A novel mechanism of fluconazole: fungicidal activity through dose-dependent apoptotic responses in *Candida albicans*

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**Abstract**

Fluconazole (FLC) is a well-known fungistatic agent that inhibits ergosterol biosynthesis. We showed that FLC exhibits dose-dependent fungicidal activity, and investigated the fungicidal mechanism of FLC on *Candida albicans*. To confirm the relationship between fungicidal activity and the inhibition of ergosterol, we assessed membrane dysfunctions via propidium iodide influx and potassium leakage, as well as morphological change. Interestingly, while membrane disruption was not observed at all tested concentrations of FLC, potassium efflux and cell shrinkage were observed at high dosages of FLC (HDF). Low-dosage FLC (LDF) treatment did not induce significant changes. Next, we examined whether the fungicidal activity of FLC was associated with apoptosis in *C. albicans*. FLC caused dose-dependent apoptotic responses, including phosphatidylserine externalization and DNA fragmentation. It was also involved in glutathione depletion followed by oxidative damage. In particular, unlike LDF, HDF leads to the disruption of mitochondrial homeostasis, including mitochondrial membrane depolarization and accumulation of calcium and reactive oxygen species. HDF-induced mitochondrial dysfunction promoted the release of cytochrome c from mitochondria to the cytosol, and activated intracellular metacaspase. In conclusion, the dose-dependent fungicidal activity of FLC was due to an apoptotic response in *C. albicans*.

**INTRODUCTION**

*Candida* species are the most common fungal pathogens of humans and the cause of invasive and superficial candidiasis. The majority of *Candida* infections are mucosal, manifesting as oral or vaginal candidiasis that together account for an estimated 40 million infections per year [1]. These pathogenic species are also the fourth leading cause of nosocomial infections and are associated with high mortality rates [2, 3]. *Candida albicans*, an endogenous commensal, is the most common *Candida* species to cause invasive infections [4]. *C. albicans* can cause two major types of infections: life-threatening systemic infections and superficial infections, such as vaginal or oral candidiasis. They can also cause dysfunction of the adaptive immune system [5].

Several classes of compounds are used to treat *Candida* infections. Among various antifungals, the azole class is the most commonly prescribed antifungal against the majority of *C. albicans* infections [6]. The growth of *C. albicans* is inhibited in the presence of azole antifungals; therefore, these agents are known to be fungistatic against this microorganism [2]. Recently, it has been reported that certain azoles are involved in additional biological processes that inhibit ergosterol biosynthesis. For example, reactive oxygen species (ROS) produced by miconazole have a substantial role in antifungal activity [7], and imidazole-induced cell death is associated with intracellular acidification and caspase-3 activation [8]. An improved understanding of the biological activities of well-known conventional azole agents is needed to clarify their mechanisms of action.

Fluconazole (FLC) is an azole antifungal agent that is widely used against *Candida* species [2]. Recently, other biological mechanisms of FLC besides the inhibition of sterol biosynthesis have attracted attention, such as transcriptional regulator disruption and metabolic injury [2, 9]. Although it is well known that FLC exerts a fungistatic effect on *C. albicans* by disrupting the biosynthesis of ergosterol [9], little is known about its fungicidal effects. Understanding the mechanisms of action of FLC may assist the development of novel antifungal agents. Therefore, we investigated the mechanism underlying the response of *C. albicans* to an increased concentration of FLC.
METHODOLOGY

Preparation of organisms and test compounds
C. albicans (ATCC 90028) was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were grown on agar plates containing yeast extract peptone dextrose broth (YPD; Difco, Sparks, MD, USA) and cultured for 15 h at 28°C in YPD broth before use. FLC, amphotericin B and H$_2$O$_2$ (Sigma-Aldrich, St Louis, MO, USA) were used in dimethyl sulfoxide (for FLC and amphotericin B) and sterile distilled water (for H$_2$O$_2$) according to the manufacturer’s instructions.

Time-kill kinetic analysis
C. albicans (1×10$^7$ cells ml$^{-1}$) was incubated with FLC at various concentrations (16–128 µg ml$^{-1}$) in YPD broth at 28°C. After incubation for 0, 2, 4, 8, 12 and 24 h, the cultures were spread onto YPD agar plates. Subsequently, after incubation for 24 h at 28°C, the colony-forming units (cfu) were counted [10]. All time-kill curve experiments were performed in triplicate.

