Antimicrobial peptide AMP-17 exerts anti-Candida albicans effects through ROS-mediated apoptosis and necrosis

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Abstract

There is a need for new anti-*Candida albicans* drugs owing to the emergence of drug resistance in recent years. AMP-17, an antimicrobial peptide from *Musca domestica*, is known to be an effective inhibitor of many fungal pathogens, including *C. albicans*. In this study, we investigated the potential mechanism underlying the anti-*C. albicans* effects of AMP-17 using flow cytometry, transmission electron microscopy, fluorescent probes, fluorescence microplate reader, and confocal laser microscopy. Transmission electron microscopy showed that, following AMP-17 treatment, the shape of *C. albicans* cells became irregular, and vacuoles could be seen in the cytoplasm. Further, AMP-17 treatment resulted in an increase in reactive oxygen species (ROS) levels, depolarization of the mitochondrial membrane potential (MMP), and changes in the cell cycle, leading to the apoptosis and necrosis, which ultimately contributed to the death of *C. albicans* cells.

Introduction

Candida albicans is an opportunistic fungal pathogen with a dimorphic phenotype, i.e., it can grow in either a yeast or hyphal form (Lass-Flörl et al. 2021; Tan et al. 2021). When the immune system is functioning normally, *C. albicans* can be present on the skin, oral cavity, gastrointestinal tract, and genitourinary tract of an individual as a commensal, without causing infection (Wiederhold. 2017). When the immune system is impaired, such as in AIDS patients, patients using immunosuppressive agents for an organ transplant, and patients with microbial flora disorders, *C. albicans* can readily become pathogenic (Wubulikasimu et al. 2020). Such patients with specific risk factors are generally susceptible to *C. albicans*, and almost all organs can be infected (Boniche et al. 2020). Over recent years, owing to the widespread use of traditional antifungal agents such as triazoles, the isolation rate of resistant *C. albicans* strains has increased, which has brought great challenges to the treatment of *C. albicans* infections (Costa-de-Oliveira and Rodrigues 2020; Wang et al. 2021). Consequently, there is a critical need to develop effective antifungal agents with unique structures and mechanisms of action targeting this pathogen.

Antimicrobial peptides (AMPs) are a class of small, biologically active proteins produced by a variety of organisms as a first line of defense against pathogens. When the hosts are infected or subject to immune stimulation, AMPs can be quickly synthesized in some tissues and cells. In addition to inhibiting pathogens such as bacteria and fungi, AMPs also have varying degrees of killing effects on viruses, tumor cells, and parasites (Falanga et al. 2016; Kounatidis and Ligoxygakis 2012). Compared with traditional antibiotics, AMPs have a broader spectrum of antimicrobial effects, better thermal stability, and less toxicity toward human cells. In addition, they represent the most promising alternatives to traditional antibiotics because they are not easily susceptible to microbial resistance (Sheehan et al. 2018).

The housefly, *Musca domestica*, is the most abundant and widespread dipteran insect globally (Hou et al. 2007). It usually lives in extremely dirty environments and, consequently, can carry a large number of

pathogens (Gremillion and Piperno 2009). However, houseflies themselves are generally not susceptible to infection by pathogenic microorganisms, mainly owing to their strong innate immune system, within which the AMP response constitutes a particularly important and effective defense strategy. AMP-17 (*M. domestica* antimicrobial peptide-17) is encoded by a highly expressed gene identified from the *M. domestica* transcriptome database constructed after a 12-h exposure to microbial infection. We have previously produced the recombinant AMP-17 protein in a prokaryotic expression system and showed that it possesses excellent antifungal activity (Guo et al. 2017; Jiangfan et al. 2016). In a subsequent study, we found that AMP-17 can impair cell wall integrity, destroy cell membrane structure, and increase cell membrane permeability in *C. albicans* (Ma et al. 2020; Yang et al. 2022). However, whether AMP-17 acts on intracellular targets in *C. albicans* remains unclear. To address this, we undertook an in-depth study on the potential mechanisms underlying the anti-*C. albicans* effects of AMP-17 from an intracellular perspective, including the internal structure.

