Therapeutic efficacy of halocidin-derived peptide HG1 in a mouse model of *Candida albicans* oral infection

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**Objectives:** HG1 is an antimicrobial peptide derived from halocidin, which is naturally found in tunicates. The purpose of this study was to evaluate the therapeutic potential of HG1 as a novel antifungal agent for treating oral candidiasis.

**Methods:** The pharmacokinetic properties of HG1 were explored in mice, which were orally administered a single dose of HG1. Anti-*Candida* activity of HG1 was investigated in a time-dependent manner in the presence of saliva obtained from healthy donors or patients with oral candidiasis. In addition, HG1 was evaluated for its anti-*Candida* activity in the presence of proteins extracted from the culture supernatant of *Candida albicans*. The therapeutic potential *in vivo* and *ex vivo* of HG1 against oral candidiasis was investigated using a mouse model of oral candidiasis.

**Results:** Our data showed that absorption of HG1 into the blood did not occur following oral administration. In addition, HG1 exerted marked anti-*Candida* activity after short-term incubation at a concentration of 20 mg/L and it also caused a considerable reduction in fungal burden in the oral candidiasis mouse model when treated with 1 mg or 0.5 mg.

**Conclusions:** This study suggests that HG1, as a novel component of mouthwash, might become an alternative antifungal agent to conventional drugs used to manage oral candidiasis.

**Keywords:** antimicrobial peptides, antifungal activity, oral candidiasis

**Introduction**

The frequency of mucosal and cutaneous fungal infections has dramatically increased worldwide.¹⁻¹² As opportunistic infections, most fungal diseases can become life-threatening in immune-compromised or malnourished populations, even under optimal antifungal therapy.¹ Candidiasis is a recalcitrant infection caused by *Candida* species, mostly *Candida albicans*, which colonize human mucosal membrane surfaces. Azoles and polyenes are the most common antifungal agents available to treat a variety of *Candida* infections. However, these antifungal drugs have several defects relating to clinical usage, such as the time required for killing fungi being too long and low efficacy due to side effects.³⁻⁵ Moreover, continued extensive use of these ant mycotic drugs has resulted in the emergence of drug-resistant *Candida* species.⁶,⁷ Hence, there is an urgent need to develop new antifungal agents against *Candida* species with novel mechanisms of action.

Over the last two decades, cationic antimicrobial peptides have been considered as promising candidates for a new type of antifungal agent, because they have a broad antimicrobial spectrum and a mode of action that is distinctly different from conventional antimicrobials.⁸⁻¹⁰ Some antimicrobial peptides display excellent *in vitro* antifungal activity commensurate with that of antifungal drugs.¹¹⁻¹³ Accordingly, these molecules are being proposed as possible anti-*Candida* agents, although several aspects remain to be clarified, including their *in vivo* efficacy and structural stability upon administration on *Candida*
infection sites. This study was conducted to determine whether HG1 antimicrobial peptide is a practical treatment for Candida infections occurring in the oral cavity.

Halocidin is a natural antimicrobial peptide isolated from the tunicate Halocynthia aurantium. Antimicrobial studies performed with diverse halocidin congeners have found di-K19Hc, referred to herein as HG1 (Halocidin Group #1), to be the most appropriate candidate. HG1 shows potential antifungal activity against a variety of pathogenic fungi including C. albicans, and HG1 is resistant to host and bacterial proteases, which is a favourable property to overcome a common hurdle against the use of antimicrobial peptides as therapeutic agents.

In this study, we first evaluated the pharmacokinetic properties of HG1 following oral administration to mice and investigated the kinetics of its candidacidal action. In particular, the rapid candidacidal activity of HG1 is critical given the short period of time (<3 min) that a mouthwash agent is retained in the oral cavity. Using the hyphal form of C. albicans, which is associated with oral candidiasis, the time required for killing C. albicans was measured using confocal microscopic observation. Additionally, the resistance of HG1 to proteases secreted from C. albicans was assessed by testing its candidacidal activity in the presence of proteins extracted from the culture supernatant (CS) of C. albicans. HG1 antifungal activity was also evaluated in saliva obtained from healthy persons and patients with oral candidiasis. Finally, we verified the therapeutic potential of HG1 against oral candidiasis via ex vivo and in vivo experiments using a mouse model of C. albicans infection.

