Cellular apoptosis: An alternative mechanism of action for caspofungin against Candida glabrata

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ABSTRACT

Background and Purpose: Although the mechanism of action for echinocandins is known, the physiological mechanisms by which these antifungal agents cause cell death via the classical apoptotic pathways are not well-defined yet. Regarding this, the present study aimed to evaluate the mechanisms of caspofungin-induced Candida glabrata cell death.

Materials and Methods: For the purpose of the study, the minimum inhibitory concentration (MIC) of caspofungin against C. glabrata (ATCC 90030) was determined using the broth microdilution reference method (CLSI M27-A2 and M27-S4). The annexin V and propidium iodide staining was performed to determine the way through which caspofungin acts against C. glabrata (i.e., through the induction of apoptosis and/or necrosis). Additionally, the possible effect of caspofungin on inducing the expression of two apoptotic genes, namely MCA1 and NUC1, was studied using the real-time polymerase chain reaction assay.

Results: According to the obtained MIC value (0.5 µg/mL), C. glabrata, exposed to 0.25, 0.5, and 1 µg/mL of caspofungin, exhibited the features of late apoptosis/necrosis after 18 h of incubation. Furthermore, the use of 0.25, 0.5, and 1 µg/ml caspofungin induced apoptosis (early/late) in 14.67%, 17.04%, and 15.89% of the cells, respectively. The results showed a significant difference between the percentages of early-apoptotic cells at the three concentrations (P<0.05). In addition, the rate of necrosis was significantly greater than that of apoptosis in response to caspofungin. Accordingly, necrosis occurred in 71.26%, 71.26%, and 61.26% of the cells at the caspofungin concentrations of 0.25, 0.5, and 1 µg/mL, respectively (P<0.05). The analysis of the data in the REST software demonstrated a significant increase in the expression of MCA1 and NUC1 genes (P<0.05).

Conclusion: As the findings of the present study indicated, caspofungin promoted both necrosis and apoptosis of C. glabrata cells at concentrations higher than or equal to the MIC value.

Keywords: Candida glabrata, Caspofungin, Flow cytometry, MCA1, NUC1

Introduction

Echinocandins are the newest class of antifungal agents, which inhibit the synthesis of fungal cell wall through the noncompetitive inhibition of the Fks subunits of (1,3)-β-d-glucan synthase [1, 2]. Currently, three echinocandins, including caspofungin, micafungin, and anidulafungin, have been approved for clinical applications [3-6]. The caspofungin is the most widely used echinocandin in clinical settings, which has fungicidal activity against the majority of Candida species. Nevertheless, C. parapsilosis and C. guilliermondii are relatively insensitive to caspofungin [7, 8]. Resistance to echinocandins has also increased significantly in C. glabrata with the expanded use of these agents in therapy. Susceptibility testing on 1,380 isolates of C. glabrata collected within 2008-2013 showed that 3.3% of the isolates were resistant to caspofungin [9]. Resistance to echinocandins in C. glabrata and...
different *Candida* species is explained by the occurrence of mutations in the genes encoding glucan synthases (e.g., *FKS1* and *FKS2*) [10]. Therefore, the development of more effective antifungal agents directly depends on understanding their mechanism of action and the basis of cell death decisions in fungi. Although the spectrum of activity for echinocandins is known, the physiological mechanisms by which these antifungal drugs cause cell death via the classical apoptotic pathways are not identified well.

Apoptosis is a conserved cell biochemical process of cell death that plays a key role in normal development. Apoptotic cells are characterized by a specific series of morphological and biochemical properties that set apoptosis apart from the accidental cell death and are observed in eukaryotes and prokaryotes [11, 12]. The principal morphological feature of apoptosis is the shrinkage of the cell and its nucleus [13, 14]. Necrosis, on the other hand, is the death resulting from direct cellular injury, which is best defined by the cell and organelle swelling and lysis [14].

Notably, *C. albicans* cells show apoptotic markers with high similarity to those of mammalian cells, including phosphatidylserine externalization, reactive oxygen species accumulation, mitochondrial membrane potential dissipation, and DNA condensation and fragmentation [15]. Apoptosis is elucidated by two distinct routes, namely caspase-dependent and caspase-independent manners. According to the evidence, apoptosis-inducing factor (AIF) [16], AIF-homologous mitochondrion-associated inducer of death [17], and endonuclease G (EndoG) [18, 19] can all induce apoptotic cell death in a caspase-independent manner. With this background in mind, the present study was conducted to investigate the mechanisms of *C. glabrata* cell death caused by caspofungin. To this aim, we reported both apoptosis and necrosis in the caspofungin-treated *C. glabrata* cells.