An antimicrobial peptide can act through multiple mechanisms. Studies have shown that in addition to altering membrane permeability, AMPs can also target cellular contents, thereby inhibiting processes such as transcription and translation, among others (Lan et al. 2010). Mitochondria are organelles that produce reactive oxygen species (ROS). ROS accumulation is a hallmark of apoptosis. Papiliocin, a 37-residue peptide isolated from *Papilio xuthus*, can induce mitochondrial membrane damage through ROS accumulation, leading to cell dysfunction and, ultimately, cell apoptosis or necrosis (Hwang et al. 2011). In this research, we examined the ultrastructure of *C. albicans* by transmission electron microscopy (TEM) and assessed *C. albicans* cell cycle progression using flow cytometry. To further clarify the mode of the antifungal action of AMP-17, fluorescent probes were used to detect ROS accumulation in *C. albicans* cells and analyze the correlation between ROS levels and cell necrosis in *C. albicans*. In addition, a JC-1 kit was used to measure the effect of AMP-17 on the intracellular MMP of *C. albicans* cells. Finally, AMP-17-induced apoptosis and necrosis were investigated using Annexin V-FITC/PI staining. Our goal was to identify the targets of the antifungal effects of AMP-17, which is the key to enhancing its therapeutic potential.

Materials And Methods

Peptides and Strains

The recombinant AMP-17 protein was obtained using a prokaryotic expression system and purified by nickel ion metal chelator affinity chromatography. The minimal inhibitory concentration (MIC) of recombinant AMP-17 toward *C. albicans* was 20 μg/mL as detected using the micro-liquid dilution method (Ma et al. 2020).*C. albicans* (ATCC10231) was stored in 30% glycerol at −80 °C and subcultured twice on Sabouraud dextrose agar (SDA) plate. Before each experiment, cells were cultured in Sabouraud dextrose broth (SDB) for 18 h on a shaking incubator (200 rpm) at 37 °C.

Analysis of cell morphology

The effect of AMP-17 on the ultrastructure of *C. albicans* cells was analyzed using TEM. *C. albicans* cells at an initial density of 1.0~5.0 × 10^6 colony-forming units (CFU)/mL were treated with 40 μg/mL AMP-17 at 37 °C for 16 h, and then collected by centrifugation (5,000 rpm for 10 min). Untreated cells served as the control. Samples from each group were washed twice with phosphate buffer saline (PBS) and fixed in 1 mL of 2.5% glutaraldehyde at 4 ℃ overnight. The fixed samples were washed twice with PBS and dehydrated in 50%, 75%, and 100% ethanol. Finally, the cells were observed under a Hitachi H-7650 TEM (Tokyo, Japan).

Cell cycle analysis by flow cytometry using PI

C. albicans cells (1.0~5.0 × 10^6 CFU/mL) were treated with AMP-17 at a final concentration of 0 (control), 20, 40, or 80 μg/mL. The cells were incubated at 37 °C for 12 h, collected by centrifugation at 3,000 rpm for 5 min, and washed twice with PBS. Each group of cells was resuspended in precooled 70% ethanol and fixed at 4 °C for more than 18 h. The fixed cells were collected by centrifugation at 3,000 rpm for 5 min at 4 °C and washed twice with sterile PBS. After incubation with ribonuclease A (RNAse A) at 37 °C for 2 h, Propidium iodide (PI) staining solution was added to the cells at a final concentration of 0.1 mg/mL, followed by incubation at 4 °C for 18 h in the dark. The cell cycle was then analyzed by flow cytometry.

