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Antifungal activity and mechanism of novel peptide GmAMP against fluconazole-resistant *Candida tropicalis*

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Background. The overusing of antifungal drugs leads to an increase in the number of clinically isolated fluconazole-resistant Candida spp. so it is an urgent need to develop novel alternative therapeutic strategies. GmAMP is a novel peptide screened by us using artificial intelligence modeling techniques, and pre-tests showed its strong antimicrobial activity against clinically fluconazole-resistant Candida tropicalis. Methods. The study aimed to comprehensively investigate the antimicrobial activity and mechanisms of GmAMP against fluconazole-resistant C. tropicalis. The antifungal activity of GmAMP against fluconazole-resistant C. tropicalis was measured by broth microdilution method, growth and fungicidal kinetics, hypha transformation, and antibiofilm assay. To further uncover the potential mechanisms of action of GmAMP, we performed scanning electron microscopy, flow cytometry, cell membrane potential probe DiSC3(5), and reactive oxygen species probe DCFH-DA detection to assess the cellular morphology and structure, membrane permeability, membrane depolarization, and ROS accumulation, respectively. At the same time, we evaluated the toxicity of GmAMP in vitro through erythrocyte hemolysis degree and cytotoxicity assays. Cytotoxicity and therapeutic efficacy in vivo were assessed by the Galleria mellonella larvae infection model. Results. GmAMP exhibited significant antifungal activity against fluconazole-resistant C. tropicalis with a MIC of 25 μM and demonstrated fungicidal effects at 100 μM within 2 h. inhibited the transition from yeast to hypha morphology, restrained the biofilm formation rate of 88.32%, and eradicated the mature biofilm rate of 58.28%. Furthermore, treatment of fluconazole-resistant C. tropicalis with GmAMP at a concentration of 100 μM resulted in cell structure damage while treatment with GmAMP at concentrations ranging from 25 ~ 100 μM all caused membrane permeability, depolarization of cell membrane potential, and

intracellular ROS accumulation, Moreover, GmAMP enhanced the survival rate of 75% for G. mellonella with fluconazole-resistant C. tropicalis infection as well as reduced fungal burden in vivo by approximately 1.0×102 CFU per larva. Conclusion. GmAMP can disrupt the cell membrane of fluconazole-resistant C. tropicalis and also shows favorable safety and therapeutic efficacy in vivo. Accordingly, GmAMP has the potential to be an agent against drug-resistant fungi.

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ABSTRACT

Background. The overusing of antifungal drugs leads to an increase in the number of clinically isolated fluconazole-resistant *Candida* spp. so it is an urgent need to develop novel alternative therapeutic strategies. GmAMP is a novel peptide screened by us using artificial intelligence modeling techniques, and pre-tests showed its strong antimicrobial activity against clinically fluconazole-resistant *Candida tropicalis*.

Methods. The study aimed to comprehensively investigate the antimicrobial activity and mechanisms of GmAMP against fluconazole-resistant *C. tropicalis*. The antifungal activity of GmAMP against fluconazole-resistant *C. tropicalis* was measured by broth microdilution method, growth and fungicidal kinetics, hypha transformation, and antibiofilm assay. To further uncover the potential mechanisms of action of GmAMP, we performed scanning electron microscopy, flow cytometry, cell membrane potential probe $DiSC_3(5)$, and reactive oxygen species probe DCFH-DA detection to assess the cellular morphology and structure, membrane permeability, membrane depolarization, and ROS accumulation, respectively. At the same time, we evaluated the toxicity of GmAMP in vitro through erythrocyte hemolysis degree and cytotoxicity assays. Cytotoxicity and therapeutic efficacy in vivo were assessed by the *Galleria mellonella* larvae infection model.

Results. GmAMP exhibited significant antifungal activity against fluconazole-resistant *C. tropicalis* with a MIC of 25 μM and demonstrated fungicidal effects at 100 μM within 2 h. inhibited the transition from yeast to hypha morphology, restrained the biofilm formation rate of 88.32%, and eradicated the mature biofilm rate of 58.28%. Furthermore, treatment of

fluconazole-resistant *C. tropicalis* with GmAMP at a concentration of 100 μM resulted in cell structure damage while treatment with GmAMP at concentrations ranging from 25 ~ 100 μM all caused membrane permeability, depolarization of cell membrane potential, and intracellular ROS accumulation, Moreover, GmAMP enhanced the survival rate of 75% for *G. mellonella* with fluconazole-resistant *C. tropicalis* infection as well as reduced fungal burden in vivo by approximately 1.0×10^2 CFU per larva.

Conclusion. GmAMP can disrupt the cell membrane of fluconazole-resistant *C. tropicalis* and also shows favorable safety and therapeutic efficacy in vivo. Accordingly, GmAMP has the potential to be an agent against drug-resistant fungi.

Keywords: Antimicrobial peptide; GmAMP; Drug-resistance; Antifungal activity; *Candida tropicalis*

INTRODUCTION

Opportunistic fungal pathogens can cause cutaneous infections and invasive infections, leading to substantial illness and death (Thomas-Rüddel et al. 2022). Among these pathogens, invasive *candidiasis* emerges as a prominent concern, characterized by mortality rates exceeding 40% (Sasani et al. 2021; Tsay et al. 2020). *C. tropicalis* is a part of the human microbiomes but is also capable of causing invasive infection and it is widely regarded as the second to fourth most virulent genus of *Candida* species (Contreras Martínez et al. 2022). Azole antifungal agents, including fluconazole, itraconazole, voriconazole, posaconazole, and others, constitute the primary therapeutic resources against *C. tropicalis* infection. However, the indiscriminate and excessive use of azoles has engendered the emergence of azole-resistant strains of *C. tropicalis*

from clinical isolates. In addition, epidemiologic studies have shown that *C. tropicalis* has a higher rate of resistance to fluconazole compared to other *Candida* species (Tseng et al. 2022). Consequently, invasive infection due to high rates of azole resistance in *C. tropicalis* clinical isolates has attracted increasing attention (Fan et al. 2023). In 2022, the World Health Organization (WHO) issued the first list of fungal priority pathogens aimed at developing a strategic framework for research, development, and public health interventions, with *C. tropicalis* listed as a high-priority pathogen (Fisher & Denning 2023; World Health Organization (WHO) 2022).

It is widely recognized that azoles, especially fluconazole, are crucial therapeutic and protective agents for the controlling of *Candida* infections. However, due to the frequent and widespread use of fluconazole, there is an increasing trend of resistance, which eventually leads to the emergence of cross-resistance to other antifungal compounds (Chai et al. 2010; Jia et al. 2019; Pappas et al. 2016). Studies have shown that resistance rates of *C. tropicalis* to azoles such as fluconazole, itraconazole, voriconazole, and posaconazole have recorded resistance rates as high as 40% to 80% (World Health Organization (WHO) 2022). In addition, more than 21% of *C. tropicalis* isolates in China are resistant to fluconazole and even 21.7% of *C. tropicalis* isolates were two to four times more resistant to multi-azoles in Iran (Badiee et al. 2022; Liu et al. 2022). This resistance phenomenon may stem from various factors, including alterations in drug targets, upregulation of drug target expression, and increased expression of efflux pumps (Lee et al. 2021). As a result, antifungal drug therapies may be inefficient in treating drug-resistant candidiasis, which poses significant challenges in clinical management (McCarthy & Walsh

2017; Zhang et al. 2017). Therefore, the exploitation of novel and potential antifungal agents as well as alternative therapeutic approaches is essential to address the current problem of fungal drug resistance.

Antimicrobial peptides (AMPs) represent critical components of the immune defense system in organisms, with broad-spectrum antimicrobial activity, low drug resistance, and diverse bactericidal mechanisms (Li et al. 2020). The mechanism by which antimicrobial peptides interact with cell membranes to fulfill their antimicrobial and bactericidal effects has long been accepted (Fernández de Ullivarri et al. 2020; Zhou et al. 2023). This unique mechanism makes it not easily susceptible to the development of resistance. In earlier period research work, We employed multitasking adaptive modeling and model adaptation to establish a prediction model and screening protocol for antifungal peptides based on antimicrobial peptide databases such as APD, DRAMP, CAMP, antifp, etc (Zhang et al. 2022). Then we predicted more than three million unknown functional sequences in the UniProt database from the established model and screened out several hundreds of peptides that might be antifungally active then synthesized them by solid-phase organic synthesis method, and the antimicrobial activity was verified by wet experiments. Among them, the novel antimicrobial peptide SPGKKKKKKKKKKTKKKKKK showed strong antimicrobial activity against fluconazole-resistant *C. tropicalis* in the pre-test, with a MIC of 25 μM (MIC of fluconazole against this isolate >3343 μM).

According to the sequence homology nomenclature in the antimicrobial peptide APD3 database (http://aps.unmc.edu/AP/, main.html), The novel peptide was named GmAMP. To more fully assess GmAMP, we first determined the MIC of GmAMP against four strains of clinically

fluconazole-resistant *C. tropicalis* and the standard strain of *C. tropicalis* ATCC 20962, for which GmAMP showed excellent antimicrobial activity. Besides, to assess the antifungal activity and mechanism of GmAMP, we performed experimental validation on fluconazole-resistant *C. tropicalis*. As a result, the antifungal mechanism of GmAMP against fluconazole-resistant *C. tropicalis* was investigated in terms of physicochemistry and morphology, and its in vivo efficacy was evaluated by the *G. mellonella* larvae infection model. Thus, the findings of this study provide a certain experimental basis for the exploration and utilization of novel peptide antimicrobial drugs.