**Materials and Methods**

*Candida glabrata* strain and growth conditions

For the purpose of the study, *C. glabrata* ATCC90030 was grown on the Sabouraud dextrose agar medium (Difco, USA) and incubated at 30°C for 24 h. The strain had been previously identified by the sequencing of the complete ribosomal DNA internal transcribed spacer region.

**Antifungal susceptibility testing**

The minimum inhibitory concentration (MIC) of caspofungin was determined using the broth microdilution reference method as recommended by the Clinical and Laboratory Standards Institute (CLSI) document M27-A2 and M27-S4 [20, 21]. Caspofungin (Pfizer Central Research, Sandwich, Kent, UK) was obtained from the respective manufacturers in the form of reagent-grade powders for the preparation of the CLSI microdilution trays. To get the two times of the caspofungin concentration, it was diluted in the standard RPMI-1640 medium (Sigma Chemical Co.) buffered at a pH of 7.0 with 0.165 M morpholinepropanesulfonic acid (Sigma Chemical Co.) and L-glutamine without bicarbonate.

The antifungal agent was dispensed into 96-well microdilution trays with a final concentration of 0.016-16 μg/ml. Conidial suspensions were prepared from the isolates grown for 24 h, suspended in sterile saline solution, and adjusted by spectrophotometric measurements at a wavelength of 530 nm to a percent transmittance range of 75-77. A working suspension was made by a 1:10 dilution, followed by a 1:100 dilution of the stock suspension, with RPMI medium, which resulted in 2.5-5×10⁶ CFU/ml. In the next stage, the microdilution plates were incubated at 35°C and examined visually after 24 h; in this regard, the drug concentration that elicited 50% growth inhibition was compared with a drug-free control. The MIC values for caspofungin were compared with the CLSI (M27-A2 and M27-S4) interpretative guidelines on antifungal susceptibility testing. Briefly, *C. glabrata* with the MICs of ≥ 0.12, 0.25, and ≥ 0.5 μg/ml were considered susceptible, susceptible dose-dependent, and resistant to caspofungin, respectively. *Candida krusei* (ATCC 6258) and *C. parapsilosis* (ATCC 22019) were used as quality controls.

**Annexin V and propidium iodide staining**

The concentration of *C. glabrata* cells exposed to 1, 0.5, and 0.25 μg/ml of caspofungin was adjusted by spectrophotometric measurements at 600 nm wavelength to an absorption range of 0.2-0.3. The yeasts were then washed twice in sorbitol solution (1 M sorbitol, 0.25 m MEDTA, and 20 m MDTT), and then incubated at 30°C for 70 min in 0.01 mg/ml lyticase in 10 mM sodium citrate buffer to disrupt the cell wall. Cell apoptosis and necrosis were determined using the annexin V/propidium iodide (PI) kit according to the manufacturer’s instruction (eBioscience, USA). Following incubation with the appropriate concentrations of annexin V and PI, sample acquisition was performed using the Partec flow cytometry system. The obtained data were analyzed using the Flomax software (Partec, Germany). Nonstained cells were used as controls for background determination. For each sample, a minimum of 10,000 events were counted and then subjected to analysis. Pilot experiments were first performed to ensure that lyticase did not cause the achievement of false-positive annexin V or PI staining. All assays were performed at least in triplicate and repeated at least three times.