Measurement of ROS generation in *C. albicans*

The dye 2',7'-dichlorofluorescein diacetate (DCFH-DA) from Sigma Chemicals (St Louis, MO, USA) was used to detect the generation of intracellular ROS (Chang et al. 2011; Li et al. 2015). *C. albicans* cells at an initial density of 1.0~5.0 × 10^6 CFU/mL were treated with 0 (control), 20, 40, or 80 μg/mL AMP-17 at 37 °C for 12 h. Cells treated with fluconazole (FLC) under the same conditions served as the positive control. Following treatment, the cells were collected, fixed, and stained with DCFH-DA at a final concentration of 20 μg/mL. After 30 min of incubation at 30 °C, the cells were collected, washed twice, and then diluted to 1 × 10^6 CFU/mL with SDB. The fluorescence intensity (excitation and emission at 485 and 530 nm, respectively) of the cells was measured with a microplate reader (Berthold Biotechnologies, Bad Wildbad, Germany) and fluorescence images were obtained by confocal laser scanning microscopy (CLSM) using an FITC filter (Olympus FV1000, Olympus, Tokyo, Japan). To test the effect of antioxidants on AMP-17-induced ROS accumulation, these experiments were also conducted in the presence of 5 and 50 mM ascorbic acid (AA) and glutathione (GSH). To determine whether the ROS production induced by AMP-17 is involved in the antifungal activity of the peptide, we examined the anti-*C. albicans* activity of AMP-17 without and with the ROS scavengers, AA and GSH. The MIC was determined using the Clinical and Laboratory Standards Institute (CLSI) method (Ma et al. 2020). All samples were analyzed in triplicate, and the experiment was repeated three times.

Detection of the correlation between AMP-17-induced ROSaccumulation and necrosis using DCFH-DAand PI double staining

C. albicans cells (1.0~5.0 × 10^6 CFU/mL) were cultured in SDB containing 80 μg/mL AMP-17 at 37 °C for 12 h. Sterile water was used as a negative control. The cells were centrifuged for 10 min at 5,000 rpm, incubated with 10 μg/mL DCFH-DA (a probe for detecting intracellular ROS) and 20 μg/mL PI (an indicator dye for cell necrosis) for 30 min in dark, and then analyzed by CLSM.

Measurement of the MMP

A JC-1 kit (Beyotime, Shanghai, China) was used to analyze changes in MMP. *C. albicans* cells (1.0~5.0 × 10^6 CFU/mL) were treated with 0 (control), 20, 40, or 80 μg/mL AMP-17 at 37 °C for 12 h. The positive control sample was incubated with carbonyl cyanide m-chlorophenyl hydrazone (CCCP) under the same conditions. The cell suspensions were then stained with 5 μmol/L JC-1 at 37 °C for 30 min in the dark. JC-1 fluorescence (green and red) was monitored at Ex/Em = 490/525 nm and 490/590 nm with a microplate reader (Berthold Biotechnologies, Bad Wildbad, Germany). The results were presented as the mean values of triplicate measurements from three independent experiments.

Analysis of apoptosis and necrosis induced by AMP-17

C. albicans cells at a concentration of 1.0~5.0 × 10^6 CFU/mL were treated with 0 (control), 20, 40, or 80 μg/mL AMP-17 and incubated at 37 °C for 12 h. The cells were then collected by centrifugation (5,000 rpm for 10 min) and washed twice in PBS. Each sample was processed according to the instructions of the Annexin V/FITC Kit (BD pharmingen, NJ, USA). The stained cells were observed by CLSM.

Statistical significance

All data were analyzed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Differences were analyzed by one-way ANOVA using Dunnett's analysis. Data are expressed as mean±standard deviation (Mean±SD), and p value of <0.05 was considered statistically significant.

Results

Transmission electron microscopy

We first utilized TEM to identify changes in the ultrastructural features of *C. albicans* in response to AMP-17 treatment. Untreated *C. albicans* cells were normal and intact, with a plump appearance, a smooth surface, and clear cell boundaries. The cells were uniform in size and showed a round or oval shape (Fig. 1A, D). After treatment with 40 μg/mL AMP-17 for 16 h, the shape of *C. albicans* cells became highly irregular, and some vacuoles could be seen at the junction of the cell membrane and cytoplasm. Importantly, the electron density in the cytoplasm region was abnormal. The edges of intracellular organelles were irregular and vacuoles appeared around the nucleus (Fig. 1B, C, E, F).

