

Silver nanoparticles induce reactive oxygen species-mediated cell cycle delay and synergistic cytotoxicity with 3-bromopyruvate in *Candida albicans*, but not in *Saccharomyces cerevisiae*

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Background: Silver nanoparticles (AgNPs) inhibit the proliferation of various fungi; however, their mechanisms of action remain poorly understood. To better understand the inhibitory mechanisms, we focused on the early events elicited by 5 nm AgNPs in pathogenic *Candida albicans* and non-pathogenic *Saccharomyces cerevisiae*.

Methods: The effect of 5 nm and 100 nm AgNPs on fungus cell proliferation was analyzed by growth kinetics monitoring and spot assay. We examined cell cycle progression, reactive oxygen species (ROS) production, and cell death using flow cytometry. Glucose uptake was assessed using tritium-labeled 2-deoxyglucose.

Results: The growth of both *C. albicans* and *S. cerevisiae* was suppressed by treatment with 5 nm AgNPs but not with 100 nm AgNPs. In addition, 5 nm AgNPs induced cell cycle arrest and a reduction in glucose uptake in both fungi after 30 minutes of culture in a dose-dependent manner ($P < 0.05$). However, in *C. albicans* only, an increase in ROS production was detected after exposure to 5 nm AgNPs. Concordantly, an ROS scavenger blocked the effect of 5 nm AgNPs on the cell cycle and glucose uptake in *C. albicans* only. Furthermore, the growth-inhibition effect of 5 nm AgNPs was not greater in *S. cerevisiae* mutant strains deficient in oxidative stress response genes than it was in wild type. Finally, 5 nm AgNPs together with a glycolysis inhibitor, 3-bromopyruvate, synergistically enhanced cell death in *C. albicans* ($P < 0.05$) but not in *S. cerevisiae*.

Conclusion: AgNPs exhibit antifungal activity in a manner that may or may not be ROS dependent, according to the fungal species. The combination of AgNPs with 3-bromopyruvate may be more useful against infection with *C. albicans*.

Keywords: metal nanoparticles, yeasts, anti-infective agents, antimetabolites

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Introduction

Silver nanoparticles (AgNPs) are defined as silver-containing particles with a diameter of 1–100 nm and are produced through chemical, physical, or biological methods.¹ Silver precursors such as silver nitrate are reduced to AgNPs by chemical reducing agents (eg, borohydride) or by physical techniques (eg, heating in an oxygen-restricted environment).¹ AgNPs are biologically synthesized by the reduction of silver ions using biological reducing agents (eg, enzymes).¹ The biological synthesis of AgNPs is called green synthesis as it does not utilize toxic chemicals as reducing agents, but instead uses proteins, nucleotides, and carbohydrates produced by plants, microorganisms, or algae.²

AgNPs exert anticancer and antimicrobial activities.³ The cytotoxicity of AgNPs has been observed in various human cancer cells including colon cancer, glioblastoma, lung cancer, breast cancer, and acute myeloid leukemia.^{1,4} The antibacterial activities of AgNPs have been demonstrated against various pathogenic Gram-positive and Gram-negative bacteria.^{5–7} AgNPs even inhibit the growth of antibiotic-resistant pathogens such as *Bacillus cereus*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*.⁸ AgNPs also suppress the growth of various pathogenic fungi such as *Candida*, *Aspergillus*, and *Cryptococcus* species.^{9,10} Even antifungal drug-resistant *Candida albicans* was killed by AgNPs.¹¹ AgNPs have potential applications in the treatment of cancer and infectious diseases.

