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Antifungal Activities of Peptides with the Sequence 10-17 of Magainin 2 at the N-termini against *Aspergillus fumigatus*

Myung Kyu Lee, Dong Gun Lee, Song Yub Shin, Sung Gu Lee, Joo Hyun Kang and Kyung-Soo Hahm*

Peptide Engineering Research Unit, Korea Research Institute of Bioscience and Biotechnology, KIST, P.O. Box 115, Yusong, Taejon 305-600, Korea.

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Two peptides, MA-inv and MA-ME, with the sequence 10-17 of magainin 2 at their N-termini were designed and synthesized. The peptides had higher antifungal activities against *Aspergillus fumigatus* without hemolytic activities. The minimal inhibition concentratory (MIC) values of both peptides against *A. fumigatus* were 5 μg/ml, whereas those of the native peptides, magainin 2 and melittin, were 10 μg/ml. At 3 μg/ml, MA-inv and MA-ME inhibited the mycelium growth of *A. fumigatus* by 94.6% and 97.3%, respectively, whereas magainin 2 and melittin inhibited by 62.2% and 32.4%, respectively. MA-inv showed up to 80% inhibition of (1,3)-β-D-glucan synthase activity of *A. fumigatus*. The peptides also showed antifungal activities for other fungi of *Aspergillus sp.* However, the antibiotic activities of MA-ME against *Escherichia coli, Bacillus subtilis* and *Fusarium oxysporum* were more effective than those of MA-inv, suggesting that the C-terminal sequences of MA-inv and MA-ME may also influence their antibiotic activities. These results suggest that the N-terminal sequence of the designed peptides, KKFGKAFV, is important for their antifungal activities against *A. fumigatus* and their C-terminal sequences are related to the organism selectivity.

Key words: Antifungal activity, magainin 2 derived peptide, *Aspergillus fumigatus*, synthetic peptide, glucan synthase

A. fumigatus causes a diverse spectrum of human diseases, including allergic bronchopulmonary aspergillosis, asthma, aspergilloma, and invasive infection in immunocompromised hosts (1). Aspergillosis is the second most common fungal infection requiring hospitalization of patients. The respiratory tract of patients with chronic pulmonary diseases, particularly cystic fibrosis (2), is frequently colonized by *A. fumigatus*. The symptoms of acute form of wood-trimmers' disease, generally called extrinsic allergic alveolitis, is reported to be caused by tremorgenic mycotoxins from *A. fumigatus* (3).

Magainins are known to be toxic to both Gram-positive and Gram-negative bacteria, fungi, protozoa (4), and tumor cell lines (5), but are not hemolytic (4). The bee venom toxin, melittin, has a variety of toxic properties (6) and also has antibacterial activity (7). The amphipathic natures of magainins are found to be correlated with the antibiotic activity by acting on the phospholipid of the plasma membrane (8). Although the antifungal activity of magainins was first reported in 1987 (4), little studies have been reported.

In this study, two synthetic peptides (MA-inv and MA-ME) with the sequence 10-17 of magainin 2 (MA2) at their N-termini were designed in order to have the amphipathic-flexible-hydrophobic structure. This structure was originally designed as the hybrid of cecropin A (CA) and melittin (ME) (9). The hybrid peptides showed more potent antibacterial activities for both Gram-positive and Gram-negative bacteria (9-11), and also had a potent antimalarial activity (10). Here we report a successful design of two synthetic peptides which show higher antifungal activities against *A. fumigatus* than their native peptides, MA2 and ME. To elucidate the property, the growth inhibitory activities of MA-inv and MA-ME against *A. fumigatus* were examined. We also examined inhibition effects of the synthetic peptides on the (1,3)-(β-D-glucan synthase (GS) activity of *A. fumigatus* which is essential for the fungal mycelium growth (12). The antibiotic activities against *F. oxysporum, E. coli* and *B. subtilis* and the human red blood cell (hRBC) hemo-