The effect of AMP-17 on the cell cycle of *C. albicans*

Because the proliferation rate of *C. albicans* is related to the cell cycle, we then investigated the effect of AMP-17 on the cell cycle of this fungus. As shown in Fig. 2, following AMP-17 treatment, the percentage of *C. albicans* cells in the S phase gradually increased from 30.94% to 64.63% (Table 1), indicating that AMP-17 treatment led to a greater accumulation of cells in the S phase, and that most of the cells did not progress to the M phase.

AMP-17 exerts its antifungal activity by inducing ROS accumulation

ROS is produced under normal physiological conditions and is involved in multiple biological processes. However, excessive ROS production can cause oxidative damage to cells (Haque et al. 2019). In this study, DCFH-DA was used as a ROS indicator to study the effect of AMP-17 on intracellular ROS production. The results showed that AMP-17 promoted cellular ROS generation in a dose-dependent manner. Quantification of cellular ROS generation in AMP-17-treated *C. albicans* cells revealed that, compared with control cells, ROS levels were significantly increased after incubation with 80 μg/mL AMP-17 for 12 h, comparable with that seen in the FLC-treated group (Fig. 3A). CLSM analysis revealed that the numbers of cells with green fluorescence increased with increasing AMP-17 concentration, indicative of enhanced ROS production (Fig. 3B). The effects of the antioxidants AA and GSH on AMP-17-induced ROS accumulation in *C. albicans* are shown in Fig. 3C. Without these antioxidants, AMP-17 treatment induced ROS accumulation in *C. albicans* cells in a dose-dependent manner; however, in the presence of AA and GSH, AMP-17-mediated ROS production was significantly inhibited. In addition, ROS scavenging agents reduces the antifungal activity of AMP-17 against C. albicans. In the absence of AA and GSH, the MIC of AMP-17 against *C. albicans* was 20 μg/mL; however, the addition of the two antioxidants significantly reduced the activity of AMP-17 against *C. albicans*. The MIC values of AMP-17 against *C. albicans* increased by 2-fold (from 20 to 40 μg/mL) with 5 mM AA and GSH treatment and by 8- and 4-fold, respectively, with treatment at 50 mM (Fig. 3D). These results indicated that the anti-*C. albicans* activity of AMP-17 may be mediated through inducing ROS accumulation.

The correlation between AMP-17-induced ROS accumulation and necrosis in *C. albicans*

Increased levels of ROS within the cellular environment are known to contribute to cell necrosis (Chang et al. 2011; Ding et al. 2016). To elucidate the relationship between AMP-17-induced cell necrosis and ROS accumulation, we stained AMP-17-treated *C. albicans* cells with DCFH-DA and PI and then examined them by CLSM. After treatment with 80 μg/mL of AMP-17, there was a significant increase in the number of *C. albicans* cells stained by PI and DCFH-DA compared with that in the control group (Fig. 4). The result indicated that AMP-17-induced *C. albicans* cell necrosis is related to the accumulation of intracellular ROS.

AMP-17 treatment induced alterations in the mitochondrial membrane potential of *C. albicans* cells

Mitochondria play an important role in maintaining energy metabolism and regulating cell growth, differentiation, and death. As a stable MMP is essential for normal cellular function (Golstein et al. 2003;

Jia et al. 2019), we evaluated the effect of AMP-17 on the MMP of *C. albicans* cells. The results showed that AMP-17 decreased the MMP of *C. albicans* in a dose-dependent manner. Compared with the control group, AMP-17 treatment at 20, 40, and 80 μg/mL reduced the MMP of *C. albicans* from 4.2 ± 0.36 to 3.933 ± 0.15, 3.133 ± 0.42, and 2.767 ± 0.15, respectively (Fig. 5). The result indicated that AMP-17 affects the integrity of *C. albicans* mitochondria, leading to mitochondrial membrane depolarization.

