Antimicrobial peptides enhance the candidacidal activity of antifungal drugs by promoting the efflux of ATP from *Candida* cells

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Received 10 March 2005; returned 24 May 2005; revised 21 September 2005; accepted 5 October 2005

**Objectives:** To establish a novel strategy of fungal infection control.

**Methods:** We examined the influences of antimicrobial peptides including a synthesized short lactoferrin peptide (FKCRRWQWRM, Peptide 2; Pep2) on the synthesis of *Candida* cell wall polysaccharides, ergosterol synthesis, membrane permeability and the efflux of ATP.

**Results:** Colony formation of *Candida albicans* was synergistically suppressed by a combination of low concentrations of each drug and peptide. All peptides and amphotericin B, but not itraconazole, revealed weak inhibitory activities against ergosterol synthesis and the peptides weakly suppressed the synthesis of *Candida* cell wall components, glucan, mannan and chitin. Cell membrane permeability was not only increased by these peptides but also clearly increased by both amphotericin B and itraconazole. ATP efflux was however up-regulated by low concentrations of the peptides, especially by Pep2 and Hst5, although both antifungal drugs did not exert any influence on ATP efflux. The expression of the *Candida* drug resistance genes 1 and 2 (CDR1 and CDR2) was increased by both drugs, but this increase was suppressed by each peptide. In addition, larger amounts of amphotericin B and itraconazole remained in *Candida* cells in the presence of Pep2 or Hst5 due to the lower excretion. The effects of both peptides on ATP efflux and increase of intercellular amphotericin B and itraconazole were blocked by anion channel inhibitors 4,4'-disothiocyanato-stilbene-2, 2'-disulphonic acid and 5-nitro-2-(3-phenylpropylamino) benzoic acid.

**Conclusions:** The examined peptides, especially Pep2 and Hst5, enhance the candidacidal activity of antifungal drugs by promoting anion channel-associated ATP efflux from *Candida* cells and decreasing efflux of the drugs, which could be useful clinical applications.

**Keywords:** α-defensin 1, ATP efflux, *Candida albicans*, histatin 5, Peptide 2

**Introduction**

Fungal infection is becoming a serious medical problem because of the difficulty of its control in immunocompromised individuals and because of the emergence of multidrug-resistant fungi, although a variety of antifungal drugs has been developed. Most antifungal drugs have been synthesized with the aim to inhibit the synthesis of cell membrane and cell wall components. However, these inhibitory activities are generally insufficient for completely controlling fungal infection. In addition, antifungal drugs often exert multiple adverse effects and are occasionally dose-limiting. In such circumstances, a novel strategy is required for the control of fungal infections.

Aspergillosis and candidiasis, which are usually caused by *Aspergillus fumigatus* and *Candida albicans*, respectively, are the most frequent of fungal infections. Fungi possess both cell membrane and cell wall, and their cell wall mainly consists of polysaccharides such as glucan, mannan and chitin. In *Candida* cells, glucan is most plentiful in the wall, composing 60–65% of the total polysaccharides and mannan makes up ~20–25%. Chitin composes a small amount, ~5% of the total saccharides in the cell wall. The external layer of the cell wall is mainly composed of mannan. For the *Candida* membrane, the inner layer of cell wall consists of lipids (phospholipids and neutral lipids) and proteins. Ergosterol is the most neutral lipid component and most important for the life of fungi. Among antifungal drugs, azole derivatives inhibit the activity of the ergosterol-synthesizing enzyme cytochrome P450. Recently, a new series of drugs including echinocandin and nikkomycin Z have been developed and their antifungal activities clinically studied. However, the control of...
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deep candidiasis and non-*Candida albicans* such as *Candida dubliniensis*, which was isolated from patients with AIDS,1,10 presents a new problem.