MATERIALS AND METHODS

Materials

The peptide GmAMP (SPGKKKKKKKKKKTKKKKKK) was synthesized by solid phase chemical synthesis method by Gil Biochemical Co., Ltd (Shanghai, China), purified by reversed-phase high-performance liquid chromatography (RP-HPLC) (Fig. S. 1A), and the purity was >95%. They were dissolved in deionized water at a stock concentration of 5 mg/mL before use. Then the molecular weight of GmAMP was determined using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Fig. S. 1B).

Strains and cell culture conditions

The fluconazole-resistant *C. tropicalis* 4171, 4252, 6984, and 8402 were collected from infected patient's blood in the affiliated hospital of Guizhou Medical University. *C. tropicalis* ATCC 20962 was purchased from the Shanghai Conservation Biotechnology Center. All strains were grown in yeast extract peptone dextrose medium (YPD, Solarbio, Beijing, China) at 35℃ with

shaking at 200 rpm until the cultures reached the exponential growth phase. RPMI-1640 (Invitrogen, Carlsbad, CA, United States) supplemented with 15% fetal bovine serum (FBS) (Sigma-Aldrich) was used as the culture medium for hypha growth of *C. tropicalis*. The mouse monocyte-macrophage cell line RAW 264.7 was donated by Jiahong Wu from the Key and Characteristic Laboratory of Modern Pathogen Biology, Guizhou Medical University, and cells were cultured in DMEM medium (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin (Gibco, USA), and 100 μg/mL streptomycin (Gibco, USA) and maintained at 37℃ in a humidified 5% CO2 incubator.

Antifungal Activity

The minimum inhibitory concentration (MIC) of GmAMP for four fluconazole-resistant *C. tropicalis* from clinical isolates, a standard strain of *C. tropicalis* ATCC 20962 was determined by using broth microdilution method according to the Standards of Clinical and Laboratory Standards Institute (CLSI) (CLSI Clinical and Laboratory Standards Institute; 2023). In brief, these yeasts of fungal strains were cultured in YPD broth medium at 35℃ to the logarithmic growth stage, and the cultures were washed by phosphate-buffered saline (PBS, 10 mM, pH 7.4) three times and resuspension to 0.5×10^3~2.5×10^3 CFU/mL. Then 100 μL above fungal suspension was added to a 96-well plate with a series concentration of GmAMP and fluconazole (Yuan ye, Shanghai, China) and co-cultured with fungal suspension at 35℃ for 24 h, and 10 mM PBS and medium were used as the negative and blank controls, respectively. The drug concentration corresponding to the well without visible fungal growth was regarded as MIC and the experiment was performed in triplicate and repeated three times.

Growth and fungicidal kinetics

To analyze the antifungal or fungicidal process of GmAMP against fluconazole-resistant *C. tropicalis*, the growth kinetics and time-kill kinetics of GmAMP on fluconazole-resistant *C. tropicalis* were further investigated at different times after GmAMP treatment as ~~to~~ previously described (Melo et al. 2024). The concentration of the prepared fungal cells was 1.0×10^6 CFU/mL according to the previously mentioned method and incubated with 25, 50, and 100 μM of GmAMP at 35℃ for 48 h. During co-culture, the OD_{630nm} was recorded every 2 h by a microplate reader (Thermo Scientific, USA). Meanwhile, the cultured yeasts were taken at specific time intervals (0、2、4、6、8、10、12 h), then the agar solid plate experiment was carried out after gradient dilution, and 10 mM PBS and medium was used as the negative and blank controls in the experiment. Fungal colonies were counted after incubation at 35℃ for 24 h. Finally, the results were presented as the average of triplicate measurements from three independent assays.

Effect of GmAMP on hypha formation

To analyze the effect of GmAMP on the transition of yeast-to-hyphal phase in fluconazole-resistant *C. tropicalis* as described previously (Lochenie et al. 2024). The concentration of the prepared fungal cells was 1.0×10^6 CFU/mL in RPMI 1640 medium (Gibco, USA) which contained 15% fetal bovine serum (Gibco, USA) according to the previously mentioned method. Then 500 μL of fungal suspension was incubated with GmAMP at concentrations (25, 50, and 100 μM) in 24 well plates and 10 mM PBS as the negative control. Next the plate after cultivation at 37 °C for 3, 6, 9, 12, and 24 h, the hypha formation was observed and

photographed under an inverted microscope.

Antibiofilm Assay

The fungal cells cultured to logarithmic phase were adjusted to 1.0×10^6 CFU/mL by using RPMI-1640 liquid medium and were added to the 96-well polypropylene plate and then incubated at 37℃ for 90 mins (early biofilm formation) or 48 h (mature biofilm formation) according to previous method (Zou et al. 2024). And 100 μL newly prepared 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl) 2H-tetrazolium5-carboxamide sodium salt (XTT) solution (Yuan ye, Shanghai, China) was added to each well for 2 h at 37℃ after different concentrations of GmAMP were added and continued to co-incubate for 24 h. Absorbance was measured by using a microplate reader at OD_{490nm}. Next, the sterile poly-lysine cell crawling tablets were placed at the bottom of the 24-well plate, and fungal suspension with the above concentration of 500 μL was added. The early and mature biofilms were prepared according to the XTT method, and 500 μL of SYTO 9 (Invitrogen, USA) and propidium iodide (PI, Sigma, USA) solution with the final concentration of 10 μM were added and incubated for 20 mins while 10 mM PBS was used as the negative control. Using nail polish to seal the cover glass, laser confocal microscopy was used to observe and obtain images.

Scanning electron microscope

The concentration of the prepared fungal cells was 1.0×10^6 CFU/mL based on the previous description with minor modifications (Alfaro-Vargas et al. 2022), incubated in GmAMP with culture medium at 35℃ for 2 h, and 10 mM PBS was used as the negative control, then the suspension was centrifuged at 5000 rpm for 10 mins, fixed with 2.5% glutaraldehyde overnight

at 4℃, and dehydrated with 50%, 75%, 95% and 100% series of ethanol solutions for 10 minutes. It was then dried in a vacuum evaporator and coated with a thin layer of gold-palladium. The samples were observed by scanning electron microscopy and the image acquisition was performed using a Hitachi Regulus SU8100 (Tokyo, Japan)

Flow cytometry analysis

The concentration of the prepared fungal cells was 1.0×10^6 CFU/mL according to the previous description with minor modifications (Torres et al. 2023), GmAMP with different concentrations was incubated at 35℃ for 1 h, and 10 mM PBS was used as the negative control. After that, the fungal suspension was incubated with SYTO 9 and PI staining with a final concentration of 10 μM at 35℃ for 15 mins, and the stained cells were analyzed by flow cytometry.

Membrane potential

The concentration of the prepared fungal cells was 1.0×10^6 CFU/mL as described in the previous study (Decker et al. 2024), then added to 96-well plates while the membrane potential $DiSC_3(5)$ probe was added into the fungal suspension, then treated with different concentrations of GmAMP as well as 10 mM PBS was used as the negative control to be measured fluorescence intensity. The change of fluorescence intensity in 1 h was continuously and dynamically monitored with by RF-5301PC sectrofluoro-photometer (Bio-Tek Synergy HTX, United States).

Reactive oxygen species level

The levels of ROS were determined by using 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Yuan ye, Shanghai, China) according to previously described with minor modifications (Shaban et al. 2024). The 1.0×10^6 CFU/mL of fungal suspension was incubated with 10 μM

DCFH-DA, and PBS was used as the negative control. The change of fluorescence intensity in 1 h was continuously and dynamically monitored by RF-5301PC sectrofluoro-photometer (Bio-Tek Synergy HTX, United States).

Cytotoxicity Assays

Mouse RAW 264.7 cells were used to evaluate the cytotoxicity of GmAMP on mammalian cells as previously described (de Oliveira et al. 2023). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco, USA), 1% penicillin-streptomycin (Gibco, USA), and maintained at 37°C in a humidified 5% CO_2 incubator. Firstly, 100 μL of RAW 264.7 cells suspension (2×10^4 cells/mL) was added to 96-well plates for cultivating overnight. 100 μL with different concentrations of GmAMP solution was added and then incubated at 37°C for 24 h. 10 mM PBS and complete medium were used as the negative and blank controls, respectively. After the end of incubation, 10 μL of CCK8 solution was added to each assay well and incubated for 1 h. Absorbance values were detected at $OD_{450nm,}$ and the percentage of cell survival was counted.

$$\text{Cell viability}(\%) = \left(\frac{\text{Abs}_{450\text{nm}}\text{of GmAMP solution} - \text{Abs}_{450\text{nm}}\text{of blank control}}{\text{Abs}_{450\text{nm}}\text{of PBS control} - \text{Abs}_{450\text{nm}}\text{of blank control}}\right) \times 100\%$$

Hemolysis of Human Red Blood Cells

The human red blood cells (hRBCs) were used to evaluate the hemolytic activity of GmAMP on the basis of previous descriptions with minor modifications (Chiramba et al. 2024). The

GmAMP with different concentrations and 2% hRBCs were added to the 96-well plate and incubated for 1 h in 37℃. 1% Triton X-100 (Solarbio, Beijing, China) was the positive control, and 10 mM PBS was used as the negative control. After the end of incubation, the samples were taken out and centrifuged at 1000 rpm for 10 min, the supernatant of samples was transferred to a new 96-well plate, and the percentage of hemolysis was calculated by the OD_{540nm} absorbance value.