AMP-17 induces the apoptosis and necrosis of *C. albicans* cells

Annexin V-FITC/PI staining was used to confirm that AMP-17 did indeed promote apoptosis and necrosis in *C. albicans*. CLSM analysis showed a concentration-dependent increase in both green and red fluorescence in *C. albicans* cells after 12 h of AMP-17 treatment when compared with that of control cells (Fig. 6), suggesting that AMP-17 had induced apoptosis and necrosis.

Discussion

Candida albicans, displaying both yeast and mycelial growth forms, is the most frequently isolated fungus from bloodstream infections (Tong et al. 2021).Although significant progress has been made in the diagnosis and treatment, *C. albicans* infections continue to pose a significant challenge for intensive care units worldwide. The yeast state of *C. albicans*, with its unique structure and characteristics, is more likely to spread through the blood circulation of debilitated patients (Chang et al. 2011; Costa-de-Oliveira and Rodrigues 2020). Based on this diffusion property, the inhibition of the proliferation of *C. albicans* at this stage is an effective treatment method. The proliferation rate of *C. albicans* has been reported to be closely related to the cell cycle. Consequently, the development of drugs that can prolong the cell cycle has become an effective strategy for the treatment of *Candida* infections (Singh et al. 2007). In this study, we found that AMP-17 can block the cell cycle of *C. albicans* in the S phase. In this phase, cells undergo DNA replication, and cell cycle arrest in the S phase prevents cells from entering the M phase, thereby limiting their further growth.

ROS are by-products of cellular metabolism generated primarily by mitochondria. When *C. albicans* cells encounter external stimuli, such as oxidants, heat shock, and metal ions, among other factors, they enhance ROS generation to resist the pressure of the external environment (Chang et al. 2011; Ding et al. 2016; Golstein et al. 2003). If the ROS produced by the cell exceeds its metabolic capacity, the excess intracellular ROS induces oxidative damage to lipids, DNA, proteins, and other cellular components, such as the plasma membrane, which may lead to apoptotic or necrotic cell death (Haque et al. 2019; Jia et al. 2019). Here, we found that AMP-17 treatment increased ROS generation and showed a good concentration-dependent effect; however, when the *C. albicans* cells were pretreated with GSH and AA, two antioxidants, AMP-17-mediated ROS accumulation in *C. albicans* was significantly inhibited. In addition, pretreatment with GSH and AA markedly reduced the anti-*C. albicans* activity of AMP-17, indicating that ROS accumulation indeed resulted from the antifungal activity of AMP-17 and was not a stress response by the cells to changes in the external environment. Thus, these results indicated that AMP-17 exerts its antifungal activity by inducing ROS accumulation.

As mentioned above, excessive intracellular ROS accumulation can have fatal consequences for cells. Necrosis is a form of cell death induced by extreme physical, chemical, or other pathological stimuli (Haque et al. 2019; Jamieson 1995). In our study, using DCFH-DA and PI double staining, we showed that the cell necrosis induced by AMP-17 was associated with an increase in intracellular ROS production. In addition, impaired cell membrane integrity is a hallmark of cell necrosis (Haque et al. 2019). We have previously demonstrated that AMP-17 treatment can cause the loss of *C. albicans* cell membrane integrity by destroying the structure of the plasma membrane and increasing its permeability (Ma et al. 2020; Yang et al. 2022). TEM analysis indicated the presence of abnormal vacuoles in the cytoplasm, which is also a typical feature of cell necrosis. Together, these results revealed that cell necrosis is an important feature underlying the anti-*C. albicans* effect of AMP-17.

Mitochondria are the energy-producing organelles of eukaryotic cells. Because they are also where most ROS are generated, mitochondria are both the origin and the target of ROS (Nunnari and Suomalainen 2012; Terman et al. 2006; Tong et al. 2021). When cellular ROS accumulation is excessive or the antioxidant defense system is weakened, free radicals in the mitochondria cannot be effectively eliminated. Excess ROS can oxidize the corresponding redox-sensitive sites on mitochondrial permeability transition pores, leading to mitochondrial damage (Pereira et al. 2008). In addition, ROS accumulation can result in lipid peroxidation, which impairs the performance of mitochondrial membranes and triggers changes in membrane potential (Curtin et al. 2002). In this study, changes in the MMP of *C. albicans* cells were assessed using the JC-1 fluorescent reagent. We found that the MMP gradually decreased with AMP-17 treatment, reaching a significant depolarization at 80 μg/mL.