Multiple genes are involved in *Candida* growth and virulence. Of these, *Candida* drug resistance genes 1 and 2 (CDR1, CDR2) and multidrug resistance gene 1 (MDR1) are closely associated with drug resistance.11–13 *Candida* strains expressing high levels of CDR1 and CDR2 are resistant to drugs by utilizing ATP-binding cassette transporters,12 and new antimicrobial agents which are not excreted by the ATP-dependent efflux pump may permit new treatments for fungal and bacterial infections. One of the strategies for preventing drug resistance is therefore to decrease the level of intracellular ATP, which induces inactivation of the ATP-dependent efflux pump. However, such agents have not been clinically used.

The innate immune system has been thoroughly studied in insects, animals and humans, along with the signal pathways of external toxins of Gram-positive and -negative bacteria.14–17 Correspondingly, antimicrobial peptides have been discovered and their structures and biological activities have been well studied.18,19 Among them, short lactoferrin peptides with potent antimicrobial activities have been synthesized.20 Our research group synthesized such a peptide, FKCKRWQWRM (Peptide 2, Pep2), following the N-terminal amino sequence of bovine lactoferrin.21 We previously reported that the survival duration of *Candida*-injected mice is greatly prolonged by the combined injection of Pep2 and amphotericin B, suggesting a new approach for inhibiting fungal infection.21–23 For this novel strategy, the influence of antimicrobial peptides on neutrophils and fungi should be further clarified. In the present study, we examined the influences of Pep2, α-defensin 1 (HNP1) and histatin 5 (Hst5) on the synthesis of *Candida* cell wall polysaccharides, ergosterol synthesis, membrane permeability and the efflux of ATP. Our results support the use of combinations of these peptides and antifungal drugs for treating fungal infection.

Materials and methods

Cell preparation and culture

*C. albicans* strain TIMM0134 was supplied by the Department of Microbiology at our Kochi Medical School. *Candida* cells were grown in Sabouraud’s dextrose agar (Difco, Detroit, MI, USA) at 37°C and inoculated into Sabouraud’s broth medium. After cultivation for 12–18 h, yeast-form cells in the exponential growth phase were used in all experiments. Before use, cells were washed with distilled water and suspended in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal bovine serum (pH 7.0). Viability was confirmed by inoculating cells into serially diluted suspensions on Sabouraud’s dextrose agar plates.

Peptides and antifungal drugs

Pep2 was synthesized by Iwaki Glass Biolab Co. (Chiba, Japan) by a solid-phase method and was purified by HPLC on reverse-phase C18 column. The level of purity was >95%, as analysed from the peak integration with high-performance liquid chromatograms at 214 nm. HNP1 was purchased from Peptide Institute Inc. (Osaka, Japan). Hst5, amphotericin B and itraconazole were obtained from Sigma (Steinheim, Germany).

*Candida* growth inhibition

The candidacidal activities of Pep2, HNP1, Hst5, amphotericin B and itraconazole were examined. *Candida* cells (2 × 10^6 cells/mL) were cultivated for 1–12 h in the presence of the indicated concentration of these agents and washed twice. The cells were then plated on Sabouraud’s dextrose agar plates at 100 μL per plate of 5 cm in diameter. After cultivation for 48 h at 37°C, the colonies were counted. For the assessment of synergistic activities of each peptide with antifungal drugs, cells were treated with weak candidacidal concentrations of antifungal drugs and each peptide. The colony count suppression rate (%) was calculated using the following formula: \([1 - (\text{colonies from suspension with each agent/colonies from suspension without each agent})] \times 100\). Our definition of synergistic effect corresponds to that of a greater effect than the sum of the individual effects caused by the antifungal drug and each peptide in combination. From the colony count suppression rates, curves were drawn and MIC_{50} was determined for each peptide and drug. MIC_{50} was defined as the minimum concentration of antifungal drugs and peptides that completely inhibits the growth of 50% of cultures of *C. albicans*.

