Biochimica et Biophysica Acta 1848 (2015) 2365–2373

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Antimicrobial peptide protonectin disturbs the membrane integrity and induces ROS production in yeast cells

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ARTICLE INFO

Article history:
Received 28 January 2015
Received in revised form 11 July 2015
Accepted 19 July 2015
Available online 21 July 2015

Keywords:
Protonectin
Antifungal
ROS production
Biofilm formation

ABSTRACT

Candidiasis is often observed in immunocompromised patients and is the 4th most common cause of bloodstream infections. However, antifungals are limited, so novel antifungal agents are urgently needed. Antimicrobial peptides (AMPs) are considered as potential alternatives of conventional antibiotics. In the present study, antimicrobial peptide protonectin was chemically synthesized and its antifungal activity and mode of action were studied. Our results showed that protonectin has potent antifungal activity and fungicidal activity against the tested fungi cells. Its action mode involved the disruption of the membrane integrity and the inducing of the production of cellular ROS. Furthermore, protonectin could inhibit the formation of biofilm and kill the adherent fungi cells. In conclusion, with the increase of fungal infection, protonectin may offer a new strategy and be considered as a potential therapeutic agent against fungal disease.

1. Introduction

Candida is a causal trigger of opportunistic oral and genital infections that frequently coexists in the human oral cavity and gastrointestinal tract. In general, *Candida* is innocuous although overgrowth of *Candida* results in candidiasis. Candidiasis is often observed in immunocompromised patients (e.g., AIDS) and is the 4th most common cause of bloodstream infections, with an estimated mortality rate upwards of 25% [1]. However, treatment against *Candida* infections involving triazoles (i.e. fluconazole and itraconazole) is often inoperative because *Candida* cells could acquire resistance after long periods of treatment, leading to higher susceptibility to fungal infections [2]. The severe adverse effects of antifungals like liver damage, allergic reactions or disorder of estrogen levels also made the traditional antifungals have limited clinical application [3]. In addition, the formation of highly drug-tolerant biofilm by *Candida* cells further exacerbates the antifungal therapeutic challenge [4,5]. So a novel strategy is eagerly needed for the control of fungal infections

It is believed that antimicrobial peptides could be potential alternatives of conventional antifungals [6]. AMPs are an evolutionarily conserved component of the innate immunity and exist among all classes of life, ranging from prokaryotes to plants, insects, amphibians and mammals [7–11]. They have a broad spectrum of antimicrobial activities against bacteria, mycobacteria, enveloped viruses, fungi and even transformed or cancerous cells. So far, lots of AMPs demonstrating antifungal activities have been isolated and characterized. The antifungal action modes of AMPs are diverse, including disrupting fungal cell membrane and inhibiting intracellular targets. So it is believed that the possibility for fungal cells to develop resistance toward AMPs is relatively low [12].

Protonectin was originally isolated from the venom of the neotropical social wasp *Agelaia pallipes pallipes*, with an amino acid sequence of ILGTILGLLKGL-NH_2 (1210.57 Da) [13]. It has potent antimicrobial activity against both gram positive and negative bacteria, but weak hemolytic activity [13,14]. However, the effect of protonectin on fungi cells has not been reported. In this study, protonectin was chemically synthesized and antifungal activity against *Candida* cells was determined. Then, the effects of protonectin on the integrity of fungal cell membrane and some intracellular events were investigated. Our results showed that protonectin had potent antifungal activity against *Candida* cells and the action mode involved membrane disturbance and the inducing of ROS production.

2. Material and methods

2.1. Peptide synthesis

Protonectin (ILGTILGLLKGL-NH_2) was synthesized by a stepwise solid-phase method on rink amide MBHA resin using N-9-fluorenylmethoxycarbonyl (F-moc) chemistry [15]. After cleavage, protonectin was desalted using a Sephadex gel column and purified by reverse-phase high-performance liquid chromatography (RP-HPLC,

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http://dx.doi.org/10.1016/j.bbamem.2015.07.008
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Waters) using a μBondapak C_{18} 19 mm by 300 mm column with gradient elution of 20% to 80% CH_3CN/H_2O with 0.1% trifluoroacetic acid (TFA) at a flow rate of 8 ml/min. The atomic mass of the synthetic peptide was confirmed by electrospray ionization-mass spectrometry (ESI-MS, MaXis 4G, Bruker, Germany). Antimicrobial peptides melittin and magainin-2 also were synthesized and purified. The peptide was dissolved in double-distilled water and stored at −20 °C before use.

2.2. Fungi and materials

Candida albicans (ATCC 14053), *Candida glabrata* (ATCC 2001), *Candida parapsilosis* (ATCC 22019), *Candida tropicalis* (ATCC 750), and *Candida krusei* (ATCC 6258) used in this study were purchased from the American Type Culture Collection (ATCC) and the *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 phosphate-buffer solution (PBS), shaken 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 get logarithmic phase. The fresh culture was centrifuged, washed and re-suspended in 25% $C_3H_8O_3/H_2O$ and stored at −80 °C before use. Agents used for peptide synthesis were purchased from GL Biochem Ltd (Shanghai, China) with desired purity.

2.3. Antifungal assays

2.3.1. The minimum inhibition concentration assay (MIC)

The antifungal assay was determined according to the EUCAST method with minor modifications [16]. Briefly, cells activated in Sabouraud dextrose broth medium were washed, diluted and inoculated into 96 well flat-bottom plates containing serially two fold dilutions of protonectin. The plates with an inoculum size of 0.5×10^5 to 2×10^5 CFU were statically placed in an incubator at 35 °C overnight. Minimal inhibitory concentration (MIC) of protonectin was defined as the lowest concentration at which no visible turbidity was observed compared with drug-free group. The MICs assay was repeated in triplicate.

2.3.2. The minimum fungicidal concentration assay (MFC)

For minimal fungicidal concentration (MFC) determination, 50 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 to drug-free group. The MFC assay was repeated in triplicate.

