Antifungal effect and action mechanism of antimicrobial peptide polybia-CP

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The incidence of life-threatening invasive fungal infections increased significantly in recent years. However, the antifungal therapeutic options are very limited. Antimicrobial peptides are a class of potential lead chemical for the development of novel antifungal agents. Antimicrobial peptide polybia-CP was purified from the venom of the social wasp Polybia paulista. In this study, we synthesized polybia-CP and determined its antifungal effects against a series of Candidian species. Our results showed that polybia-CP has potent antifungal activity and fungicidal activity against the tested fungal cells with a proposed membrane-active action mode. In addition, polybia-CP could induce the increase of cellular reactive oxygen species production, which would attribute to its antifungal activity. In conclusion, the present study suggests that polybia-CP has potential as an antifungal agent or may offer a new strategy for antifungal therapeutic option. Copyright © 2015 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimicrobial peptide; polybia-CP; candidiasis; antifungal; ROS production

Introduction

Antimicrobial peptides (AMPs) have been found almost in all kinds of organism, including bacteria [1], plants [2], insects [3], amphibians [4–6], mammals, and humans [7]. Some of them play an important role in the immune system to defend the invasive infection of microbial organism. AMPs were believed to be the first defensive line of living creature. It has been shown that antimicrobial peptides have extensive biological activities, such as antibacterial, antifungal, anti-inflammatory, antiviral, and antitumor activities [8,9]. The possible mechanism of AMPs involves physically interfering the integrity of biological membrane, causing the depolarization of bio-membrane, interfering the transcription of genome DNA, and so on [10,11]. Notably, the membrane-active action mode was believed to be the superiority of antimicrobial peptides against conventional antibiotics, which target at specific cellular molecules. Because it is difficult for microbial organism to change the basic component of bio-membrane, the development of resistance to AMPs is thought to be considerably reduced compared with conventional antibiotics. Furthermore, the effect of conventional multidrug-resistant mechanism on the activity of AMPs was limited. Recently, the increase of infection of microbial organism, especially the acquired multidrug-resistant microbial organism, has resulted in a significant threat to human health, even life. So the search of the alternatives of conventional antimicrobials is urgently needed. As ideal candidates, AMPs attract more and more attention for the development of new class of therapeutic agents against microbial invasion.

Antimicrobial peptide polybia-CP (ILGLTILGLKSL-NH₂) was originally isolated from the venom of the social wasp Polybia psulista [12]. It has been shown to exhibit antibacterial activity and antitumor activity with a membrane-active action mode [13,14]. However, its antifungal activity has not been reported in all the literature. As we know, fungi infections have increased dramatically during the past three decades, especially the life-threatening invasive fungal infection. Candida infections have the highest incidence in the invasive fungal infection. Candidiasis encompasses infections that range from superficial, such as oral thrush and vaginitis, to systemic and potentially life-threatening diseases [15]. Frustratingly, the therapeutic option is far more limited, because there are a few classes of antifungal agents against fungi infection.

In the present study, the antifungal effect of polybia-CP against some Candida species was determined. Meanwhile, the possible action mode of polybia-CP also was investigated. We found that polybia-CP exhibits its antifungal activity through a multi-action mode-involved mechanism, not only disturbing the integrity of membrane but also interfering the function of mitochondrion. Our results support that AMPs may be potential candidates for clinical therapeutic use.

Materials and Methods

Peptide Synthesis

Polybia-CP was synthesized by a stepwise solid-phase method on rink amide MBHA resin using N-9-fluorenylmethoxycarbonyl (Fmoc).