Inhibition of ergosterol synthesis

Briefly, *Candida* cells (1 × 10^6 cells/mL) were incubated for 3 h at 37°C in DMEM with Na[^14]C acetate (1 μCi: PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA) and various concentrations of each antifungal agent. After incubation, the cells were saponified in 90% ethanol solution containing 15% potassium hydroxide and 0.1% pyrogallol at 90°C for 1 h. Non-saponifiable lipids were extracted and separated by thin-layer chromatography using precoated silica gel plates (Merck, Darmstadt, Germany) and heptane/diisopropylether/acetic acid/ethyl acetate (60 : 40 : 4 : 34.7, v/v) as a developing solvent. The radioactivity of the ergosterol fraction was then quantified using a BAS2000 bioimaging analyser (Fuji Film, Tokyo, Japan). The percentage inhibition of ergosterol synthesis was calculated as \([1 - (\text{radioactivity of treated cells with each agents/radioactivity of non-treated cells})] \times 100\).

Glucan, mannan and chitin synthesis

*C. albicans* (1 × 10^6 cells/mL) were treated with each antifungal agent for 1, 2 and 3 h at 37°C, and those in DMEM were pulse-labelled for 30 min with 1 μCi of [^3]H[^14]C-acetyl glucosamine and [^14]C-glucose (PerkinElmer Life and Analytical Sciences, Inc.). Radiolabelling was then quenched by the addition of an equal volume of 12% NaOH. NaOH-insoluble pellets containing β-(1,3)-glucan and β-(1,6)-glucan linked to chitin were subsequently treated with 100 U/mL of chitinase and centrifuged at 5000 g for 10 min at 4°C. Chitin synthesis activity was determined from the [^3]H radioactivity in the supernatant and the NaOH-insoluble glucan level was determined from the [^14]C radioactivity of each pellet. The NaOH-soluble supernatant contained mannan and NaOH-soluble glucan. Mannan in the supernatant was precipitated with Fehling’s reagent and [^14]C radioactivity in the supernatant was measured. NaOH-soluble glucan was isolated by the addition of 10 U/mL glucanase to the supernatant and extracted with 70% ethanol, and the NaOH-soluble glucan level was determined from the [^14]C radioactivity. Total glucan synthesis activity was calculated by adding together the NaOH-insoluble and NaOH-soluble glucan levels. The percentage inhibition of glucan, mannan and chitin synthesis was evaluated as \([1 - (\text{radioactivity of treated cells with each agent/radioactivity of non-treated cells})] \times 100\).

Cell membrane permeability

*C. albicans* (1 × 10^7 cells/mL) were incubated with 5 μL of 1 mM calcein acetoxymethyl ester (Sigma) at 5 μM for 2 h at room temperature. The cells were then washed four times to remove unincorporated dye and 100 μL (10^6 cells) was transferred to Eppendorf tubes. After treatment with Pep2, HNP1, Hst5, amphotericin B or itraconazole at
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the indicated concentration for 1, 2 and 3 h at 37°C, the fluorescence intensity of the supernatant (released calcein) was measured at emission wavelengths of 485 and 530 nm using a Hitachi F-2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Calcein release was calculated as the percentage fluorescence release, which is released calcein/inciporated calcein × 100. Incorporated calcein denotes the fluorescence value after boiling the calcein-loaded cells.

ATP efflux

ATP efflux of *C. albicans* was measured using an ATP determination kit (Molecular Probes, USA). Briefly, *C. albicans* (1 × 10⁶ cells/mL) in Eppendorf tubes were treated with Pep2, HNP1, Hst5, amphotericin B or itraconazole for 1, 2 or 3 h at 37°C after pre-cultivation for 30 min in the presence or absence of each channel inhibitor [4-AP, 4-aminopyridine; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; or DIDS, 4,4′-disothiocyanatostilbene-2,2′-disulphonic acid] (Sigma). Each tube was then centrifuged at 5000 g for 5 min and 25 µL of each supernatant was mixed with 225 µL of boiled TE buffer (pH 7.8). The mixture was boiled for 2 min and cooled down at 4°C and stocked until examination. ATP efflux was subsequently determined by the ATP concentration in each supernatant, which was calculated following the manufacturer’s recommendations.