2.3.3. Radial diffusion assay

The fungicidal activity of protonectin was evaluated by a modification of the sensitive radial diffusion assay. Briefly, the fungi cells were cultured as described above. 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 protonectin to each well, the plates were incubated at 35 °C for 18 to 24 h. 25 μl of sterile water was added as a control.

2.3.4. Time-killing kinetics of protonectin against Candida cells

Time-killing kinetics of protonectin against *Candida* cells were performed according to standard microbiological techniques with minor modifications [17]. Logarithmic cultures diluted to an inoculum size of 10^4–10^5 CFU in fresh Sabouraud dextrose broth medium were incubated with protonectin at the required concentration, and the mixture was incubated at 35 °C with shake. 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 9 cm in diameter for CFU counting. The original number of colonies was confirmed at time zero. The killing activity of protonectin was illustrated by plotting the $\log_{10}$ CFU per milliliter against incubation time. Sabouraud dextrose broth medium was run as negative control.

2.4. Protonectin binding assay with Candida cells

Binding of protonectin to the surface of *Candida* cells was examined by assessing the effect of representative fungal cell wall polysaccharides on killing activity of protonectin described before [18]. In brief, protonectin (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 % = $100 \times (1 - Ft / Fo)$, in which Fo and Ft represented the CFU of polysaccharide-free group and polysaccharide-treated group. The figure was plotted as killing rate against polysaccharide concentration.

2.5. Localization of FITC-protonectin (FITC-pro) in Candida cells

2.5.1. Confocal laser scanning microscopy

C. glabrata cell cultures at logarithmic phase with an inoculum size of 10^6–10^7 CFU were exposed to 64 μM in Sabouraud dextrose broth medium for timed duration at 35 °C. Microscopic analysis was done with a laser confocal scanning microscope (Zeiss LSM 710 META) after 60 min incubation.

2.5.2. Flow cytometric analysis

For FACS assay, *C. glabrata*cell cultures with an inoculum size of 10^6–10^7 CFU were exposed to FITC-pro in Sabouraud dextrose broth medium at different concentrations for 2 h at 35 °C with shaking. After incubation, the cells were centrifuged, washed and resuspended with PBS. Flow cytometric analysis was conducted by a FACS Calibur flow cytometer (Becton–Dickinson, San Jose, CA, USA).

Table 1
MIC values of protonectin against the tested *Candida* cells.

MIC (μM &μg/ml)[b]									
Peptide	*C. glabrata* ATCC 2001	*C. albicans ATCC 14053*	*C. parapsilosis ATCC 22019*	*C.tropicalis ATCC 750*	*C. krusei ATCC 6258*	*C. glabrata 14-1*	*C. albicans* 14-2	*C. albicans 14-3*	*C. albicans 14-4*
Protonectin[a]	32/38.8[c]	32/38.8[c]	128/155[c]	8/9.7[c]	32/38.8[c]	32/38.8[c]	32/38.8[c]	32/38.8[c]	32/38.8[c]
Melittin	32/91[c]	4/11.4[c]	64/182[c]	1/2.8[c]	16/45.5[c]	32/91[c]	8/22.8[c]	32/91[c]	16/45.5[c]
Magainin2	32/79[c]	32/79[c]	128/315.8[c]	16/39.5[c]	16/39.5[c]	32/79[c]	32/79[c]	64/158[c]	64/158[c]

[a] Initially obtained by Maria Anita Mendes et al. The sequence is ILGTILGLLKGL-amide. The calculated monoisotopic mass value (M_{calc})is 1210.57, while the observed monoisotopic mass value (M_{obs}) is 1211.3. M_{obs} was deduced from the protonated molecule ($M + H^+$).
[b] Both the values in μM &μg/ml were provided.
[c] The left of diagonal mark (/) is the MIC value in μM, while the right is the MIC value in μg/ml.