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Antifungal Activity Assays

(1) Fungi strains and culture. Fungal strains used in this study were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) as follows: Candida albicans (ATCC 14053), Candida glabrata (ATCC 2001), Candida parapsilosis (ATCC 22019), Candida tropicalis (ATCC 750), and Candida krusei (ATCC 6258). C. glabrata 14-1, C. albicans 14-2, C. albicans 14-3, and C. albicans 1-4 were clinically isolated. Dry powder of Candida strains was dissolved in sterile 10 mM phosphate-buffer solution (PBS) (pH 7.4), vortexed, and spread on Sabouraud dextrose broth plate. After overnight incubation at 35 °C, a colony was cultured into Sabouraud dextrose broth medium at 35 °C for 12 h with shaking to acquire logarithmic phase. The fresh culture was centrifuged, washed, re-suspended in 25% C3H8O3/H2O, and stored at –80 °C before use.

(2) The minimum inhibition concentration (MIC) assay. The anti-fungal assay was determined according to the EUCAST method with minor modifications [18]. Briefly, cells activated in Sabouraud dextrose broth medium were washed, diluted, and inoculated into 96-well flat-bottom plates containing serially twofold dilutions of polybia-CP. The plates with an inoculum size of 0.5 × 10^3 to 2 × 10^3 colony-forming unit (CFU) were statically placed in an incubator at 35 °C overnight. MIC of polybia-CP was defined as the lowest concentration at which no visible turbidity was observed compared with drug-free group.

(3) The minimum fungicidal concentration (MFC) assay. For MFC determination, 100 μl cultures at concentration equal to or above MIC were plated on Sabouraud dextrose broth plate for CFU counting. After incubation at 35 °C for 24 h, the MFC was defined as the lowest concentration that resulted in 99.9% killing compared with drug-free group.

(4) Radial diffusion assay. The fungicidal activity of polybia-CP was evaluated by a modification of the sensitive radial diffusion assay. Briefly, the fungi cells were cultured as described earlier. One milliliter of the fungi 10^7 CFU was added to 100 ml of previously autoclaved, warm Sabouraud dextrose agar. After rapid dispersion of the fungi, the agar was poured into an agar plate to form a layer approximately 5 mm deep and was punched with a 3-mm-diameter gel punch to make evenly spaced wells. Following the addition of 25 μl with different concentrations of polybia-CP to each well, the plates were incubated at 35 °C for 18–24 h. Twenty microfilters of sterile water was also added as a control.

(5) Time-killing kinetics of polybia-CP against Candida cells. Time-killing kinetics of polybia-CP against Candida cells were performed as follows. Logarithmic cultures diluted to an inoculum size of 10^5–10^6 CFU in fresh Sabouraud dextrose broth medium were incubated with polybia-CP at different concentrations. To determine the viabilities of the cultures, the cells were then taken and serially diluted at different intervals after treatment and plated on Sabouraud's dextrose agar plates of 5 cm in diameter for CFU counting. The original number of colonies was confirmed at time zero. The killing activity of polybia-CP was illustrated by plotting the log_{10} CFU per milliliter against incubation time. Sabouraud dextrose broth medium was run as a negative control.

Hemolysis of Human Red Blood Cells

Hemolytic activity was investigated according to Yan with a minor modification [19]. Fresh red blood cells were collected in heparinized tube and centrifuged at 800 rpm for 10 min. The pellet was washed three times with PBS (10 mM, pH 7.4) and suspended in the same buffer to a final erythrocyte concentration of 8%. The red blood cell suspension (100 μl) was added to a 96-well microtiter plate. Polybia-CP dissolved in water was added to the wells of a 96-well plate by serially diluted twofold (100 μl per well) and incubated for 60 min at 37 °C. One per cent Triton-X 100 and PBS were used as positive and negative controls, respectively. The release of hemoglobin of the supernatants was measured by microplate reader (Bio-Rad Laboratories) at 490 nm after centrifugation (1200 rpm, 15 min).