RT–PCR

*Candida* cells (1 × 10⁶ cells/mL) were treated with each antifungal agent and total cellular RNA was extracted using an RNasey Mini Kit (Qiagen Inc., Valencia, CA, USA). The RNA concentration was quantified by measuring the optical density at 260 nm, and RT–PCR analysis was performed using 1 µg of extracted RNA. The following primers were used: *CDR1* (sense: 5′-GCCAAGGGGGAAATTGTITT-3′, antisense: 5′-ATCCATTCTGCTGGATTGC-3′) and *CDR2* (sense: 5′-GGGTATATGCTGGCTCTAATGTTGATC-3′, antisense: 5′-CTGCCCCAAGCCAGTAAAAGAAAATAGTAA-3′), and the PCR cycling conditions were 18 cycles of 2 min at 94°C, 2 min at 54°C, and 2 min at 72°C (*CDR1*) and 30 s at 94°C, 30 s at 58°C and 90 s at 72°C (*CDR2*). mRNA expression levels were evaluated by densitometric analysis of band intensity on agarose gel stained with ethidium bromide.

125I-labelling of antifungal drugs

Amphotericin B and itraconazole were labelled with 125I-diiodinated Bolton–Hunter reagent (PerkinElmer Life and Analytical Sciences, Inc.). Briefly, amphotericin B and itraconazole dissolved in 50 mM NaBO₃ were added to dried Bolton–Hunter reagent (1 µCi), and the mixture was left at 4°C until use. The 125I-labelled antifungal drugs were stored at 4°C until use.

Measurement of the intracellular antifungal drug concentrations

(i) *Candida* cells (1 × 10⁶ cells/100 µL) were poured into 96-well microplates and treated with 125I-labelled antifungal drugs concomitantly with or without Pep2, HNP1 or Hst5 for 1, 2 and 3 h at 37°C. (ii) *Candida* cells (1 × 10⁶ cells/100 µL) were treated first with 125I-labelled antifungal drugs for 1 h, washed twice and suspended in the medium, and then transferred to 96-well microplates. Second, the cells were treated with each peptide for 1, 2 and 3 h at 37°C. After these treatments, *Candida* cells were washed twice with distilled water and harvested on glass fibre filters. The radioactivities in the harvested cells were measured and the intracellular antifungal drug concentrations were determined by the radioactivity. As the control, *Candida* cells treated only by 125I-labelled antifungal drug were used.

Results

MIC₅₀ of peptides, amphotericin B and itraconazole

Among the examined agents, amphotericin B exhibited the lowest MIC₅₀ against the *Candida* strain TIMM0134 from 1–12 h (Table 1). The MIC₅₀ of amphotericin B for 1 h treatment was 0.9 ± 0.2 mg/L in TIMM0134 and decreased along with cultivation time, being reduced to 0.1 ± 0.1 mg/L by 12 h treatment. The MIC₅₀ of itraconazole was almost the same as the MIC₅₀ of amphotericin B throughout the treatment. Among the peptides, Hst5 showed the strongest *Candida* growth inhibitory activity; the MIC₅₀ was 2.0, 1.5, 0.8 and 0.3 µM for 1, 3, 6 and 12 h treatment, respectively. The MIC₅₀ of HNP1 was 1.25- to 3-fold higher than that of Hst5, and the MIC₅₀ of Pep2 was 2.5- to 5-fold higher than Hst5.