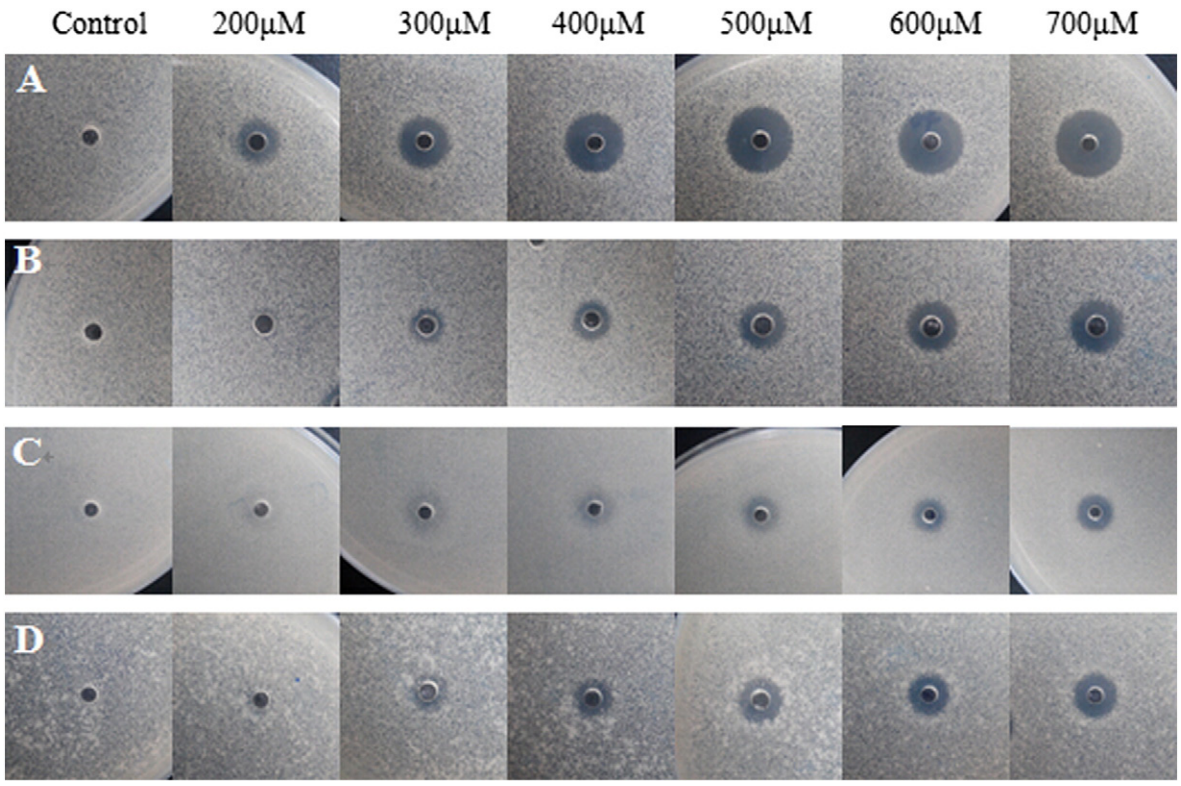

Fig. 1. The fungicidal activity of protonectin (0, 200, 300, 400, 500, 600, and 700 μM) against *C. tropicalis* ATCC 750 (A), C. *albicans* ATCC 14053 (B), *C. glabrata* ATCC 2001 (C) and *C. krusei* ATCC 6258 (D) was determined by radial diffusion assay. Protonectin made fungi-free zones around the wells in a concentration dependent manner. (The diameter of wells was 3 mm).

2.6. Determination of fungal cell integrity using PI uptake

2.6.1. Confocal laser scanning microscopy

The propensity of protonectin to cause loss of membrane integrity was measured by PI uptake assay using a laser confocal scanning microscope and FACS. Briefly, freshly collected logarithmic *C. glabrata* (ATCC 2001) cultures with an inoculum size of 10^6–10^7 CFU were incubated with protonectin at 128 μM (4 × MIC) or PBS as negative control. After incubation at 35 °C for 60 min, PI solution was added to get 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 phosphate-buffer solution (PBS) and microscopic analysis was done with a laser confocal scanning microscope (Zeiss LSM 710 META).

2.6.2. Flow cytometry analysis

To quantitatively analyze the loss of fungal membrane integrity after peptide treatment, *C. glabrata* cells (10^6–10^7 CFU) were treated with an increasing concentration of protonectin, 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).

2.7. Electron microscopy

To view the morphological change of fungal cells after treatment with protonectin, electron microscopy was employed [19]. In brief, *C. glabrata* cells grown to logarithmic phase with an inoculum size of 10^6–10^7 CFU were incubated with protonectin 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% (wt/v) uranyl acetate. The grids were examined by using a scanning electron microscope (JSM-6380Lv, Japan).

Table 2
MFC values of protonectin against the tested *Candida* cells.

MFC (μM &μg/ml)[a]									
Peptide	*C. glabrata* ATCC 2001	*C. albicans ATCC 14053*	*C. parapsilosis ATCC 22019*	*C.tropicalis ATCC 750*	*C. krusei ATCC 6258*	*C. glabrata 14-1*	*C. albicans* 14-2	*C. albicans 14-3*	*C. albicans 14-4*
Protonectin	64/77.5[b]	128/155[b]	256/310[b]	16/19.9[b]	32/38.8[b]	32/38.8[b]	64/77.5[b]	64/77.5[b]	32/38.8[b]
Melittin	64/182 [b]	32/91 [b]	64/182 [b]	2/5.7 [b]	32/91 [b]	32/91 [b]	32/91 [b]	64/182 [b]	32/91 [b]
Magainin2	32/79 [b]	64/158 [b]	>128/315.8 [b]	32/79 [b]	16/39.5 [b]	32/79 [b]	32/79 [b]	64/158 [b]	64/158 [b]

[a] Both the values in μM &μg/ml were provided.
[b] The left of diagonal mark (/) is the MIC value in μM, while the right is the MIC value in μg/ml.