* To whom correspondence should be addressed

Table 1. Amino acid sequences of the peptides used in this study

Peptides	Sequences	Remarks
MA2	GIGKFLHSAKKFGKAFVGEIMNS	Native magainin 2
ME	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2[a]	Native melittin
MA-inv[b]	KKFGKAFV GIGKFLHSA-NH_2[a]	MA : 10-17-MA : 1-9
MA-ME[b]	KKFGKAFV GIGAVLKVLTTG-NH_2[a]	MA : 10-17-ME : 1-12

[a]: C-terminal amidated peptides.
[b]: The designed peptides in this study.

lytic activity of these peptides were also examined.

Materials and Methods

Strains

A. fumigatus, *A. nidulans*, *A. oryzae*, *F. oxysporum*, *E. coli* and *B. subtilis* were obtained from the Korean Collection for Type Cultures (KCTC), and hRBC was obtained from the blood center of Korean Red Cross. The fungi were grown in the YPD medium (2% glucose, 1% peptone and 0.5% yeast extract, pH 5.5) at 30 ℃, and the bacteria were incubated in the LB medium at 37 ℃.

Peptide syntheses

Peptide syntheses were performed by solid phase methods (13) using Fmoc as the Nα-amino protecting group. All peptides were purified by HPLC on a reverse phase C_{18} column. The molecular weights (MWs) of the purified peptides were determined by the matrix-assisted laser desorption ionization (MALDI) mass spectroscopy (14). The sequence data of the synthetic peptides used in this study are summarized in Table 1.

Antifungal activity assays

Minimal inhibitory concentrations (MICs) against *A. fumigatus* were determined by a broth dilution method (15). MA2, ME, MA-inv and MA-ME were used in concentrations varying between 20 μg/ml and 1.25 μg/ml, in two-fold serial dilutions. The final spore concentration was 1×10^4 colony forming units (CFUs/ml).

Growth inhibition activities of *A. fumigatus* and *F. oxysporum* were tested at fixed peptide concentrations, 3 μg/ml and 5 μg/ml in 10 ml volume, respectively. The final spore concentration was 1×10^4 CFUs/ml. After incubating for 3 days at 30℃ under constant shaking (140 rpm), the growing mycelia were harvested and the dry weight was measured (16).

Antibacterial and hemolytic assays

Antibacterial activity was determined by the lethal concentration (LC) as reported (11). *E. coli* HB101 and *B. subtilis* ATCC6633 were chosen as models of Gram-negative and Gram-positive bacteria, respectively. Three μl of serially diluted peptides were placed in the 3 mm wells of the thin agarose plates seeded with $1\text{-}2\times10^5$ CFUs/6 ml and the plates were incubated overnight at 37℃. The lethal concentration was estimated by the regression of the plot, the square diameter of the clear zone versus the peptide concentration, as described by Boman *et al.* (9)

Hemolytic activity was determined in the agarose plates prepared with 6 ml of a medium containing 1% agarose, 0.9% sodium chloride and 10% (v/v) human red blood cell (hRBC) suspended in Alsever's solution (11). Three μl of serially diluted peptides were applied in the 3 mm wells and the plates were incubated overnight at 37℃. The hemolytic concentration was estimated by the regression of the plot, the square diameter of the clear zone versus the peptide concentration, as described by Boman *et al.* (9).

