significantly increased at both 30°C (P < 0.001) and 37°C (P < 0.05) in the presence of protease inhibitors. However, the high candidacidal activities of Hst 5 4–15-Spd in saliva were similar with or without protease inhibitors; showing the activity was protease independent. These results confirm that Hst 5 degradation and loss of candidacidal activity occur in whole saliva but remarkably showed that Hst 5 4–15-Spd is resistant to salivary proteases and retains high candidacidal activity in whole human saliva.

Next, we examined whether C. albicans germinated cells were equally susceptible to the fungicidal activity of Hst 5 4–15-Spd as yeast cells. There was no significant difference in the sensitivity to Hst 5 4–15-Spd to germinated cells compared with yeast cells, although Hst 5 had slightly higher activity with germinated cells (P = 0.0167) compared to yeast cells (Fig. 6A). To determine if Hst 5 4–15-Spd retained fungicidal activity in serum, we tested the fungicidal activity of Hst 5 and Hst 5 4–15-Spd in the presence of 10% fetal bovine serum (FBS). As expected, Hst 5 had very low candidacidal activity in serum, killing ≈25% of either germinated or yeast cells even at very high (120 μM) concentrations. In contrast, Hst 5 4–15-Spd had significantly (P < 0.001) higher activity than Hst 5 in 10% FBS against both yeast cells and germinated cells (Fig. 6B). Although a 2-fold-higher concentration of Hst 5 4–15-Spd in 10% FBS was needed for comparable fungicidal activity in NaPB alone (Fig. 6A), its killing could be increased up to 90% with higher peptide concentrations— unlike Hst 5, which retained low killing activity even at high doses in the presence of 10% FBS. Thus, Hst 5 4–15-Spd exhibits significant fungicidal activity even in the presence of serum in the environment.

Antifungal efficacy of Hst 5 4–15-Spd in a murine model of oral candidiasis. Prior to in vivo experiments, the toxicity of these peptide conjugates was evaluated by using hemolysis assays. No hemolytic activity was detected at any concentration (1 to 250 μM) of conjugate examined (data not shown). Next, we tested the efficacy of Hst 5 4–15-Spd as a topical application to an established infection in a murine model of oral candidiasis (34). Mice do not produce salivary histatins, so native
Novel and selective candidacidal activity of Hst 5\textsubscript{4-15}-Spd conjugates. Instead of amino acid substitutions that enhance overall amphipathicity of the peptide and broaden its antimicrobial activity, our approach has been through rational selection of a carrier molecule to improve fungus-specific Hst 5 killing based upon our discovery that Hst 5 utilizes polyamine transporters for its uptake. Previously we found that Hst 5 uptake, rather than binding, is the key element limiting its fungicidal activity, since Hst 5 is not a lytic peptide but instead targets selective functional processes once it has reached the cytosol. This is the first report in which modification of a conjugate peptide based upon its mechanism of action in fungal cells has resulted in significantly improved and specific fungicidal activity. Previously, polyamines were used as vectors for delivery of anticancer drugs. For example, improved cytotoxicity towards cancer cell lines in vitro was found with artemisinin-spermidine conjugates due to enhanced polyamine transport and uptake (35). Interestingly, the same polyamine analogues and conjugates were found to be effective inhibitors of Plasmodium falciparum due to their uptake through parasite polyamine transporters (35), thus pointing toward their use as antimalarial drugs. Recently, anthracene and benzene-polyamine conjugates were shown to be effective against Pneumocystis pneumonia, since these conjugates had higher affinities toward polyamine transporters and inhibited the uptake of native polyamines, thus reducing the severity of infection and lung inflammation (36).