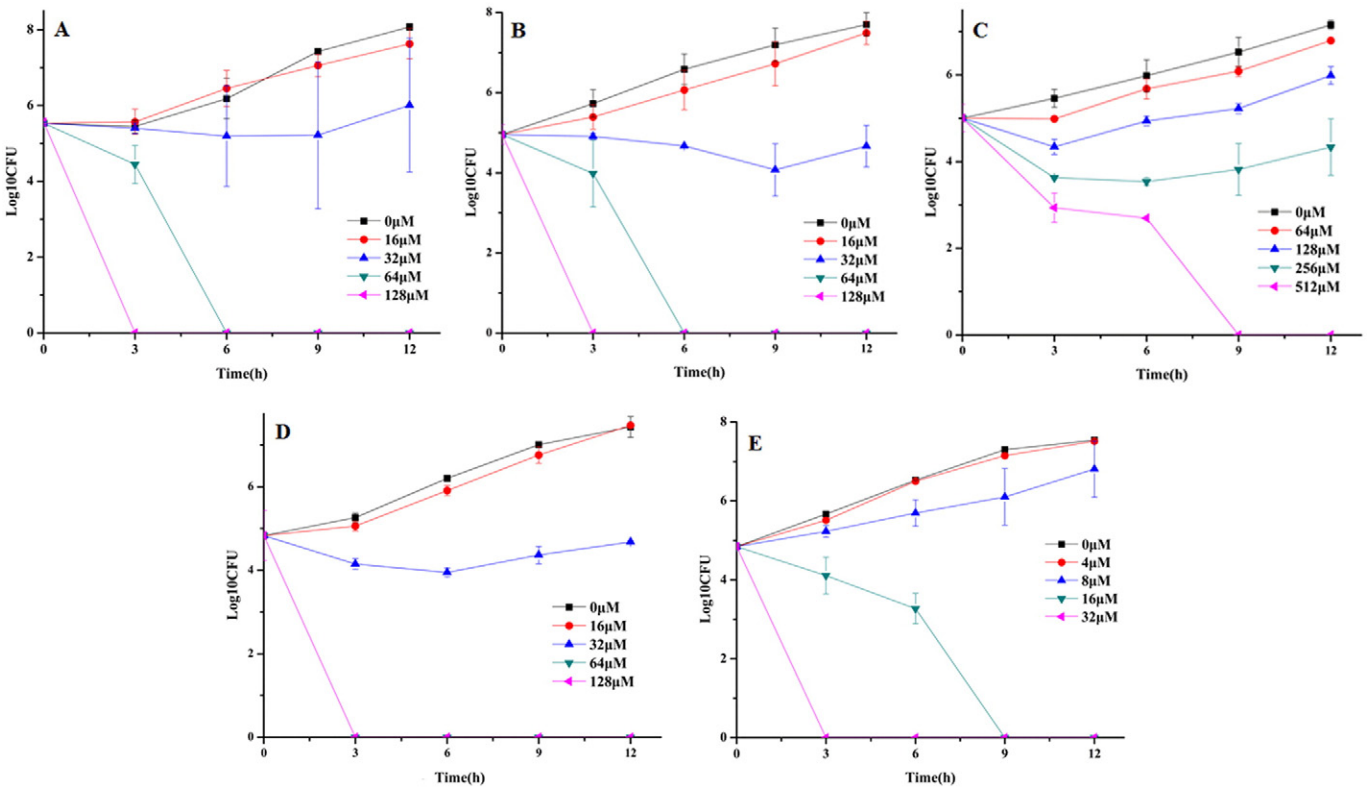

Fig. 2. Time-killing kinetics of protonectin against the tested *Candida* cells: (A), *C. glabrata* (ATCC 2001); (B), *C. albicans* (ATCC 14053); (C), *C. parapsilosis* (ATCC 22019); (D), *C. krusei* (ATCC 6258) and (E), *C.tropicalis* (ATCC 750). Cells were cultured in the presence of protonectin at indicated concentrations or Sabouraud's dextrose broth (0 μM protonectin) 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 intervals after treatment. Error bars represent standard error from mean cell proliferation as determined by repeated experiments.

2.8. Measurement of cellular ROS production

Endogenous amounts of ROS were measured by fluorometric assay with 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as described earlier [20]. Briefly, the logarithmic *C. glabrata* cells were centrifuged, washed and re-suspended in PBS to an OD_{600} of 1. The cultures were then treated with protonectin alone or in combination with ascorbic acid or left untreated for 1 h at 35 °C. After incubation, 10 μM 2′,7′-dichlorofluorescin diacetate in PBS was added. The fluorescence intensities (excitation 488 nm and emission 525 nm respectively) of the treated cells were measured with a spectrofluorometer at different intervals.

2.9. Biofilm killing test

The biofilm was prepared according to the previous method with minor modifications [21]. Briefly, the logarithmic *C. glabrata* cultures grown in Sabouraud dextrose broth for 18 h at 35 °C in a rotary shaker

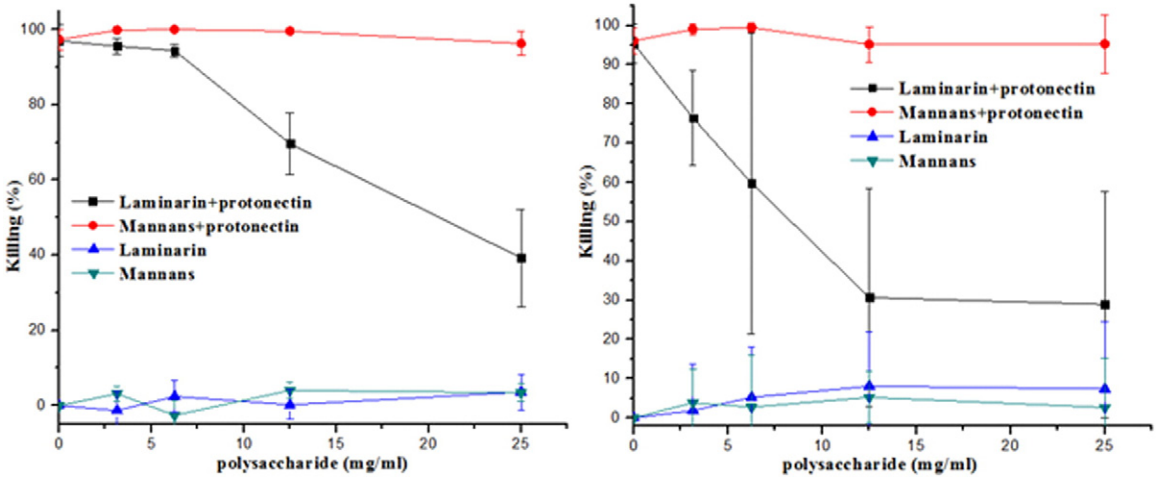

Fig. 3. Effect of representative *Candida* cell wall polysaccharides (laminarin (β-1,3-glucan) and mannans) on the killing rate of protonectin against tested *Candida* cells. Laminarin and mannans were pre-incubated with protonectin for 1 h, then incubated with *Candida* cells (left panel: *C. albicans*; right panel: *C. glabrata*) for another 1 h. Laminarin and mannans served as the negative control. The binding effects of protonectin with cell wall polysaccharides were reflected by the reduction of fungicidal activity. Error bars represent standard error from mean cell proliferation as determined by repeated experiments.