Maintenance of the MMP is a prerequisite for normal mitochondrial function (Haque et al. 2019; Sharon et al. 2009; von der Haar et al. 2017), while MMP depolarization is considered to be the earliest event in cells undergoing apoptosis. Apoptosis is a form of programmed cell death, and plays a vital role in the maintenance of organismal homeostasis through the elimination of damaged, unwanted, mutated, or otherwise redundant cells (Jia et al. 2019; Rockenfeller and Madeo 2008; Singh et al. 2007). In addition to multicellular organisms, apoptosis also exists in unicellular organisms such as bacteria and yeast (Eisenberg et al. 2007; Kwun et al. 2021). In this study, using Annexin V-FITC and PI co-staining, we found that AMP-17 can induce both apoptosis and necrosis in *C. albicans* cells, which likely explains the observed mitochondrial dysfunction and ROS accumulation.

Conclusions

In the present study, we found that AMP-17 inhibits the growth and proliferation of *C. albicans* cells by affecting cell cycle progression. In addition, we confirmed that AMP-17 treatment led to a notable and dose-dependent increase in intracellular ROS production, resulting in oxidative damage to the mitochondria and other cellular components. Mitochondrial dysfunction and ROS accumulation were identified as the main AMP-17-related triggers for the apoptosis and necrosis of *C. albicans* cells. That AMP-17 affects multiple targets in *C. albicans* highlights its potential as a novel treatment option for the prevention and control of clinical fungal infections.

Declarations

Authors' contributions

HM, JP and GG conceived and designed the experiments. HM, LY, ZT and LZ performed the experiments. HM, JP, JX and PF analyzed the data. JP and LY contributed reagents/ materials/ analysis tools. HM and GG wrote the paper. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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Tables

Table 1 The percentage of *C. albicans* DNA content after treatment with AMP-17

AMP-17	Cell cycle ratio (%)		
(μg/mL)	G0/G1	S	G2/M
0	33.75	30.94	32.91
20	5.04	54.76	38.51
40	27.72	65.70	6.59
80	4.79	64.63	30.57