These and other studies show that the structural requirements for uptake of polyamine conjugates are not stringent; thus, a wide variety of analogs may be transported by polyamine uptake systems, as illustrated by our findings demonstrating uptake of Hst 5 by C. albicans Dur3/31 polyamine transporters (24). Although it might be anticipated that spermidine conjugated to a 12-amino-acid cationic peptide might hinder its uptake, instead we found that the conjugate molecule had an accelerated rate of uptake by C. albicans cells. Hst 5 fungicidal activity is not dependent upon small changes in net charge or upon chirality (19); thus, it is possible that the Hst 5\textsubscript{4-15} peptide when conjugated to spermidine allows it to assume a more compact or globular conformation that is a more favorable substrate for fungal polyamine transporters. This result was reproducible in additional independent experiments, and at even higher control infection levels (\(10^8\) C. albicans cells/g), Hst 5\textsubscript{4-15} Spd topical treatment significantly reduced tongue tissue infection by 3- to 4-log fold (\(5 \times 10^4\) C. albicans cells/g) compared to a 2-log fold reduction with Hst 5 (4.3 \(\times 10^4\) C. albicans cells/g). Histological examination of tongues after sacrifice on day 5 substantiated the clinical appearance. Control group tongue tissue surfaces were heavily colonized by C. albicans, including invasive hyphae penetrating the mucosal epithelial layer. These tissues had extensive inflammatory cell infiltration, as well as gross damage to oral epithelia with loss of rete peg structures (Fig. 7D). Hst 5-treated mice showed comparatively less C. albicans surface colonization with little epithelial invasion and only partial loss of rete peg structures. Tongues of mice treated with Hst 5\textsubscript{4-15}-Spd showed no evidence of infection in the areas treated topically and had completely normal rete peg structure and epithelial morphology. Thus, these experiments showed Hst 5\textsubscript{4-15}-Spd was highly effective as a topical treatment for murine oral candidiasis compared to Hst 5.

FIG 6 Hst 5\textsubscript{4-15}-Spd showed higher candidacidal activity in serum against C. albicans yeast and germinated cells. (A) C. albicans yeast or germinated cells (10\textsuperscript{6} cells) were exposed to Hst 5 or Hst 5\textsubscript{4-15}-Spd without serum. Both Hst 5 and Hst 5\textsubscript{4-15}-Spd 5 were equally active against germinated C. albicans cells compared to yeast cells, although the candidacidal activity of Hst 5\textsubscript{4-15}-Spd 5 was significantly greater (\(P < 0.001\)). (B) C. albicans yeast or germinated cells were incubated with serial dilutions of Hst 5 or Hst 5\textsubscript{4-15}-Spd in the presence of 10% serum. Hst 5\textsubscript{4-15}-Spd showed significantly (\(P < 0.001\)) higher candidacidal activity at all concentrations in serum compared to Hst 5.

secretions will not confound exogenous application of Hst 5. Before infection, we confirmed that mice were not carrying C. albicans cells in the oral cavity, as assessed by culturing oral swabs for each animal. BALB/c mice were immunosuppressed with cortisone, and oral infection with C. albicans was established prior to the first treatment with the PBS control, Hst 5, or Hst 5\textsubscript{4-15}-Spd (250 \(\mu\)M in NaPB) (\(n = 5\) per experimental group) (Fig. 7A). Extensive white lesions typical of oral candidiasis were found on the dorsal surfaces of tongues of mice in the control group by day 3 and became nearly confluent by day 5 (Fig. 7B). In contrast, mice treated with Hst 5 had reduced but still visible white lesions on day 3, while tongues of Hst 5\textsubscript{4-15}-Spd-treated animals had negligible white lesions within the area of topical application. The clinical appearance of tongues was more striking at day 5 in that Hst 5\textsubscript{4-15}-Spd-treated regions appeared pink and free of lesions, while Hst 5-treated tongues had light lesions, and underlying tissues appeared more reddened.

Tongues from PBS-treated mice contained \(5 \times 10^7\) to \(5 \times 10^8\) C. albicans cells per g of tongue tissue, whereas tongues from Hst 5-treated mice had about 2-log fold reductions in cells (\(\approx 10^6\) C. albicans cells/g) (Fig. 7C). Topical treatment with Hst 5\textsubscript{4-15}-Spd resulted in highly significant (\(P < 0.0001\)) reduction in C. albicans cells compared to the control group (average of \(10^6\) C. albicans cells/g), and several tongues had no recoverable C. albicans. This Secretary at all concentrations in serum compared to Hst 5.
is the likely reason for the somewhat higher fungicidal activity of Spd when conjugated at the C terminus compared with the N terminus, as a result of differences in charge clustering due to a less-ordered secondary structure. However, the presence of spermidine plays a role in recognition of both Hst 54-15-Spd conjugates by Dur transporters to drive its uptake.