### Materials and methods

**Peptides, antifungal agents, Candida species and animal experiments**

We employed P113 as a control antimicrobial peptide, which is an antifungal peptide derived from histatins, a group of antimicrobial peptides found in human saliva.

Two antimicrobial peptides (HG1 and P113) were synthesized in accordance with their amino acid sequences using an automated solid-phase peptide synthesizer (Pioneer Applied Biosystems, Foster City, CA, USA) at AnyGen, Inc. (Jeonnam, South Korea) (Table 1). In the case of HG1, synthetic monomers were exposed to 0.1 M ammonium bicarbonate (pH 8.7) for 3 days at room temperature and then subjected to C_{18} reversed-phase HPLC (Vydac 218TP54; The Separation Group, Hesperia, CA, USA) to purify the resultant dimer peptide. The commercial antifungal drug, nystatin (N3503; Sigma, St Louis, MO, USA), was used as a positive control. C. albicans was obtained from the Culture Collection of Antibiotic-Resistant Microbes (CCARM) for animal experiments using a mouse model of C. albicans infection.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence(s)</th>
<th>Mass (Da)</th>
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<td>KWLALLHHGLNCAKGVLA-NH₂</td>
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<td>P113</td>
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*The vertical bar in the HG1 sequence signifies a disulphide bond between two cysteine residues.*

### HG1 pharmacokinetics

Single-dose pharmacokinetics of HG1 was assessed through oral administration, skin application and intravenous injection. HG1 was given orally (1 mg/mouse in 0.1 mL of sterile saline) to mice (n=4/group) and blood samples were collected at 1 min, 30 min and 1, 2, 4, 8, 16, 24 and 48 h after oral administration. For skin application, the shaved back skin of each mouse was scratched with no. 600 sandpaper (Deerfos, Incheon, South Korea) and a plastic cylinder with a diameter of 1.65 cm was affixed to the abrasion site using cyanoacrylate adhesive. The HG1 solution was prepared at a concentration of 2 mg/mouse in 0.02 mL of PG-NaP buffer (45% polyethylene glycol 400/45% glycerol/10% 10 mM sodium phosphate (NaP) buffer, pH 7.4) and poured into the cylinder chamber (n=4/group). Blood samples were collected at 1 min, 30 min and 1, 2, 4, 8, 16, 24 and 48 h after HG1 application. For a positive control, HG1 was administered via a single intravenous bolus injection (0.1 mg/mouse in 0.1 mL of sterile saline) to mice (n=4/group) and blood samples were also collected at 1 min, 30 min and 1, 2, 4, 8, 16, 24 and 48 h after intravenous injection. The experiments were repeated three times on different days. All blood samples were centrifuged at 10000 g for 10 min to obtain plasma. Plasma samples were then stored at −20°C until use.

### Bioanalytical assay

To analyse HG1 levels in plasma, 0.15 mL of acetonitrile was added to 0.1 mL of plasma sample. The mixture was vortex mixed for 1 min and centrifuged at 10000 g for 5 min. The supernatants were dried in a speed-vacuum system and resuspended in 0.4 mL of 10 mM NaP buffer (pH 7.4) and subjected to C_{18} reversed-phase HPLC, using a gradient of acetonitrile in 0.1% trifluoroacetic acid. The acetonitrile concentration was kept at 0% for 10 min and then increased by 1%/min over the next 60 min. Fractions in the range of the 0.5 min before and the 0.5 min after the HG1 elution time were pooled and electrophoresed by acid urea-PAGE (AU-PAGE). The AU-PAGE gel was transferred to nitrocellulose membranes in transfer solution (1% acetic acid, 10% methanol in distilled water) at 110 mA for 1 h. After transfer, the membranes were equilibrated in Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5; TBS) for 10 min and then incubated for 1 h in blocking solution (8% BSA in TBS). After a brief wash in TBS containing 0.05% Tween 20 (TBBS), the membrane was incubated overnight in a primary antibody solution (anti-HG1 anti-rabbit serum diluted 500-fold with blocking solution). The membrane was washed twice in TBBS and then incubated for 2 h in blocking solution containing a 1000-fold diluted secondary antibody (rabbit anti-goat IgG Horseradish Peroxidase; Invitrogen). Finally, the membrane was washed twice in TBBS, once in TBS and developed by enhanced chemiluminescence. In the case of the control sample, HG1 was prepared in normal mouse serum at 100 mg/L and was 2-fold serially diluted in serum to 3.1 mg/L.