Figures

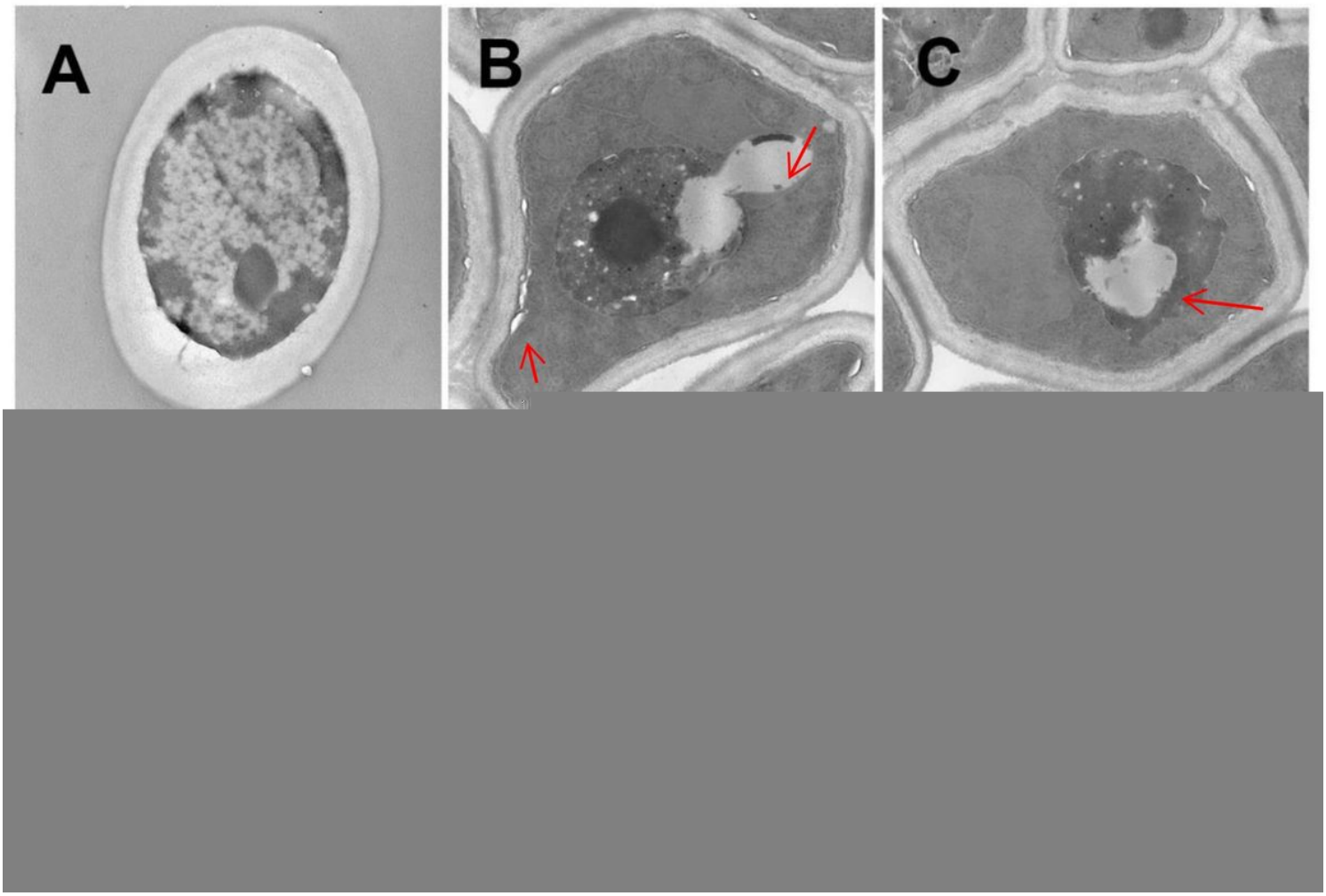

Figure 1

TEM showing the effect of AMP-17 on the internal structure of *C. albicans*. (A: ×40,000, D: ×50,000) Untreated *C. albicans* cells are intact, displaying a plump appearance, clear cell boundaries, and normal electron density in the cytoplasm. (B, C: ×40,000; E, F: ×50,000) *C. albicans* cells treated with AMP-17 (40 μg/mL) showed severe irregularities in shape, and cells adhered tightly to each other. Importantly, abnormal vacuoles of different sizes could be seen in the cytoplasm of the treated cells (red arrowheads).

Figure 2

Cell cycle analysis of *C. albicans* assayed by flow cytometry. (A) Untreated cells. (B–D) *C. albicans* cells treated with 20, 40, or 80 μg/mL AMP-17 at 37 °C for 12 h.

Figure 3

The effect of AMP-17 on ROS generation in *C. albicans*. Cells were incubated with 0 (control), 20, 40, and 80 μg/mL AMP-17 at 37 °C for 12 h. After staining with 20 μg/mL DCFH-DA, fluorescence was detected using a microplate reader (A) and a CLSM (B). The effects of the antioxidants AA and GSH on AMP17-induced ROS generation (C). The MIC of AMP-17 against *C. albicans* was significantly increased in the presence of the antioxidants(D). The bars in (A, C, D) indicate standard deviations. Bars in (B) = 5 μm. **P*<0.05, ***P*<0.01 compared with the negative control.