**DNA damage and chromatin condensation**

To investigate the features of late apoptosis in response to caspofungin, we utilized 406-diamidino-2-phenylindole dihydrochloride (DAPI) staining. The basic protocol for the DAPI staining of nuclei was carried out as previously described [22] using 1 mg of DAPI/ml (molecular probes). To this end, two drops of
Table 1. Primers used for MCA1, NUC1, and RDN5.8 gene expression

| Name               | Reference gene Accession No/ identifier | Primer’s Sequence (5’ –> 3’) | PCR product length |
|--------------------|----------------------------------------|------------------------------|--------------------|
| MCA1-F             | KU739085.1                             | AGCTCGGTTACGAAAAAGCA         | 121                |
| MCA1-R             |                                        | CGAACCTGTCGCTGTAGTGT         |                    |
| NUC1-F             | MF113057                               | GCGGGTTTTTTCAATGATGGA        | 192                |
| NUC1-R             |                                        | TTCAGGAATGGCCTTCATCC         |                    |
| RDN5.8-F           | AB032177.1                             | CTTGCTTCGCGCATGATGGA         | 98                 |
| RDN5.8-R           |                                        | GCAGAAATGGCCTGTCCA          |                    |

PCR: polymerase chain reaction

DAPI were applied on the sections, and then the coverslip was carefully lowered on the sections. After keeping the medium in darkness for a few minutes, DAPI stain was observed with ultraviolet excitation.

RNA extraction and quantitative real-time reverse transcription polymerase chain reaction

Total RNA was extracted from C. glabrata strain under both caspofungin-treated and -untreated conditions. Briefly, Candida glabrata cells were treated with 0.25 µg/ml using a method recommended by CLSI M27-A2. However, in order to get a large mass of C. glabrata cells, the test was performed in 24-well trays. A positive control (i.e., untreated C. glabrata) was also run for each isolate in each plate. RNA extraction was performed, and the strain was grown to the mid-logarithmic phase, by using the RNAX plus kit (Sina clone, Karaj, Iran) following the instructions of the manufacturer. RNA concentrations and purity were determined by spectrophotometric measurements (Biochrom WPA Biowave II, UK). An equal amount of RNA treated with DNase was subjected to cDNA synthesis by using the PrimeScript RT reagent kit (Vivantis, Malaysia).

Primers were designed on the basis of the published sequence of the relevant genes in C. glabrata (Table 1). The Ribosomal 5.8s RNA gene (RDN5.8) was used as the endogenous reference gene. Standard curves for each gene were established with four-fold serially diluted cDNA obtained from the cells grown to the mid-logarithmic phase at 37°C by using specific primers under appropriate PCR conditions. Real-time reverse transcription PCR was performed with the ABI Step One real-time PCR system (Applied Biosystem, USA); in addition, SYBR Premix Ex Taq II was used as a reagent specifically designed for intercalator-based real-time PCR.

All PCR reaction mixtures contained 10 µl SYBR Premix Ex Taq II (2x), 2 µl of first strand cDNA, 0.4 µM of each primer, and dH2O at a final volume of 20 µl. The amplification program included an initial denaturation step at 95°C for 30 sec, followed by 40 cycles, each of which entailed two steps performed at 95°C for 5 sec and 60°C for 30 sec. The negative controls were also included in each run. The expression of all genes was normalized to the housekeeping gene RDN5.8s and analyzed by means of the REST software (2009). This software uses the comparative Ct method (ΔΔCt) to analyze data. Experiments under each condition were performed in duplicate, and each experiment was repeated twice on two different days to assess reproducibility.

Statistical analysis

Statistical analysis was performed in the REST software. The software uses a pairwise fixed reallocation randomization test. The results were presented as mean and standard deviation. Differences in the results obtained at two caspofungin concentrations were assessed using the Student’s t-test. Comparison of the results at more than two caspofungin concentrations were made using ANOVA. A P-value less than 0.05 was considered statistically significant.

Results

Antifungal susceptibility testing

The MIC for C. glabrata isolate was obtained as 0.5 µg/ml indicating the susceptibility of this species to caspofungin.

Induction of Candida glabrata apoptosis and necrosis by caspofungin

Annexin V and PI staining was performed to determine if killing by caspofungin occurred through the induction of apoptosis and/or necrosis. Annexin V and PI assays were performed on yeast spheroplasts that were prepared using lyticase, a cell wall-active lytic enzyme that hydrolyzes (1, 3)-β-D-glucan linkages. These assays showed no evidence of apoptosis or necrosis until reaching a lyticase concentration of 0.1 mg/ml (data not shown). Thereafter, the assays were performed in the presence of caspofungin using 0.02 mg/ml lyticase.