Measurement of membrane damages by FLC
Harvested C. albicans cells (1×10$^7$ cells ml$^{-1}$) were resuspended in phosphate-buffered saline (PBS), treated with 16, 64 µg ml$^{-1}$ FLC and 1.3 µM H$_2$O$_2$, and incubated at 28°C for 2 h. After incubation and centrifugation, the cells were resuspended in PBS. Membrane disruption was measured using propidium iodide (PI) staining [11]. The 9 µM PI-stained cells were analysed using a FACSVerse flow cytometer (Becton Dickinson, NJ, USA), measuring 10 000 events for each sample.

We measured external potassium levels to detect the movement of potassium ions. After incubation with 16, 64 µg ml$^{-1}$ FLC and 1.3 µM H$_2$O$_2$ for 2 h, the samples were centrifuged and the supernatants were transferred to 24-well plates. The cells were preincubated for 15 min with 5 mM tetraethylammonium chloride (TEA) as a potassium ion channel blocker. A potassium ionic strength adjuster and distilled water were added to produce a volume of 3 ml. After 10 min of incubation for stabilization, the potassium voltage was measured using an ion selective electrode meter (Orion Star A214, Thermo Scientific, Singapore), and the external potassium level (expressed in percentage points) was calculated using the following formula: external potassium level (%) = 100 × ([K$^+$] - [K$^+$]$_0$)/([K$^+$]$_i$ - [K$^+$]$_0$), where [K$^+$] and [K$^+$]$_0$ represents the potassium voltage of the FLC-treated and untreated samples, respectively, and [K$^+$]$_i$ denotes the potassium voltage of the sonicated samples for total external potassium [12].

Morphological changes to the cells (1×10$^7$ cells ml$^{-1}$) were determined using the FACSVerse flow cytometer to confirm the effect of FLC on cell volume. A total of 10 000 events were counted. After the cells (1×10$^7$ cells ml$^{-1}$) were incubated with 16, 64 µg ml$^{-1}$ FLC and 1.3 µM H$_2$O$_2$, they were investigated by determining their position on forward scatter (FS) versus side scatter (SS) contour plots [13].

Detection of apoptotic responses
In yeast, the externalization of phosphatidylserine (PS) can be observed with a FACSVerse flow cytometer using a fluorescent isothiocyanate (FITC) Annexin V apoptosis detection kit (BD Pharmingen) according to the manufacturer’s instructions [14]. During the FACS analysis 10 000 events were collected. DNA fragmentation was analysed using a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay via an in situ cell death detection kit (Roche) at wavelengths of Ex/Em=495/519 nm using a spectrofluorophotometer [15]. To confirm the cell cycle, the harvested cells (1×10$^7$ cells ml$^{-1}$) were treated with 16, 64 µg ml$^{-1}$ FLC at 28°C for 2 h. After incubation, the cells incubated in the dark for 20 min with 1 mM SYTOX green, which is a DNA-sensitive fluorescent probe, as previously described, using the FACSVerse flow cytometer [16], and measuring 10 000 events for each sample.

Analysis of cytosolic and mitochondrial calcium levels
Fura-2AM and Rhod-2AM (Molecular Probes) were used to determine the calcium levels in the cytosol and mitochondria, respectively. C. albicans cells (1×10$^7$ cells ml$^{-1}$) were incubated with 16, 64 µg ml$^{-1}$ of FLC and 1.3 µM H$_2$O$_2$ for 2 h and then washed twice in Krebs buffer (pH 7.4) before being treated with 1% bovine serum albumin and 0.01% pluronic F-127 (Molecular Probes). The cells were stained with 5 µM Fura-2AM or 10 µM Rhod-2AM, and incubated for 40 min at 37°C according to the manufacturer’s instructions. The cells were then washed twice with calcium-free Krebs buffer. The fluorescence intensities were monitored using a spectrofluorophotometer (Shimadzu RF-5301PC; Shimadzu, Kyoto, Japan); the excitation wavelength/emission wavelength (Ex/Em) was 340/515 nm for Fura-2AM and 550/580 nm for Rhod-2AM [17].