Cooperative suppression of *Candida* colony formation by the antifungal drugs and peptides

Pep2, Hst5 and HNP1 synergistically cooperated with amphotericin B and itraconazole to suppress *Candida* colony formation (Figure 1). When *Candida* cells were cultured in the presence of amphotericin B (0.15 mg/L) and Pep2 (0.88 µM), the colony counts at 3 h were decreased from 215 ± 12 colonies/plate to 50 ± 8 colonies/plate, although their own colony formation inhibitory activities at 3 h were very weak (Figure 1a). Through the addition of 0.88 µM of Pep2 and 0.3 mg/L of amphotericin B, *Candida* colony formation was almost completely suppressed at 2 h. The synergistic cooperation of Pep2 with itraconazole was also observed. Hst5 revealed synergistic effects against colony formation cooperating with both antifungal drugs (Figure 1b). The synergism between HNP1 and the drugs was weaker than that in the combination of other peptides and the antifungal drugs and the cooperative suppression of colony formation was additive for the combination of HNP1 and itraconazole but a weak synergistic suppression of colony formation was observed between HNP1 and amphotericin B (Figure 1c).

Inhibitory activity of peptides against ergosterol synthesis

Among the agents examined, only itraconazole strongly inhibited ergosterol synthesis in a dose-dependent manner (Figure 2). The percentage inhibition by 0.1, 0.2 and 0.4 mg/L of itraconazole was

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<th>Table 1. MIC₅₀ of antifungal drugs and peptides</th>
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<td>Agent</td>
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<td>AMB (mg/L)</td>
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40, 48 and 60%, respectively. For amphotericin B and three peptides, dose-dependent inhibition was observed but their inhibition levels were <23% even at high concentrations of these agents. The combination of Pep2 (3.5 μM) with amphotericin B additively inhibited ergosterol synthesis.

**Influence of peptides on synthesis of the cell wall components, glucan, mannan and chitin**

The antifungal drugs amphotericin B and itraconazole strongly suppressed the synthesis of glucan, mannan and chitin (Figure 3).
By treatment with amphotericin B (0.3 mg/L) or itraconazole (0.4 mg/L), the synthesis of glucan was suppressed to 45% of the control value. For mannan and chitin, both drugs suppressed their synthesis to the same degree. The inhibition of glucan and mannan synthesis by Hst5 (1.5 μM) and Pep2 (3.5 μM) was ~20%. Among these, suppression by HNP1 was lowest; the maximal inhibition rate for 1.8 μM of HNP1 was <13%. Pep2 (3.5 μM) and amphotericin B (0.3 mg/L) additively inhibited glucan, mannan and chitin synthesis.

Influence of peptides on cell membrane permeability
Calcein release was highly increased by amphotericin B (0.3 mg/L) and itraconazole (0.4 mg/L) (Figure 4). By treatment of Candida cells with these drugs for 1, 2 and 3 h, the release of calcein was increased by 17–19, 26–28 and 32–35% more than the control, respectively. The percentage increase of calcein release in HNP1 (1.8 μM)-treated cells was 5% after 3 h treatment. Hst5 (1.5 μM) increased the release up to 3% after 3 h treatment than the control. Compared with these agents, Pep2 induced a very weak increase of the cell membrane permeability. Pep2 did not enhance amphotericin B-induced calcein release.

Influence of peptides on ATP efflux
A high level of ATP efflux from Candida cells was induced by treatment with Pep2 or Hst5 (Figure 5a). By 1 h treatment with 3.5 μM Pep2 or 1.5 μM Hst5, 52 and 68 pM of ATP were released, respectively. In Pep2-treated cells, ATP efflux gradually increased with the treatment time and the efflux reached 65 pM after 3 h treatment with 3.5 μM of Pep2. The level of ATP efflux from HNP1-treated cells was the lowest among the three peptides; 41 ± 6, 46 ± 8 and 48 ± 9 pM at 1, 2 and 3 h, respectively (1.8 μM of HNP1). Both antifungal drugs least increased the efflux; the efflux levels by amphotericin B (0.3 mg/L)- and itraconazole (0.4 mg/L)-treated cells were near the control level, 9 and 10 pM, respectively, at 3 h. The increase of ATP efflux by Pep2 was not suppressed by the addition of amphotericin B; the ATP efflux level from cells treated with a combination of Pep2 (3.5 μM) and amphotericin B (0.3 mg/L) was similar to that from cells treated with Pep2 alone. Similarly, the increased ATP efflux by Hst5 and HNP1 was not suppressed by amphotericin B and itraconazole (data not shown). On the other hand, the increase of ATP efflux induced by Pep2 and Hst5 were almost completely abrogated by the anion channel inhibitor DIDS and Cl– channel inhibitor NPPB (Figure 5b and c). The K+ channel inhibitor strongly inhibited the up-regulation of efflux by Pep2 but did not inhibit the up-regulation by Hst5.