As summarized in Table 2, caspofungin induced apoptosis (early/late) in 14.67%, 17.04%, and 15.89% of the cells at 0.25, 0.5, and 1 µg/ml, respectively. On the other hand, the rate of necrosis was significantly greater than that of apoptosis following the exposure to caspofungin as observed in 71.26%, 71.26%, and 61.26% of the cells at the caspofungin concentrations of 0.25, 0.5, and 1 µg/ml, respectively (P<0.05). The results of ANOVA revealed a significant difference among the three different concentrations of caspofungin in terms of the percentage of apoptotic/necrotic cells (P<0.05; Figure 1 and Figure 2).

DNA damage and chromatin condensation

Chromatin is damaged during the late stages of apoptosis due to the proteolysis of nuclear proteins, a process which results in DNA damage and chromatin condensation [23]. As depicted in Figure 3, C. glabrata cells exposed to caspofungin (0.125 µg/ml) had
Induction of apoptosis in *Candida glabrata* using caspofungin

**Table 2.** Effects of caspofungin on early apoptosis, late apoptosis, and necrosis as determined by annexin V and propidium iodide staining

| Caspofungin Conc (µg/ml) | % of cells<sup>a</sup> | Annexin V+/PI- (early apoptosis) | Annexin V+/PI+ (late apoptosis/necrosis) | Annexin V-/PI+ (necrosis) |
|-------------------------|-----------------------|----------------------------------|----------------------------------------|--------------------------|
| Control                 |                       | 0.13±0.006                       | 0.4±0.03                               | 17.61±0.4                |
| 0.25                    |                       | 0.46±0.05                        | 14.21±0.7                              | 61.26±0.5                |
| 0.5                     |                       | 0.56±0.2                         | 16.48±0.4                              | 71.26±0.1                |
| 1                       |                       | 0.86±0.04                        | 15.3±0.6                               | 71.26±0.6                |

<sup>a</sup>The data are presented as mean percentage±standard deviation.

**Figure 3.** Cell apoptosis determined by annexin V-fluorescein isothiocyanate staining

(Flow cytometer analysis of the apoptotic and necrotic cells [Q1: necrotic; Q2: late apoptotic; Q3: live; Q4: early apoptotic] after 18 h of incubation with 0.25 (B), 0.5 (C) and 1(D) µg/ml caspofungin and control (A). Results are expressed as mean±standard deviation [n=3].)

**Figure 4.** Effect of different concentrations of caspofungin on early apoptosis, late apoptosis, and necrosis

(Effects of different concentrations of caspofungin on early apoptosis, late apoptosis, and necrosis, as determined by annexin V and propidium iodide staining after 18 h of incubation. Necrosis is the predominant response induced after drug exposure. Nevertheless, a higher rate of apoptosis was observed at a caspofungin concentration of 0.5 µg/ml.)
evidence of DNA damage and chromatin condensation. In this regard, the cells exposed to caspofungin exhibited irregular fragmented DNAs (single arrow), which are typical of nuclear abnormalities associated with DNA damage during apoptosis. In the control cells, on the other hand, diamidino-2-phenylindole dihydrochloride staining revealed a single, bright, round nucleus and peripheral cell spots corresponding to stained mitochondria.

Quantitative real-time reverse transcription polymerase chain reaction

The possible effect of caspofungin on inducing expression in the two apoptotic genes (e.g., MCA1 and NUC1) was studied using real-time PCR assay. In addition, RDN5.8 gene was applied as an endogenous reference gene. Based on the results, MCA1, NUC1, and RDN5.8s primers had similar efficiency in a titration experiment using C. glabrata cDNA (1000 ng-1000 pg) in serial dilutions (data not shown). Expression of each gene was indicated as the ratio of expression relative to that of untreated logarithmic-phase-grown yeasts (Table 3).

The REST software was employed to represent the relative expression between the treated and untreated (control) samples of all studied genes. The boxplot given by REST 2009 is a very informative way to visualize the gene expression data. It includes the smallest observation (sample minimum), lower quartile, median, upper quartile, and largest observation (sample maximum). In this boxplot, values of 0-1 and > 1 indicate underexpression and overexpression, respectively. On the basis of the REST output, the expression of the MCA1 and NUC1 genes increased significantly (P<0.05). Figure 4 illustrates the ratio of expression under the highest concentration of caspofungin-treated condition relative to that of the untreated condition.