Glutathione level assay
The harvested cells (1×10$^7$ cells ml$^{-1}$) were washed with PBS and incubated with FLC at 28°C for 2 h. To determine the total glutathione content, the cell pellets were resuspended in 5% 5-sulfosalicylic acid (SSA) solution, which causes the precipitation of proteins, and exposed to three freeze-thaw cycles to lyse the cells. The extract was incubated for 5 min at 4°C, and the supernatant was then used to measure the total glutathione levels. The glutathione levels were measured at 415 nm using a spectrophotometer and the intracellular protein levels were normalized by the Bradford method. The glutathione assay was based on the glutathione reductase enzymatic recycling method previously described by Bergmeyer et al. [18].

Measurement of intracellular ROS levels and mitochondrial specific ROS accumulation
Intracellular ROS levels were investigated using the fluorescent dye H$_2$DCFDA (Molecular Probes, Eugene, OR, USA) [19]. MitoSOX Red (Molecular Probes) was used for the mitochondria-specific ROS measurements [20]. After treatment of the harvested cells (1×10$^7$ cells ml$^{-1}$) with 16 and
64 µg ml⁻¹ of FLC for 2 h at 28 °C, the suspension was washed with PBS. The cells were then incubated with 10 mM H₂DCFDA for 1 h, or 5 M MitoSOX Red for 30 min, at 28 °C, respectively, and analysed via a FACSVerse flow cytometer, measuring 10 000 events for each sample.

Assessment of lipid peroxidation

We used the thiobarbituric acid reactive substances (TBARS) assay to determine the level of lipid peroxidation in the cells. The cells (1 × 10⁷ cells ml⁻¹) were treated with 16 and 64 µg ml⁻¹ of FLC for 2 h at 28 °C, and washed with PBS after separation by centrifugation. The pellets were sonicated twice on ice with 5 % trichloroacetic acid (TCA; Merck). After the mixture had been centrifuged, the supernatant was added to an equal volume of 0.365 % (w/v) TBA and incubated for 1 h at 95 °C. The absorbance of the sample was measured at 532 nm using a spectrophotometer (DU530, Beckman). A standard curve was generated using malondialdehyde (MDA; Sigma) to determine the concentration of TBARS [21].

Measurement of mitochondrial membrane potential, cytochrome c release and metacaspase activity

JC-1 (Molecular Probes) was used to investigate changes in mitochondrial membrane potential (MMP), as previously described [22]. The mean of the fluorescence intensities at FL2 and FL1 was determined using the FACSVerse flow cytometer. During FACS analysis 10 000 events were collected. The ratio of the fluorescence intensities of FL2 (aggregated JC-1) to FL1 (monomer) was calculated.

Western blot analysis was used to estimate the levels of cytosolic and mitochondrial cytochrome c (cyt c). The cells were homogenized in buffer A (50 mM Tris, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 7.5). To eliminate impurities, 2 % glucose was added and the mixture was centrifuged. The supernatants were collected to detect released cytoplasmic cyt c. To obtain pure mitochondria, the pellet was washed in buffer B (50 mM Tris, 2 mM EDTA, pH 5.0) by centrifugation. The mitochondria were then suspended in 2 mg ml⁻¹ Tris-EDTA buffer. The protein contents were quantified using the standard Bradford method. Each cytoplasmic and mitochondrial sample of protein was separated on a 12 % polyacrylamide gel and transferred to a nitrocellulose membrane. After the protein was transferred to the membrane, we confirmed the total by Ponceau S staining to check for transfer problems and equal loading of the total proteins. The transfer membranes were probed using rabbit polyclonal anti-yeast cytochrome c antibody and the secondary antibody was horseradish peroxidase-linked goat anti-rabbit immunoglobulin G [23].

Caspases were detected by FITC-VAD-FMK staining [15]. After the cells (1 × 10⁷ cells ml⁻¹) were incubated with 16, 64 µg ml⁻¹ FLC and 1.3 µM H₂O₂, the samples were washed twice and incubated with CaspACE FITC-VAD-FMK for 20 min. The fluorescence intensity was determined using the FACSVerse flow cytometer, measuring 10 000 events for each sample.

Statistical analysis

Measurements were performed in triplicate, and values are reported as means ± standard deviations (SDs). After the normality of distribution was confirmed using the Shapiro–Wilk test, group statistical comparisons were assessed by analysis of variance (ANOVA). This was followed by post hoc analysis with Tukey’s test for three-group comparisons using SPSS software (version 23; SPSS/IBM). Differences between samples were considered to be significant for P-values < 0.05.