### Saliva collection

Normal saliva was collected from three orally healthy donors at least 1 h after their last meal. Immediately after collection, whole saliva was cleared from particulate matter such as bacteria and desquamated epithelial cells by centrifugation at 14 000 g for 10 min at 4°C. The supernatant was used as the normal saliva sample. Saliva was collected from three patients with oral candidiasis who consented to its use.
Protein extraction from C. albicans CS

To obtain proteins extracted from the C. albicans CS, a culture of C. albicans (CCARM 144-87) was incubated for 20 h in Sabouraud dextrose broth (Difco, Detroit, MI, USA) and centrifuged for 30 min at 10000 g. The CS was concentrated by ultrafiltration using a 10 kDa cut-off membrane (Vivavflow 200, no. 05VF2004-5, Viva Science Ltd, Gloucestershire, UK). Proteins in the concentrated CS were obtained via 60% saturation with ammonium sulphate.

Antifungal assay against C. albicans hyphae

Yeast-form cells grown to the stationary phase were harvested in Winge medium (0.3% yeast extract, 0.2% glucose) and suspended to a final density of 1×10^8 cfu/mL in Winge medium containing N-acetylglucosamine (1 g/L). A 0.04 mL aliquot of cell suspension was placed in a sterile Eppendorf tube and incubated for 3 h at 37°C to form C. albicans hyphae, which were then confirmed using a light microscope. Candida hyphae were collected by centrifugation at 12000 g and 0.04 mL of antifungal samples (HG1 or nystatin, 20 mg/mL) was added to the sediment. After incubations for predetermined times (0.5, 1, 2, 4, 8, 16, and 30 min) at 37°C, each mixture was directly plated on tryptic soy agar (TSA) consisting of 1.5% agarose and 3% tryptic soy broth powder (Difco). The resultant colonies were then counted after a 24 h incubation at 37°C.

Live cell imaging of HG1 killing kinetics by confocal microscopy

Microscopic data were obtained using images of living C. albicans hyphal cells. Briefly, 1×10^7 hyphae were attached to a cover-glass coated with Concanavalin A (1 g/L solution in water) in chambered wells (Lab-Tek, 171080, Thermo Scientific) for 30 min at room temperature. After washing the chambered wells three times with NaP buffer, 0.1 mL of NaP buffer containing 0.005 mg of propidium iodide (PI; Sigma) was added to each well. Images of hyphae were adjusted optimally under an FV500 laser-scanning confocal microscope (Olympus, Tokyo, Japan). Then, 0.1 mL of HG1 or nystatin (final concentration, 20 mg/L) was added to the wells containing hyphal cells. Confocal images were automatically acquired at predetermined intervals. Nystatin was selected as the control, because it has known surface activity against fungi.

Antifungal assay against C. albicans in saliva

First, 0.2 mL of normal saliva containing C. albicans (1×10^7 cfu/mL) was mixed with 0.8 mL of HG1 or P113 at different concentrations (5, 20, 80 or 312.5 mg/mL). After incubations for predetermined times (2, 4, 8, 30 and 60 min) at 37°C, each mixture was directly plated on TSA. The resultant colonies were then counted after an overnight incubation at 37°C. Alternately, 0.2 mL of clinically obtained saliva was mixed with 0.8 mL of HG1 or P113 at different concentrations (0.312, 0.625, 1.25 or 2.5 g/L). At the indicated intervals, aliquots were spread on TSA containing 100 mg/L ampicillin (A1593, Sigma) and 25 mg/L kanamycin (G60165, Sigma). Recovered colonies were counted after an overnight incubation at 37°C.

Anti-Candida activity of HG1 in the presence of proteins extracted from C. albicans CS

Anti-Candida activity of HG1 or P113 was investigated in the presence of proteins extracted from C. albicans CS. A 0.05 mL aliquot of HG1 or P113 solution (400 mg/mL) was mixed with 0.05 mL of C. albicans CS protein (4 g/L) that was already treated or not treated with pepstatin A (Sigma, P5318), which is a known specific inhibitor of secreted aspartyl protease (SAP) from C. albicans. After a 30 min incubation, 0.1 mL of C. albicans suspension (2×10^6 cfu/mL) was added to each mixture and incubated for 10 min. A 0.05 mL aliquot of each sample was spread on TSA and the resultant colonies were counted after an overnight incubation at 37°C. The structural stability of HG1 or P113 upon attack by SAP was already treated or not treated with pepstatin A solution (400 mg/L) was mixed with 0.2 mg of C. albicans CS protein in a total volume of 0.1 mL, followed by a 30 min incubation at 37°C. The reaction was halted by adding 0.1% trifluoroacetic acid (0.9 mL), after which 0.1 mL of each mixture was subjected to AU-PAGE analyses.