Discussion

As the findings of the present study indicated, caspofungin and other echinocandins can compromise C. glabrata cell viability via both necrosis (i.e., inhibiting cell wall integrity) and apoptosis (i.e., inducing the initiation of programmed cell death). In the present study, the evaluation of apoptosis was accomplished by the implementation of annexin V staining. This assay aims at detecting the externalization of plasma membrane phosphatidylserine, which is a critical event in the apoptotic procedure. Annexin V stains phosphatidylserine, which is a negatively charged phospholipid that is translocated from the inner leaflet of the plasma membrane to the outer part during early apoptosis [24].

On the other hand, since the PI stain does not permeate the cells with undamaged membranes, it facilitates the identification of the necrotic cells. Therefore, staining patterns discriminate between the live cells (annexin V), negative (annexin V-/PI-) and early apoptosis (annexin V+/PI-), necrosis (annexin V-/PI+), and late apoptosis/necrosis (annexin V+/PI+). In
the present study, apoptosis was not induced within the first hour of caspofungin exposure (early caspofungin exposure). Therefore, in the present study, the incubation time of 18 h was applied to investigate the early apoptosis and late apoptosis/necrosis. The rate of late apoptosis was demonstrated by nuclear condensation (DAPI staining).

In each of the assays, apoptosis was evident at the caspofungin concentrations lower than or equal to the MIC value (0.5 µg/mL). Moreover, the real-time assay performed on the cells exposed to 0.125 µg/mL of caspofungin revealed the overexpression of two major apoptotic genes, namely MCA1 and NUC1. Caspases are the classes of cysteine-aspartic acid proteases regulated at the posttranslational level. These proteases convey a signal in a proteolytic cascade that induces apoptosis when cleaved, and lead to cell death [25]. A caspase-like protein, fitting into the type I category of metacaspases, named MCA1, was first identified in *Saccharomyces cerevisiae* [26, 27]. The recent studies demonstrated that following an apoptotic stimulus, the product of MCA1 was processed by the proteolytic removal of a 14-kDa peptide leading to the activation of metacaspase as in mammalian caspases [28].

Chromatin condensation and DNA fragmentation are both the key features of apoptosis. Caspase-activated DNase is the best characterized EndoG in charge of the caspase-dependent apoptotic process [29]. *Nuc1p* has been the best-conserved yeast homologue of a mammalian apoptosis regulator to date. In a study, upon treatment with either acetic acid or H2O2, the overexpression of *EndoG (Nuc1p)* led to the elevation of apoptosis, compared with that in the control. In the mentioned study, this effect was reported to be depended on nuclease activity [30].

In *C. glabrata*, programmed cell death was characterized by the upregulation of *MCA1* and *NUC1* upon treatment with glabridin [31, 32]. According to the literature, several drugs, including ciclopiroxolamine, osmotin, pradimicin, dermaseptin, histatin [33], silver-coumarin complexes [34], farsenol [35], and amphoterin B [36], cause *C. albicans*/*Saccharomyces cerevisiae* cells to undergo apoptosis. Moreover, apoptosis induction was reported in the *C. albicans* exposed to caspofungin. In a study, apoptosis was induced within the first hour of caspofungin exposure. In this regard, the early apoptosis and late apoptosis/necrosis were apparent in 20-25% and 5-7% of *C. albicans* cells, respectively, following 3 h of exposure [37].

However, our findings revealed that most of *C. glabrata* cells were viable after 3 h of caspofungin treatment, and no sign of early apoptosis was observed in the samples. According to our results, direct toxicity due to the loss of cell wall integrity and cell lysis and the induction of programmed cell death were the responses of *C. glabrata* cells to caspofungin. However, it is well-established that nonlethal exposures to caspofungin rapidly induces the upregulation of chitin synthase genes and leads to the activation of the protein kinase C cell wall integrity signaling pathway [38]. This compensatory response is the result of cell exposure to caspofungin. Programmed cell death may be regarded as a strategy to conserve the resources for healthier cells that are more likely to survive and replicate efficiently.

**Conclusion**

The findings of the present study demonstrated that caspofungin promoted both necrosis and apoptosis of *C. glabrata* cells at concentrations greater than or equal to the MIC value. Nevertheless, the real-time assay revealed that apoptosis was induced at concentrations lower than the MIC value.