Preparation and activity assay of *A. fumigatus* GS

The membrane fraction containing GS of *A. fumigatus* was prepared according to the method described by Beaulieu *et al.* (17). *A. fumigatus* mycelial cells were grown in YPD broth, inoculated with 1 to 2×10^4 spores/ml, and incubated at 37℃ for 24 h. Cells were harvested by filtration on 0.22 μM membrane filter, and then washed with cold deionized water. Two to 2.5 g of the filtered cells were frozen at -70℃ and then broken by grinding with mortar. These freezing and grinding steps were repeated five times. The ground cells were resuspensed in 15 ml of cold extraction buffer (50 mM Hepes pH 7.7, 1 M Sucrose, 50 mM NaCl, 1 mM dithiothreitol, 5 mM EDTA, 10 mM sodium fluoride, and 0.1 mM GTP), then homogenized with a teflon homogenizer. Clear lysates were obtained by low speed centrifugation (2,000×g, 4℃, 15 min). The plasma membranes were precipitated by ultracentrifugation (100,000×g, 4℃, 60 min). The pellets were homogenized in 20 ml of cold extraction buffer with a teflon homogenizer, and then ultracentrifuged as above. The pellets were suspended in the extraction buffer (final protein concentration 2 mg/ml) and used for GS activity assay.

Polymerization activity of GS present in membrane preparations was also assayed by the method of Beaulieu

Table 2. Antifungal and Hemolytic activities of the peptides

Peptides	MIC[a](μg/ml) for *A. fumigatus*	% growth Inhibition[b] for *A. fumigatus* at 3 μg/ml of peptide	% growth Inhibition[b] for *F. oxysporum* at 5 μg/ml of peptide	Hemolytic concentration[c] (μM)
MA2	10	62.2	0	ND[d]
ME	10	32.4	100	50
MA-inv	5	94.6	0	ND[d]
MA-ME	5	97.3	100	ND[d]

[a]: The minium peptide concentration which completely inhibited the fungal growth in liquid culture.

[b]: Percentage growth inhibition was determined by comparison of the dry weights of the fungus in the peptide treated group with no peptide group.

[c]: The hemolytic concentration was calculated by the regression of the plot, the square diameter of the clear zone versus the peptide concentration, as described by Boman *et al.* (9)

[d]: Hemolytic activity was not detected up to 1000 μM of the peptide.

et al. (17). One hundred μl of the membrane fraction (containing 200 μg protein) was mixed with 33 μl of a reaction mixture (2 mg/ml α-amylase, 3 mg/ml bovine serum albumin, 0.4 mM UDP-glucose, and UDP-[U-^{14}C] glucose (1 μCi/ml) in extraction buffer). Reaction was performed at room temperature for 15 min and stopped by adding 1 ml of cold 5% trichloroacetic acid (TCA), which precipitated the glucan polymer, under the conditions with or without 10 μg of the peptides. The precipitated polymers were recovered by vacuum filtration on 0.45 μM membrane filters. Filters were rinsed with 5% TCA, dried, and measured radioactivity.

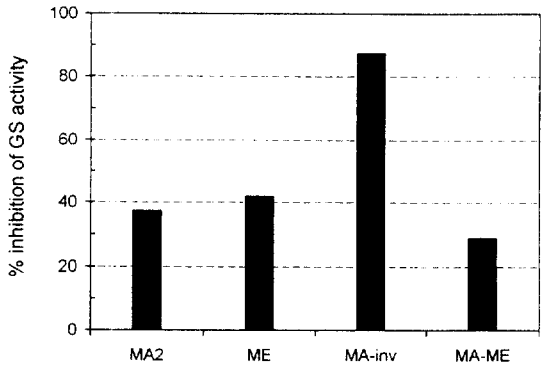

Fig. 1. Percent inhibition of the *A. fumigatus* (1,3)-β-D-glucan synthase by 10 μg/ml of peptides. Percent inhibition value was obtained by the formular; the radioactivity count of reaction with the peptide/the radioactivity count of reaction without the peptide.

Results

The purities of the purified peptides were judged up to 95% by analytical HPLC, and the observed MWs of the peptides were consisted with the expected MWs (MA2: expected MW 2468.6, observed 2469.6; ME: expected MW 2848.4, observed 2850.6; MA-inv: expected MW 1834.8, observed 1834.4; and MA-ME: expected MW 2034.2, observed 2034.0).