Influence of peptides on the mRNA expression of CDR1 and CDR2
The mRNA expression levels of CDR1 and CDR2 were increased by 3 h treatment with amphotericin B or itraconazole (Figure 6). Pep2 and Hsp5 slightly suppressed the expression of CDR1 mRNA and HNP1 also weakly suppressed the expression of CDR2 mRNA. All peptides suppressed the amphotericin B- and itraconazole-induced up-regulation of the CDR1 and CDR2 mRNA expression.

Influence of peptides on the intra-candidal concentrations of amphotericin B and itraconazole
Candida cells were cultured in the presence of 125I-labelled amphotericin B or itraconazole with or without each peptide for the
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Figure 3. The influence of peptides on synthesis of the cell wall components, glucan, mannan and chitin. *Candida* cells (1 x 10^6 cells/mL) were labelled with [3H]-N-acetylglucosamine and [14C]glucose in DMEM for 30 min at 37°C and cultured in the presence or absence of Pep2 (1.75 µM, closed circles; 3.5 µM, open circles), HNP1 (0.9 µM, closed diamonds; 1.8 µM, open diamonds), Hst5 (0.75 µM, closed squares; 1.5 µM, open squares), amphotericin B (0.3 mg/L, open triangles), itraconazole (0.4 mg/L, closed triangles) and Pep2 + AMB (3.5 µM and 0.3 mg/L, closed inverted-triangles) for the indicated times at 37°C. Each cell wall fraction was then obtained and the % inhibition of glucan, mannan and chitin was calculated according to the method and formula shown in the Materials and methods section. (a) Percentage inhibition of glucan synthesis. (b) Percentage inhibition of mannan synthesis. (c) Percentage inhibition of chitin synthesis. Each bar indicates the mean ± 1 SD of triplicate assays. *P < 0.05 versus control (by t-test).

Figure 4. The influence of peptides on cell membrane permeability. *Candida* cells (1 x 10^6 cells/mL) were cultured in the presence of calcein acetoxymethyl ester (5 mM/mL) for 2 h at room temperature. After washing four times, the cells were cultured in the presence or absence of each peptide or antifungal drug for the indicated times at 37°C. Control cells were treated without each agent. The amount of calcein released was then quantified by the method shown in the Materials and methods section, and the membrane permeability was determined by the calcein release. Each bar is the mean ± 1 SD of triplicate assays. *P < 0.05 versus control (by t-test).

Discussion

Recently, the biological activities of antimicrobial peptides have been studied and their roles in protection against infection, especially in the local immune system against microbial invasion, has been explored. A variety of epithelial and non-epithelial cells generate antimicrobial peptides, which protect the skin and mucosa from the growth and invasion of bacteria, fungi and viruses. For example, saliva maintains the integrity of the oral mucosa and protects it from dryness and microbial growth and invasion by possessing antimicrobial peptides such as histatins, α- and β-defensins, cystatins, lactoferrin and mucin. Generally, the antimicrobial activities of these peptides depend on destruction (rupture) of bacterial and fungal cell membranes, suppression of mitochondrial respiration or glucose utilization, and activation of neutrophils and macrophages. Recently, the indicated times, and the intracellular isotope activity was measured (Figure 7a). The intracellular isotope activities at 1 h were almost the same as the control level but the influence of the peptides became visible after 2 h. Compared with the control cells (without any peptides), cells cultured in the presence of Pep2 (0.88 µM) or Hst5 (0.33 µM) revealed higher intracellular isotope activities at 2 and 3 h. HNP1 (0.45 µM) also increased the intracellular activity but the increase was smaller than those in other peptides. The anion channel blocker DIDS (5 mg/L) almost abrogated the increase of isotope activities. When *Candida* cells were treated with each peptide after incubation with [125I]-labelled amphotericin B or itraconazole, the levels of isotope were largely increased at 1, 2 and 3 h, and the increase was inhibited by DIDS (Figure 7b).
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The influence of peptides on ATP efflux. Candida cells were treated with each peptide or antifungal drug for the indicated times after pre-cultivation for 30 min in the absence (a) or presence (b and c) of each channel inhibitor. Each bar indicates the mean ± 1 SD of triplicate assays. *P < 0.05 versus control (by t-test). AMB, amphotericin B; ITC, itraconazole.