$$\text{Hemolysis}(\%) = \left(\frac{\text{Abs}_{540\text{nm}} \text{ of GmAMP solution} - \text{Abs}_{540\text{nm}} \text{ of PBS control}}{\text{Abs}_{540\text{nm}} \text{ of (TritonX} - 100) - \text{Abs}_{540\text{nm}} \text{ of PBS control}}\right) \times 100\%$$

Galleria mellonella infection model

The larvae used in the experiment were purchased from Huiyude Biotechnology Co., Ltd.,(Tianjin, China), each weighing 250~300 mg and about 2~3 cm in length. As previously described with slight modifications (Fernandes et al. 2020). All larvae were placed in a dark incubator at 35℃ overnight before the experiment. Ten larvae were randomly divided into each group to inject 10 μL of GmAMP solution with a concentration of 8~32 mg/kg into the last left proleg of larvae to evaluate the toxicity of GmAMP. The negative control was given an equal volume of sterile PBS. To evaluate the efficacy of GmAMP, 12 larvae were randomly divided into each group and injected 10 μL into the last left proleg of larvae with approximately 5.0×10^8 CFU/mL of fungal suspension. After 1 h in the incubator, the same volume of GmAMP was injected into the last right proleg using the same method. Incubated at 35℃ for 5 days, live and

dead counts were performed every 24 h, and larvae were considered dead when they turned black or soft and had no obvious tactile response. 24 h after injection of GmAMP, 3 larvae were randomly selected from each group and placed in a 1.5 mL sterile PBS solution for high-speed homogenization grinding. 10 μL of gradient dilution was followed by dripping on a sterile solid YPD plate for culturing 24 h. The number of fungal single colony was recorded, and the *C. tropical* burden of each larva was counted.

Data processing

Statistical mapping and data analysis were performed using GraphPad Prism 8.0 software (GraphPad, Software). Data were expressed as mean ± SD and analyzed by One-Way ANOVA. Long-rank test was used for the analysis of Mantel-Cox survival curves for the *G. mellonella* survival experiment. The $P < 0.05$ was considered statistically significant.

RESULTS

GmAMP chemical characteristic

The secondary structure of antimicrobial peptides plays an important role in antimicrobial activity. α-helical structure promotes the interaction of antimicrobial peptides with the cell membrane to enhance the antimicrobial activity (Personne et al. 2023). The antimicrobial peptide GmAMP, composed of 21 amino acids, is predicted to have an α-helical structure (Figs. 1A and B). The molecular weight (MW) of GmAMP was confirmed to be 2539.33 Da by mass spectrometry. Furthermore, GmAMP has a net charge of +17 and a hydrophobicity value of -0.757 (Fig. 1C), and these characteristics suggest that GmAMP is a hydrophilic cationic peptide.

Antifungal activity

The results of the antifungal activity assay showed that GmAMP had strong antimicrobial effects against clinical fluconazole-resistant *C. tropicalis*, with MICs ranging from 25 to 50 μM. In comparison to this, the MIC values of fluconazole against clinical isolates exceeded 3343 μM (Table 1). Consequently, to better understand the effect of GmAMP on fluconazole-resistant *C. tropicalis* clinical isolate, 4252 isolate (MIC 25 μM) was selected for further study.

Growth kinetics and fungicidal kinetics

To elucidate the effect of GmAMP on the growth process of fluconazole-resistant *C. tropicalis*. The growth curve of fluconazole-resistant *C. tropicalis* under GmAMP treatment was further plotted, as shown in Fig. 2A, the fungal cells in the control group entered the logarithmic phase within 6 h and reached the stabilization phase within 18 h. In comparison, GmAMP treatments at concentrations of 25 μM and 50 μM were found to slow down the proliferation rate of fluconazole-resistant *C. tropicalis* and prolong the time to reach the logarithmic phase. When treated with GmAMP at a concentration of 100 μM, GmAMP showed an inhibitory effect on fluconazole-resistant *C. tropicalis* and prevented the natural growth and reproduction of fluconazole-resistant *C. tropicalis*. The time-fungicidal kinetic curve was further plotted to clarify the fungicidal effect of GmAMP (Fig. 2B). Compared with the control group, GmAMP exhibited a powerful inhibitory effect on fluconazole-resistant *C. tropicalis* when the concentrations of GmAMP were 25 μM and 50 μM. Notably, dealing with the concentration at 100 μM of GmAMP, the fluconazole-resistant *C. tropicalis* was killed within 2 h. These results indicate that GmAMP manifests effective antimicrobial activity and fungicidal effect against

fluconazole-resistant *C. tropicalis*.

The transformation of yeast to the mycelial phase

The transformation of *Candida* mycelial morphology is closely related to its pathogenicity. Morphological changes during the transformation of the fluconazole-resistant *C. tropicalis* yeast phase to the mycelial phase were observed by using an inverted microscope which is shown in Fig. 2C. The length of the mycelium of the fungus in the control group increased with incubation time, and 9 h of incubation, yeast cells can be seen growing to form bundles of hyphae while forming branches of various sizes and lengths and intertwining with each other to form a net structure, and a more tightly netted biofilm is formed over time. Interestingly, the development of fluconazole-resistant *C. tropicalis* from yeast cells to mycelial morphology was completely inhibited after treatment with different concentrations of GmAMP. GmAMP treatments at concentrations of 25 μM and 50 μM not only inhibited morphological transformation but also 100 uM concentration of GmAMP reduced the number of yeast cells, allowing only a minor number of yeast cells to be observed. These results suggest that GmAMP inhibited the morphological transformation process of fluconazole-resistant *C. tropicalis* from the yeast phase to the mycelial phase.

Inhibition of biofilm formation and eradication of mature biofilm

We visualized the results by using confocal laser scanning microscopy. In the control group of the biofilm formation assay, a compact and intact biofilm was observed to emit predominantly green fluorescence. Nevertheless, after GmAMP treatment, intact biofilms could no longer be formed and only dispersed incomplete membranes of different sizes were observed, while only

single yeast cells could be seen in the high-concentration group (Fig. 3A). In the control group of the biofilm eradication assay, it was observed that the tightly structured and network-interwoven biofilms mainly emitted green fluorescence. After GmAMP treatment, we found that the tightly structured biofilm became loose and the network structure was reduced and thinned, while a reduction in the number of yeasts and an increase in the number of fungal deaths were observed, with a predominantly red fluorescence (Fig. 3B). In addition, the biofilm activity of *C. tropicalis* was determined by XTT quantitative method. Compared with the control group, GmAMP at concentrations of 50, 100, and 200 μM inhibited biofilm formation by 49.75%, 71.28%, and 88.32%, respectively (Fig. 3C), and eliminated mature biofilm by 17.53%, 42.07%, and 58.28%, respectively (Fig. 3D). These results indicated that GmAMP inhibited biofilm formation and eradicated a certain amount of mature biofilm in fluconazole-resistant *C. tropicalis*.

Antifungal mechanism

To investigate the impact of GmAMP on the cell morphology of fluconazole-resistant *C. tropicalis*, the morphological changes induced by GmAMP treatment of fungal cells for 2 h were directly observed by scanning electron microscopy. Untreated fungal cells were morphologically intact, with cell surfaces remaining round and smooth (Fig. 4A). When cells were exposed to 100 μM of GmAMP for 2 h, the cells were destroyed and the surface appeared rough and irregular. (Fig. 4B). Thus, these outcomes suggested that the cellular structural integrity of fluconazole-resistant *C. tropicalis* has been impaired. To further investigate the interaction of GmAMP on cell membrane of fluconazole-resistant *C. tropicalis*, PI and SYTO9 fluorescence staining were used to determine the effect of GmAMP on the integrity of cell membrane. PI and STOY9 ~~as~~

DNA-binding dyes ~~emitted~~ red and green fluorescence, respectively, and the former only penetrated the membrane-damaged cells, while the latter ~~emitted green fluorescence and~~ stained both live and dead cells (Jin et al. 2005). As a result, when cells were exposed to 25, 50, and 100 μM of GmAMP, 45.02%, 76.88%, and 85.02% of the cells stained positively for PI, respectively. Moreover, PI staining positivity showed a dose-dependent correlation with GmAMP (Fig. 4C), which indicated that GmAMP disrupted the cell membrane integrity of fluconazole-resistant *C. tropicalis*. The membrane depolarization was detected using the membrane potential probe $DiSC_3(5)$. As shown in Fig. 4D, compared with the control group, we found that GmAMP treatment caused depolarization of the cell membrane potential of fluconazole-resistant *C. tropicalis* and the level of membrane potential rose significantly with increasing concentration of GmAMP. Reactive oxygen species (ROS) generally maintain low levels within normal cells, but the accumulation of higher levels of ROS can damage cellular structures (Huang et al. 2020). Here, different concentrations of GmAMP induced ROS accumulation in fluconazole-resistant *C. tropicalis*, and ROS levels showed a time-dose dependence (Fig. 4E). These results suggest that the presence of GmAMP induced ROS production, which contributed to the crucial factor in the antimicrobial effect of GmAMP.

Hemolytic and Cytotoxicity

To evaluate mammalian cytotoxicity, we assessed the safety of GmAMP on human red blood cells and RAW 264.7 cells. The hemolysis experiment was performed using 2% human red blood cells. At a concentration of 200 μM, GmAMP exhibited slight hemolysis with a hemolysis rate of 35.64% (Fig. 5A). Moreover, GmAMP showed no obvious cytotoxicity to RAW 264.7

cells, and the cell viability of mouse macrophage RAW 264.7 remained above 80% at 200 μM concentration of GmAMP (Fig. 5B).