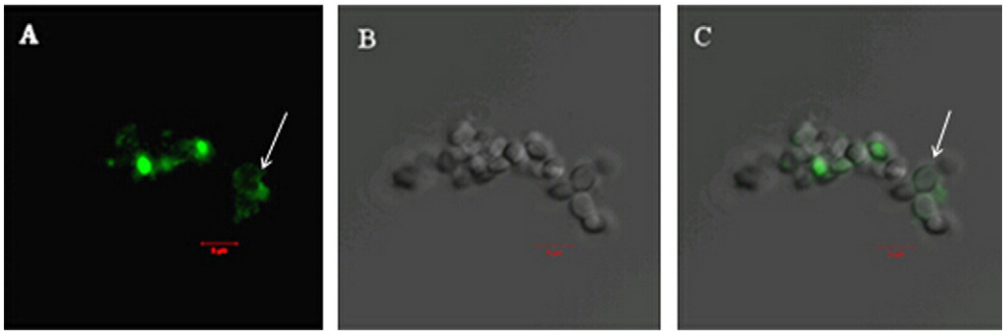

Fig. 4. Cellular localization of protonectin in *C. glabrata* cells. The confocal micrographs of *C. glabrata* cells treated with FITC-pro. (A) Fluorescence microscopy; (B) DIC microscopy; (C) merged pictures. Bars, 1 μm.

at 150 rpm were centrifuged, washed twice with PBS, counted using a hemacytometer and inoculated into polystyrene 96-well microplates with an inoculum size of 1×10^6 to 2×10^6 CFU. The plates were incubated at 35 °C with 120 rpm shaking to provide shear forces. Biofilms were formed over a series of time intervals to get adhesion-stage biofilms (1 h), early-stage biofilms (5 h) and mature-stage biofilms (18 h). At the end of incubation time, the wells containing *C. glabrata* biofilms were washed with PBS twice to remove non-adhered *C. glabrata* cells and exposed to different concentrations of protonectin or Sabouraud dextrose broth as negative control. After incubation at 35 °C for 24 h, the cells were washed three times with 0.05% Tween 20 in Tris-buffered saline (TBS). Biofilm formation was evaluated by observation in an inverted microscope.

3. Results

3.1. Protonectin showed potent antifungal activity against Candida cells

In this study, protonectin was chemically synthesized by a stepwise solid-phase chemosynthesis assay and its antifungal activity was investigated. As shown in Table 1, protonectin showed potent antifungal activity against *C. glabrata*, *C. albicans*, *C. parapsilosis*, *C.tropicalis*, and *C. krusei* with the MICs of 32 μM, 32 μM, 128 μM, 8 μM and 32 μM respectively. The fungicidal activity also was determined. As shown in Fig. 1, protonectin exhibited its fungicidal activity in a dose dependent manner. The MFCs of protonectin against the tested Candidian cells were shown in Table 2, which were 2–4 times of their MICs. The time-killing kinetics also was carried out to evaluate their fungicidal activity against

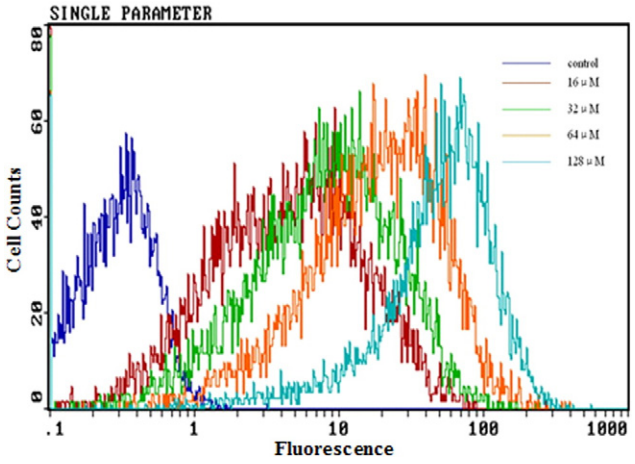

Fig. 5. Flow cytometric analysis of FITC-pro staining in *C. glabrata* cells. Cells showed an increased fluorescent intensity. *C. glabrata* cells treated with FITC-pro (16 μM, 32 μM, 64 μM and 128 μM) for 2 h at 37 °C. Then cells were washed three times in 10 mM sodium phosphate buffer (pH 7.4) and determined by FACS.

time. Our results showed that at the concentration of 2 × MIC or 4 × MIC, protonectin could reduce inoculum by 100% within 12 h, while at the concentration of MIC, it exhibited fungistatic activity (Fig. 2).

3.2. Protonectin could specifically bind with β-1,3-glucan

As we know, fungal species has cell wall to protect the cell against harsh extracellular environment [22]. However, the role of fungal cell wall in the process of peptide–cell interaction is unintelligible. So in the present study, in order to investigate whether protonectin could bind with fungal cell surface, the interaction of protonectin with the main component of cell wall was determined. Protonectin at the concentration of 256 μM was incubated with different concentrations of the polysaccharides laminarin and mannans. Then, the fungicidal activity of the mixture was tested. As shown in Fig. 3, pre-incubation of protonectin with laminarin at high concentration of 12.5 or 25 mg/ml for 1 h decreased the killing activity dramatically, while mannans have a little effect on the fungicidal activity of protonectin. It revealed that the relative specificity of binding between protonectin and laminarin played a crucial role in the binding of protonectin with *Candida* cells.

3.3. Protonectin could accumulate on the fungal cell surface

As protonectin could specifically bind to β-1, 3-glucan, a polysaccharide representative of fungal cell wall that is abundant in fungal cell wall, FITC-pro was used to reveal the interaction between protonectin and *Candida* cells. As shown in Fig. 4, FITC-pro was attached to the surface of *C. glabrata* cells. The accumulation on the surface was ensued by the influx of protonectin into cytoplasm. As shown in Fig. 5, the increase of fluorescence intensity caused by increasing concentration of protonectin indicated that the interaction between protonectin and *C. glabrata* cells was in a dose-dependent manner, as monitored by FACS. Contrastively, the accumulation could be detained by the pre-incubation of FITC-pro and β-1, 3-glucan (Fig. S1).