The antifungal mechanisms of AgNPs remain to be elucidated. Treating *C. albicans* with AgNPs for 24 hours leads to cell wall damage and disruption.^{12,13} AgNPs have been reported to exert toxicity through an increase in reactive oxygen species (ROS) production; however, this is still subject to debate. ROS are involved in the AgNP-induced cell death of *C. albicans*, although ROS scavengers do not completely suppress the cytotoxic activity of AgNP on *C. albicans*.^{14,15} In *S. cerevisiae*, the toxicity of AgNPs was not attributed to oxidative stress response or increased cell wall/membrane permeability, based on testing with single-gene mutant strains deficient in the ability to detoxify ROS or maintain cell wall/membrane integrity.¹⁶ However, the role of genes regulating the various stress responses, such as mitogen-activated protein kinase (MAPK) *HOG1*, oxidative stress-activated transcription factors, *SKN7* and *YAP1*, and the cell wall integrity maintaining factor, MAPK *SLT2*, in the antifungal activity of AgNPs has not been well studied.^{17–21} Furthermore, we noted that AgNP-mediated structural changes and ROS generation in yeast cells were mostly observed 3–24 hours after the exposure to AgNPs in vitro.

In this study, we focused on the early events and ROS generation induced by 5 nm AgNPs in *C. albicans* and *S. cerevisiae*. We observed the cell cycle delay, reduced glucose uptake, and increased ROS production in *C. albicans* after 30 minutes' exposure to AgNPs. Similarly, 5 nm AgNPs inhibited the growth, cell cycle progression, and glucose uptake of *S. cerevisiae*; however, increases in ROS production and cell death were not observed in *S. cerevisiae*. Furthermore, we showed that ROS may contribute to the antifungal activity of AgNPs against *C. albicans* but not against *S. cerevisiae*.

Materials and methods

AgNPs and chemical reagents

Polyvinylpyrrolidone (PVP)-coated AgNPs of mean sizes 5 and 100 nm were purchased from I&C Technology (Seoul, Republic of Korea). Characterization of AgNPs was carried out as previously reported.²² In brief, using transmission electron microscopy (model JEM-1011; JEOL, Tokyo, Japan), the mean size was determined as 7.9 ± 5.3 nm for 5 nm AgNPs and 70.9 ± 71.3 nm for 100 nm AgNPs. By dynamic light scattering analysis (Malvern Instruments, Novato, CA, USA), the average size of AgNPs was determined as 3.7 nm for 5 nm AgNPs and 95.9 nm for 100 nm AgNPs. N-acetylcysteine (NAC), 3-bromopyruvate (BrPA), and nocodazole were purchased from Sigma-Aldrich (St Louis, MO, USA). Propidium iodide was purchased from Merck Millipore (Billerica, MA, USA). Cell-permeable reagents 2',7'-dichlorofluorescein diacetate (DCFDA) and MitoSOX Red were purchased from Molecular Probes (Eugene, OR, USA).

Yeast strains and AgNPs treatment conditions

Candida albicans was provided by the National Culture Collection for Pathogens (Cheongju-si, Republic of Korea). Wild-type and six deletion mutants (Δ skn7, Δ yap1, Δ skn7 and Δ yap1 double mutant, Δ hog1, Δ SLT2, and Δ hog1 and Δ SLT2 double mutant) of *S. cerevisiae* BY4741 were purchased from Invitrogen (Carlsbad, CA, USA).

Candida albicans was grown in Sabouraud dextrose broth at 37°C for 6 hours on a rotary shaker at 140 rpm. The culture was diluted with fresh medium until it exhibited an OD of 0.1 at 600 nm and then either 5 nm or 100 nm AgNPs were added to the culture to a final concentration of 2, 20, or 50 μ g/mL. Each *S. cerevisiae* strain was plated on YPD (1% yeast extract, 2% peptone, and 2% dextrose) agar medium and incubated at 30°C for 2 days. Then, a single colony was cultured in the YPD broth at 30°C for 1 day in the shaker at 140 rpm. The culture was diluted with fresh medium to an OD₆₀₀ of 0.1 and then either 5 nm or 100 nm AgNPs were added to the culture to a final concentration of 2, 20, or 50 μ g/mL.