Therapeutic effect on fluconazole-resistant C. tropical infection in vivo

The *Galleria mellonella* larvae infection model was used to study the in vivo therapeutic effect of GmAMP. In the peptide toxicity test (Fig. 6B). All larvae survived and were undead in the experimental model, suggesting that GmAMP did not show significant toxicity in the concentration range of 32 mg/kg. Survival of larvae infected with fluconazole-resistant *C. tropicalis* was increased by injecting various concentrations of GmAMP, with 75% survival in the 32 mg/kg group ($P<0.05$), whereas the control group has 40% survival rate at 5 days post infection (Fig. 6C). These results were also reflected by the fungal burden of *Galleria mellonella* larvae, after treating larvae with GmAMP for 24 hours, and the number of colonies pre larvae was significantly reduced in all treatment groups while 32 mg/kg of GmAMP reduced fungal burden from 5.27×10^8 to 6.37×10^6 CFU per larva (Fig. 6D). Consequently, GmAMP has the potential for clinical application as it effectively treats fluconazole-resistant *C. tropicalis* infection and reduces the fungal burden in vivo.

DISCUSSION

Currently, the irrational use of antifungal drugs is leading to a rapid development of resistance to antifungal drugs resulting in a surge in morbidity and mortality from invasive fungal infection (Fan et al. 2024). The isolation rate of *Candida* species, such as *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, and *Candida parapsilosis* is steadily increasing in hospitals (Falagas et al. 2010; Lee et al. 2022). Recent reports show that the proportion of

fluconazole-resistant *C. tropicalis* isolates continues to rise in China (Wang et al. 2021). The development of new antifungal drugs to address this problem is imminent. Widely distributed in animals, plants, and other organisms, AMPs are a fast and effective barrier against pathogens in humans. AMPs exert their antimicrobial activity through a unique membrane-targeting mechanism that avoids the development of drug resistance and is regarded as a novel alternative to synthetic antibiotics (Mulukutla et al. 2024).

In this study, we determined in vitro the antimicrobial activity of GmAMP against four clinical isolates of fluconazole-resistant *C. tropicalis* and the standard strain of *C. tropicalis* ATCC 20962, and GmAMP showed good antimicrobial effect against clinical isolate of fluconazole-resistant *C. tropicalis* 4252, with a MIC value of 25 μM. GmAMP not only delayed the proliferation rate and inhibited the growth and reproduction of fungi, but also effectively killed GmAMP within 2 h. These results revealed that GmAMP is an effective antimicrobial agent, and there is a need to further investigate the antifungal efficacy of GmAMP.

The process of transformation from the yeast phase to the mycelial phase, termed "biphasic", is considered the most important pathogenic characteristic of *Candida*, and is also recognized as a key stage in biofilm formation and maturation (Zhu et al. 2024). Furthermore, the hyphae formed during the morphological transformation can penetrate cells and invade the bloodstream, expressing a wide range of virulence factors, and are therefore considered to be a more virulent phenotype than yeast (Khamzeh et al. 2023). Herein, GmAMP inhibited the transition of yeast phase cells to mycelial phase morphology, preventing the process of mycelial development, which demonstrates that GmAMP plays a key role in inhibiting the formation and maturation of

fluconazole-resistant *C. tropicalis* biofilm by preventing mycelial development. The yeast cells of *C. tropicalis* are characterized by a high capacity to form biofilms compared to other *Candida* species (Zuza-Alves et al. 2017), which may be related to an increased amount of biomass in the membranes and extracellular matrix, leading to a denser structure (Chandra & Mukherjee 2015; Desai & Mitchell 2015). Here, the 50 μM (2×MIC) of GmAMP inhibits biofilm formation and has an eradicative effect on mature biofilms. Moreover, GmAMP inhibited and eliminated biofilms of fluconazole-resistant *C. tropicalis* in a concentration-dependent manner. Therefore, GmAMP showed good bioactivity in inhibiting morphological transformation and anti-biofilm processes.

It has been reported that most antimicrobial peptides exert their antimicrobial effects mainly by targeting cell membranes (Aguiar et al. 2020; Buda De Cesare et al. 2020; Hu et al. 2022). In the present study, the results of the scanning electron microscopy assay demonstrated that GmAMP disrupts the morphology and structure of the fluconazole-resistant *C. tropicalis*. A similar phenomenon was also found by Ma, et al (Ma et al. 2020; Zhang et al. 2023). Moreover, we speculated the exact reason for the morphological damage and subsequent cell death is correlated with increased membrane permeability resulting from electrostatic interactions between the positively charged peptide GmAMP and the negatively charged components of the fungal cytoplasmic membrane (Boparai & Sharma 2020; Jayasinghe et al. 2023; Kodedová et al. 2019). The experimental result verified membrane permeability by a significant increase in the number of PI-stained positive cells of the fungi treated with GmAMP. Changes in cell membrane permeability usually trigger variation in cell membrane potential, which is closely related to

cellular function (D'Auria et al. 2022). When membrane-modifying compounds (e.g., peptides) depolarize the membrane then the potential is lost. $DiSC_3(5)$ is released into the solution, causing fluorescence enhancement, which indicates that the cytoplasmic membrane is altered because of the cell membrane depolarization by the action of GmAMP in a concentration-dependent manner, which suggests that the dissipation of membrane potential might be involved in the formation of channels or pores, then allowed the passage of ions or macromolecules, to lead cytoplasmic membrane dysfunction (Bezerra et al. 2022; Venkatesh et al. 2017). It has been found that aerobic metabolism-generated ROS are usually present in cells that are in equilibrium with antioxidant enzymes, and excess ROS have certain deleterious effects on the basic structure of fungi, such as damage to nucleic acids, DNA, amino acid residues, and cell membranes (Perrone et al. 2008). We found that GmAMP induced the accumulation of reactive oxygen species in a dose-dependent manner(Taveira et al. 2022). In brief, we hypothesized that the cationic peptide GmAMP can interact with certain negatively charged substance molecules on the cell membrane through electrostatic interactions, it leads to a series of consequences such as increased membrane permeability, altered depolarization of the membrane potential, structural loss of membrane integrity, accumulation of ROS, and further leakage of intracellular contents, which finally leads to cytoplasmic membrane dysfunction and cell death.

The excellent antimicrobial activity of antimicrobial peptides is usually associated with strong hemolytic activity and cytotoxicity, and assessment of the in vitro safety of AMP is paramount for further consideration as a potential clinical candidate (Zhang et al. 2024). In this study, our results showed that GmAMP showed little cytotoxicity and low hemolytic effect. Although some cytotoxicity

and hemolytic activity were observed at higher concentrations, considering the MIC value of GmAMP was 25 μM (Table 1), which was much lower than its cytotoxicity concentration, GmAMP safety is also guaranteed under the premise of ensuring its activity. However, the potential toxicity of GmAMP to other mammalian cells remains to be studied.

Cytotoxicity and hemolytic assays confirmed the safe and effective dosage range of GmAMP, laying the foundation for its application in animal studies. In this study, the therapeutic efficacy of GmAMP was tested in vivo using the *Galleria mellonella* larvae infection model, which showed a significant improvement in survival. GmAMP treatment significantly reduced the fungal burden of *Galleria mellonella* larvae in vivo, and these findings suggest that GmAMP may exhibit a strong safety and certain therapeutic potential.

CONCLUSIONS

This work describes the antifungal activity and mechanism of antimicrobial peptide GmAMP and therapeutic efficacy in vivo. GmAMP possesses potent antimicrobial activity, anti-biofilm formation, and eradication ability, and may play an antimicrobial role by disrupting the structure of fungal cytomembrane. Here, GmAMP displays low cytotoxicity and low hemolytic activity in vitro experiments. Furthermore, GmAMP exhibits a therapeutic effect against fluconazole-resistant *C. tropical* infection and reduces the number of fungi in vivo. These properties make GmAMP a potential treatment for fluconazole-resistant *C. tropical* infection, which is worthy of further optimization and development. In addition, GmAMP offers more possibilities for the clinical application of antimicrobial peptides for safety and therapeutic efficacy in the development of drug resistance.

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Ruxia Cai conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Na Zhao conceived and designed the experiments, performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.

- Chaoqin Sun performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
- Mingjiao Huang performed the experiments, prepared figures and/or tables, and approved the final draft.
- Zhenlong Jiao performed the experiments, analyzed the data, and approved the final draft.
- Jian Peng performed the experiments, prepared figures and/or tables, and approved the final draft.
- Jin Zhang analyzed the data, prepared figures and/or tables, and approved the final draft.
- Guo Guo conceived and designed the experiments, authored or reviewed drafts of the article, explore experimental procedures and supervise the experiment, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw measurements are available in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found in supplemental files.