RESULTS

FLC-induced fungicidal effects at higher concentrations

The growth of C. albicans was monitored for 24 h using kill curves (Fig. 1). The results showed that FLC had fungistatic activity at 16 and 32 µg ml⁻¹, whereas it had a fungicidal effect at 64 and 128 µg ml⁻¹. In the following experiments, C. albicans was treated with low dosage FLC (LDF; 16 µg ml⁻¹) for fungistatic activity and high dosage FLC (HDF; 64 µg ml⁻¹) for fungicidal activity.

Cell volume decrease without membrane disruption in HDF-treated cells

When there are severe lesions on the membrane, PI fluorescence intensity increases [11]. Increased fluorescence was not observed compared with that for an untreated sample in the cells exposed to LDF or HDF (Fig. 2a). This result indicated that FLC did not influence membrane permeability. While membrane disruption was not induced, significant potassium leakage was observed with HDF (Fig. 2b). In TEA-pretreated cells with HDF, the voltage was reduced compared to HDF treatment alone, but not restored to the voltage of untreated cells. In contrast, in the LDF-treated sample, the voltage remained similar to that in the untreated and the TEA-pretreated sample with LDF.

Intracellular ion movement causes morphological changes, such as loss of cell volume or cell shrinkage [24]. As shown in Fig. 2c, HDF treatment decreased SS and FS, whereas it had a fungicidal effect at 64 and 128 µg ml⁻¹. In the following experiments, C. albicans was treated with low dosage FLC (LDF; 16 µg ml⁻¹) for fungistatic activity and high dosage FLC (HDF; 64 µg ml⁻¹) for fungicidal activity.

HDF triggers phosphatidylserine externalization and DNA damage in C. albicans

As cell shrinkage is considered to be a unique feature of apoptosis, known as apoptotic volume decrease (AVD) [13], we investigated the apoptotic features of HDF-treated cells. PS externalization is regarded as an apoptosis marker [15], and was confirmed using FITC Annexin V staining. In
HDF-treated cells there was a significant increase in externalized PS (28.96±3.12 %) compared with the untreated cells (17.10±0.99 %) (Fig. 3a). At apoptosis, several DNA-related types of damage, such as DNA fragmentation and cell division arrest, were observed. TUNEL staining was used to analyse DNA fragmentation by FLC, indicating apoptosis [15]. Cells exposed to HDF exhibited increased TUNEL fluorescence intensity (240.73±2.94) compared with the untreated cells (178.57±1.28) (Fig. 3b). This result indicates that treatment with HDF causes DNA fragmentation. When cells undergo apoptosis, apoptosis-related endonucleases cause DNA degradation, indicating a sub-G1 population [25, 26]. As shown in Fig. 3c, the sub-G1 cells, which are associated with DNA fragmentation, comprised 0.67±0.30 and 13.83 ±1.05 % of the total number of cells after treatment with LDF and HDF, respectively, compared with the control values (0.40±0.05). These results demonstrate that treatment of C. albicans with FLC causes PS externalization and DNA damage, as apoptotic markers.

Cytosolic and mitochondrial calcium levels increase in HDF-treated cells

Cytosolic free calcium in yeast is finely regulated and maintained at low levels through calcium storage in several compartments [27]. Disruption of calcium homeostasis stimulates potassium efflux [13, 28]. An increase of intracellular calcium is involved in the main events of yeast apoptosis [27]. When LDF was used, the intracellular calcium levels were similar to those in the untreated sample. However, after treatment with HDF, the calcium levels increased in the cytosol and mitochondria (Fig. 4). Therefore, HDF caused an unexpected movement of calcium, which accumulated in the mitochondria and cytosol.

HDF-treated cells exhibited glutathione depletion

Glutathione is the most abundant intracellular thiol, and reaches millimolar concentrations in most cell types [29]. It plays an important role in maintaining redox homeostasis, which is critical for the proper function of cellular processes, including apoptosis [29–31]. Our results showed that LDF treatment did not significantly change the glutathione levels or the GSSG/GSH ratio (230.60±7.11 and 0.24±0.02) compared with those for the untreated cells (221.12±8.94 and 0.23±0.03). However, lower glutathione levels were observed following treatment with HDF (187.95±7.11). Furthermore, an increased glutathione disulfide/glutathione (GSSG/GSH) ratio was observed after treatment with HDF (0.41±0.04 %).
(Fig. 5a, b). Thus, HDF led to reduce intracellular glutathione and a relative increase in GSSG compared to GSH.