The designed peptides, MA-inv and MA-ME, showed 2-fold lower MIC values against *A. fumigatus* as compared with the native peptides, MA2 and ME (Table 2), even though MA-inv (17 mer) and MA-ME (20 mer) have shorter sequence than MA2 (23 mer) and ME (26 mer). MICs of both MA-inv and MA-ME were 5 μg/ml, whereas MICs of both MA2 and ME were 10 μg/ml. At the fixed peptide concentration, 3 μg/ml, the growth inhibitions of MA-inv and MA-ME were also much greater than those of MA2 and ME (Table 2). MA-inv and MA-ME showed 94.6% and 97.3% growth inhibitions, respectively, whereas MA2 and ME showed 62.2% and 32.4% growth inhibitions, respectively. These results suggest that MA-inv and MA-ME inhibit growth of *A. fumigatus* more effectively than their native forms do. These peptides also inhibit growth of other two *Aspergillus* species, *A. nidulans* and *A. oryzae* effectively (data not shown). However, at 5 μg/ml, ME and MA-ME completely inhibited the growth of *F. oxysporum*, but MA-inv and MA2 showed no antifungal activity against *F. oxysporum* (Table 2). The designed peptides, MA-inv and MA-ME, as well as MA2 had no hemolytic activity up to 1000 μM like, whereas ME showed strong hemolytic activity as previously reported (6).

The antifungal lipopeptide, cilofungin, is known to inhibit the GS activity, and this property is related to its antifungal activity (17). To find whether the peptides used in this study inhibit the GS activity, the plasma membrane fraction with GS of *A. fumigatus* was prepared and the enzyme activity was assayed by measuring the ^{14}C labeled glucan polymer. All the peptides inhibited the GS activity. Among them, ME-inv was the most effective. This peptide showed 87% inhibition at the 10 μg/ml concentration, whereas other peptides showed 29% to 42% inhibitions (Fig. 1).

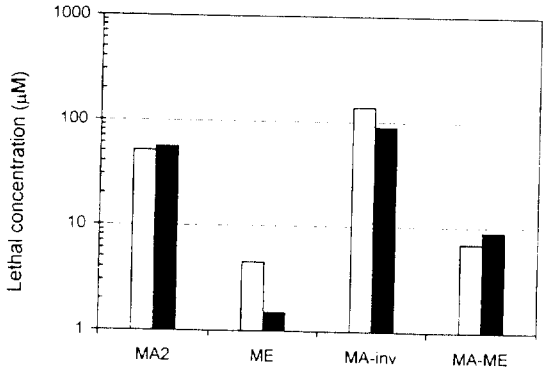

Fig. 2. Lethal concentrations of the peptides against *E. coli* HB101 (open bar) and *B. subtilis* ATCC6633 (closed bar).

The antibacterial activities of the peptides for both *E. coli* HB101 and *B. subtilis* ATCC6633 were also examined. The result (Fig. 2) shows that ME has the strongest antibacterial activity against both these two bacterial species, and MA-ME displays stronger antibacterial activity than MA2 and MA-inv.

Discussion

The aim of this study is the development of shortened peptides with potent antifungal activity against *A. fumigatus* relative to aspergillosis (1). Since MA2 is known to have an antifungal activity (4), the MA2-derived peptides based on the amphipathic-flexible-hydrophobic sequence (9, 11) were designed. Because the amphipathic α-helical structure is critical for antimicrobial activity (8), the amphipathic sequence 10-17 of MA2 was placed at the N-termini of the designed peptides, MA-inv and MA-ME (Table 1). The flexible-hydrophobic sequences of MA-inv and MA-ME were derived from the sequence 1-9 of MA2 and 1-12 of ME, respectively (Table 1). Both designed peptides have similar amphipathic α-helical patterns in their helical-wheel diagrams within an arc of 160° positive, whereas the patterns of MA2 and ME are much different from those of MA-inv and MA-ME (data not shown).