Cell growth analysis

Candida albicans and *S. cerevisiae* were cultured in the presence or absence of AgNPs as described in “Yeast strains and AgNPs treatment conditions”, for 12 and 28 hours, respectively. The absorbance of the culture at 600 nm was

measured every 2 hours in the case of *C. albicans* or 4 hours in the case of *S. cerevisiae* using a Beckman-Coulter (Brea, CA) DU730 spectrophotometer. After 12 hours of culture of *C. albicans*, a spot assay was performed by inoculating 5 μ L of each 10-fold serially diluted culture on Sabouraud dextrose agar plates and then incubating them at 37°C for 2 days. Similarly, after 20 hours of culture time of *S. cerevisiae*, a spot assay was performed by inoculating 5 μ L of each 10-fold serially diluted culture on the YPD agar plates and then incubating them at 30°C for 2 days.

Cell death analysis

Candida albicans and *S. cerevisiae* were cultured in the presence or absence of AgNPs as described in “Yeast strains and AgNPs treatment conditions” for 24 hours. In the glycolysis inhibitor condition, BrPA co-treatment along with AgNPs, BrPA was added to the culture to a final concentration of 20, 80, or 200 μ M. After 2, 4, 6, and 24 hours, 1 mL of the culture was centrifuged at 11,400 \times g for 5 minutes and the harvested cells were washed twice with PBS. The cells were labeled with propidium iodide (250 ng/mL) in PBS for 30 minutes at room temperature and then analyzed using a FACSCanto II (BD Biosciences, San Diego, CA, USA).

Cell cycle analysis

Candida albicans and *S. cerevisiae* were cultured in the presence of AgNPs as described in “Yeast strains and AgNPs treatment conditions”. As a positive control for cell cycle analysis, *C. albicans* was cultured in the presence of a cell cycle arresting agent, nocodazole, with a final concentration of 15 μ g/mL. At 30 minutes and 1, 2, and 4 hours, cells were harvested by centrifugation of 1 mL culture at 11,400 \times g for 5 minutes. The cells were washed twice with PBS, fixed in 75% ethyl alcohol, and then incubated in 200 μ L of PBS containing 100 μ g/mL of RNase A for 3 hours at 37°C. After washing twice with PBS, the cells were resuspended in 200 μ L of PBS containing propidium iodide (10 μ g/mL), and then incubated for 4 hours at room temperature. The cell cycle was analyzed using a flow cytometer. In the case of the ROS scavenger co-treatment, NAC was added to the culture to a final concentration of 5 mM and 1 hour later AgNPs were added.

Analysis of glucose uptake

Candida albicans and *S. cerevisiae* were cultured as described in “Yeast strains and AgNPs treatment conditions” and diluted with fresh media containing half the amount of

dextrose of the original Sabouraud dextrose broth and YPD broth, respectively, to an OD₆₀₀ of 0.2. *Candida albicans* and *S. cerevisiae* were cultured for 1 hour at 37°C and at 30°C, respectively, in the presence or absence of 5 mM NAC. Then, 5 nm AgNPs and tritium-labeled 2-deoxyglucose (2-DG) (final 2.5 μ Ci/mL; Perkin Elmer, Boston, MA, USA) were added to the culture. After 30 minutes, the cells in 1 mL of culture were harvested by centrifugation and washed three times with cold PBS containing the glucose transporter inhibitor cytochalasin B (2.5 μ g/mL; Sigma-Aldrich) by centrifugation at 11,400 \times g, at 4°C for 5 minutes. The cells were then lysed in 400 μ L RIPA buffer containing 1% SDS and mixed with 8 mL of scintillation solution. The radioactive count was determined using a liquid scintillation analyzer (Perkin Elmer).