3.4. Protonectin could cause the loss of cell integrity

In the present study, the effect of protonectin on the integrity of the fungal cell membrane was determined by PI uptake assay. PI is membrane impermeable and generally excluded from viable cells. When the membrane integrity lost, PI could pass through plasma membrane and bind with genomic DNA and give a red fluorescence with the excitation of 480 nm laser. As shown in Fig. 6 under the view of a confocal fluorescence microscope, *C. glabrata* cells treated by protonectin showed red fluorescence while the untreated group showed no fluorescence. This indicated that protonectin could disturb the integrity of the tested fungal cells. Then the FACS assay was employed to verify the uptake of PI. As shown in Fig. 7, protonectin could induce the shift of

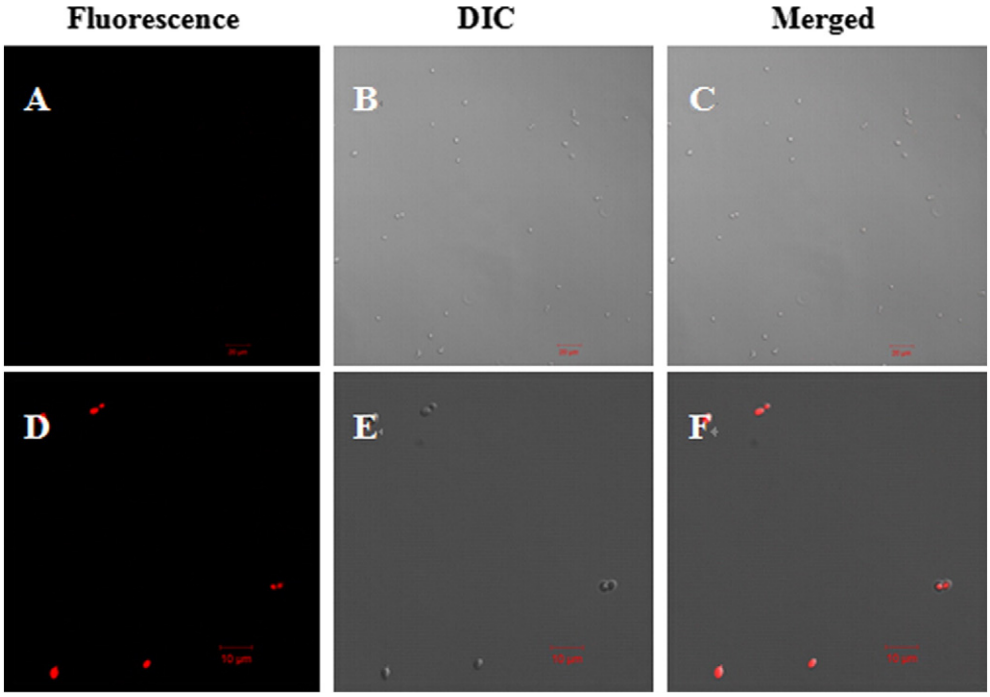

Fig. 6. Microscopy analysis of membrane permeabilization detected by propidium iodide uptake assay in *C. glabrata* cells after incubation with 0uM peptides (A–C) and 128 μM peptides (D–E) for 1 h at 35 °C.

fluorescence intensity in a dose dependent manner. This result was consistent with the confocal microscopy assay.

3.5. Protonectin caused morphological change of Candida cells

In this study, scanning electron microscopy was employed to determine the effect of protonectin on the morphology of the fungal cells. As shown in Fig. 8, *C. glabrata* cells treated by protonectin showed an anomalous morphological structure with inflated, uneven and discrete cell wall. The cells were surrounded by numerous vesicular structures. However, in the control group, the untreated cells have continuous, smooth and intact cell wall with little vesicular structure.

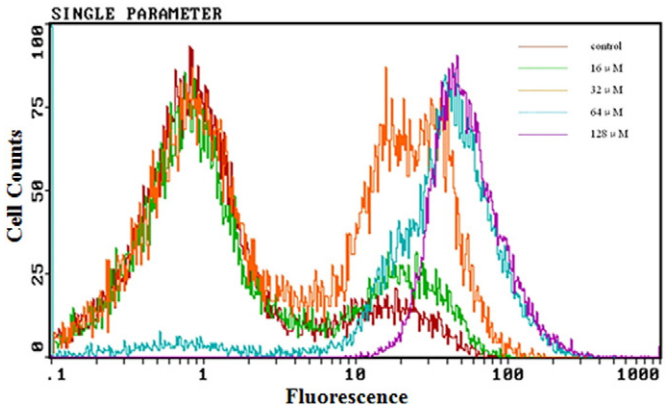

Fig. 7. Cytometric analysis of membrane permeabilization. *C. glabrata* cells treated with protonectin, exhibit increased PI uptake. *C. glabrata* cells were incubated for 2 h at 37 °C with protonectin (16 μM, 32 μM, 64 μM and 128 μM), followed by a 30 min incubation with 50 mg/ml PI. Cells were washed three times in 10 mM sodium phosphate buffer (pH 7.4) and analyzed by FACS.

3.6. Protonectin could induce ROS production in a time- and dose-dependent manner

In the present study, DCFH-DA was employed to monitor the production of ROS in the *Candida* cells after treatment by protonectin. The cell-permeant dye DCFH-DA is oxidized to yield the fluorescent molecule 2′,7′-dichlorofluorescein, which can be monitored by a FlexStation 3 benchtop multi-mode microplate reader (FlexStationIII) with the maximum excitation wavelength of 488 nm and the maximum emission wavelength of 525 nm. Compared with the control cells, protonectin induced a rapid increase in fluorescence in a dose- and time-dependent manner. No fluorescence increase was detected when the cells were treated with protonectin in combination with a well-known antioxidant ascorbic acid (Fig. 9).