**Acknowledgments**

The authors would like to thank the Graduate University of Mazandaran University of Medical Sciences, Sari, Iran, for providing laboratory facilities.

**Author’s contribution**

M. M. and H. A. conceived the study, M. N. and A. A. prepared the strains, P. A., N. A. and H. H. performed the experiments. M. M., T. S., and H. B. prepared the manuscript. All authors read and approved the final manuscript.

**Conflicts of interest**

The authors declare no conflicts of interest regarding the publication of this paper.

**Financial disclosure**

No financial interests related to the material of this manuscript have been declared.

**References**

1. Douglas CM, D’ippolito JA, Shieh GI, Meinz M, Onishi J, Marrinan IA, et al. Identification of the FKS1 gene of *Candida albicans* as the essential target of 1, 3-beta-D-glucan synthase inhibitors. Antimicrob Agents Chemother. 1997; 41(11):2471-9.
2. Denning DW. Echinocandin antifungal drugs. Lancet. 2003; 362(9390):1142-51.
3. Chandrasekar P, Sobel J. Micafungin: a new echinocandin. Clin Infect Dis. 2006; 42(8):1711-8.
4. Kartsonis NA, Nielsen J, Douglas CM. Caspofungin: the first in a new class of antifungal agents. Drug Resist Updat. 2003; 6(4):197-218.
5. Walker LA, Munro CA, De Brujin I, Lenardon MD, McKinnon A, Gow NA. Stimulation of chitin synthesis rescues *Candida albicans* from echinocandins. PLoS Pathog. 2008; 4(4):e1000040.
6. Walker LA, Gow NA, Munro CA. Fungal echinocandin resistance. Fungal Genet Biol. 2010; 47(2):117-26.
7. Barchiesi F, Spreghini E, Tomassetti S, Della Vittoria A, Arzeni D, Manso E, et al. Effects of caspofungin against *Candida guilliermondii* and *Candida parapsilosis*. Antimicrobial Agents Chemother. 2006; 50(8):2719-27.
8. Pfäffl M, Diekema D, Andes D, Arendrup M, Brown S, Lockhart S, et al. Clinical breakpoints for the echinocandins and *Candida* revisited: integration of molecular, clinical, and microbiological data to arrive at species-specific interpretive criteria. Drug Resist Updat. 2011; 14(3):164-76.
9. Pham CD, Iqbal N, Bolden CB, Kuykendall RJ, Harrison LH, Farley MM, et al. Role of FKS mutations in *Candida glabrata*: MIC values, echinocandin resistance, and multidrug resistance. Antimicrobial Agents Chemother. 2014; 58(8):4690-6.
10. Arendrup MC, Perlin DS. Echinocandin resistance: an emerging
clinical problem? Curr Opin Infect Dis. 2014; 27(6):484-92.

11. Aerts AM, Carmona-Gutiérrez D, Lefèvre S, Govaert G, François IE, Madeo F, et al. The antifungal plant defense RxAFP2 from radish induces apoptosis in a metacaspase independent way in Candida albicans. FEBS Lett. 2009; 583(15):2513-6.

12. Aerts AM, Bammens L, Govaert G, Carmona-Gutiérrez D, Madeo F, Cammue BP, et al. The antifungal plant defense HsAFP1 from Heuchera sanguinea induces apoptosis in Candida albicans. Front Microbiol. 2011; 2:47.

13. Diaz L, Chiong M, Quest AF, Lavandero S, Stutzen A. Mechanisms of cell death: molecular insights and therapeutic perspectives. Cell Death Differ. 2005; 12(11):1449-56.

14. Collins JA, Schandl CA, Young KK, Vesely J, Willingham MC. Major DNA fragmentation is a late event in apoptosis. J Histochem Cytochem. 1997; 45(7):923-34.

15. Munoz AJ, Wanichthanarak K, Meza E, Petranovic D. Systems biology of yeast cell death. FEMS Yeast Res. 2012; 12(2):249-65.

16. Candé C, Ceconi F, Dessen P, Kroemer G. Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death? J Cell Sci. 2002; 115(24):4727-34.

17. Wu M, Xu LG, Li X, Zhai Z, Shu HB. AIFM, an apoptosis-inducing factor-homologous mitochondrion-associated protein, induces caspase-independent apoptosis. J Biol Chem. 2002; 277(28):25617-23.