Figure 5. The influence of peptides on ATP efflux. Candida cells were treated with each peptide or antifungal drug for the indicated times after pre-cultivation for 30 min in the absence (a) or presence (b and c) of each channel inhibitor. Each bar indicates the mean ± 1 SD of triplicate assays. *P < 0.05 versus control (by t-test). AMB, amphotericin B; ITC, itraconazole.

mechanism of antifungal activities of antimicrobial peptides has been explored but much remains to be clarified.

The Candida cell wall mainly consists of polysaccharides such as glucan, mannan and chitin. Among these, glucan is the highest constituent and gives the wall its mechanical strength in cooperation with chitin. Mannan exists on the surface layer of the cell wall and plays an important role in the adhesion between Candida cells and dermal and mucosal surfaces. Several antifungal drugs were recently developed aimed at inhibition of glucan and chitin synthesis. It was reported that amphotericin B and itraconazole induced increase of membrane permeability in C. albicans and stopped glucose metabolism. In the present study, the examined peptides and amphotericin B revealed a weak polysaccharide synthesis-inhibitory activity, and these agents exhibited only weak suppression of ergosterol synthesis. However, the peptides synergistically cooperated with amphotericin B and itraconazole in low concentrations compared with their individual MIC₅₀ by inhibiting Candida colony formation. In the combination, the cooperation between HNPI and itraconazole was additive but Pep2 and both drugs as well as HNPI and amphotericin B, and Hst5 and both drugs revealed a synergistic suppression against the colony formation. Therefore, we aimed to explore the mechanism of these synergistic effects.

Cell membrane permeability was not increased by the examined peptides, although both antifungal drugs increased cell permeability. Conversely, the ATP efflux was not increased by treatment with amphotericin B or itraconazole but was increased by the peptides. A large amount of ATP was excreted by Candida cell treatment with each peptide in a time-dependent manner. The increased ATP efflux was almost completely suppressed by DIDS and NPPB but only weakly by 4-AP in Pep2-treated cells, while the increase of ATP efflux was not inhibited by 4-AP in Hst5-treated cells. Therefore, the increased ATP efflux induced by these peptides appears to be closely associated with anion channels, especially Cl⁻ channels and weakly associated with Na⁺/K⁺ channels in Pep2. There are multiple studies concerning the mechanism of ATP efflux. It was previously reported that ATP efflux induced by the mast cell degranulator compound 48/80 was mediated by intracellular Ca²⁺, which was increased in association with the activation of guanine-binding protein and phospholipase C. According to another series of our experiments, Pep2 activates pertussis toxin-insensitive and cholera toxin-sensitive G-protein, and the activated signals down-stream through phosphatidylinositol 3-kinase to protein kinase C. Taken together with these reports and results, the present study appears to show that Pep2-induced ATP efflux is mediated by G-protein activation.