3.7. Protonectin demonstrated antifungal activity against adhered fungal cells

Biofilms were dynamic communities of microorganisms tenaciously attached to biological and nonbiological surfaces and were enclosed in self-produced matrix to allow fungal cells survive in harsh environment and disperse to colonize new foothold [23]. The kinetics of biofilm formation revealed that there were three phases, including adhesion phase (1–3 h), intermediate phase (4–8 h) and maturation phase (18–36 h) when planktonic cells were cultivated into wells of polystyrene 96-well plates. At adhesion phase, the cells adhere to the bottom of the wells in a monolayer cell manner and undergo a morphological change from yeast like phenotype to filamentous type. With a further incubation for 4 h, the cells multiply and group in clusters in a filamentous form. At maturation phase, adherent cells are embedded within a self-produced matrix generally composed of extracellular DNA, proteins and polysaccharides. At this point, the biofilm always were consisted of several layers of cells [21]. Our results showed that protonectin could inhibit the growth of adhesion-stage or intermediate-stage biofilm completely at the concentration of 1–2 × MIC. While at the

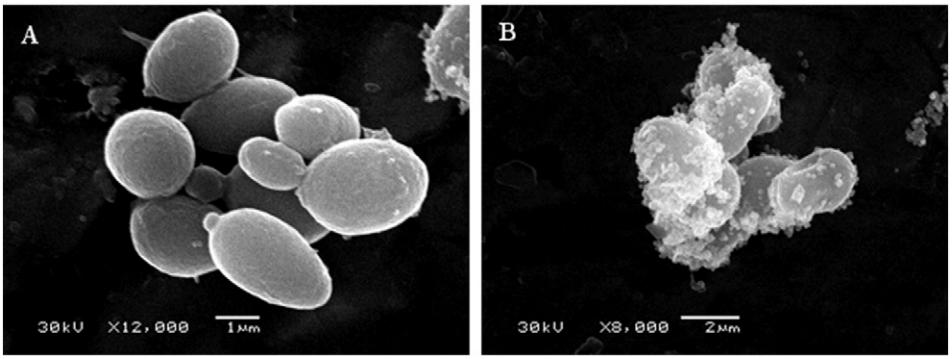

Fig. 8. Scanning electron microscopy of protonectin treated *C. glabrata* cells. Mid-growth-phase *C. glabrata* were incubated with protonectin (at the concentration of 2 × MIC) for 4 h at room temperature. The surface of untreated *C. glabrata* cells was smooth (A) and the surfaces of the protonectin-treated cells were ruffled, and became disrupted (B).

concentration below MIC, the growth of biofilm was partially inhibited. For the maturation phase biofilm, protonectin had a weaker inhibition effect. The inhibition concentration was up to 4 × MIC (Fig. 10).

4. Discussion

The increasing multidrug resistance of microorganisms to available traditional antibiotics and antifungal agents is becoming a global puzzle, resulting in enormous efforts to develop new antibiotics and antifungal agents with more effective efficacy. One of the promising alternatives of traditional agents is host-defense cationic antimicrobial peptides. AMPs are an integral component of the innate immune system of all biological organisms, functioning in the mode of physically permeating and rapidly disrupting the microorganism cell membrane. Therefore, the possibility of microbial resistance toward AMPs is limited. Based on these properties, AMPs attracted more and more attention.

In the present study, we investigated the antifungal activity of cationic antimicrobial peptide protonectin. Our results showed that protonectin had a potent antifungal activity against *C. albicans* and *C. glabrata*. As our previous study reported, membrane active action mode was involved in the mechanism of protonectin against bacteria. So the effect of protonectin on the integrity of fungal cell membrane also was determined in this study. Our results from PI uptake assay and SEM assay showed that protonectin also could disrupt the integrity of the fungal cell membrane. However, as we know, fungal cells have a polysaccharide cell wall as a boundary to protect them from the harsh environment. Accounting for 80–90% of the cell wall of *Candida* is constituted by carbohydrate that envelops cells with an elastic and multifunctional barrier and maintains structural integrity [22]. In *Candida* cells, glucan is a maximum in the cell wall, accounting for 60–65% of the total polysaccharides and mannan accounts for 20–25% [24]. The effect of the cell wall on the exertion of antifungal activity of protonectin also was determined. Our results revealed that the incubation of protonectin with laminarin (β-1,3-glucan) could decrease the antifungal activity dramatically, indicating that protonectin could bind with the fungal cell wall. This was further confirmed by the location of FITC-pro under the CLMS view. Then we could speculate that protonectin first bound to the fungal cell by the peptide–laminarin interaction, then it passed through the cell wall and interacted with the lipid membrane to exert its antifungal activity.

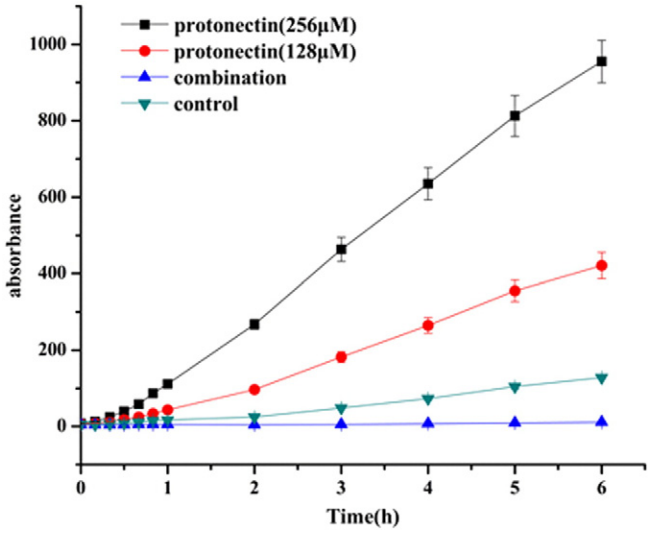

Fig. 9. Effect of protonectin on the cellular ROS formation of *C. glabrata* cells. The *C. glabrata* cells were incubated in the presence of protonectin at indicated concentration, or in the presence of the combination of protonectin and ascorbic acid for 1 h at 35 °C, then 10 μM 2′,7′-dichlorofluorescin diacetate (ROS-sensitive probe) was added. The fluorescence was monitored at different intervals. Error bars represent standard error from mean cell proliferation as determined by repeated experiments.

Notably, fungal infection is becoming an increasingly life-threatening medical problem not only because of the emergence of multidrug resistant fungi but also the obstacle of fungal treatment in immunocompromised patients. The formation of biofilm was a kind of obstacle for treatment and always made the situation worse [23]. Biofilm formation represents the most common growth pattern of microorganisms in nature, a phase that presumably resists the invasion of innate immune system and antifungal agents [25]. The misuse of antifungal agents made the biofilm becomes more and more resistant to the innate immune system and chemotherapy [21]. Our results showed that protonectin has the potential to be used to inhibit the formation of biofilm or eliminate the formed biofilm.