Ex vivo experiment for therapeutic effect of HG1 on oral candidiasis

An ex vivo model was developed to demonstrate the therapeutic effect of HG1 on oral candidiasis. Tongues were excised from sacrificed mice and each tongue sample was placed in 1 mL of NaP buffer containing 10^7 C. albicans cells. After being incubated at 30°C for 12 h with gentle rotation, the tongues were washed three times with NaP buffer and transferred to new tubes containing 0.9 mL of 22.2% human saliva solution (0.2 mL of saliva and 0.7 mL of NaP buffer), followed by a further incubation at 30°C for 6 h with gentle rotation. Then, 0.1 mL of NaP buffer containing 0.5 mg of HG1 or nystatin was added to each mixture and incubated at 30°C for 12 min with gentle rotation. After washing three times with NaP buffer, each tongue was weighed and homogenized in NaP buffer (10 mL/g of tongue). A 0.01 mL aliquot of each homogenate sample was subjected to a colony count assay. The number of recovered colonies was converted into cfu/mg of tongue. Alternatively, to mimic a procedure for treatment via an oral rinse, the mouse tongue samples were soaked in 1 mL of 20% human saliva solution containing 0.5 mg of HG1 or nystatin for 3 min, followed by washing in NaP buffer for 10 min to remove residual HG1 or nystatin from the surface of the tongue tissue. After this procedure was repeated four times, the number of C. albicans recovered from each tongue sample was enumerated as described above.

Mouse model of oral candidiasis

Experimental oral candidiasis of mice was established as described previously with some modifications. Briefly, to maintain neutropenia for at least 72 h, mice were immunosuppressed by two subcutaneous injections of cyclophosphamide monohydrate (200 mg/kg of body weight) at 1 and 3 days before the C. albicans infection. Teicoplanin (Dongkook Pharm, South Korea) and ceftriaxone sodium (Hanmi Pharm, South Korea) at a concentration of 1 g/L each were given with drinking water throughout the experiment to eradicate potential bacterial competitors. Mice were anaesthetized via intramuscular injection of tiletamine hydrochloride/zolazepam hydrochloride (Zoletil; Virbac, France) at a dosage of 50 mg/kg of body weight and then challenged orally with 10^8 C. albicans cfu in 0.02 mL of saline. Treatment was initiated at day 3 post-infection when we observed white patches on the tongue. A 0.02 mL aliquot of 5% (weight/volume) HG1 solution in PG-NaP was applied orally and therapy was continued three times daily for 3 days (n=10). The PG-NaP solution and nystatin in PG-NaP were used as a negative and positive control, respectively (n=10). After the final administration of HG1, the mice were sacrificed and tongues were excised. Each tongue sample was then placed in 0.3 mL of PBS and homogenized in a tissue grinder. Serial dilutions of the homogenate were spread on TSA and incubated for 24 h at 37°C. The resultant colonies were counted and cfu/mg of tongue was calculated.

Histochemical analyses

Tongue samples were obtained from animals that were sacrificed 12 h after the last treatment. The tissues were directly fixed in 4%
paraformaldehyde for 24 h and embedded in paraffin. Five micrometre-thick sections were prepared. The slides were stained with periodic acid-Schiff and observed using light microscopy.

Results

**HG1 pharmacokinetics**

HG1 has been found to infiltrate into the dermis upon topical administration onto mouse skin without being subjected to degradation, which was confirmed by HPLC and immunoblotting analyses. Here we investigated whether or not HG1 was detected in the plasma following a single oral and topical administration of HG1. As shown in the immunoblotting analyses performed with anti-HG1 antibody solution, oral administration and skin application of HG1 did not result in detectable plasma HG1 concentrations, indicating that HG1 is rarely absorbed into the bloodstream following oral administration and skin application (Figure 1). Indeed, transdermal delivery systems of peptide drugs are limited because they do not permeate skin layers. Additionally, orally administering peptide drugs is more difficult because peptide drugs must penetrate the mucous and epithelial layers in the intestine to enter into cells and avoid proteolysis by the action of gastric and pancreatic enzymes. Therefore, oral administration of HG1 should not present therapeutic effects or a risk of severe systemic toxic side effects via redistribution through the systemic circulation.