The designed peptides showed stronger antifungal activities against *A. fumigatus* than both their native peptides, MA2 and ME, but did not have hemolytic activity (Table 2). It appeares that the N-terminal sequence 10-17 of the designed peptides, KKFGKAFV, was considered to be important for antifungal activity for *A. fumigatus*. When the sequence of MA-ME was changed into the other amphipathic sequences such as 11-18 of bombinin, KVGLKGLA, or 20-29 of bovine lactoferrin, RRWQWRMKKL, the resulting peptides no longer showed antifungal activity against *A. fumigatus* even though they still have stronger antibacterial activities (data not shown). Both MA-inv and MA-ME had similar antifungal activities against *Aspergillus* sp., but different activity against *F. oxysporum* which probably is due to the differences in their C-terminal sequences (Table 2). The C-terminal effects of the peptides were also found in the antibacterial activities against both *E. coli* and *B. subtilis* (Fig. 2).

A fungal GS is important to the fungal mycelium growth by synthesizing (1,3)-β-D-glucan, a crucial (up to 50~60%) component of the fungal cell wall. The lipopeptides such as cilofungin and aucleacin A inhibit *in vitro* GS activity of *A. fumigatus*, and the inhibition of GS activity by cilofungin is well correlated with its *in vivo* activity against *A. fumigatus* (17). This study (Fig. 1) also suggests that inhibition of (1,3)-glucan synthesis by the peptides could be one of the possible ways of their antifungal activity against *A. fumigatus*, and in case of MA-inv this may be more closely related to the antifungal activity against *A. fumigatus*. Since GS is a membrane enzyme and the peptides could interact with the plasma membrane, the inhibitions of GS activity by the peptides could be resulted from two possible mechanisms: 1) the plasma membrane can be disrupted by the peptides, thus GS conformation may be changed to the lower active form and/or 2) the peptides directly bind to GS and then inhibit competitively or noncompetitively. To define this, further studies are needed.

It is generally considered that the final targets of antibiotic peptides with amphipathic α-helical structures are the plasma membranes of the target organisms (18, 19). Therefore the cell wall and cell surface components of the fungi could be barriers which inhibit the access of the antibiotic molecule to the target molecules on the plasma membrane. It has been reported that the mutants of *Saccharomyces cerevisiae* resistant to a lipopeptide, aculeacin A, are no different from the parent strain with respect to cell wall polysaccharide composition and *in vitro* GS activity, but that they have differences in cell surface hydrophobicity (20). Another study showed that the antifungal lipopeptide, cilofungin, have the higher MIC value for *A. fumigatus in vitro*, even though it is effective *in vivo* (17), and the authors suggested that the results are caused by the differences of cell surface hydrophobicities between *in vitro* and *in vivo*. Our designed peptides, MA-inv and MA-ME, have 25-fold lower MIC values against *A. fumigatus* (Table 2) than the lipopeptide, cilofungin, reported previously (17), indicating that our designed peptides may therefore have higher accessibility to the plasma membrane of *A. fumigatus* than lipopeptides *in vitro*, suggesting that these peptides are good candidate peptides for drug development against *A. fumigatus*.

In conclusion, we designed the peptides, MA-inv and

MA-ME, with more potent activity against *A. fumigatus* without hemolytic activity. Their N-terminal sequence, KKFGKAFV, was important to their activity against *Aspergillus sp.*, and their different C-terminal sequences were related to their different activities against *F. oxysporum*, *E. coli* and *B. subtilis*. The inhibition activity against GS on the plasma membrane of *A. fumigatus* was greatly appeared when treated with MA-inv, and this was considered to be one of possible mechanisms of its antifungal activity against *A. fumigatus*. Although we could not conclude the general antibiotic mechanisms of our designed peptides against *Aspergillus sp.*, our data suggest that, comparing to the native forms, the synthetic peptides may have a better ability to penetrate the cell wall, to interact with the plasma membrane, and/or to inhibit the GS activity.

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