REFERENCES

Aguiar FLL, Santos NC, de Paula Cavalcante CS, Andreu D, Baptista GR, and Gonçalves S. 2020. Antibiofilm Activity on Candida albicans and Mechanism of Action on Biomembrane Models of the Antimicrobial Peptide Ctn[15-34]. *Int J Mol Sci* 21. http://dx.org/10.3390/ijms21218339

Alfaro-Vargas P, Bastos-Salas A, Muñoz-Arrieta R, Pereira-Reyes R, Redondo-Solano M, Fernández J, Mora-Villalobos A, and López-Gómez JP. 2022. Peptaibol Production and Characterization from Trichoderma asperellum and Their Action as

Biofungicide. *J Fungi (Basel)* 8. http://dx.org/10.3390/jof8101037

Badiee P, Boekhout T, Haddadi P, Mohammadi R, Ghadimi-Moghadam A, Soltani J, Zarei Mahmoudabadi A, Ayatollahi Mousavi SA, Najafzadeh MJ, Diba K, Salimi-Khorashad AR, Amin Shahidi M, Ghasemi F, and Jafarian H. 2022. Epidemiology and Antifungal Susceptibility of Candida Species Isolated from 10 Tertiary Care Hospitals in Iran. *Microbiol Spectr* 10:e0245322. http://dx.org/10.1128/spectrum.02453-22

Bezerra LP, Freitas CDT, Silva AFB, Amaral JL, Neto NAS, Silva RGG, Parra ALC, Goldman GH, Oliveira JTA, Mesquita FP, and Souza PFN. 2022. Synergistic Antifungal Activity of Synthetic Peptides and Antifungal Drugs against Candida albicans and C. parapsilosis Biofilms. *Antibiotics (Basel)* 11. http://dx.org/10.3390/antibiotics11050553

Boparai JK, and Sharma PK. 2020. Mini Review on Antimicrobial Peptides, Sources, Mechanism and Recent Applications. *Protein Pept Lett* 27:4-16. http://dx.org/10.2174/0929866526666190822165812

Buda De Cesare G, Cristy SA, Garsin DA, and Lorenz MC. 2020. Antimicrobial Peptides: a New Frontier in Antifungal Therapy. *mBio* 11. http://dx.org/10.1128/mBio.02123-20

Chai LY, Denning DW, and Warn P. 2010. Candida tropicalis in human disease. *Crit Rev Microbiol* 36:282-298. http://dx.org/10.3109/1040841x.2010.489506

Chandra J, and Mukherjee PK. 2015. Candida Biofilms: Development, Architecture, and Resistance. *Microbiol Spectr* 3. http://dx.org/10.1128/microbiolspec.MB-0020-2015

Chiramba CK, Möller DS, Lorenz CD, Chirombo RR, Mason AJ, Bester MJ, and Gaspar ARM. 2024. Tryptophan End-Tagging Confers Antifungal Activity on a Tick-Derived Peptide by Triggering Reactive Oxygen Species Production. *ACS Omega* 9:15556-15572. http://dx.org/10.1021/acsomega.4c00478

CLSI. Clinical and Laboratory Standards Institute; 2023. *Performance Standards for Antimicrobial Susceptibility Testing*.

Contreras Martínez OI, Angulo Ortíz A, and Santafé Patiño G. 2022. Mechanism of Antifungal Action of Monoterpene Isoespintanol against Clinical Isolates of Candida tropicalis. *Molecules* 27. http://dx.org/10.3390/molecules27185808

D'Auria FD, Casciaro B, De Angelis M, Marcocci ME, Palamara AT, Nencioni L, and Mangoni ML. 2022. Antifungal Activity of the Frog Skin Peptide Temporin G and Its Effect on Candida albicans Virulence Factors. *Int J Mol Sci* 23. http://dx.org/10.3390/ijms23116345

de Oliveira AS, de Oliveira JS, Kumar R, Silva FBA, Fernandes MR, Nobre FD, Costa ADC, Albuquerque P, Sidrim JJC, Rocha MFG, Santos FA, Srivastava V, Romeiro LAS, and Brilhante RSN. 2023. Antifungal activity of sustainable histone deacetylase inhibitors against planktonic cells and biofilms of Candida spp. and Cryptococcusneoformans. *Med Mycol* 61. http://dx.org/10.1093/mmy/myad073

Decker T, Rautenbach M, Khan S, and Khan W. 2024. Antibacterial efficacy and membrane mechanism of action of the Serratia-derived non-ionic lipopeptide, serrawettin W2-FL10. *Microbiol Spectr*:e0295223. http://dx.org/10.1128/spectrum.02952-23

Desai JV, and Mitchell AP. 2015. Candida albicans Biofilm Development and Its Genetic Control. *Microbiol Spectr* 3. http://dx.org/10.1128/microbiolspec.MB-0005-2014

Falagas ME, Roussos N, and Vardakas KZ. 2010. Relative frequency of albicans and the various non-albicans Candida spp among candidemia isolates from inpatients in various parts of the world: a systematic review. *Int J Infect Dis* 14:e954-966. http://dx.org/10.1016/j.ijid.2010.04.006

Fan X, Dai RC, Zhang S, Geng YY, Kang M, Guo DW, Mei YN, Pan YH, Sun ZY, Xu YC, Gong J, and Xiao M. 2024. Author Correction: Tandem gene duplications contributed to high-level azole resistance in a rapidly expanding Candida tropicalis population. *Nat Commun* 15:587. http://dx.org/10.1038/s41467-024-44825-y

Fan X, Tsui CKM, Chen X, Wang P, Liu ZJ, and Yang CX. 2023. High prevalence of fluconazole resistant Candida tropicalis

among candiduria samples in China: An ignored matter of concern. *Front Microbiol* 14:1125241. http://dx.org/10.3389/fmicb.2023.1125241

Fernandes KE, Weeks K, and Carter DA. 2020. Lactoferrin Is Broadly Active against Yeasts and Highly Synergistic with Amphotericin B. *Antimicrob Agents Chemother* 64. http://dx.org/10.1128/aac.02284-19

Fernández de Ullivarri M, Arbulu S, Garcia-Gutierrez E, and Cotter PD. 2020. Antifungal Peptides as Therapeutic Agents. *Front Cell Infect Microbiol* 10:105. http://dx.org/10.3389/fcimb.2020.00105

Fisher MC, and Denning DW. 2023. The WHO fungal priority pathogens list as a game-changer. *Nat Rev Microbiol* 21:211-212. http://dx.org/10.1038/s41579-023-00861-x

Hu N, Mo XM, Xu SN, Tang HN, Zhou YH, Li L, and Zhou HD. 2022. A novel antimicrobial peptide derived from human BPIFA1 protein protects against Candida albicans infection. *Innate Immun* 28:67-78. http://dx.org/10.1177/17534259221080543

Huang Y, Fujii K, Chen X, Iwatani S, Chibana H, Kojima S, and Kajiwara S. 2020. Fungal NOX is an essential factor for induction of TG2 in human hepatocytes. *Med Mycol* 58:679-689. http://dx.org/10.1093/mmy/myz105

Jayasinghe JNC, Whang I, and De Zoysa M. 2023. Antifungal Efficacy of Antimicrobial Peptide Octominin II against Candida albicans. *Int J Mol Sci* 24. http://dx.org/10.3390/ijms241814053

Jia C, Zhang J, Zhuge Y, Xu K, Liu J, Wang J, Li L, and Chu M. 2019. Synergistic effects of geldanamycin with fluconazole are associated with reactive oxygen species in Candida tropicalis resistant to azoles and amphotericin B. *Free Radic Res* 53:618-628. http://dx.org/10.1080/10715762.2019.1610563

Jin Y, Zhang T, Samaranayake YH, Fang HH, Yip HK, and Samaranayake LP. 2005. The use of new probes and stains for improved assessment of cell viability and extracellular polymeric substances in Candida albicans biofilms. *Mycopathologia* 159:353-360. http://dx.org/10.1007/s11046-004-6987-7

Khamzeh A, Dahlstrand Rudin A, Venkatakrishnan V, Stylianou M, Sanchez Klose FP, Urban CF, Björnsdottir H, Bylund J, and Christenson K. 2023. High levels of short chain fatty acids secreted by Candida albicans hyphae induce neutrophil chemotaxis via Free Fatty Acid Receptor 2. *J Leukoc Biol*. http://dx.org/10.1093/jleuko/qiad146

Kodedová M, Valachovič M, Csáky Z, and Sychrová H. 2019. Variations in yeast plasma-membrane lipid composition affect killing activity of three families of insect antifungal peptides. *Cell Microbiol* 21:e13093. http://dx.org/10.1111/cmi.13093

Lee JK, Park S, Kim YM, Guk T, Choi JK, Kim JY, Lee MY, Jang MK, and Park SC. 2022. Antifungal and Anti-Inflammatory Activities of PS1-2 Peptide against Fluconazole-Resistant Candida albicans. *Antibiotics (Basel)* 11. http://dx.org/10.3390/antibiotics11121779

Lee Y, Puumala E, Robbins N, and Cowen LE. 2021. Antifungal Drug Resistance: Molecular Mechanisms in Candida albicans and Beyond. *Chem Rev* 121:3390-3411. http://dx.org/10.1021/acs.chemrev.0c00199

Li R, Zhao J, Huang L, Yi Y, Li A, Li D, Tao M, and Liu Y. 2020. Antimicrobial peptide CGA-N12 decreases the Candida tropicalis mitochondrial membrane potential via mitochondrial permeability transition pore. *Biosci Rep* 40. http://dx.org/10.1042/bsr20201007

Liu Y, Chen Z, Li J, Zhu Z, Pang S, Xu J, and Wu J. 2022. Extensive Diversity and Prevalent Fluconazole Resistance among Environmental Yeasts from Tropical China. *Genes (Basel)* 13. http://dx.org/10.3390/genes13030444

Lochenie C, Duncan S, Zhou Y, Fingerhut L, Kiang A, Benson S, Jiang G, Liu X, Mills B, and Vendrell M. 2024. Photosensitizer-Amplified Antimicrobial Materials for Broad-Spectrum Ablation of Resistant Pathogens in Ocular Infections. *Adv Mater*:e2404107. http://dx.org/10.1002/adma.202404107