**Time-dependent candidacidal activity of HG1**

In our colony count assay against *C. albicans* hyphae, it was determined that HG1 retained much stronger activity than nystatin during the entire incubation (Figure 2a). This type of test was not sufficient to evaluate the exact time required to kill *C. albicans*, as an antifungal agent could continue to exert activity in a condition of conjugation with hyphae after being plated on TSA. Thus, we performed a visual experiment using confocal microscopy. The PI uptake into *C. albicans* cytosol was observed at predetermined times after treatment of *C. albicans* hyphae with HG1 or nystatin. As shown in Figure 2(b), PI was translocated into all hyphae within 1 min after HG1 treatment. In contrast, PI uptake by hyphae was not detected until 36 min after treatment with nystatin. Considering that mouthwash should be kept in the oral cavity for <3 min, these results strongly suggest that HG1 could be useful as an active ingredient in gargle for treating oral candidiasis.

**Antifungal activity of HG1 in the presence of normal and clinically isolated saliva**

The colony count assay was performed using agar plates containing antibiotics after incubation with two types of saliva to test HG1 antifungal activity. In the case of normal saliva, cultured *C. albicans* was added before testing, as no fungus was detected in normal saliva samples. As shown in Figure 3, HG1 displayed rapid and marked antifungal activity compared with that of P113, although its activity was somewhat affected in saliva. HG1 at >250 mg/L exerted antifungal activity against fungi in the clinically isolated saliva, suggesting that it may significantly reduce fungal burden in the oral cavity of patients with candidiasis.

**Antimicrobial activity of HG1 in the presence of *C. albicans* proteins**

The candidacidal activity of HG1 and P113 was tested in the presence of proteins secreted from *C. albicans* (Figure 4a). After 30 min of incubation with the *C. albicans* CS proteins, HG1 maintained significant candidacidal activity. In contrast, P113 totally lost its activity in the presence of the *C. albicans* CS proteins. Additionally, P113 candidacidal activity was little affected by the CS proteins pre-treated with pepstatin A, which is a specific inhibitor of *C. albicans* SAP. Therefore, it was concluded that P113, unlike HG1, was degraded by SAP and its candidacidal activity was severely damaged. The structural stability of the two peptides upon incubation with CS proteins was also tested by AU-PAGE analysis. As shown in Figure 4(b), HG1 maintained its intact form after the incubation with CS proteins. In contrast, the P113 band faded from the gel upon incubation with *C. albicans* CS proteins.

**Therapeutic effect of HG1 in ex vivo experiments**

The results of the ex vivo studies are shown in Figure 5. When *C. albicans*-infected mouse tongue was treated with 0.05%
(weight/volume) HG1 or nystatin in the presence of 20% human saliva for 12 min, the cfu of recovered C. albicans decreased >10 times compared with that in a control sample treated with buffer (Figure 5a). Alternatively, the therapeutic efficacy of HG1 on oral candidiasis was evaluated in a manner similar to actual treatment with mouth rinse. C. albicans-infected tongues were treated four times with 0.05% (weight/volume) HG1 or nystatin for 3 min per treatment. As a result, HG1 showed significant therapeutic efficacy, whereas nystatin showed little candidacidal effect (Figure 5b). In short, a marked difference in the candidacidal effect was observed between HG1 and nystatin upon their application in an oral rinse.

**Therapeutic efficacy of HG1 in mouse models of oral candidiasis**

The antifungal efficacy of HG1 was further investigated using a murine model of oral candidiasis. The tongues of mice were covered with many white patches after the C. albicans infection. The mean value of recovered C. albicans cfu in the HG1-treated group (n=10) with a high dosage (1 mg) and a low dosage (0.5 mg) was ~100 times lower than that in the vehicle-treated group (control) (Figure 6a). The mean value of recovered C. albicans cfu in the HG1-treated group treated with a low dosage was a little different from that in the HG1-treated group with a high dosage, but the difference between the two groups was not significant (P=0.2455). Compared with nystatin, which is a known antifungal agent in currently available mouthwash, the therapeutic effect of HG1 was almost equivalent to that of nystatin in both dosage groups. To further assess the in vivo therapeutic effect of HG1, tongue samples stained with periodic acid-Schiff were observed with light microscopy (Figure 6b). Histopathological sections of tongue tissue from vehicle-treated mice showed numerous C. albicans infiltrating the epithelium of the dorsal surface of the tongue. In contrast, tongues of HG1-treated or nystatin-treated mice showed only very limited colonization by C. albicans in lingual papillae, which was similar to that of a healthy tongue. These results indicate that HG1 and nystatin exerted therapeutic activity in a mouse model of oral candidiasis.