Binding Effects of Polybia-CP to the Cell Wall Component of Candida Cells

Binding of polybia-CP to the surface of Candida cells was examined by assessing the effect of representative fungal cell wall polysaccharide mannan (Sigma-Aldrich, St. Louis, MO, USA, catalog no. M7504) and laminarin (Sigma-Aldrich, catalog no. L9634) on killing activity of polybia-CP described before [20]. In brief, polybia-CP (final concentration, 256 μM) reacted with increasing concentration of each representative cell wall polysaccharide at 35 °C for 1 h. After incubation, 100 μl of samples was collected directly into tubes containing an equal volume of Candida cultures with an inoculum size of 2 × 10^5 CFU for 1 h and serially diluted in Sabouraud dextrose broth medium. Aliquots of 100 μl were spread onto Sabouraud dextrose broth agar plates and incubated for 48 h at 35 °C. The killing rate % = 100 × (1 – F/F_0), in which F_0 and F represented the CFU of polysaccharide-free group and polysaccharide-treated group. The figure was plotted as killing rate against polysaccharide concentration.

Determination of Fungal Cell Integrity Using PI Uptake

(1) Confocal laser scanning microscopy. The propensity of polybia-CP to cause loss of membrane integrity was measured by propidium iodide (PI) uptake assay using laser confocal scanning microscope and fluorescence-activated cell sorting (FACS). Briefly, freshly collected logarithmic C. glabrata (ATCC 2001) cultures with an inoculum size of 10^6–10^7 CFU were incubated with polybia-CP at 32 μM (2 × MIC) or PBS as negative control. After incubation at 35 °C for 3 h, PI solution was added to acquire a final concentration of 50 μg/ml, and a further incubation was conducted for 15 min. At the end of incubation time, cells were centrifuged, washed, and re-suspended with PBS, and microscopic analysis was performed with laser confocal scanning microscope (LSM 710 META; Zeiss, Oberkochen, Germany).
(2) Flow cytometric analysis. To quantitatively analyze the loss of fungal membrane integrity after peptide treatment, C. glabrata cells (10^6–10^7 CFU) were treated with increasing concentration of polybia-CP and incubated for 2 h at 35°C. After incubation, the cells were stained with PI (final concentration = 50 μg/ml) and incubated for 15 min at room temperature in dark place. Subsequently, the cells were harvested by centrifugation, washed, and suspended in PBS. Flow cytometric analysis was conducted by a FACS Calibur flow cytometer (Becton–Dickinson, San Jose, CA, USA).

Scanning Electron Microscopy

To view the morphological change of fungal cells after treatment with polybia-CP, electron microscopy was employed. In brief, C. glabrata cells grown to logarithmic phase with an inoculum size of 10^6–10^7 CFU were incubated with polybia-CP at the concentration of 125 μM or Sabouraud dextrose broth medium as negative control. After incubation for 2 h at 35°C with shaking, the cells were centrifuged, washed twice with PBS, re-suspended, and fixed with an equal volume of 5% (v/v) glutaraldehyde in 0.2 M sodium cacodylate buffer at 4 °C overnight. The pellet was re-suspended. A drop containing treated C. glabrata cells was deposited onto a carbon-coated grid and negatively stained with 1% uranyl acetate. The grids were examined by using a scanning electron microscope (JSM–6380Lv, Tokyo, Japan).

Detection of Polybia CP-induced Cellular Reactive Oxygen Species Production

Cellular amounts of reactive oxygen species (ROS) were measured by fluorometric assay with 2',7'-dichlorofluorescin diacetate (DCFH-DA) as described earlier [21]. Briefly, the logarithmic cultures were centrifuged, washed, and re-suspended in PBS. Then the cultures were treated with polybia-CP alone or in combination with ascorbic acid or left untreated for 1 h at 35°C. After incubation, 10 μM DCFH-DA in PBS was added. The fluorescence intensities of the treated cells were measured with a spectrofluorometer at different intervals.