Analysis of ROS production

Candida albicans and *S. cerevisiae* were treated with AgNPs for 30 minutes as described in “Yeast strains and AgNPs treatment conditions”. When the ROS scavenger was included, NAC (final concentration of 5 mM) was added into the culture and 1 hour later AgNPs were added. As a positive control, the ROS inducer H₂O₂ was added to the culture of *C. albicans* and *S. cerevisiae* to a final concentration of 100 μ M and 580 μ M, respectively, and then incubated for 30 minutes. The cells were then harvested and washed in PBS twice by centrifugation at 11,400 \times g, at 4°C for 5 minutes. The cells were resuspended in PBS and labeled with cellular ROS-indicating fluoroprobe DCFDA (5 μ M) for 30 minutes or mitochondrial superoxide indicator MitoSOX Red (2 μ M) for 10 minutes at 37°C. The cells were washed three times, and the geometric mean fluorescence intensity (gMFI) of the cells was measured using flow cytometry.

Statistical analysis

The Kruskal–Wallis test with Dunn’s multiple comparison test was used for evaluating the differences among three or more groups. Two-way ANOVA with Bonferroni correction was used to analyze the difference between two groups. Differences were considered significant when the *P*-value was lower than 0.05.

Results

Treatment with 5 nm AgNPs inhibits cell proliferation of both *C. albicans* and *S. cerevisiae*

We first analyzed the effect of various concentrations of 5 nm and 100 nm AgNPs on the proliferation of *C. albicans* and

S. cerevisiae. Compared to the control (culture without AgNPs), *C. albicans* proliferation significantly decreased in the presence of 20 $\mu\text{g/mL}$ or 50 $\mu\text{g/mL}$ of 5 nm AgNPs; however, cell proliferation was not affected in the presence of 2 $\mu\text{g/mL}$ of 5 nm AgNPs or at any concentration of 100 nm AgNPs (Figure 1A) ($P < 0.005$). A spot assay using a 12-hour culture showed concordant results (Figure 1C). Similarly, *S. cerevisiae* proliferation was suppressed in the presence of 20 $\mu\text{g/mL}$ or 50 $\mu\text{g/mL}$ of 5 nm AgNPs (Figure 1B); however, it was not affected in the presence of 2 $\mu\text{g/mL}$ of 5 nm AgNPs or any concentration of 100 nm AgNPs ($P < 0.005$). A spot assay using a 20-hour culture showed concordant results (Figure 1D).

Treatment with 5 nm AgNPs enhances cell death in *C. albicans* but not in *S. cerevisiae*

We then examined cell death in AgNP-treated *C. albicans* and *S. cerevisiae* cultures (Figure 2). The cell death

frequency in *C. albicans* in the presence of 50 $\mu\text{g/mL}$ of 5 nm AgNPs was significantly increased at 2, 4, 6, and 24 hours compared to that of the control (Figure 2A) ($P < 0.05$). The cell death frequency in *C. albicans* treated with 20 $\mu\text{g/mL}$ of 5 nm AgNPs was also increased compared to that of the control but statistical significance was observed only at 6 and 24 hours ($P < 0.05$). In the case of the cell death frequency in *C. albicans* treated with 2 $\mu\text{g/mL}$ of 5 nm AgNPs, a statistically significant increase was found only at 6 hours compared to that of the control ($P < 0.05$). The cell death frequency in *C. albicans* in the presence of 20 and 50 $\mu\text{g/mL}$ of 5 nm AgNPs increased with time, whereas in the presence of 2 $\mu\text{g/mL}$ of AgNPs it decreased at 24 hours, probably owing to the proliferation of live *C. albicans*. The treatment of *C. albicans* with 100 nm AgNPs did not change cell death frequency compared to that of the control (Figure 2A). In contrast to *C. albicans*, there was no significant difference in the cell death frequency in *S. cerevisiae* cultured in the presence of 5 nm and 100 nm AgNPs for 2–24 hours, compared to that