Ma H, Zhao X, Yang L, Su P, Fu P, Peng J, Yang N, and Guo G. 2020. Antimicrobial Peptide AMP-17 Affects Candida albicans by Disrupting Its Cell Wall and Cell Membrane Integrity. *Infect Drug Resist* 13:2509-2520. http://dx.org/10.2147/idr.S250278

McCarthy MW, and Walsh TJ. 2017. Drug development challenges and strategies to address emerging and resistant fungal pathogens. *Expert Rev Anti Infect Ther* 15:577-584. http://dx.org/10.1080/14787210.2017.1328279

Melo AKV, da Nóbrega Alves D, Gomes da Costa PCQ, Lopes SP, de Sousa DP, Guerra FQS, Sobral MV, Gomes Moura AP, Scotti L, and de Castro RD. 2024. Antifungal Activity, Mode of Action, and Cytotoxicity of 4-chlorobenzyl p-coumarate: a Promising New Molecule. *Chem Biodivers*:e202400330. http://dx.org/10.1002/cbdv.202400330

Mulukutla A, Shreshtha R, Kumar Deb V, Chatterjee P, Jain U, and Chauhan N. 2024. Recent advances in antimicrobial peptide-based therapy. *Bioorg Chem* 145:107151. http://dx.org/10.1016/j.bioorg.2024.107151

Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, Reboli AC, Schuster MG, Vazquez JA, Walsh TJ, Zaoutis TE, and Sobel JD. 2016. Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America. *Clin Infect Dis* 62:e1-50. http://dx.org/10.1093/cid/civ933

Perrone GG, Tan SX, and Dawes IW. 2008. Reactive oxygen species and yeast apoptosis. *Biochim Biophys Acta* 1783:1354-1368. http://dx.org/10.1016/j.bbamcr.2008.01.023

Personne H, Paschoud T, Fulgencio S, Baeriswyl S, Köhler T, van Delden C, Stocker A, Javor S, and Reymond JL. 2023. To Fold or Not to Fold: Diastereomeric Optimization of an α-Helical Antimicrobial Peptide. *J Med Chem* 66:7570-7583. http://dx.org/10.1021/acs.jmedchem.3c00460

Sasani E, Khodavaisy S, Rezaie S, Salehi M, and Yadegari MH. 2021. The relationship between biofilm formation and mortality in patients with Candida tropicalis candidemia. *Microb Pathog* 155:104889. http://dx.org/10.1016/j.micpath.2021.104889

Shaban S, Patel M, and Ahmad A. 2024. Antifungal activity of human antimicrobial peptides targeting apoptosis in Candida auris. *J Med Microbiol* 73. http://dx.org/10.1099/jmm.0.001835

Taveira GB, de Oliveira Mello É, Simão T, Cherene MB, de Oliveira Carvalho A, Muzitano MF, Lassounskaia E, Pireda S, de Castro Miguel E, Basso LGM, Da Cunha M, da Motta OV, and Gomes VM. 2022. A new bioinspired peptide on defensin from C. annuum fruits: Antimicrobial activity, mechanisms of action and therapeutical potential. *Biochim Biophys Acta Gen Subj* 1866:130218. http://dx.org/10.1016/j.bbagen.2022.130218

Thomas-Rüddel DO, Schlattmann P, Pletz M, Kurzai O, and Bloos F. 2022. Risk Factors for Invasive Candida Infection in Critically Ill Patients: A Systematic Review and Meta-analysis. *Chest* 161:345-355. http://dx.org/10.1016/j.chest.2021.08.081

Torres R, Barreto-Santamaría A, Arévalo-Pinzón G, Firacative C, Gómez BL, Escandón P, Patarroyo MA, and Muñoz JE. 2023. In Vitro Antifungal Activity of Three Synthetic Peptides against Candida auris and Other Candida Species of Medical Importance. *Antibiotics (Basel)* 12. http://dx.org/10.3390/antibiotics12081234

Tsay SV, Mu Y, Williams S, Epson E, Nadle J, Bamberg WM, Barter DM, Johnston HL, Farley MM, Harb S, Thomas S, Bonner LA, Harrison LH, Hollick R, Marceaux K, Mody RK, Pattee B, Shrum Davis S, Phipps EC, Tesini BL, Gellert AB, Zhang AY, Schaffner W, Hillis S, Ndi D, Graber CR, Jackson BR, Chiller T, Magill S, and Vallabhaneni S. 2020. Burden of Candidemia in the United States, 2017. *Clin Infect Dis* 71:e449-e453. http://dx.org/10.1093/cid/ciaa193

Tseng KY, Liao YC, Chen FC, Chen FJ, and Lo HJ. 2022. A predominant genotype of azole-resistant Candida tropicalis clinical strains. *Lancet Microbe* 3:e646. http://dx.org/10.1016/s2666-5247(22)00179-3

Venkatesh M, Barathi VA, Goh ETL, Anggara R, Fazil M, Ng AJY, Harini S, Aung TT, Fox SJ, Liu S, Yang L, Barkham TMS, Loh XJ, Verma NK, Beuerman RW, and Lakshminarayanan R. 2017. Antimicrobial Activity and Cell Selectivity of Synthetic and Biosynthetic Cationic Polymers. *Antimicrob Agents Chemother* 61. http://dx.org/10.1128/aac.00469-17

Wang Y, Fan X, Wang H, Kudinha T, Mei YN, Ni F, Pan YH, Gao LM, Xu H, Kong HS, Yang Q, Wang WP, Xi HY, Luo YP, Ye LY, and Xiao M. 2021. Continual Decline in Azole Susceptibility Rates in Candida tropicalis Over a 9-Year Period in China. *Front Microbiol* 12:702839. http://dx.org/10.3389/fmicb.2021.702839

World Health Organization (WHO) ARDA, Control of Neglected Tropical Diseases (NTD), Global Coordination and Partnership (GCP). 2022. WHO fungal priority pathogens list to guide research, development and public health action.

Zhang H, Chen Q, Xie J, Cong Z, Cao C, Zhang W, Zhang D, Chen S, Gu J, Deng S, Qiao Z, Zhang X, Li M, Lu Z, and Liu R. 2023. Switching from membrane disrupting to membrane crossing, an effective strategy in designing antibacterial polypeptide. *Sci Adv* 9:eabn0771. http://dx.org/10.1126/sciadv.abn0771

Zhang H, Zhang S, Wang P, Qin Y, and Wang H. 2017. Forecasting of particulate matter time series using wavelet analysis and wavelet-ARMA/ARIMA model in Taiyuan, China. *J Air Waste Manag Assoc* 67:776-788. http://dx.org/10.1080/10962247.2017.1292968

Zhang J, Yang L, Tian Z, Zhao W, Sun C, Zhu L, Huang M, Guo G, and Liang G. 2022. Large-Scale Screening of Antifungal Peptides Based on Quantitative Structure-Activity Relationship. *ACS Med Chem Lett* 13:99-104. http://dx.org/10.1021/acsmedchemlett.1c00556

Zhang LM, Zhou SW, Huang XS, Chen YF, Mwangi J, Fang YQ, Du T, Zhao M, Shi L, and Lu QM. 2024. Blap-6, a Novel Antifungal Peptide from the Chinese Medicinal Beetle Blaps rhynchopetera against Cryptococcus neoformans. *Int J Mol Sci* 25. http://dx.org/10.3390/ijms25105336

Zhou Y, Meng X, Chen F, Xiong M, Zhang W, and Wang KJ. 2023. Newly Discovered Antimicrobial Peptide Scyampcin(44-63) from Scylla paramamosain Exhibits a Multitargeted Candidacidal Mechanism In Vitro and Is Effective in a Murine Model of Vaginal Candidiasis. *Antimicrob Agents Chemother* 67:e0002223. http://dx.org/10.1128/aac.00022-23

Zhu X, Jin F, Yang G, Zhuang T, Zhang C, Zhou H, Niu X, Wang H, and Wu D. 2024. Mitochondrial Protease Oct1p Regulates Mitochondrial Homeostasis and Influences Pathogenicity through Affecting Hyphal Growth and Biofilm Formation Activities in Candida albicans. *J Fungi (Basel)* 10. http://dx.org/10.3390/jof10060391

Zou K, Yin K, Ren S, Zhang R, Zhang L, Zhao Y, and Li R. 2024. Activity and mechanism of action of antimicrobial peptide ACPs against Candida albicans. *Life Sci*:122767. http://dx.org/10.1016/j.lfs.2024.122767

Zuza-Alves DL, Silva-Rocha WP, and Chaves GM. 2017. An Update on Candida tropicalis Based on Basic and Clinical Approaches. *Front Microbiol* 8:1927. http://dx.org/10.3389/fmicb.2017.01927

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Table 1(on next page)

Antifungal activity of GmAMP.

The determination of the antifungal activity of GmAMP against four strains of *C. tropicalis*.

Table 1：

Antifungal activity of GmAMP.

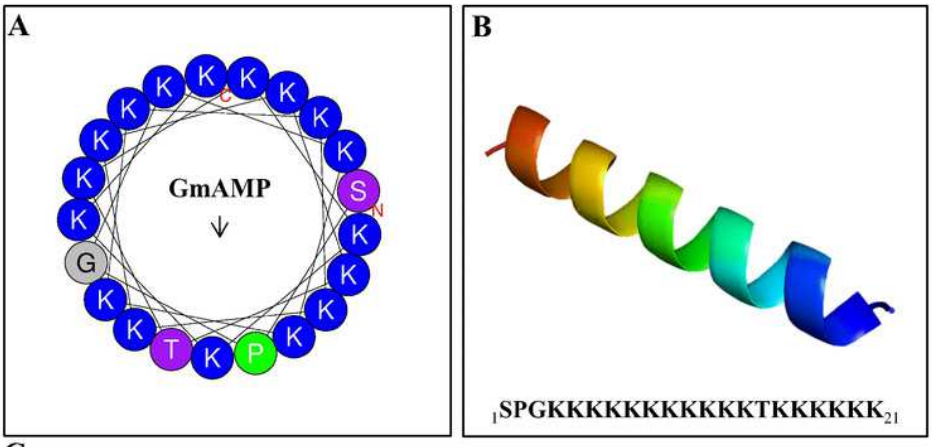
The determination of the antifungal activity of GmAMP against four strains of *C. tropicalis*.