![Figure 2. Killing kinetics of HG1 and nystatin against C. albicans hyphae.](image-url)
Discussion

Current antimycotic therapies are limited by fast-emerging drug resistance, a limited choice of antifungals and increased fungal infections in immunocompromised patients.3,5 As seen in many

Figure 3. Antifungal activity against fungi in saliva from healthy human volunteers or patients with oral candidiasis. (a) Each peptide at different concentrations (4, 16, 64 and 250 mg/L) was mixed with 20% normal saliva containing C. albicans for a predetermined time (2, 4, 8, 30 and 60 min). Aliquots were spread onto TSA at the indicated intervals. (b) A 20% clinically obtained saliva sample was treated with HG1 or P113 at different concentrations (0.25, 0.5, 1 and 2 g/L) for a predetermined time (2, 4, 8, 30 and 60 min) and then spread on TSA containing 100 mg/L ampicillin and 25 mg/L kanamycin. Mean values from triplicate experiments were used to derive the data shown.

Figure 4. Resistance of antimicrobial peptides to proteases secreted from C. albicans. (a) Each peptide was incubated with CS or CS pre-treated with pepstatin A (Ptn A) and then tested for remaining candidacidal activity via a colony count assay. Experiments were repeated three times and the mean values were used to make the graph. (b) After incubation with CS for 30 min, part of each sample was subjected to AU-PAGE analysis. The symbols + and – indicate treatment and no treatment of the corresponding protein with each peptide, respectively. Gels were stained with Coomassie blue.

Figure 5. Therapeutic effect of HG1 in an ex vivo model of mouse tongue infected with C. albicans. Results were analysed using analysis of variance (ANOVA). (a) C. albicans-infected tongues were treated with 0.05% HG1, 0.05% nystatin or NaP buffer in 20% (volume/volume) human saliva for 12 min. ANOVA revealed a significant mean difference among the three groups, $F=18.16; P<0.001$. (b) Treatment was performed four times for 3 min each. Numbers of C. albicans recovered are expressed in terms of cfu/mg of tongue. ANOVA test, $F=27.43; P<0.001$. Horizontal bars signify the mean value of each group.

Discussion

Current antimycotic therapies are limited by fast-emerging drug resistance, a limited choice of antifungals and increased fungal infections in immunocompromised patients.3,5 As seen in many
fungal infections that occur in poorly vascularized or avascular structures, the systemic management of oral candidiasis also poses a therapeutic problem because of the distribution and retention of adequate amounts of antifungal at the site of infection. Moreover, most antifungals cause undesirable side effects upon systemic administration.28,29 Accordingly, topical treatment using a mouthwash may become a more plausible therapeutic regimen in the management of oral candidiasis. We have conducted this study to assess HG1 as an antifungal component of mouthwash for treating oral candidiasis. We evaluated the pharmacokinetic properties of HG1 via an oral route and examined its anti-

Candida activity through five different experiments: (i) time-dependent candidacidal test; (ii) an antifungal assay in the presence of normal or clinically obtained saliva; (iii) a colony count assay in the presence of proteases secreted from C. albicans; (iv) an ex vivo antifungal test using mouse tongues infected with C. albicans; and (v) a therapeutic assay in a mouse model of oral candidiasis.

An antifungal drug is normally retained in the oral cavity for 2–3 min during a mouthwash regimen for treating oral candidiasis but its contact with oral mucosa is interrupted by the cleaning action of the oral musculature. Therefore, the rapid action of an antifungal agent is an important property that should be considered when developing a mouthwash treatment; the more rapidly an antifungal agent kills C. albicans, the more effective it should be. The antifungal mechanism of HG1 is different from those of currently available antifungals and the peptide has an effective minimum inhibitory concentration against a variety of fungal strains including C. albicans.16 It has been demonstrated that HG1 exerts its candidacidal activity by binding to the cell wall component (β1,3-glucan) of C. albicans and attacking the cell membrane. In the present study, HG1 exerted profound activity against the pathogenic form of C. albicans (hyphae) within 1 min. Based on its distinct and rapid mode of action, it was suggested that HG1 might retain its potent activity against many Candida spp. that are resistant to conventional antifungal