Commensal oral bacteria are important to the health of the mouth and prevention of overgrowth of *C. albicans*. For example, oral administration of the probiotic *Streptococcus salivarius* K12 to mice with oral candidiasis reduced the severity of candidiasis (37). Hst 5 itself has no microbicidal activity with commensal human oral bacteria, including *Streptococcus mutans* and *Streptococcus sanguis* (16), and we found that the conjugate Hst 54-15-Spd was also inactive against *S. gordonii*, *S. sanguinis*, and *S. parasanguinis*. Although it is not known whether the lack of microbicidal activity against these streptococcal species is because Hst 5 is not taken up by cells or if bacteria lack intracellular targets, studies with *Staphylococcus aureus* showed that Hst 5 was not taken up by these bacteria (38). Prokaryotic cells express two types of polyamine transporters, namely, spermidine-preferential (PotABCD) and putrescine-specific (PotFGHI) ATP-binding cassette transporters, which differ substantially from yeast cells (39, 40). Such differences between yeast and bacterial polyamine transporters suggest that bacteria do not take up Hst 5 or its conjugate molecule. In either case, the specificity of Hst 54-15-Spd for fungal cell uptake makes it advantageous as a drug selective for treatment of oral candidiasis, so that even large dosages might not disturb the protective commensal microbiome in the oral cavity.

Modifications of Hst 5 primary structure have been investigated extensively in order to achieve a peptide with higher activity than the native Hst 5. Single or multiple amino acid substitutions were employed to increase both the lateral amphipathicity and helical conformation of the synthesized Hst peptide, in order to increase its propensity to associate with membranes (16). Using this strategy, multisite-substituted Hst 5 analogues (dhvar1 and dhvar2) were constructed and exhibited increased fungicidal activity over Hst 5 (16); however, both dhvar1 and dhvar2 analogues showed increased membranolytic and hemolytic activities (41, 42) and inhibited the growth of the oral commensal bacterium *Streptococcus sanguis* (16). Unexpectedly, dhvar2 was found to increase
HIV-1 replication by promoting the envelope-mediated cell entry process, likely as a result of its membrane activity (43). Thus, although these Hst 5 modifications improved fungidal activity, they also resulted in broader-spectrum antibacterial and hemolytic activities as well as enhanced HIV-1 replication, so that their clinical utility is limited. In contrast, the Hst 5_4–15-Spd conjugate has none of the drawbacks associated with nonspecific membrane-nolytic activity.

**Stability of Hst 5_4–15-Spd conjugates in saliva and serum.**

A major disadvantage to using Hst 5 in treatment of oral candidiasis is that its antifungal activity is rapidly lost in saliva due to proteolytic degradation by salivary enzymes (15) and by secreted C. albicans proteolytic enzymes (44). Among the family of antifungal Hst proteins, Hst 5 is the most fungidical, but it is also the most susceptible to proteolytic activity in whole saliva (45, 46).

Thus, topical treatment of ex vivo murine tongue tissues with Hst 5 was no more effective than treatment with saliva alone in preventing C. albicans colonization of tissues (47). To overcome this problem, another approach has been to stabilize its structure through introduction of a single disulfide bond (48) or by N- to C-terminal cyclization (49). Cyclization of Hst 5 increased its activity by 1,000-fold (49), while cyclization of Hst 3 using disulfide bonds or a lactam bridge increased its killing of yeast by 100-fold without an increase in hemolysis (47). Importantly, these modifications induced a more compact folded structure that increased the Hst half-life in serum (47), presumably by masking proteolytic cleavage sites. Similarly, we found that Hst 5_4–15-Spd was less sensitive to protease degradation in saliva, suggesting that this conjugate has a more compact or folded structure with fewer exposed protease substrate sites. Furthermore, our results from topical application in vivo point to an enhanced half-life at the site of application, since only three doses were needed to treat the infection.

Topical antifungal therapy is the recommended first line of treatment for uncomplicated oral candidiasis or denture stomatitis (50). The advantages of topical therapy for oropharyngeal candidiasis (OPC) are direct drug exposure at the oral mucosa site of infection as well as the lack of adverse systemic effects or drug interactions (51). Although azole antifungals are commonly used as topical (and systemic) therapeutics for oral candidiasis, their use is contraindicated in patients with recurrent oral yeast infections due to a risk of selection and enrichment of drug-resistant strains (52). As an alternative to azole therapeutics, we report that topical treatment with Hst 5_4–15-Spd was highly effective at doses well below those of systemic fluconazole (53), as tongues of treated animals had negligible white lesions within the area of topical application. Similar to native Hst 5, spermidine conjugates of the active fragment of Hst 5 are nontoxic and have a higher clinical half-life, enhanced uptake into Candida cells, and greater candiacidal efficacies than Hst 5. Although further studies regarding potential toxicity of these conjugate peptides in humans need to be done, this study highlights the potential of histatins conjugated with polyamines as potent antifungal drugs.

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**REFERENCES**


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