Strains	MIC（μM）	
	GmAMP	Fluconazole
Fluconazole-resistant *C. tropicalis* 4252	25	>3343
Fluconazole-resistant *C. tropicalis* 4171	50	>3343
Fluconazole-resistant *C. tropicalis* 6984	50	>3343
Fluconazole-resistant *C. tropicalis* 8402	50	>3343
C. tropicalis ATCC 20962	12	13

Figure 1

Physicochemical properties of GmAMP.

(A) Heliquest software (https://heliquest. ipmc.cnrs.fr/) was used to draw the spiral wheel diagram, positively charged amino acids are indicated in blue, uncharged polar residues are shown in purple the red ‘N’ represents the starting position and the arrow represents the hydrophobic moment. (B) The structure of GmAMP was predicated by AlphFold2. (C) The physical and chemical properties of GmAMP were analyzed via the Expasy ProtParam website (http://web.expasy.org/protparam/).



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Peptide	Hydrophobicity <H>	Hydrophobic moment <μH>	Net charge	Theoretical MW	Measured MW
GmAMP	-0.757	0.142	+17	2539.33	2539.35

Figure 1 Physicochemical properties of GmAMP. (A) Heliquest software (https://heliquest. ipmc.cnrs.fr/) was used to draw the spiral wheel diagram, positively charged amino acids are indicated in blue, uncharged polar residues are shown in purple，the red 'N' represents the starting position and the arrow represents the hydrophobic moment. (B) The structure of GmAMP was predicated by AlphFold2. (C) The physical and chemical properties of GmAMP were analyzed via the Expasy ProtParam website (http://web.expasy.org/protparam/).

Figure 2

Effect of GmAMP on the growth of fluconazole-resistant *C. tropicalis.*

(A) Growth kinetics of fluconazole-resistant *C. tropicalis*. (B) Time-killing kinetics of fluconazole-resistant *C. tropicalis*. (C) The transformation from yeast phase to mycelial phase of fluconazole-resistant *C. tropicalis*. Scale bar, 25 μm.

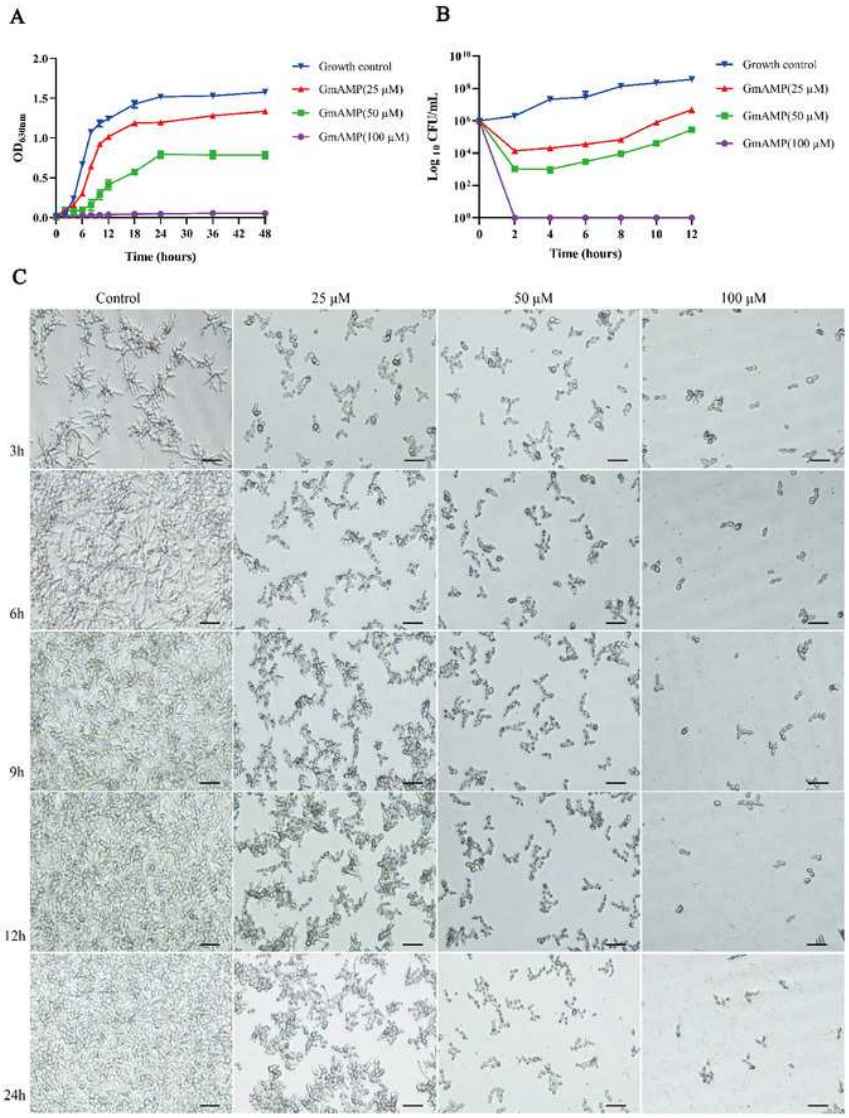

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Figure 3

Effect of GmAMP on the biofilm of fluconazole-resistant *C. tropicalis*.

Inhibition (A) and eradication (B) effects of fluconazole-resistant *C. tropicalis* biofilms treated with GmAMP at different concentrations observed by confocal laser scanning microscopy. Images obtained by live/dead staining (SYTO 9, green; PI, red). Scale bar, 20 μm. The activity level of biofilm under different concentrations of GmAMP was determined by the XTT reduction method (C and D), and the colorimetric absorbance was measured at OD490nm. The error bar represents the standard deviation of the three independent experiments. ***$P < 0.001$ compared with the control group.

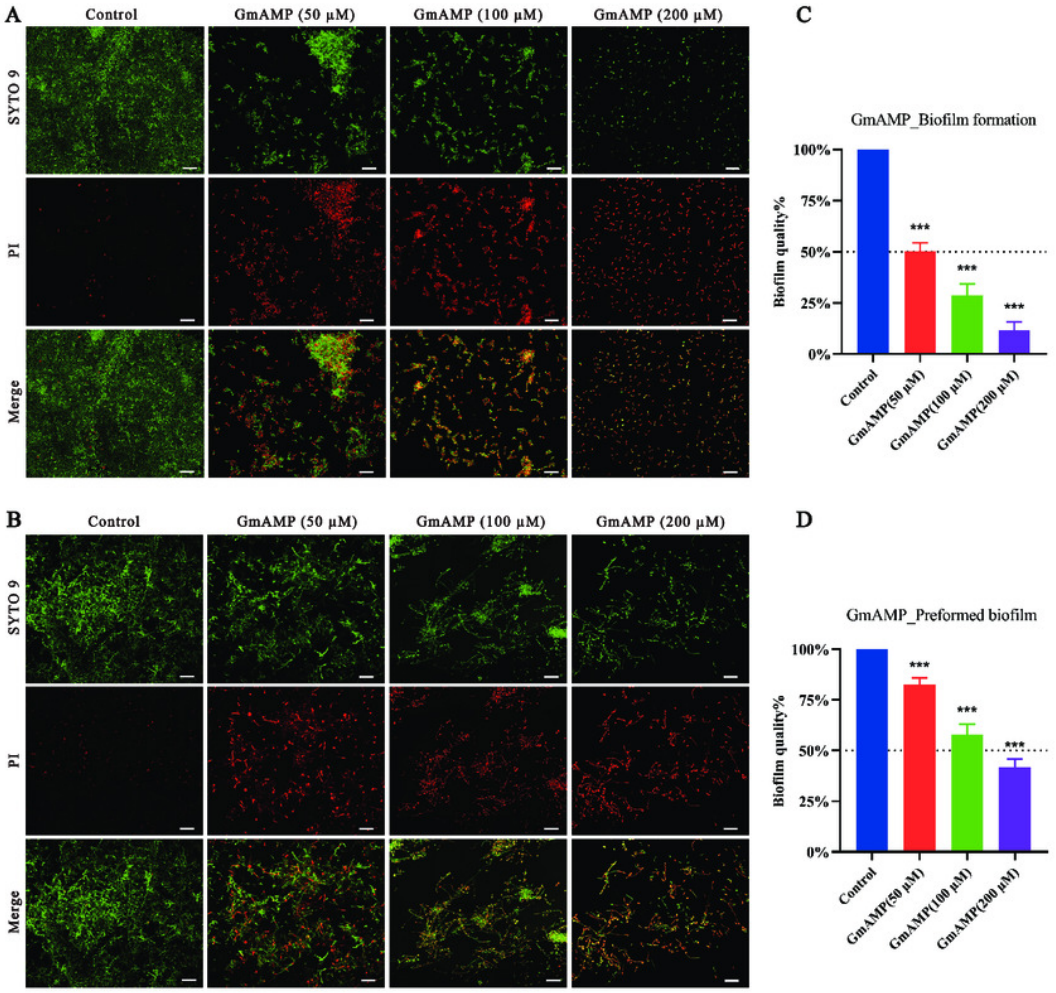

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Figure 4

Effects of GmAMP cell morphology and cell membranes of fluconazole-resistant *C. tropicalis*.