Furthermore, as we know, ROS generated as a natural byproduct of the normal metabolism of oxygen and had important roles in cell signaling and maintaining internal homeostasis [26]. However, under the environmental stress, ROS levels could increase dramatically, resulting in significant damage to cell structures. Recent studies demonstrate that endogenous reactive oxygen species is an important mediator of antifungal effect [27]. So in the present study, the effect of protonectin on the production of ROS also was tested. Our results showed that protonectin could induce the production of ROS in a time- and concentration-dependent manner. The mitochondrion is the site of oxidative metabolism in the cell, which is involved in the production and degradation of highly reactive and toxic oxidizing agent. So protonectin might affect the function of mitochondrion and result in the production of oxygen radicals, which lead to the cell death. It could be concluded that mitochondrion might be involved in the action mechanism of protonectin.

In conclusion, as the systemic fungal infections are becoming a major cause of morbidity and mortality in debilitated patients, and the currently available antifungal therapies exhibit limited effectiveness and

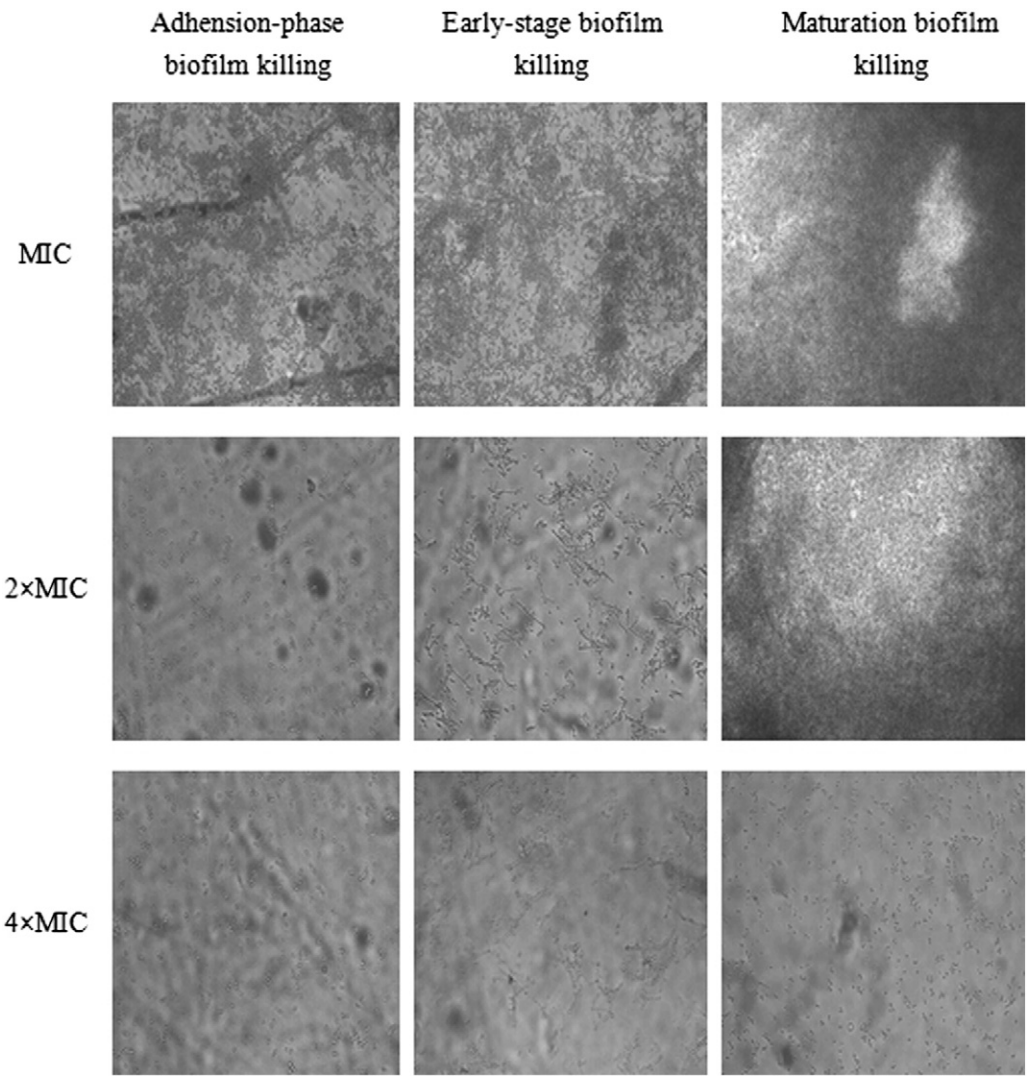

Fig. 10. Effects of protonectin on the biofilm formation of *C. albicans*. Cells of indicated stage were treated by protonectin at the indicated concentration for 18 h. The biofilm morphology was assessed by phase contrast microscopy. Protonectin could inhibit the growth of adhesion-stage or intermediate-stage biofilm completely at the concentration of 1–2 × MIC. While at the maturation phase biofilm, the inhibition concentration was up to 4 × MIC. The inhibition effects were in a concentration dependent manner.

severe side effects, the search for better therapeutic strategies is in an urgent need. Our results showed that protonectin had potent antifungal activity, with membrane active and mitochondrion involved action mode. Together with the inhibition of the growth of biofilm, protonectin would act as a potential alternative of conventional antifungal agents.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2015.07.008.

Conflict of interest

The authors disclose no conflicts.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (nos. 31200584, 21272108, 21432003, 91213302, 81473095); the Key National S&T Program "Major New Drug Development" of the Ministry of Science and Technology (2012ZX09504001-003); the Research Fund for the Doctoral Program of Higher Education of China (20120211120045) and the Fundamental Research Funds for the Central Universities (lzujbky-2015-155).

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