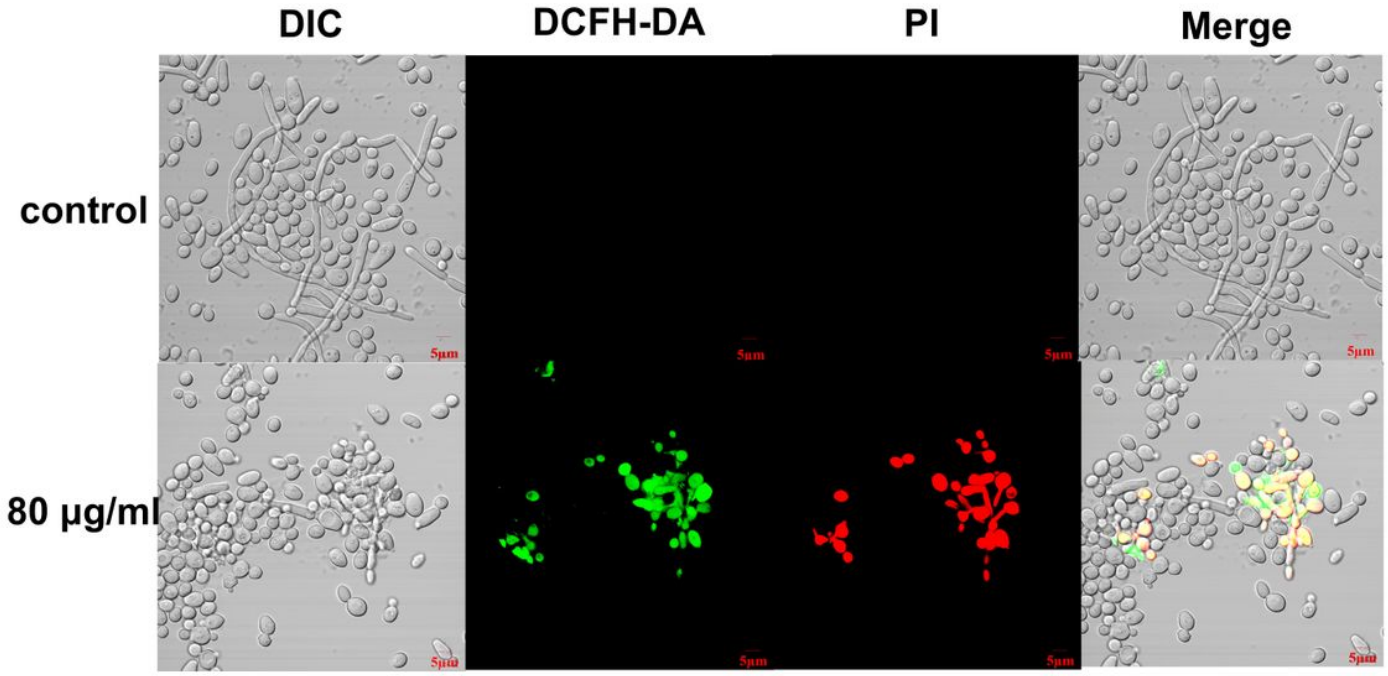

Figure 4

The correlation between reactive oxygen species (ROS) accumulation and necrosis (×1200). *Candida albicans* cells were incubated with 0 (control) or 80 μg/mL AMP-17 at 37 °C for 12 h. After collection, the samples were stained with 2',7'-dichlorofluorescin diacetate (DCFH-DA) and propidium iodide (PI) and detected by confocal laser scanning microscopy after 30 min of incubation in the dark. Scale bars, 5 μm.

Figure 5

The effect of AMP-17 on the mitochondrial membrane potential of *Candida albicans* cells. Cells were treated with various concentrations of AMP-17 or carbonyl cyanide m-chlorophenyl hydrazone (CCCP; positive control) for 12 h followed by JC-1 staining for spectrofluorophotometric detection. Error bars indicate standard deviations. **P*<0.05, ***P*<0.01 compared with the negative control. FI: fluorescence intensity.

Figure 6

Observation of AMP-17-induced early apoptosis in *Candida albicans* by confocal laser scanning microscopy (×600). Cells treated with 0, 20, 40, and 80 μg/mL AMP-17 at 12 h were stained with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI). FITC, excitation at 488 nm; PI, excitation at 555 nm. Scale bars, 10 μm.