The control group (A) and GmAMP group treated with 100 μM (B) of fluconazole-resistant *C. tropicalis* morphological images by scanning electron microscopy. (C) Cell membrane permeability of GmAMP on the fluconazole-resistant *C. tropicalis* was determined by flow cytometry, and with SYTO 9 and PI as pore formation mechanism marker. (D) DiSC3(5) was used to detect the cell membrane depolarization of fluconazole-resistant *C. tropicalis*. (E) The ROS-induced accumulation of DCFH-DA is a pore formation mechanism marker

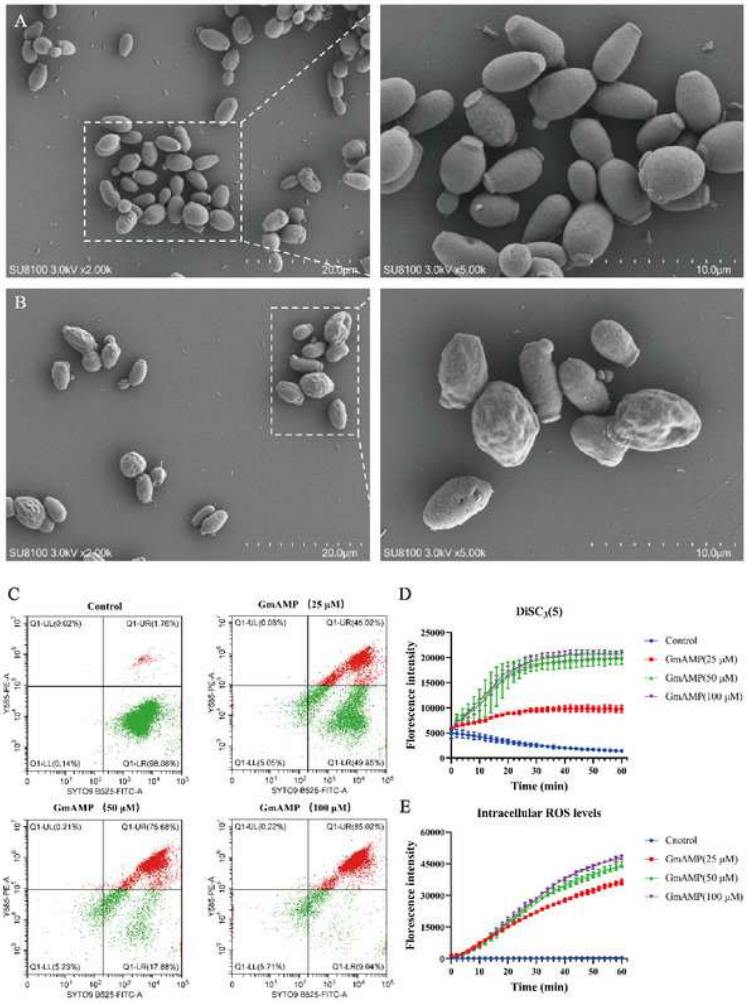


Figure 4 Effects of GmAMP cell morphology and cell membranes of fluconazole-resistant *C. tropicalis*. The control group (A) and GmAMP group treated with 100 μM (B) of fluconazole-resistant *C. tropicalis* morphological images by scanning electron microscopy. (C) Cell membrane permeability of GmAMP on the fluconazole-resistant *C. tropicalis* was determined by flow cytometry, and with SYTO 9 and PI as pore formation mechanism marker. (D) $DiSC_3(5)$ was used to detect the cell membrane depolarization of fluconazole-resistant *C. tropicalis*. (E) The ROS-induced accumulation of DCFH-DA is a pore formation mechanism marker.

Figure 5

The hemolytic and cytotoxicity effects of GmAMP.

(A) The cytotoxicity of GmAMP against RAW 264.7 cells. (B) The hemolysis rate of 2% human red blood cells.

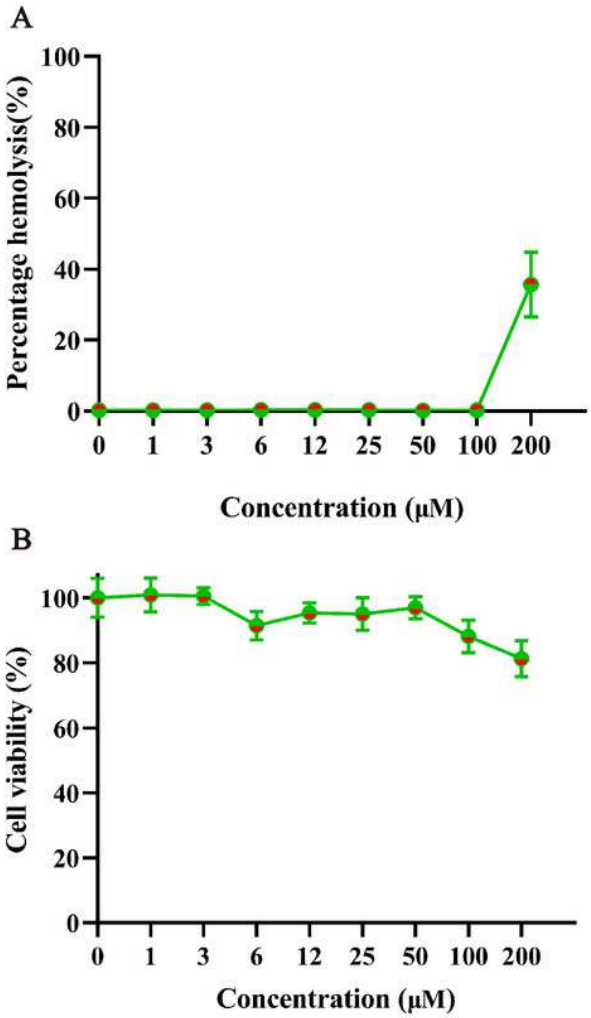
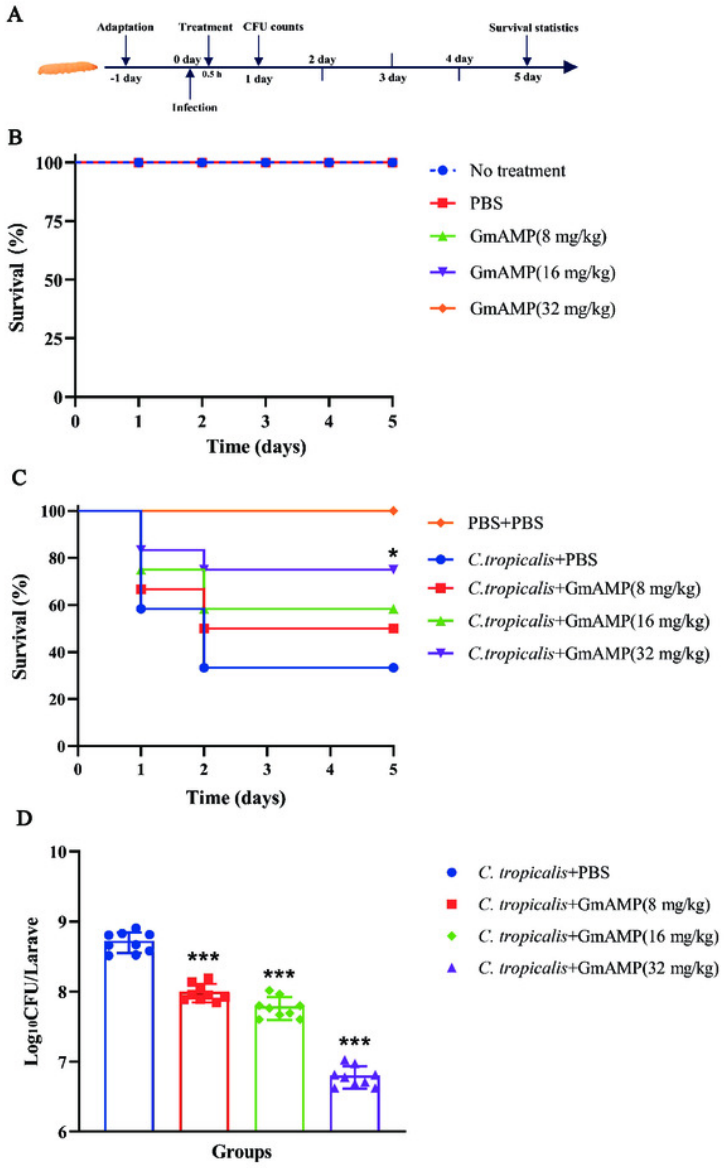


Figure 5 The hemolytic and cytotoxicity effects of GmAMP. (A) The cytotoxicity of GmAMP against RAW 264.7 cells. (B) The hemolysis rate of 2% human red blood cells.

Figure 6

In vivo toxicity and therapeutic activity of GmAMP in the *G. mellonella* model.

(A) Schematic diagram of the GmAMP treatment. (B) The toxicity of GmAMP in *G. mellonella* larvae model. (C) Survival of larvae after treatment with GmAMP. (D) Fungal burden of larvae after treatment with GmAMP. *$P < 0.05$; ***$P < 0.001$ compared with the group of fluconazole-resistant *C. tropicalis* + PBS.



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