| Table 1. MFC values of polybia-CP against the tested Candida cells |
|-------------------|-------------------|-------------------|-------------------|-------------------|
| Peptide          | MFC (μM)          |                  |                  |                  |
| Candida glabrata  | Candida albicans  | Candida tropicalis | Candida krusei    | Candida glabrata  |
| ATCC 2001        | ATCC 14053        | ATCC 750          | ATCC 6258        | 14-1              |
| Polybia-CP       | 32                | 64                | 8                | 32                |
| Candida albicans  | Candida albicans  | Candida albicans  | Candida albicans  | Candida albicans  | Candida albicans |
| ATCC 2001        | ATCC 14053        | ATCC 750          | ATCC 6258        | 14-2              | 14-3              | 1-4               |
| Polybia-CP       | 32                | 64                | 32               | 64                | 32                |

| Table 2. MIC values of polybia-CP against the tested Candida cells |
|-------------------|-------------------|-------------------|-------------------|-------------------|
| Peptide          | MIC (μM)          |                  |                  |                  |
| Candida glabrata  | Candida albicans  | Candida tropicalis | Candida krusei    | Candida glabrata  |
| ATCC 2001        | ATCC 14053        | ATCC 750          | ATCC 6258        | 14-1              |
| Polybia-CP^a     | 16                | 16                | 4                | 32                |
| Candida albicans  | Candida albicans  | Candida albicans  | Candida albicans  | Candida albicans  | Candida albicans |
| ATCC 2001        | ATCC 14053        | ATCC 750          | ATCC 6258        | 14-2              | 14-3              | 1-4               |
| Polybia-CP       | 16                | 16                | 16               | 32                | 16                |

Results

Peptides

We focus on investigating the biological activity and action mechanism of antimicrobial peptides with short sequence and its potential as therapeutic agents. In the present study, short 12-mer antimicrobial peptide polybia-CP was synthesized by stepwise solid-phase method.

Antifungal Activity of Polybia-CP

In the present study, the antifungal effects antimicrobial peptide polybia-CP against five standard fungal strains and four hospital isolated strains were determined. As shown in Table 1, polybia-CP had antifungal activity, with MIC values in the range of 4–64 μM. Polybia-CP exhibited lower MIC value against C. tropicalis than that of other Candida species tested. Fungicidal activity of polybia-CP also was accessed by MFC assay, time-killing assay, and radical diffusion assay. As shown in Table 2 and Figures 1 and 2, our result showed that polybia-CP exerted candidacidal activities. The MFC value ranges from 2 * MIC to 4 * MIC. The fungicidal activity of polybia-CP appeared to be concentration and time dependent.

The Hemolytic Activity

The hemolytic activity of polybia-CP was determined by the lysis of human erythrocytes. As shown in Figure 3, it caused less than 20% lysis of the human erythrocytes at the effective concentration of 30 μM under which polybia-CP exhibited potent antifungal activity. So it could be considered that polybia-CP has low hemolytic activity at its work concentration.

The Binding of Polybia-CP to the Component of Fungal Cell Wall

The specific binding effects of polybia-CP toward fungal cell surface were examined by determining the effects of two main polysaccharides component of fungal cell wall on the antifungal activity of polybia-CP. As shown in Figure 4, the incubation of polybia-CP with...
Figure 1. The fungicidal activity of polybia-CP (0, 100, 200, 300, and 400 μM) against Candida krusei ATCC 6258 (A), Candida glabrata ATCC 2001 (B), Candida albicans ATCC 14053 (C), and Candida tropicalis ATCC 750 (D) was determined by radial diffusion assay. Polybia-CP made fungi-free zones around the wells in a concentration-dependent manner (the diameter of wells was 3 mm).

Figure 2. Time-killing kinetics of polybia-CP against the tested Candida cells: (A) Candida glabrata ATCC 2001, (B) Candida albicans (ATCC 14053), (C) Candida krusei (ATCC 6258), and (D) Candida tropicalis (ATCC 750). Cells were cultured in the presence of polybia-CP at indicated concentrations or Sabouraud's dextrose broth (0 μM polybia-CP) as negative control with shaking. The residual viable cells were monitored as the numbers of CFU on Sabouraud's dextrose agar plates of 5 cm in diameter at different point-in-time after treatment.
increased amount of laminarin could significantly reduce its antifungal activity in both of tested Candida strains. By contrast, the incubation of polybia-CP with mannans had limited effects on its antifungal activity. It could be concluded that polybia-CP could bind to Candida cell surface by interaction with the polysaccharides component of cell wall and then exert its antifungal activity.