The membrane transportation of substances is essential for the maintenance of cell activity. Na⁺/K⁺ and Cl⁻ channels in fungal cell membranes play a role essential for cell life as in mammalian cells. There are two modes of substance transportation through these channels, passive as well as active transportation which requires ATP. Hst5 binds heat shock protein 70 (HSP70) on the membrane, and this is inhibited by Ca²+. HSP70 possesses an ATPase domain and Hst5-binding domain, suggesting that the decrease in intracellular ATP in Hst5- and probably Pep2-treated Candida cells partially results from the binding of ATPase to these peptides. The influence of these peptides on ATP synthesis from glycolysis may be involved in the regulation of ATP level, but the utilization of glucose was only weakly decreased by the peptides although the examined peptides strongly down-regulated the expression of enolase (data not shown). Therefore, the increase of ATP efflux and decrease in intracellular ATP appears to largely depend on the opening of anion channels.

There are multiple mechanisms that confer drug-resistance in bacteria and fungi. Active drug transportation (efflux) is, however, the main cause of drug resistance. In Candida cells,
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Figure 6. The influence of peptides on the mRNA expression of CDR1 and CDR2. Candida cells (1 × 10⁶ cells/mL) were cultured in the presence or absence of amphotericin B (AMB, 0.3 mg/L) or itraconazole (ITC, 0.4 mg/L) with or without Pep2 (0.88 µM), Hst5 (0.33 µM) or HNP1 (0.45 µM) for 3 h at 37°C. Total RNA was then extracted and the mRNA expression of CDR1 and CDR2 was examined by using RT-PCR as shown in the Materials and methods section. Each Arabic number indicates the proportion of each mRNA expression relative to the control level.

Figure 7. The influence of peptides on the intracellular concentration of antifungal drugs. (a) Candida cells were cultured for 1, 2 and 3 h in the presence of [125I]-labelled amphotericin B (AMB) or itraconazole (ITC) with or without Pep2, Hst5 or HNP1. (b) After the incubation of Candida cells in the presence of [125I]-labelled amphotericin B or itraconazole for 1 h, the cells were treated with Pep2, Hst5 or HNP1 for 1, 2 and 3 h at 37°C. The Candida cells were then washed twice with distilled water and the concentrations of intracellular antifungal drugs were estimated using the method shown in the Materials and methods section. Each bar indicates the mean ± 1 SD of triplicate assays. *P < 0.05 versus control (by t-test).
the drug transporter is regulated by CDR1 and CDR2. As shown in the results, CDR1 and CDR2 expression did not increase in peptide-treated Candida cells, although amphotericin B- or itraconazole-treated Candida cells revealed an increase in the expression of these genes. However, expression of these genes did not increase in Candida cells when they were treated with these drugs in the presence of low concentrations of each peptide. In addition, the increase of ATP efflux by Pep2 and Hst5 appears to indicate an inactivation of CDR1 and CDR2 because CDR1 and CDR2 are an ATP-dependent efflux pump of antifungal agents.

The anticandidal synergism of these peptides and antifungal drugs is therefore interpreted to be dependent on the fact that in addition to the peptides’ own Candida growth inhibitory activities, these antimicrobial peptides sustain the intracellular concentrations of antifungal drugs by inhibiting the efflux pumps. This interpretation indicates the advantage of using these peptides in combination with antifungal drugs, and reveals that multidrug-resistant strains are least induced by antimicrobial peptides.

The present results suggest the role of peptides in local immunity against microbial invasion. When only a low concentration of Hst5 generated from affected epithelial cells is present at the infectious site, the administered antifungal drugs can control parasites on the body surface and mucosae even if the local concentration of antifungal drug is low. In addition, synergistic cooperation between the peptides and antifungal drugs indicates a novel strategy for the treatment of fungal and bacterial infections. The local application of each peptide appears to be useful for the establishment of this novel strategy. However, the oral administration of the peptides is required for treatment of deep organ candidiasis. The pharmacodynamics of orally administered peptides will be explored in the future for the treatment of deep candidiasis with a combination of antimicrobial peptides and antifungal drugs.

**Transparency declarations**

No declarations were made by the authors of this paper.

**References**


Antimicrobial peptides promote ATP efflux