**Intracellular ROS accumulation and lipid peroxidation induced by HDF treatment**

Glutathione depletion influences oxidative stress, for example through intracellular ROS generation and lipid peroxidation [32]. Because the treatment of *C. albicans* with FLC enhanced glutathione depletion, we hypothesized that this induced excess ROS generation, which influenced the antifungal effect. To determine FLC-induced oxidative stress, the level of intracellular and mitochondrial ROS was detected using the fluorescent dyes H$_2$DCFDA and Mitosox, respectively. When intracellular ROS accumulate, H$_2$DCFDA reacts with them and is oxidized to DCF, a fluorescent derivative [19]. HDF increased H$_2$DCFDA fluorescence...
intensity (38.91±1.02 %) compared with untreated cells (29.11±0.47 %) (Fig. 5c). We also used the mitochondrial ROS-specific fluorescent probe MitoSOX Red, a dihydroethidium derivative designed to observe mitochondrial ROS [33]. In particular, MitoSOX Red fluorescence intensity increased after HDF treatment (26.98±1.85 %)

![Image](image1.png)

**Fig. 3.** Detection of phosphatidylserine (PS) externalization and DNA injury as apoptotic markers. *C. albicans* cells (1×10⁷ cells ml⁻¹) were incubated for 2 h at 28°C with LDF (16 µg ml⁻¹) and HDF (64 µg ml⁻¹). (a) Detection of plasma membrane PS externalization detected by Annexin V-FITC fluorescence. (b) DNA fragmentation was observed by TUNEL staining. (c) Inhibition of cell cycle progression by LDF (16 µg ml⁻¹) and HDF (64 µg ml⁻¹) to analyse the DNA content for identification of sub-G₁, G₁, S, and G₂/M phase cells. The DNA content was measured using a FACSVerse flow cytometer (*P<0.05; **P<0.01 versus control; one-way ANOVA followed by post hoc Tukey test).

![Image](image2.png)

**Fig. 4.** Cytosolic and mitochondrial calcium changes after treatment with FLC. After the cells (1×10⁷ cells ml⁻¹) were treated with LDF (16 µg ml⁻¹) and HDF (64 µg ml⁻¹), the fluorescence intensity of (a) Fura-2 AM and (b) Rhod-2 AM indicated the levels of calcium in the cytosol and mitochondria, respectively (*P<0.05; **P<0.01 versus control; one-way ANOVA followed by post hoc Tukey test).
compared with that for untreated cells (12.44±4.30) (Fig. 5d). Lipid peroxidation is the main molecular mechanism involved in the induction of oxidative damage, because lipid peroxides can be formed by ROS [34]. We used the TBARS assay to estimate lipid peroxidation, which indicates oxidative damage to cells [35]. High levels of TBARS (2.62±0.30) were observed after treatment with HDF compared with those for the untreated cells (1.73±0.08). The LDF-treated cells did not show increased MDA levels (1.86±0.11) (Fig. 5e). We confirmed that HDF caused oxidative damage in cytosol, mitochondria and intracellular lipids.

**HDF results in cytochrome c release and metacaspase activation**

JC-1 fluorescence staining was used to detect MMP, a valuable indicator of mitochondrial function [36]. The FL2/FL1 ratio was shown to be lower in HDF-treated cells (FL2/FL1=50.35) than in untreated cells (FL2/FL1=86.07) and LDF-treated cells (FL2/FL1=78.80) (Fig. 6a). This suggests that the treatment with HDF changes the mitochondrial transmembrane potential. Western blotting analysis was used to investigate the levels of cyt c in the cytosol and mitochondria of FLC-treated C. albicans. The cytosolic cyt c levels increased following treatment with HDF, whereas the mitochondrial cyt c levels decreased compared with those for the untreated cells (Fig. 6b). These results indicate that HDF induces the release of cyt c from mitochondria in C. albicans. The released cyt c could trigger caspase activation followed by apoptotic signals [37]. Metacaspase activity increased in the HDF-treated (37.83±2.73 %) and LDF-treated (27.71±0.98 %) cells compared with that for the untreated cells (16.47±0.28 %) (Fig. 6c). These results indicate that HDF activates metacaspase. Taken together, HDF-induced apoptosis is related to metacaspase through cyt c release.

**DISCUSSION**

Although azoles tend to be fungistatic, some azoles, including miconazole and posaconazole, are fungicidal under certain conditions, including at high concentration or against specific strains [38, 39]. In the past decade, several studies have investigated whether FLC may have a fungicidal effect. Aspasia et al. confirmed changes in metabolite pools in C. albicans after treatment with FLC [9], and Mahl et al. assessed the oxidative damage of FLC in Candida glabrata [40]. In the present study, we examined a time–kill kinetic assay at various concentrations. The results showed that FLC exhibited a fungicidal effect in a dose-dependent manner. Recent studies reported that there was no statistically significant difference in the viability of HepG2 for any of the FLC treatments tested (≥128 µg ml⁻¹) [41]. In addition, at high concentration FLC had no cytotoxic effect on mouse embryonic fibroblast 3T9 cells or human embryonic kidney 293 cells [42]. Furthermore, our previous study demonstrated that FLC does not induce haemolytic effects at 16 and 64 µg ml⁻¹ [43]. Therefore, C. albicans was treated with LDF for fungistatic activity and HDF for fungicidal activity.
to investigate the fungicidal mechanism underlying FLC treatment.

While FLC affects the inhibition of ergosterol biosynthesis and the accumulation of toxic sterols [2], FLC does not significantly increase membrane permeability. On the other hand, potassium efflux was observed with HDF and TEA treatment reduced this efflux. This suggested that FLC-induced potassium efflux is associated with potassium channels rather than leakage due to membrane disruption in C. albicans. Because cell volume is regulated by ion movements and ion channels, a disruption of the ion balance induces changes in cell volume, leading to cell swelling or shrinkage. In particular, potassium is the most predominant intracellular ion and is necessary for the growth and development of cells, while it is an osmotically important cation [13]. The release of potassium results in cell shrinkage [44]. External potassium levels and morphological changes were significantly increased by HDF, and TEA treatment reduced these changes. This suggested that HDF induces cell shrinkage via potassium efflux.

Since cell shrinkage has been observed during apoptosis, it is considered to be a unique feature of apoptosis and is called apoptotic volume decrease (AVD) [24, 45]. There was a decrease in FS and SS by HDF, demonstrating an increased percentage of cells in the AVD quarter. The HDF-induced decrease in cell volume suggests the possibility of apoptosis. Furthermore, in the past decade apoptosis has come to be considered as a fungicidal mechanism and is regarded as the major target of antifungal compounds [46–48]. Therefore, these findings reinforce the possibility that FLC triggers apoptotic responses followed by fungicidal activity. We confirmed markers of apoptosis, including PS externalization and DNA damage, to demonstrate that FLC induces apoptosis. In yeast, most membrane PS exists in the inner leaflet of the cytoplasmic membrane. When apoptosis and cell death occur, PS translocates to the outer surface of the plasma membrane, which remains intact; this is considered to be an apoptotic marker [15]. The cells treated with HDF became annexin V+PI−, which indicated that the PS exposure on the outer layer of the membrane of C. albicans is a marker of apoptosis. During apoptosis, DNA damage and fragmentation are considered to be significant morphological features of apoptotic cells [49]. The results showed that HDF treatment induced DNA fragmentation, as a further marker of apoptosis. Taken together, these results showed that HDF has a significant effect on apoptotic cellular changes in C. albicans.

Calcium is a ubiquitous intracellular messenger that regulates many cellular actions, such as differentiation, gene transcription, cell proliferation and cell death [50]. Yeast maintains a tight control of intracellular calcium homeostasis through transport systems [27]. The intracellular calcium levels significantly increased, affecting the fungicidal activity of HDF. Increased cytosolic calcium induces mitochondrial

Fig. 6. Loss of MMP, cytochrome c release and metacaspase activation of FLC. C. albicans cells (1 ×10⁷ cells ml⁻¹) were incubated for 2 h at 28 °C with LDF (16 µg ml⁻¹) and HDF (64 µg ml⁻¹). (a) Mitochondrial depolarization was detected with the fluorescent dye JC-1 with LDF and HDF. (b) Cytochrome c released from mitochondria to cytosol after incubation. (c) Metacaspase activation after treatment with LDF and HDF using 10 mM CaspACE FITC-VAD-FMK in situ marker (*P<0.05; **P<0.01 versus control; one-way ANOVA followed by post hoc Tukey test).
and glutathione depletion contributes to a loss of cell viability. The glutathione level is an indicator of oxidative stress, with reduced glutathione levels [52]. Glutathione is a significant antioxidant that protects proteins, DNA and other biomolecules from oxidative damage. Therefore, an alteration in the glutathione level is an indicator of oxidative stress, and glutathione depletion contributes to a loss of cell viability via an apoptotic pathway [32, 53, 54]. Katragkou et al. reported that FLC decreases the level of glycine, which is an amino acid that is involved in glutathione biosynthesis in C. albicans [9]. Our results confirmed that glutathione levels were reduced following treatment with HDF, which suggests that HDF decreases the biosynthesis of intracellular glutathione. With regard to the total amount of glutathione, we observed increased an GSSG/GSH ratio for HDF. When free radicals are generated, GSH is oxidized to GSSG, resulting in a decrease in GSH and an increase in GSSG content [55]. Thus, this showed that HDF degraded the glutathione-related antioxidant system, and that HDF-induced apoptosis is associated with the decrease of total glutathione.

HDF-induced glutathione depletion leads to an imbalance between cellular antioxidant activity and the production of ROS. This imbalance induces oxidative stress, which is considered to be a general response that results in cellular dysfunction, damage and cell death [56]. Many antibiotics also induce fungicidal activity by causing intracellular ROS generation [38]. Thus, HDF induces intracellular ROS accumulation, resulting in the fungicidal activity of FLC. The generated ROS trigger the disruption of intracellular molecules such as DNA and protein [57]. They can easily initiate lipid peroxidation, leading to the accumulation of lipid peroxides [58]. The oxidation of lipids was evaluated by measuring the formation of MDA, which is a naturally occurring product of lipid peroxidation [34]. Lipid peroxidation is the main molecular response involved in the induction of oxidative damage. Moreover, ROS generation in mitochondria is associated with fatal mitochondrial changes followed by apoptotic events [59]. Taken together, our results showed that HDF increases oxidative damage via ROS generation. Significant mitochondrial ROS generation reinforces the mitochondria-dependent apoptotic cell death pathway of HDF.

Mitochondria are essential organelles and energy powerhouses, and are also linked to cellular physiology and integrity [60]. Mitochondrial damage triggers apoptosis through depolarization of MMP followed by pro-apoptotic protein, cyt c and caspase activation [61]. Unlike LDF, HDF induces mitochondrial damage, including mitochondrial calcium and ROS generation. Because these types of mitochondrial damage could lead to the dysfunction of mitochondria [62–64], we investigated MMP as a dysfunction marker. Consequently, the mitochondrial damage induced by HDF resulted in the loss of MMP and the translocation of cyt c to the cytosol from mitochondria in C. albicans. In addition, HDF triggered the movement of cyt c, indicating the possibility of caspase activation. Cytosolic cyt c triggers activating caspases and apoptotic signals [37]. Caspases conduct a signal through a proteolytic cascade that induces apoptosis, leading to cell death [65]. Caspase-like proteases and other unidentified caspases called metacaspases, which play an important role in yeast apoptosis, have been reported in C. albicans [66, 67]. The cyt c released from mitochondria following treatment with HDF induced caspase activation. Therefore, apoptotic cell death in C. albicans cells exposed to HDF proceeds, at least in part, in a metacaspase-dependent manner.

Although FLC is typically used as a fungistatic agent, in the present study we confirmed previously unrecognized fungicidal mechanisms of FLC on C. albicans. Glutathione depletion resulting from treatment with a high concentration of FLC caused oxidative damage and in particular significantly increased mitochondrial ROS. During oxidative stress, treatment with a high concentration of FLC resulted in mitochondrial dysfunction. This damage induced the release of cyt c from the mitochondria into the cytosol. Ultimately, caspase activation caused by the release of cyt c led to apoptosis. Therefore, we suggest that the dose-dependent fungicidal activity of FLC induces an apoptotic response.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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