Polybia-CP Disrupted the Integrity of Fungal Membrane

The effect of polybia-CP on the integrity of fungal membrane was analyzed by monitoring the uptake of PI. Our results showed that polybia-CP could induce PI that entered into the Candida cells and exhibited increased fluorescence intensity. As shown in Figure 5, there is no fluorescence in the control group, while there is fluorescence in the Candida cell pre-incubated with polybia-CP. This result was consistent with the results monitored by flow cytometry assay. When compared with the control group, polybia-CP induced increased fluorescence intensity shifts in the tested Candida cells in a concentration-dependent manner (Figure 6). This indicated that polybia-CP could cause membrane permeabilization.

Scanning Electron Microscopic Observation

The morphological changes induced by polybia-CP in the C. glabrata cells were evaluated by scanning electron microscopy. Our results showed that the untreated cells exhibited normal, smooth surfaces (Figure 7A), while the cell surface of the C. glabrata cells F7 in the treated group indicated significant changes, including surface roughening and disruption (Figure 7B). This would be a strong evidence of that the cell membrane of C. glabrata had been disrupted by polybia-CP. We consider that this process would be attributed to the exertion of the fungicidal effects of this kind of compounds.

Polybia-CP Could Induce Fungal Cellular Reactive Oxygen Species Production in a Time-dependent and Dose-dependent Manner

The effect of polybia-CP on the cellular ROS production was determined by DCFH-DA assay. DCFH-DA was a cell-permeant dye, which could be oxidized by ROS and turned to a fluorescent molecule 2,7-dichlorofluorescein with the maximum excitation wavelength of 488 nm and the maximum emission wavelength of 525 nm. Compared with the control cells, polybia-CP induced a rapid increase in fluorescence in a dose-dependent and time-dependent manner. No fluorescence increase was detected when the cells were treated with polybia-CP in combination with a well-known antioxidant ascorbic acid (Figure 8).

Discussion

In the past two decades, as the AIDS epidemic, the increased number of organ transplantation, and cancer therapy, the incidence of life-threatening invasive fungal infections has increased dramatically [22,23]. Among the commensal fungi, Candida species often cause invasive fungal infections in their immunocompromised host and lead to much greater morbidity and mortality [24]. However, there are few antifungal agents for therapeutic option. So there is an urgent need for the development of antifungal lead chemical structure. Nowadays, as an integral component of the innate immune system of all biological organisms, AMPs attract more and more attention for the development of antifungal agents.

More than 100 natural AMPs have been determined to exhibit antifungal activity against pathogenic fungi in the last 40 years. Antimicrobial peptide polybia-CP had been revealed to exhibit
antibacterial activity against both Gram-positive and negative bacteria [14]. So it was anticipated to exhibit antifungal effects. In the present study, we assessed the antifungal activity of polybia-CP against a panel of Candidian species, including *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*.

The fungal cell wall is made up of different components, mainly, polysaccharides, proteins, and lipids. Among the polysaccharides, glucan is the plentiful, comprising 60–65% of the total polysaccharides, and mannan makes up 20–25% [25]. In the present study, the polysaccharide-binding study was employed to elucidate the effects of polysaccharides on the exertion of antifungal activity of polybia-CP. As we know, the electrostatic interaction between cationic antimicrobial peptides and negatively charged components in the outside of bacteria may be the driven force of the binding of peptide toward bacteria. Our results revealed that polybia-CP could bind with the fungal cell wall polysaccharides, because the pre-incubation of peptides and polysaccharides could significantly decrease the antifungal effects of polybia-CP. The binding effects may be the first step of polybia-CP to exert its antifungal activity. Then, the effect of polybia-CP on the integrity of fungal cell membrane was determined by PI uptake assay. PI is a fluorescent molecule, which could bind to DNA by intercalating between the bases, with little or no sequence preference. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20- to 30-fold. It is membrane impermeable and

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**Figure 5.** Microscopy analysis of membrane permeabilization detected by propidium iodide uptake assay in *Candida glabrata* cells after incubation with 0 μM peptides (A–C) and 32 μM peptides (D–F) for 3 h at 35 °C.