18. Li LY, Lao X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature. 2001; 412(6842):95-9.

19. Parrish J, Li L, Klotz K, Ledwich D, Wang X, Xue D. Mitochondrial endonuclease G is important for apoptosis in C. elegans. Nature. 2001; 412(6842):90-4.

20. Wayne P. Clinical and Laboratory Standards Institute: reference method for broth dilution antifungal susceptibility testing of yeasts; fourth informational supplement. CLSI Document. 2008; 3:6-12.

21. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts; fourth informational supplement. Wayne, PA: Clinical and Laboratory Standards Institute; 2012.

22. Almeida B, Sampaio-Marques B, Carvalho J, Silva MT, Leão C, Rodrigues F, et al. An atypical active cell death process underlies the fungicidal activity of ciclopirox olamine against the yeast Saccharomyces cerevisiae. FEMS Yeast Res. 2007; 7(3):404-12.

23. Dobrucki J, Darzykiewicz Z. Chromatin condensation and sensitivity of DNA in situ to denaturation during cell cycle and apoptosis—a confocal microscopy study. Micron. 2001; 32(7):645-52.

24. Wu XZ, Chang WQ, Cheng AX, Sun LM, Lou HX. Plagiochin E, an antifungal active macroyclic bis (bibenzyl), induced apoptosis in Candida albicans through a metacaspase-dependent apoptotic pathway. Biochim Biophys Acta. 2010; 1800(4):439-47.

25. Ligr M, Madeo F, Fröhlich E, Hilt W, Fröhlich KU, Wolf DH. Mammalian Bax triggers apoptotic changes in yeast. FEMS Lett. 1998; 438(1-2):61-5.

26. Degterev A, Boyce M, Yuan J. A decade of caspases. Oncogene. 2003; 22(53):8543-67.

27. Uren AG, O’Rourke K, Aravind L, Pisabarro MT, Seshagiri S, Koonin EV, et al. Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. Mol Cell. 2000; 6(4):961-7.

28. Madeo F, Herker E, Maldener C, Wissing S, Lächelt S, Herlan M, et al. A caspase-related protease regulates apoptosis in yeast. Mol Cell. 2002; 9(4):911-7.

29. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature. 1998; 391(6662):43-50.

30. Iwamoto Y, Li Z, Zhou B. Caspase-independent apoptosis in yeast. Biochim Biophys Acta. 2008; 1783(7):1311-9.

31. Nabil M, Moazen M, Hedayati MT, Aryamloo P, Gohar AA, Madani SM, et al. Glabridin induces overexpression of two major apoptotic genes, MCA1 and NUC1, in Candida albicans. J Global Antimicrob Resist. 2017; 11:52-6.

32. Moazeni M, Hedayati MT, Nabil M, Mousavi SJ, Abdollahi Gohar A, Gholami S. Glabridin triggers over-expression of MCA1 and NUC1 genes in Candida glabrata: is it an apoptosis inducer? J Mycol Med. 2017; 27(3):369-75.

33. Almeida B, Silva A, Mesquita A, Sampaio-Marques B, Rodrigues F, Ludovico P. Drug-induced apoptosis in yeast. Biochim Biophys Acta. 2008; 1783(7):1436-48.

34. Hwang Is, Lee J, Hwang JH, Kim KJ, Lee DG. Silver nanoparticles induce apoptotic cell death in Candida albicans through the increase of hydroxyl radicals. FEBS J. 2012; 279(7):1327-38.

35. Shirliff ME, Krom BP, Meijering RA, Peters BM, Zha J, Scheper MA, et al. Farnesol-induced apoptosis in Candida albicans. Antimicrob Agents Chemother. 2009; 53(6):2392-401.

36. Al-Dhaheri RS, Douglas LJ. Apoptosis in Candida biofilms exposed to amphotericin B. J Med Microbiol. 2010; 59(2):149-57.

37. Hao B, Cheng S, Clancy CJ, Nguyen MH. Caspofungin kills Candida albicans by causing both cellular apoptosis and necrosis. Antimicrob Agents Chemother. 2013; 57(1):326-32.

38. Walker LA, Gow NA, Munro CA. Elevated chitin content by Candida albicans HsAFP1 from H. sanguinea induces apoptosis in Candida albicans. Front Microbiol. 2011; 2:47.