Figure 6. Therapeutic effect of HG1 in a murine model of oral candidiasis. (a) Treatment was initiated at day 3 after Candida infection in the oral cavity. A 0.02 mL aliquot of HG1 solution (0.5 mg and 1 mg) and nystatin solution (0.5 and 1 mg) was applied to the tongue and spread throughout the oral cavity three times daily for 3 days (n=10). Vehicle without HG1 or nystatin was used as a negative control (n=10). ANOVA test, F=17.70; P<0.001. Horizontal bars represent the mean values for each group. (b) Histopathological examination of tongue sections. Black arrows indicate C. albicans infiltrating the stratum corneum.
drugs and could be useful as a mouthwash ingredient to treat oral candidiasis. We also noted that SAP is a known critical C. albicans virulence factor. SAP genes are expressed by C. albicans during colonization and infection in some patients with oral candidiasis, suggesting that they may be involved in pathogenesis. A member of the SAP family degrades salivary antimicrobial peptides such as histatins, which are important immune factors in the oral environment. Similarly, there are strong concerns that antimicrobial peptides administered as pharmaceutical agents might be inactivated by proteolytic attack of SAPs from C. albicans. Therefore, the structural stability of HG1 against SAPs is a crucial factor affecting the therapeutic potential of HG1 to manage oral candidiasis. HG1 is resistant to attacks by proteases such as trypsin, chymotrypsin and metalloprotease. Moreover, the peptide maintains its antimicrobial activity in the presence of fluid from human skin wounds and proteins extracted from Staphylococcus aureus and Pseudomonas aeruginosa CS. Overall, the resistance of HG1 to proteolysis by a variety of proteases is certainly a critical advantage as a candidate for a novel peptide antibiotic. A variety of azole and polyene antifungals are commonly used for topically treating oral and skin candidiasis. Of these two antifungal groups, azoles, which were once the most widely available drugs for human mycoses, have gradually lost their potency as the result of the emergence of a number of drug-resistant Candida species. In the case of polyenes including nystatin, which is a surface-active drug against fungal cells, they currently occupy the first line of treatment, as polyene-resistant Candida strains have rarely been found. A number of topical preparations featuring nystatin are now employed to manage oral candidiasis. However, oral nystatin rinse is relatively ineffective because of its short contact with the oral mucosa. The drawback of nystatin as an antifungal agent in mouthwash was also shown in the present work (Figure 2). We verified the therapeutic effect of HG1 using a mouse model of oral candidiasis. In fact, an attempt to treat oral candidiasis using an antifungal protein is interesting and unusual, but not unprecedented. Bovine lactoferrin, which is known to have both antifungal and immunomodulatory effects, was demonstrated to have therapeutic potential in a mouse model of oral candidiasis upon its oral administration in drinking water. However, unlike HG1, it was determined that the efficacy of lactoferrin for treatment of oral candidiasis was not a result of direct antifungal action, as lactoferricin B (an antifungal peptide derived from lactoferrin) did not show similar effects in an oral candidiasis model.

In our in vivo experiment, we prepared HG1 or nystatin samples in a high viscosity formulation, as water formulations would not allow the agent to remain for more than a few seconds in the mouse oral cavity. Our formulation (PG-NaP) allowed HG1 or nystatin to adequately contact the oral mucosa of mice. Therefore, it was postulated that nystatin, because of the viscosity of the vehicle, was able to exhibit a therapeutic effect in the in vivo experiment compared with a control sample (Figure 6), although it did not display antifungal activity during a short period of time (Figure 2). In conclusion, HG1 had several advantages that suggest suitability as a novel antifungal agent for the treatment of oral candidiasis: (i) HG1 displayed antimicrobial activity against a variety of C. albicans as reported previously; (ii) it exerted its candidacidal activity quickly enough to be employed as a mouthwash component; (iii) it was capable of killing fungi in the saliva of patients with oral candidiasis; and (iv) its candidacidal activity was not interrupted by the protease (SAP) secreted from C. albicans.

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Transparency declarations
S.H.S., Y.S.L., Y.P.S. and I.H.L. are employees of Hoseo Peptide Inc. All other authors have none to declare.

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