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**Figure 6.** Flow cytometric analysis of membrane permeabilization. *Candida glabrata* cells treated with polybia-CP exhibit increased PI uptake. *Candida glabrata* cells were incubated for 2 h at 37 °C with polybia-CP (16, 32, 64, and 128 μM), followed by a 30-min incubation with 50 mg/ml PI. Cells were washed three times in 10 mM sodium phosphate buffer (pH 7.4) and analyzed by FACS.

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**Figure 7.** Scanning electron microscopy of polybia CP-treated *Candida glabrata* cells. Scanning electron micrographs revealed that the surfaces of untreated *C. glabrata* cells were smooth (A) and the surfaces of the polybia CP-treated cells were ruffled and became disrupted (B). Bars = 1 μM.
In conclusion, polybia-CP exerts a potent antifungal activity with a membrane-active action mode. In addition, it also could induce significant increase of cellular ROS production in a time-dependent and concentration-dependent manner. The excessive amounts of ROS interfered with the cellular events and lead the fungal cell to death. This may be involved in the fungistatic or fungicidal activity of polybia-CP.

Acknowledgements

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References

20. Jang WS, Bajwa JS, Sun JW, Edgerton M. Salivary histatin 5 internalization to the fungal cell, which was generally excluded from viable cells and is commonly used for identifying dead cells in a population and as a counterstain in multicolor fluorescent techniques [26]. Polybia-CP could disrupt the integrity of and induce the PI entering into the fungal cell, which was detected by LSMS and FACS. We can cautiously speculate that the following steps may be involved when polybia-CP exerts the antifungal activity: first, it binds to surface of fungal cells by interacting with the polysaccharides; then, the interaction of peptides and lipid membrane drives the exertion of antifungal activity.

In order to explore the effects of polybia-CP on the intracellular events of fungal cells, DCFH-Da assay was employed to detect the cellular ROS production induced by polybia-CP. ROS play an important role in cellular physiology. Under the environmental stress, the cellular redox balance would be disturbed, and ROS production would increase dramatically, resulting in significant damage to cellular proteins, lipids, and DNA [27]. Our results showed that polybia-CP could induce significant increase of cellular ROS production in a time-dependent and concentration-dependent manner. The excessive amounts of ROS interfered with the cellular events and lead the fungal cell to death. This may be involved in the fungistatic or fungicidal activity of polybia-CP.

Figure 8. Effect of polybia-CP on the cellular ROS formation of Candida albicans cells. The C. albicans cells were incubated in the presence of polybia-CP (128 and 256 µM) or polybia-CP (128 µM) + ascorbic acid (an antioxidant, 5 mM) or PBS solution as negative control for 1 h at 35°C. Then 10 µM 2,7′-dichlorofluorescin diacetate (ROS-sensitive probe) was added. The fluorescence was measured using a spectrofluorometer at indicated time intervals.

In conclusion, polybia-CP exerts a potent antifungal activity with a membrane-active action mode. In addition, it also could induce an increase of cellular ROS production, which may contribute to the antifungal activity of polybia-CP. Therefore, polybia-CP could be developed as a novel antifungal candidate or a lead structure for developing new therapeutic agents.


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   Marks a point in the proof where a comment needs to be highlighted.

   **How to use it**
   - Click on the Add sticky note icon in the Annotations section.
   - Click at the point in the proof where the comment should be inserted.
   - Type the comment into the yellow box that appears.
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

How to use it
- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you’d like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

6. Add stamp Tool – for approving a proof if no corrections are required.

How to use it
- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you’d like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).

7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

How to use it
- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: