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# Antimicrobial activity and stability of the D-amino acid substituted derivatives of antimicrobial peptide *polybia*-MPI

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## Abstract

Antimicrobial peptide has the potential to be developed as new kind of antimicrobial agents with novel action mechanism. However, the susceptibility to protease is a drawback for potential peptides to be clinical used. D-amino acid substitution can be one way to increase the proteolytic stability of peptides. In the present study, we synthesized the D-lysines substituted analog (D-lys-MPI) and the D-enantiomer of *polybia*-MPI (D-MPI) to improve the proteolytic resistance of *polybia*-MPI. Our results showed that, the stability of its D-amino acid partially substituted analog D-lys-MPI was increased. However, it lost antimicrobial activity at the tested concentration with the loss of  $\alpha$ -helix content. As shown in the CD spectra, after substitution, the spectra of D-MPI is symmetrical to MPI, indicated it turned into left hand  $\alpha$ -helical conformation. Excitingly, the stability of D-MPI toward the tested protease was improved greatly. Notably, the antimicrobial activity of D-MPI was comparable to its L-counterpart MPI, even improved. In addition, the hemolytic activity of D-MPI was lowered. This also indicated that the action target of antimicrobial peptide *polybia*-MPI was not chiral specific. So, D-MPI may offer a therapeutic strategy to defend the infection of microbes, considering its stability to protease and relatively lower cytotoxicity to human erythrocytes.

**Keywords:** Antimicrobial peptide, *Polybia*-MPI, Stability, D-Amino acid substitution

## Introduction

Antimicrobial peptide (AMPs) is a component of innate defense system in most multicellular organisms, from humans to plants to insects. To date more than 2200 natural or synthetic AMPs have been reported through the antimicrobial peptide database (APD) (Silva et al. 2014). Antimicrobial peptides (AMPs) are generally composed of short sequences of 10–100 amino acids residues and highly amphipathic and membrane active (Reddy et al. 2004). AMPs have a broad spectrum of antibacterial, antifungal, antiviral, and anti-tumor activity at low concentrations. Unlike traditional antibiotics, antimicrobial

peptides can regulate the host immune system and kill bacteria directly (Gottler and Ramamoorthy 2009). Furthermore, other biological activities of AMPs have also been described as following: neutralization of endotoxins, immune-modulating properties, chemokine-like activities, and induction of both angiogenesis and wound repair (Guaní-Guerra et al. 2010).

Although AMPs have broad spectrum of antimicrobial activity, the therapeutic applications of them still have many limitations, such as cytotoxicity, instability and high cost. Many antimicrobial peptides have cytotoxicity to mammalian cells at high concentrations, which may be due to membrane cracking mechanism (Marr et al. 2006). Moreover, AMPs could be degraded or removed by the endogenous protease or secreted by organism before arriving at the action sites. Therefore, their antimicrobial activities were greatly reduced or completely lost (Bowdish et al. 2005; Maisetta et al. 2008). The first commercial AMP was pexiganan acetate (MSI-78) which

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was an  $\alpha$ -helical magainin variant peptide. MSI-78 has broad spectrum activity for treating foot ulcers and has developed into phase III clinical trials (Kang et al. 2012). BPI is a cationic protein from human neutrophil-derives which has antibacterial activity has just entered phase-III clinical trials (Ge et al. 1999; Hancock and Lehrer 1998). And up to now, no one have obtained US Food and Drug Administration (FDA) approval, except gramicidin for topical administrations (Steckbeck et al. 2014).

Recently, in order to reducing the toxicity and proteolytic degradation, many strategies were developed. It has been reported that the toxicity of AMPs can be reduced by modest sequence modifications (Kim et al. 2014). Strategies used to protect AMPs from protease degradation were mainly including cyclization, incorporation of non-proteinogenic amino acids, and sequence modification such as D-amino acids to replace the L-amino acids (Braunstein et al. 2004; Choi et al. 2013; Hamamoto et al. 2002; Lee and Lee 2008). Furthermore, several studies had reported D-amino acid substitution can improve the activity of AMPs or retain its activity, and more importantly can improve their stability.

*Polybia*-MPI, was a cationic peptide originally isolated from the venom of the social wasp *Polybia paulista* (Souza et al. 2005). Its primary sequence is IDWKKLL-DAAKQIL-NH<sub>2</sub> (1654.09 Da). We have demonstrated that *Polybia*-MPI displays potent antibacterial activity against both Gram-positive and Gram-negative bacteria. Furthermore, it was also found that *polybia*-MPI has potent antifungal activity and antitumor activity, and low toxicity to human red blood cells and normal fibroblasts (Wang et al. 2008, 2013). However, it is susceptible to the protease, which limits its clinical application. In the present study, its partial D-amino acid derivative D-lys-MPI (in which all L-lys was substituted by D-lys) and D-enantiomer (D-MPI) were synthesized by solid-phase peptide synthesis. Then their antimicrobial activity, hemolytic activity and stability were determined. Furthermore, the effect of such modification on the bioactivity, secondary structure and action mechanism was discussed.

## Materials and methods

### Synthesis of peptide

*Polybia*-MPI(I-D-W-K-K-L-L-D-A-A-K-Q-I-L-NH<sub>2</sub>) (1654.09 Da) and its analogues were synthesized by the standard Fmoc solid-phase peptide synthesis (Fields and Noble 1990; Wang et al. 2008). Each of synthesized peptide was purified to over 95% by reversed-phase high performance liquid chromatography (RP-HPLC) using a  $\mu$  Bondapak C18 19- by 300-mm column. The flow rate was 8 ml/min and the gradient used was 5–95% solvent (CH<sub>3</sub>CN/H<sub>2</sub>O with 0.1% trifluoroacetic acid) over 60 min (Yan et al. 2012). The molecular weights were

verified by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (ESI-MS). All the peptides were dissolved in deionized water and diluted by PBS before use.

### Antimicrobial assays

The minimal inhibitory concentration (MIC) of the peptides was measured in triplicate by the standard broth microdilution method (Peterson et al. 1980; Wang et al. 2012). In brief, bacteria/fungi were cultured overnight to mid-logarithmic-phase in Mueller–Hinton broth (MHB)/Sabouraud dextrose broth. The cultures were diluted to a final concentration of  $2 \times 10^4$  cfu/ml in fresh Mueller–Hinton broth (MHB)/Sabouraud dextrose broth. Each well of 96-well propylene microtitre plates was filled with 100  $\mu$ l cell suspension and 100  $\mu$ l peptide solution of two-fold diluted serially. And the final concentrations of peptide mixtures ranged from 1 to 256  $\mu$ M. Controls were done without peptides. After 18 h of incubation at 37 °C (bacteria)/35 °C (fungi), the minimal inhibitory concentration (MIC) of the peptides was evaluated by visible turbidity in each well.

The minimal bactericidal concentrations (MBCs) and minimum fungicidal concentration (MFCs) of the peptides was determined at the end of the incubation period by transferring 50  $\mu$ l aliquots onto agar plates and incubated overnight at 37 °C/35 °C. The MBC/MFC was set as the lowest concentration of the number of CFUs that reduced at least 99.9%.

### Time-kill analysis

Strains was grown overnight with shaking to mid-logarithmic phases described above (Wang et al. 2015). The cultures containing  $10^4$ – $10^5$  CFU/ml was incubated with different concentration of peptides which determined by MIC assay via the broth microdilution method. A no-peptide (control) tube was also included in each run. The cultures were incubated in a 37 °C/35 °C. shaking incubator. At different time intervals over the incubation period, samples from each tube were removed respectively and serially (tenfold) diluted, and 100  $\mu$ l of the dilution were plated on agar plates. Plates were incubated at 37 °C/35 °C and determine the numbers of CFU after 24 h.

### Hemolytic activity

Fresh human blood was collected in heparinized-tube, diluted in cold PBS (pH 7.4) and centrifuged at 800g for 10 min to remove the serum and buffy coat. The erythrocytes were washed twice time and re-suspended in the same buffer to a final erythrocyte concentration of 8% for the hemolysis assay (Sharma and Sharma 2001). The RBC (red blood cell) suspension of 100  $\mu$ l was transferred into

a 96 well microtiter plate. Peptides were dissolved in PBS (pH 7.4) of different concentrations from 2 to 256 μM were incubated with 8% erythrocytes suspension for 60 min at 37 °C. Subsequently, the plate was centrifuged at 1200×g for 10 min. Then, aliquots of 100 μl supernatant were retransferred to an another 96-well plates and measured to monitor the release of hemoglobin by microplate reader (Bio-Rad 680) at 490 nm, which indicated RBC membrane damage. 100 μl RBC (red blood cell) suspension with 0.2% Triton-X 100 or PBS was used as positive and negative control, respectively. Hemolysis rate was determined as below:

$$\text{Hemolysis rate(\%)} = (A - A2)/(A1 - A2) \times 100\%$$

where A is the absorbance of indicator mixtures with different factors, A1 and A2 are the positive control, and the negative control, respectively (Kim et al. 2011).

#### Circular dichroism spectroscopy analysis

The circular dichroism (CD) spectra of peptides were measured on an Olis DSM 1000 CD spectrophotometer (USA) in spectra between 195 and 240 nm under nitrogen flush in 1 mm path length at room temperature. The scanning speed was 100 nm/min, 2 s response time and 1.0 nm spectral band width. The measurements were performed with a peptide concentration of 50 mM. The spectra were measured in 50% TFE (v/v) and 10 mM PBS (pH 7.4), and the average of four scans was taken.

#### Assessment of stability

##### Viability assay

Viability assay different concentration of trypsin (from 0.0002 to 2 mg/ml) incubated with peptides for 1 or 6 h at 37 °C, and the enzymatic activity was terminated by additional heat inactivation at 60 °C for 20 min. Then the trypsin treated peptides were incubated with *Escherichia coli* (ATCC25922) at the concentration of MIC in 96-wells microtiter plate overnight at 37 °C (Choi et al. 2013). The growth inhibitory effect was determined by measuring the absorbance at 600 nm. Then we took 100 μl samples of 6 h from each well with the concentration of 0.2 mg/ml and plated onto MH broth ager after dilution. After overnight incubation at 37 °C, photos of plates were photographed and the colonies on the plate were counted.

##### Radial diffusion assay

The enzymatic stability of *Polybia*-MPI and its analogues was evaluated by a modification of the sensitive radial diffusion assay. The bacteria were cultured until the optical density at 600 nm (OD600) of an aliquot reached 0.5. One milliliter of the bacteria suspension was added to 100 ml of

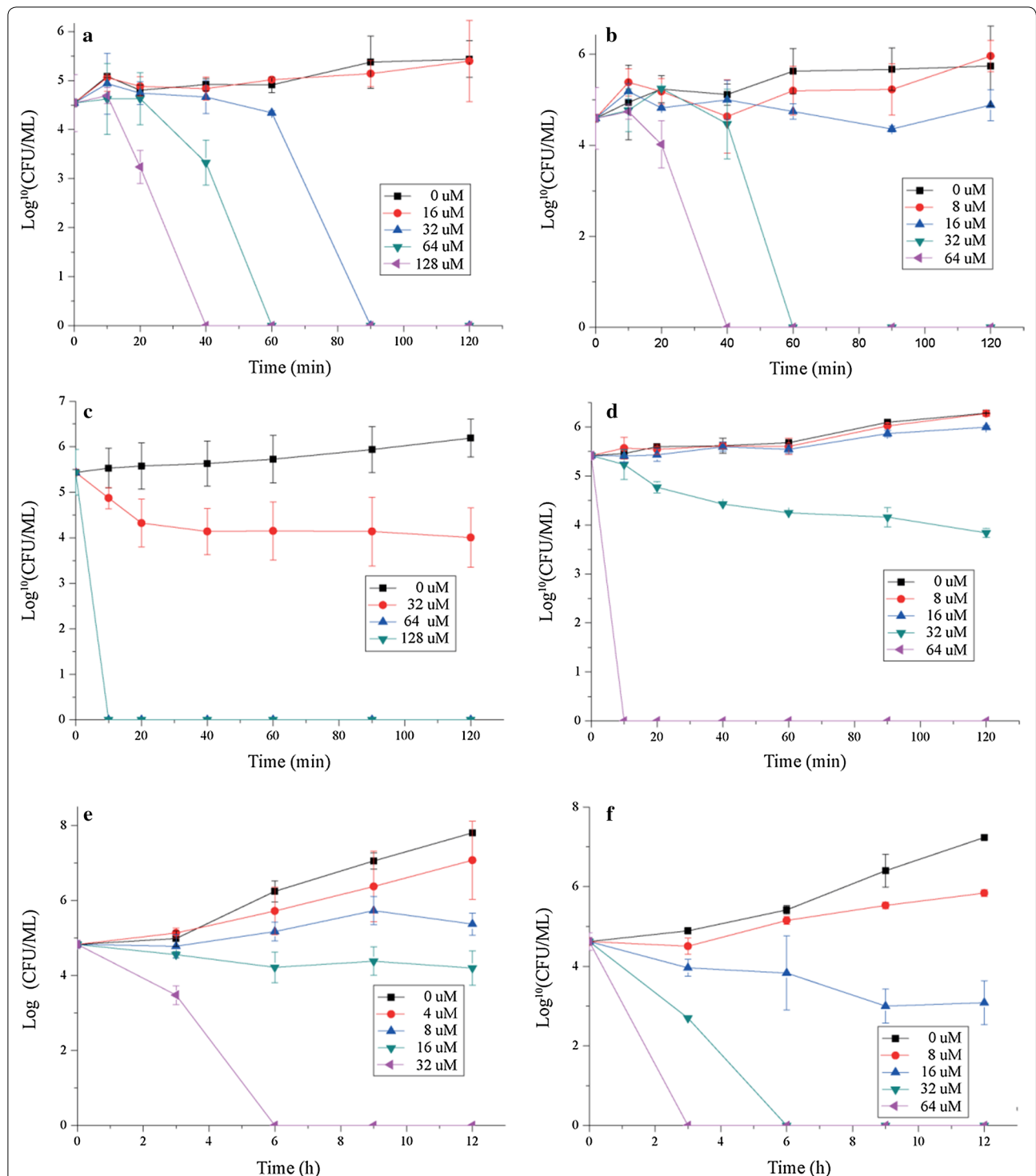
previously autoclaved, warm Mueller–Hinton agar (MHA)/Sabouraud dextrose agar. The medium were poured into layer of a 5 mm deep. Wells were performed with a sterile plastic bore of a 3-mm-diameter gel punch. Following the addition of 20 μl pre-incubated mixture of peptides and different concentrations of trypsin or chymotrypsin for

**Table 1 MIC values of *polybia*-MPI, D-MPI and D-lys-MPI against the tested Bacteria cells and Candida cells**

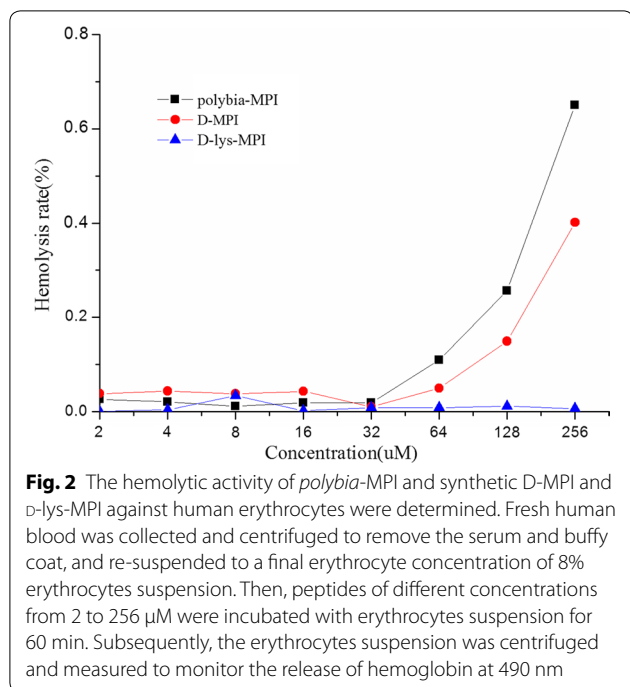
Organism	MIC(μM)		
	<i>Polybia</i> -MPI	D-MPI	D-lys-MPI
Bacteria			
<i>E. coli</i> (ATCC 2592)	32	16	>256
<i>E. coli</i> (ML-35) (ATCC 43827)	32	32	>256
<i>Klebsiella influenza</i> (ATCC 700603)	64	64	>256
<i>Pseudomonas aeruginosa</i> (ATCC 27853)	128	64	>256
<i>sakazakii</i> (ATCC 29544)	64	32	>256
<i>Staphylococcus aureus</i> (ATCC 29213)	32	16	>256
<i>Bacillus subtilis</i> (ATCC 23857)	8	8	>256
<i>Staphylococcus epidermidis</i> (ATCC 12228)	32	16	256
Fungi			
<i>Candida glabrata</i> ATCC (ATCC 2001)	8	16	128
<i>Candida albicans</i> ATCC (ATCC 14053)	16	16	256
<i>Candida parapsilosis</i> (ATCC 22019)	64	32	>256
<i>Candida tropicalis</i> (ATCC 750)	8	16	64
<i>Candida krusei</i> (ATCC 6258)	8	8	128

**Table 2 MBC/MFC values of *polybia*-MPI, D-MPI and D-lys-MPI against the tested Bacteria cells and Candida cells**

Organism	MBC/MFC(μM)		
	<i>Polybia</i> -MPI	D-MPI	D-lys-MPI
Bacteria			
<i>E. coli</i> (ATCC2592)	32	16	>256
<i>E. coli</i> (ML-35) (ATCC43827)	256	64	>256
<i>Klebsiella influenza</i> (ATCC 700603)	256	128	>256
<i>Pseudomonas aeruginosa</i> (ATCC27853)	>256	128	>256
<i>sakazakii</i> (ATCC 29544)	64	64	>256
<i>Staphylococcus aureus</i> (ATCC29213)	128	64	>256
<i>Bacillus subtilis</i> (ATCC23857)	8	8	>256
<i>Staphylococcus epidermidis</i> (ATCC 12228)	32	128	>256
Fungi			
<i>C. glabrata</i> (ATCC 2001)	32	32	256
<i>C. albicans</i> (ATCC 14053)	16	16	>256
<i>C. parapsilosis</i> (ATCC 22019)	128	32	>256
<i>C. tropicalis</i> (ATCC 750)	32	16	128
<i>C. krusei</i> (ATCC 6258)	16	8	256



**Fig. 1** Time-killing kinetics curves for polybia-MPI and D-MPI against *E. coli* (a and b), *S. aureus* (c and d) and *C. glabrata* (e and f). Standardized cells suspensions were incubated with a final concentration of peptides of 1/2 × MIC, 1 × MIC, 2 × MIC, 4 × MIC. At different time intervals, samples were taken respectively from each tube and serially diluted. Then 100 µl of the dilution were plated for colony counts. Controls were without peptide. Each data point represents mean result ± standard deviation (error bars)



4 h into wells. The plates were incubated at 37 °C for 18 to 24 h. An equal volume of 20  $\mu\text{l}$  mixture had no trypsin or chymotrypsin was used as the control. The size of the clear zones surrounding each well as indicators of the enzymatic stability of *Polybia*-MPI and its analogues.

**RP-HPLC analysis**

The stability of *polybia*-MPI and its analogues was assayed with trypsin or chymotrypsin in a volume ratio of

1:15 (peptide:trypsin) (Choi et al. 2013; Hamamoto et al. 2002; Molhoek et al. 2011). Peptides at the concentration of 10 mM were incubated with trypsin or chymotrypsin (0.2 mg/ml) at 37 °C. 40  $\mu\text{l}$  samples of the incubations were taken for the time of 0 and 360 min, respectively. Then, the samples were mixed with 80  $\mu\text{l}$  of 15% trifluoroacetic acid (TFA) and 80  $\mu\text{l}$  acetonitrile which incubated at 4 °C for at least 15 min to precipitate proteins. The supernatant was collected of each sample after centrifugation at 13,000 rpm for 10 min and were detected with reverse phase high performance liquid chromatography (RP-HPLC) analysis.

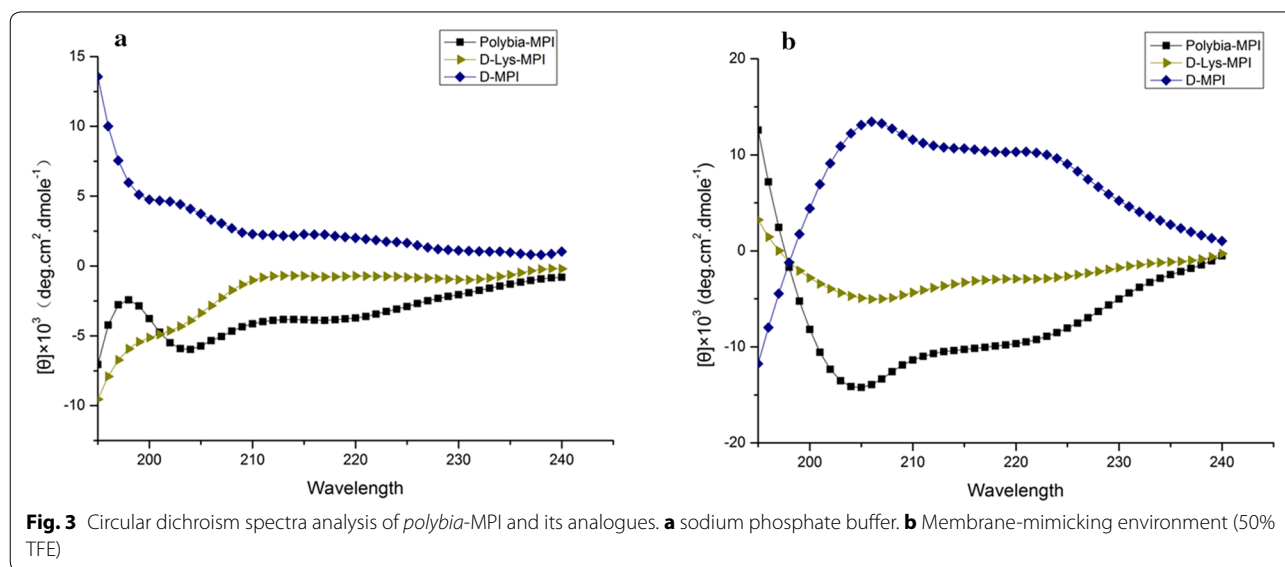
**Confocal laser scanning microscopy**

*Candida glabrata* cells were cultured overnight to mid-logarithmic-phase in Sabouraud dextrose broth. After centrifugation and suspension, the cells were incubated with *polybia*-MPI and its analogues at final concentration of 5  $\times$  MIC for 2 h at room temperature. Then, PI (50  $\mu\text{g/ml}$ ) was added and was further incubated for 5 min in the dark (Wang et al. 2012).

**Results**

**Antibacterial and bactericidal activity**

The antimicrobial activity of D-lys-MPI and D-MPI against bacteria and fungi were determined. As shown in Table 1, D-MPI could potentially inhibit the growth of all tested bacterial and fungal strains with MIC values almost in the range of 8–64  $\mu\text{M}$ . However, D-lys-MPI was inactive against bacteria and much less active against fungal strains at the tested concentration. Compared with *polybia*-MPI, the inhibitory effects of D-MPI was



improved more or less, which also could be seen in the bactericidal or fungicidal activity (Table 2). As shown in Fig. 1, the improvement of bactericidal or fungicidal activity also could be reflected in the time-killing curves of D-MPI against the tested strains.

#### Hemolytic activity

The hemolytic activity of *polybia*-MPI, D-lys-MPI and D-MPI against human erythrocytes was determined with the concentration ranging from 1 to 256  $\mu$ M. As indicated in Fig. 2, no significant hemolytic activity was found under the concentration of 100  $\mu$ M. Compared to its L-counterpart *polybia*-MPI, the hemolytic activity of D-MPI was decreased after the D-amino acid substitution. After substitution of L-lysine by D-lysine in the peptide sequence, the hemolytic activity also was abrogated.

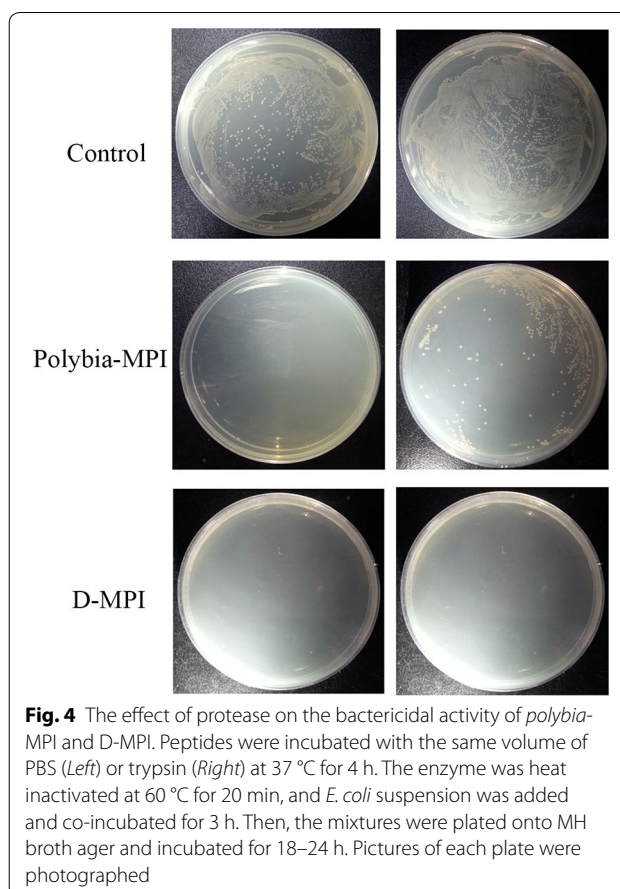
#### Circular dichroism spectroscopy analysis

The secondary structure of these peptides were determined in 10 mM sodiumphosphate buffer (PBS, pH 7.4) and a membrane-mimicking environment (50% 2,2,2-trifluoroethanol) by circular dichroism spectroscopy. As shown in Fig. 3, all the peptides displayed a unordered structure in PBS. In the membrane-mimicking environment, D-MPI showed a negative band at 195 nm, and two positive bands near 208 and 222 nm, which was mirror symmetrical to its L-counterpart *polybia*-MPI. D-MPI takes a left hand  $\alpha$ -helical conformation in the membrane-mimicking environment. However, the D-lys-MPI showed a decreased  $\alpha$ -helical conformation after the substitution of L-lysine with D-lysine for there is a quite slightly tendency of negative bands around 208 and 222 nm.

#### Stability of the peptides

##### Viability assay

One of the major limitation of AMPs for clinical use is that AMPs could be easily degraded by the endogenous proteases. To test the stability of *polybia*-MPI and D-MPI, first 1 mg/l of the peptides were incubated with trypsin and chymotrypsin for 4 h for degradation. After the enzyme was heat inactivated at 60 °C for 15 min, *E. coli* was added and co-incubated for 3 h. Then, the treated bacteria were coated to plate and incubated at 37 °C for 24 h. As shown in Fig. 4, there are no colony grown on the trypsin-D-MPI-treated plate, while there are many colonies grown on the trypsin-MPI-treated plate. D-MPI showed significant protease resistance against trypsin. To further examine the susceptibility of peptides to protease, peptides was incubated with the different concentration of trypsin (a and b) and chymotrypsin (c and d) (ranged

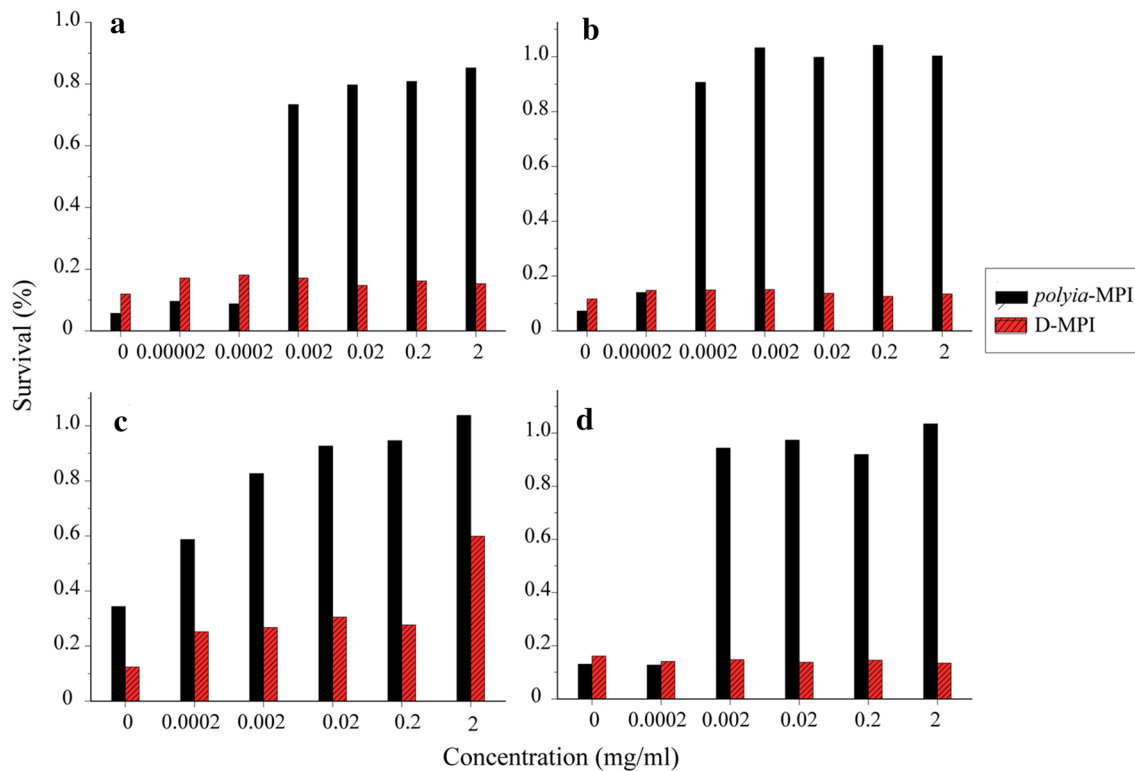


**Fig. 4** The effect of protease on the bactericidal activity of *polybia*-MPI and D-MPI. Peptides were incubated with the same volume of PBS (Left) or trypsin (Right) at 37 °C for 4 h. The enzyme was heat inactivated at 60 °C for 20 min, and *E. coli* suspension was added and co-incubated for 3 h. Then, the mixtures were plated onto MH broth agar and incubated for 18–24 h. Pictures of each plate were photographed

from 0.0002 to 2 mg/ml) for 1 and 6 h respectively, then the relative survival rate of *E. coli* were determined. As shown in Fig. 5, trypsin and chymotrypsin could totally inhibit the antimicrobial activity of *polybia*-MPI at the concentration of 0.002 mg/ml after incubation 1 h, while D-MPI retained its antimicrobial activity after incubation with 2 mg/ml of trypsin and chymotrypsin for 6 h. This result was further confirmed by radical diffusion assay. As shown in Fig. 6, the inhibition zone diameters in *polybia*-MPI treated group were decreased even disappeared after incubation with 0.02–2 mg/ml of trypsin and chymotrypsin, respectively. However, in the D-MPI treated group the inhibition zone diameters was almost the same as control after the incubation with 2 mg/ml of tested proteases.

##### RP-HPLC analysis

In the present study, HPLC was used to determine the stability of *polybia*-MPI, D-lys-MPI and D-MPI to trypsin, chymotrypsin and proteases in human serum. When comparing the HPLC profile of untreated peptides



**Fig. 5** Inhibition effect of protease on the antimicrobial activity of *polybia*-MPI and D-MPI. The proteases used were trypsin (**a** and **b**) and chymotrypsin (**c** and **d**). Peptides with proteases of different concentration which ranged from 0.0002 to 2 mg/ml (tenfold) were incubated for 1 h (left) or 6 h (right) at 37 °C, respectively. Then tested for its antimicrobial activity against *E. coli* by determine the OD values at 600 nm

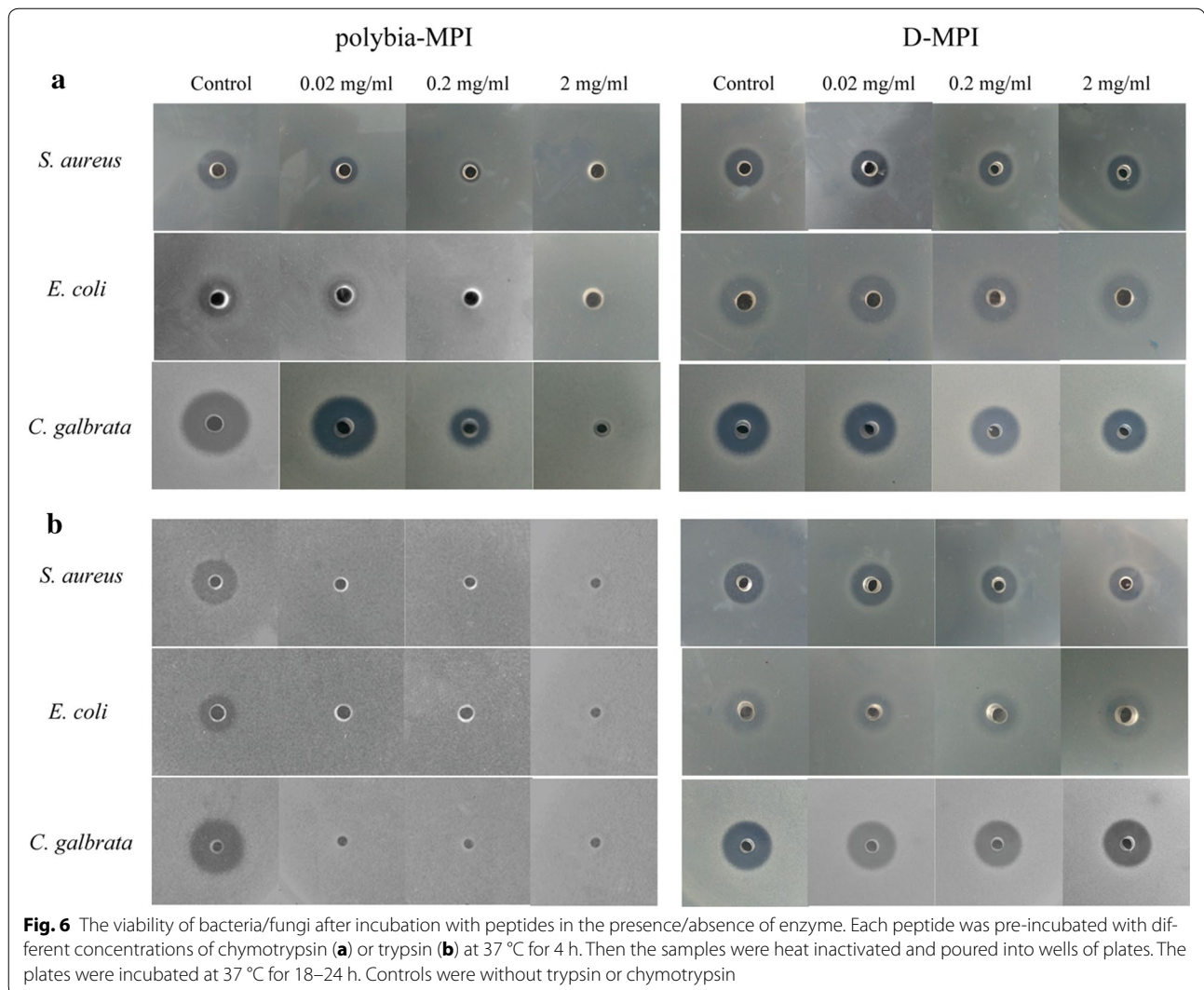
to the HPLC profile of trypsin and chymotrypsin treated peptides, it was found that *polybia*-MPI was susceptible to all the tested enzymes and serum, while its partially D-lysine substituted analogue and fully D-amino acid substituted were not or only partly degraded after treatment (Fig. 7).

#### Peptides induced cell membrane permeabilization

To determine the effect of D-MPI on the integrity of the fungal cell membrane, PI uptake assay was employed in the present study. PI was a kind of DNA intercalating fluorescent dye, which could only pass through the damaged cell membranes and combined with DNA to form a PI-DNA complex. As shown in Fig. 8, there is no fluorescence in the control group (A–C), while the D-MPI treated group (G–I) showed fluorescence as its L-counterpart treat group (D–F), indicating that D-MPI also could disrupt the integrity of the cell membrane.

#### Discussion

Since the golden age of antibiotics, antibiotics had truly become the “panacea” in the field of medicine and were being used to treat even the most common infections. However, spawned a series of multi-drug resistant bacteria with the indiscriminate use and abuse of antibiotics, traditional antibiotics have been unable to meet the needs of clinical medicine (Wang et al. 2016). Therefore, the urgent need for new antibiotics has stimulated the rapid development of antimicrobial peptides. Most antimicrobial peptides are presented with a positive charge at neutral PH and have an amphiphilic topology, so they can combined with a negatively charged phospholipid head, and ultimately damage membrane structure through interactions with cytoplasmic membrane (Wang et al. 2013). Due to these special properties, antimicrobial peptides are therefore considered to be a potential alternative to traditional antibiotics. Although

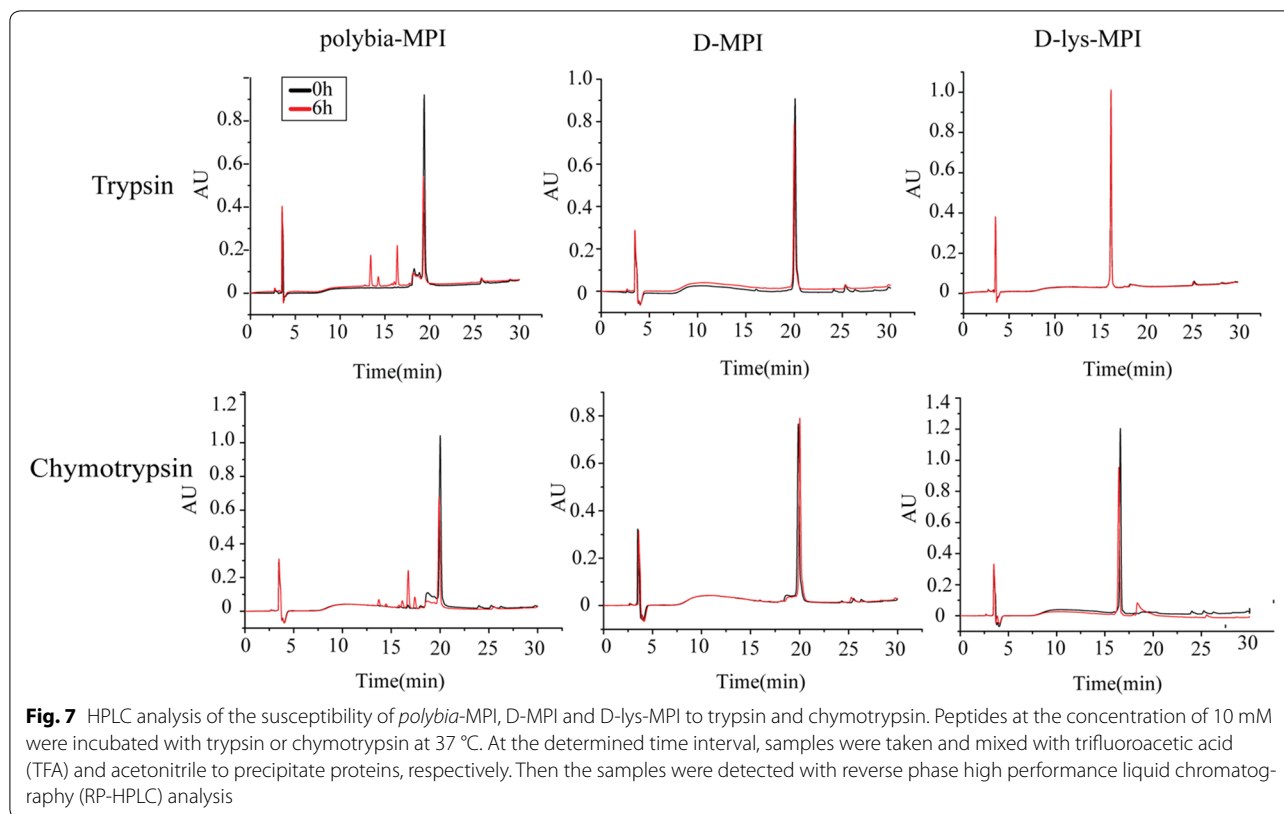


AMPs have broad spectrum antimicrobial activity *in vitro*, there are many obstacles in clinical application, such as cell toxicity, instability and high cost (Kim et al. 2014). To solve these problems, many methods have been reported. For instance, substitution Trp and Phe residues with less hydrophobic amino acids can decrease the toxicity of AMPs (Kim et al. 2014). Introduction of non-natural amino acids (mainly D-form amino acids), cyclization or modification the terminal regions by acetylation or amidation can improve the stability of peptides (Wei and Bobek 2005; Kim et al. 2014).

In nature, almost all of the proteins and peptides were composed of L-amino acid. Different enzyme could recognize specific peptide bond formed by L-amino acid and

break it (Chen et al. 2016). However, enzyme could not break the peptide bond formed by D-amino acid for the difference of the spatial configuration. Actually, there is no difference between L-amino acid and its D-counterpart in the chemical and physical property. So, D-amino acid substitution almost has no influence on the chemical and physical property of the peptides except the configuration. As the influence of D-amino acid substitution on the biological activity of peptides, it is dependent on that if the action mechanism related to the recognition of chiral targets (Braunstein et al. 2004). In the present study, when only the three Lysines in the sequence of *polybia*-MPI were substituted by their D-counterparts, the secondary structure was changed compared to



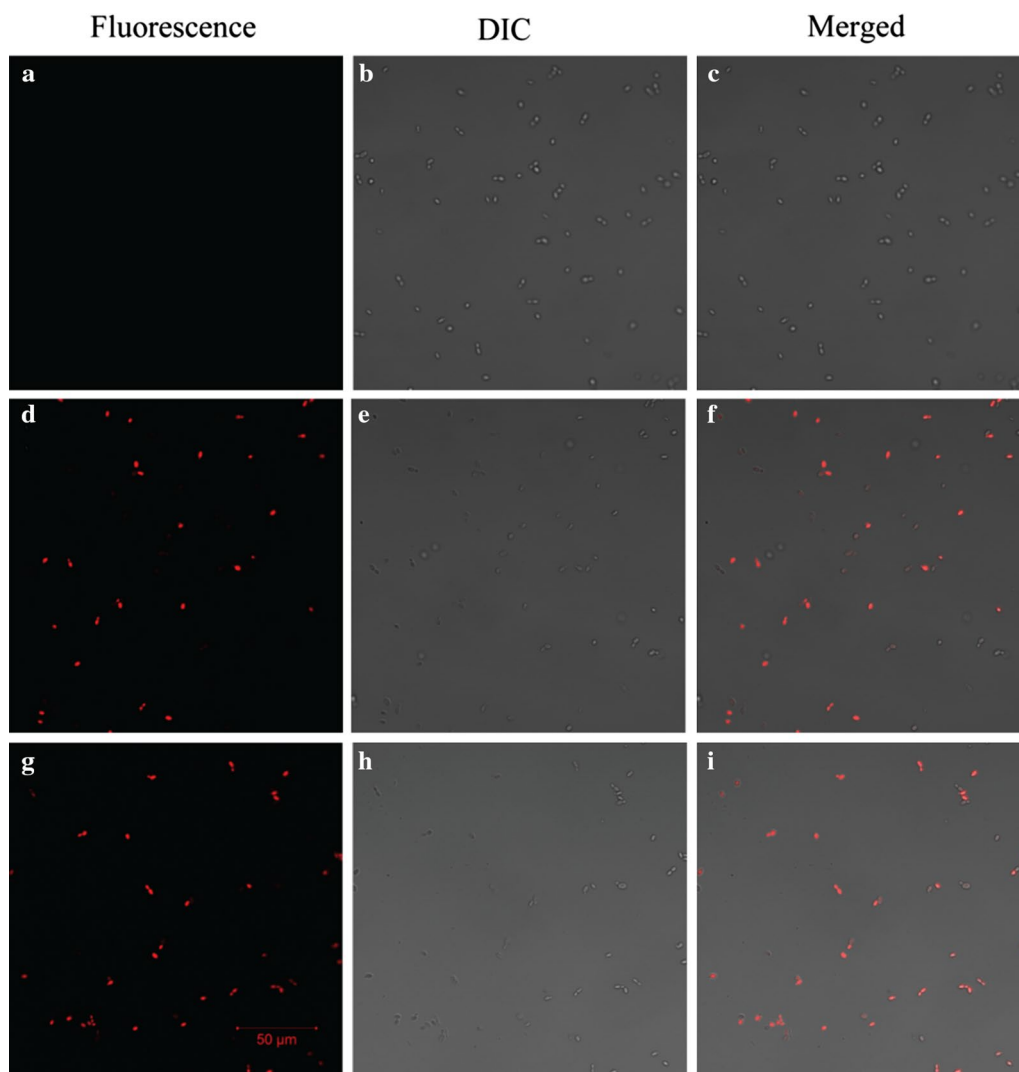


*polybia*-MPI and the peptide almost lost all the biological activity in the tested concentration. However, when all the L-amino acids in the sequence of *polybia*-MPI were substituted by D-amino acids, the configuration was reversed. The D-enantiomer of *polybia*-MPI was turned into left hand  $\alpha$ -helical conformation. More importantly, all D-amino acid substitution didn't affect its biological activity. D-MPI has the same antimicrobial activity even improved activity as *polybia*-MPI.

Furthermore, the action mechanism of D-MPI also was analyzed in the present study. The action mode of AMPs has been investigated in many studies. Many AMPs were found that they could bind with the membrane of microbes and then disrupt the integrity of cell membrane (Arnusch et al. 2012). There are mainly three models, the barrel-stave model, the toroidal pores model, and the carpet model (Chih et al. 2015), which are widely accepted to describe the membrane-active action mode. Membrane active action mode of AMPs was believed that it is difficult for microbes to develop resistance toward AMPs (Hancock and Sahl 2006). The L-counterpart of D-MPI mainly

exerts its antitumor, antibacterial and antifungal activity through the disruption of the integrity of cell membrane (Wang et al. 2008, 2009, 2013, 2014). As expected, it may also exert its antimicrobial activity through disrupting the integrity of cell membrane and lead to the death of cells. This kind of cell membrane lysis action mechanism always was not dependent on the chiral recognition, which support that D-MPI retained the activity of its L-counterpart. Interestingly, all D-amino acid substitution also decreased the hemolytic activity of *polybia*-MPI.

In conclusion, our results showed that the fully substitution of all the amino acid of *polybia*-MPI with D-amino acid could protect the peptide from the degradation of the tested proteases. This modification turned right hand  $\alpha$ -helical conformation of *polybia*-MPI to left hand conformation and retained or improved the antimicrobial activity of *polybia*-MPI. Although the partially D-lysine substitution could improve the stability of *polybia*-MPI toward the tested protease, the secondary conformation was changed after modification and its antimicrobial activity decreased greatly. So, D-MPI



**Fig. 8** Detection of peptides-induced PI uptake by LSCM. Mid-logarithmic-phase *C. glabrata* cells were incubated with 0  $\mu$ M or 5  $\times$  MIC peptides for 2 h. Then, PI (50  $\mu$ g/ml) was added and was further incubated for 10 min in the dark. PI uptake was detected by LSCM. The control was done without peptide. Control (**a–c**); *polybia*-MPI (**d–f**); D-MPI (**g–i**). Bars, 50  $\mu$ m

may offer a new strategy and will be an excellent alternative to conventional antibiotics to defend the resistant bacteria.

#### Authors' contributions

YZ, MZ, SQ, JW, JP, PZ, RZ carried out the experiments. KW, WY, RW, HW and YL participated in the design of the study, and supervised the research work. YZ and KW drafted the original manuscript. MZ, HW, YL, WY and RW helped to review and editing the manuscript. WY and RW take part in the project administration and funding acquisition. MZ, HW, YL, KW, WY and RW participated in the providing the necessary resources and funding acquisition. All authors read and approved the final manuscript.

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#### Competing interests

The authors declare that they have no competing interests.

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## References

- Arnusch CJ, Ulm H, Josten M, Shadkhan Y, Osherov N, Sahl HG, Shai Y (2012) Ultrashort peptide bioconjugates are exclusively antifungal agents and synergize with cyclodextrin and amphotericin b. *Antimicrob Agents Chemother* 56(1):1–9
- Bowdish DM, Davidson DJ, Hancock RE (2005) A re-evaluation of the role of host defence peptides in mammalian immunity. *Curr Protein Pept Sci* 6(1):35–51
- Braunstein A, Papo N, Shai Y (2004) In vitro activity and potency of an intravenously injected antimicrobial peptide and its D-L amino acid analog in mice infected with bacteria. *Antimicrob Agents Chemother* 48(8):3127–3129
- Chen HL, Su PY, Shih C (2016) Improvement of in vivo antimicrobial activity of hbcard peptides by D-arginine replacement. *Appl Microbiol Biotechnol* 100(21):9125–9132
- Chih YH, Lin YS, Yip BS, Wei HJ, Chu HL, Yu HY, Cheng HT, Chou YT, Cheng JW (2015) Ultrashort antimicrobial peptides with antiendotoxin properties. *Antimicrob Agents Chemother* 59(8):5052–5056
- Choi H, Hwang JS, Kim H, Lee DG (2013) Antifungal effect of CopA3 monomer peptide via membrane-active mechanism and stability to proteolysis of enantiomeric D-CopA3. *Biochem Biophys Res Commun* 440(1):94–98
- Fields GB, Noble RL (1990) Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int J Pept Protein Res* 35(3):161–214
- Ge Y, MacDonald D, Henry MM, Hait HI, Nelson KA, Lipsky BA, Zasloff MA, Holroyd KJ (1999) In vitro susceptibility to pexiganan of bacteria isolated from infected diabetic foot ulcers. *Diagn Microbiol Infect Dis* 35(1):45–53
- Gottler LM, Ramamoorthy A (2009) Structure, membrane orientation, mechanism, and function of pexiganan-A highly potent antimicrobial peptide designed from magainin. *Biochimica Et Biophysica Acta-Biomembranes* 1788(8):1680–1686
- Guaní-Guerra E, Santos-Mendoza T, Lugo-Reyes SO, Terán LM (2010) Antimicrobial peptides: general overview and clinical implications in human health and disease. *Clin Immunol* 135(1):1–11
- Hamamoto K, Kida Y, Zhang Y, Shimizu T, Kuwano K (2002) Antimicrobial activity and stability to proteolysis of small linear cationic peptides with D-amino acid substitutions. *Microbiol Immunol* 46(11):741–749
- Hancock RE, Lehrer R (1998) Cationic peptides: a new source of antibiotics. *Trends Biotechnol* 16(2):82–88
- Hancock REW, Sahl HG (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol* 24:1551–1557
- Kang SJ, Kim DH, Mishig-Ochir T, Lee BJ (2012) Antimicrobial peptides: their physicochemical properties and therapeutic application. *Arch Pharmacol Res* 35(3):409–413
- Kim JK, Lee E, Shin S, Jeong KW, Lee JY, Bae SY, Kim SH, Lee J, Kim SR, Lee DG, Hwang JS, Kim Y (2011) Structure and function of Papiliocin with antimicrobial and anti-inflammatory activities isolated from the Swallowtail Butterfly, *Papilio xuthus*. *J Biol Chem* 286(48):41296–41311
- Kim H, Jang JH, Kim SC, Cho JH (2014) De novo generation of short antimicrobial peptides with enhanced stability and cell specificity. *J Antimicrob Chemother* 69(1):121–132
- Lee J, Lee DG (2008) Structure-antimicrobial activity relationship between pleurocidin and its enantiomer. *Exp Mol Med* 40(4):370–376
- Maisetta G, Di Luca M, Esin S, Florio W, Brancatisano FL, Bottai D, Campa M, Batoni G (2008) Evaluation of the inhibitory effects of human serum components on bactericidal activity of human beta defensin 3. *Peptides* 29(1):1–6
- Marr AK, Gooderham WJ, Hancock RE (2006) Antibacterial peptides for therapeutic use: obstacles and realistic outlook. *Curr Opin Pharmacol* 6(5):468–472
- Molhoek EM, van Dijk A, Veldhuizen EJ, Haagsman HP, Bikker FJ (2011) Improved proteolytic stability of chicken cathelicidin-2 derived peptides by D-amino acid substitutions and cyclization. *Peptides* 32(5):875–880
- Peterson LR, Gerding DN, Johnson MM, Cherner JE, Opfer BJ, Hall WH (1980) Evaluation of a commercial microdilution system for quantitative susceptibility testing of aminoglycosides against multidrug-resistant Gram-Negative Bacilli. *Antimicrob Agents Chemother* 17(1):20–23
- Reddy KV, Yedery RD, Aranha C (2004) Antimicrobial peptides: premises and promises. *Int J Antimicrob Agents* 24(6):536–547
- Sharma P, Sharma JD (2001) In vitro hemolysis of human erythrocytes by plant extracts with antiparasitoid activity. *J Ethnopharmacol* 74(3):239–243
- Silva PM, Gonçalves S, Santos NC (2014) Defensins: antifungal lessons from eukaryotes. *Front Microbiol* 5:97
- Souza BM, Mendes MA, Santos LD, Marques MR, César LM, Almeida RN, Pagnocca FC, Konno K, Palma MS (2005) Structural and functional characterization of two novel peptide toxins isolated from the venom of the social wasp *Polybia paulista*. *Peptides* 26(11):2157–2164
- Steckbeck JD, Deslouches B, Montelaro RC (2014) Antimicrobial peptides: new drugs for bad bugs? *Expert Opin Biol Ther* 14(1):11–14
- Wang K, Dang W, Xie J, Zhu R, Sun M, Jia F, Zhao Y, An X, Qiu S, Li X, Ma Z, Yan W, Wang R (2015) Antimicrobial peptide protonectin disturbs the membrane integrity and induces ROS production in yeast cells. *Biochimica Et Biophysica Acta-Biomembranes* 1848(10):2365–2373
- Wang K, Zhang B, Zhang W, Yan J, Li J, Wang R (2008) Antitumor effects, cell selectivity and structure-activity relationship of a novel antimicrobial peptide *polybia*-MPI. *Peptides* 29(6):963–968
- Wang K, Yan J, Zhang B, Song J, Jia P, Wang R (2009) Novel mode of action of *polybia*-MPI, a novel antimicrobial peptide, in multi-drug resistant leukemic cells. *Cancer Lett* 278(1):65–72
- Wang K, Yan J, Chen R, Dang W, Zhang B, Zhang W, Song J, Wang R (2012) Membrane-active action mode of *Polybia*-CP, a novel antimicrobial peptide isolated from the venom of *Polybia paulista*. *Antimicrob Agents Chemother* 56(6):3318–3323
- Wang K, Yan J, Dang W, Liu X, Chen R, Zhang J, Zhang B, Zhang W, Kai M, Yan W, Yang Z, Xie J, Wang R (2013) Membrane active antimicrobial activity and molecular dynamics study of a novel cationic antimicrobial peptide *polybia*-MPI, from the venom of *Polybia paulista*. *Peptides* 39:80–88
- Wang K, Yan J, Dang W, Xie J, Yan B, Yan W, Sun M, Zhang B, Ma M, Zhao Y, Jia F, Zhu R, Chen W, Wang R (2014) Dual antifungal properties of cationic antimicrobial peptides *polybia*-MPI: membrane integrity disruption and inhibition of biofilm formation. *Peptides* 56:22–29
- Wang K, Jia F, Dang W, Zhao Y, Zhu R, Sun M, Qiu S, Zhu Y, Yan J, Kong Z, Yan W, Wang R (2016) Antifungal effect and action mechanism of antimicrobial peptide *polybia*-cp. *J Pept Sci* 22(1):28–35
- Wei GX, Bobek LA (2005) Human salivary mucin muc7 12-mer-I and 12-mer-D peptides: antifungal activity in saliva, enhancement of activity with protease inhibitor cocktail or edta, and cytotoxicity to human cells. *Antimicrob Agents Chemother* 49(6):2336–2342
- Yan J, Wang K, Chen R, Song J, Zhang B, Dang W, Zhang W, Wang R (2012) Membrane active antitumor activity of NK-18, a mammalian NK-lysin-derived cationic antimicrobial peptide. *Biochimie* 94(1):184–191

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