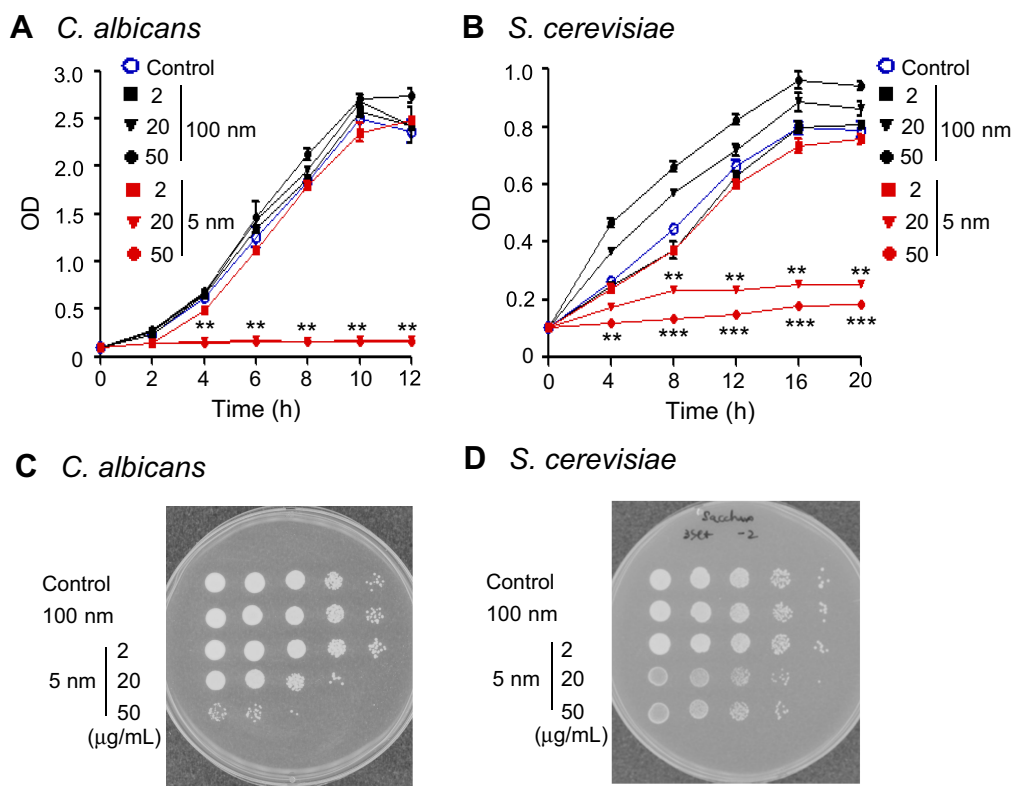


Figure 1 Growth inhibition of *Candida albicans* and *Saccharomyces cerevisiae* after treatment with 5 nm AgNPs. (A) Growth kinetics and (C) spot assay of *C. albicans* in either the absence or presence of 5 nm or 100 nm AgNPs (2, 20, and 50 $\mu\text{g/mL}$). The *C. albicans* culture was diluted with fresh Sabouraud dextrose broth and cultured for 12 hours at 37°C. Spot assay was performed by inoculation of 10-fold serial dilutions of 12-hour culture on the Sabouraud dextrose agar plates. (B) Growth kinetics and (D) spot assay of *S. cerevisiae* in either the absence or presence of 5 nm or 100 nm AgNPs. The *S. cerevisiae* culture was diluted with fresh YPD broth and cultured for 20 hours at 30°C. Spot assay was performed by inoculation of 10-fold serial dilutions of 20-hour culture on the YPD agar plates. Cell growth was measured at 600 nm. Control consists of yeast cells not treated with AgNPs. Data are presented as mean \pm SD from two independent experiments performed in triplicate. ** $P < 0.005$, *** $P < 0.001$ vs control and vs cells treated with 100 nm AgNPs.

Abbreviations: AgNPs, silver nanoparticles; BrPA, 3-bromopyruvate; YPD, 1% yeast extract, 2% peptone, and 2% dextrose.

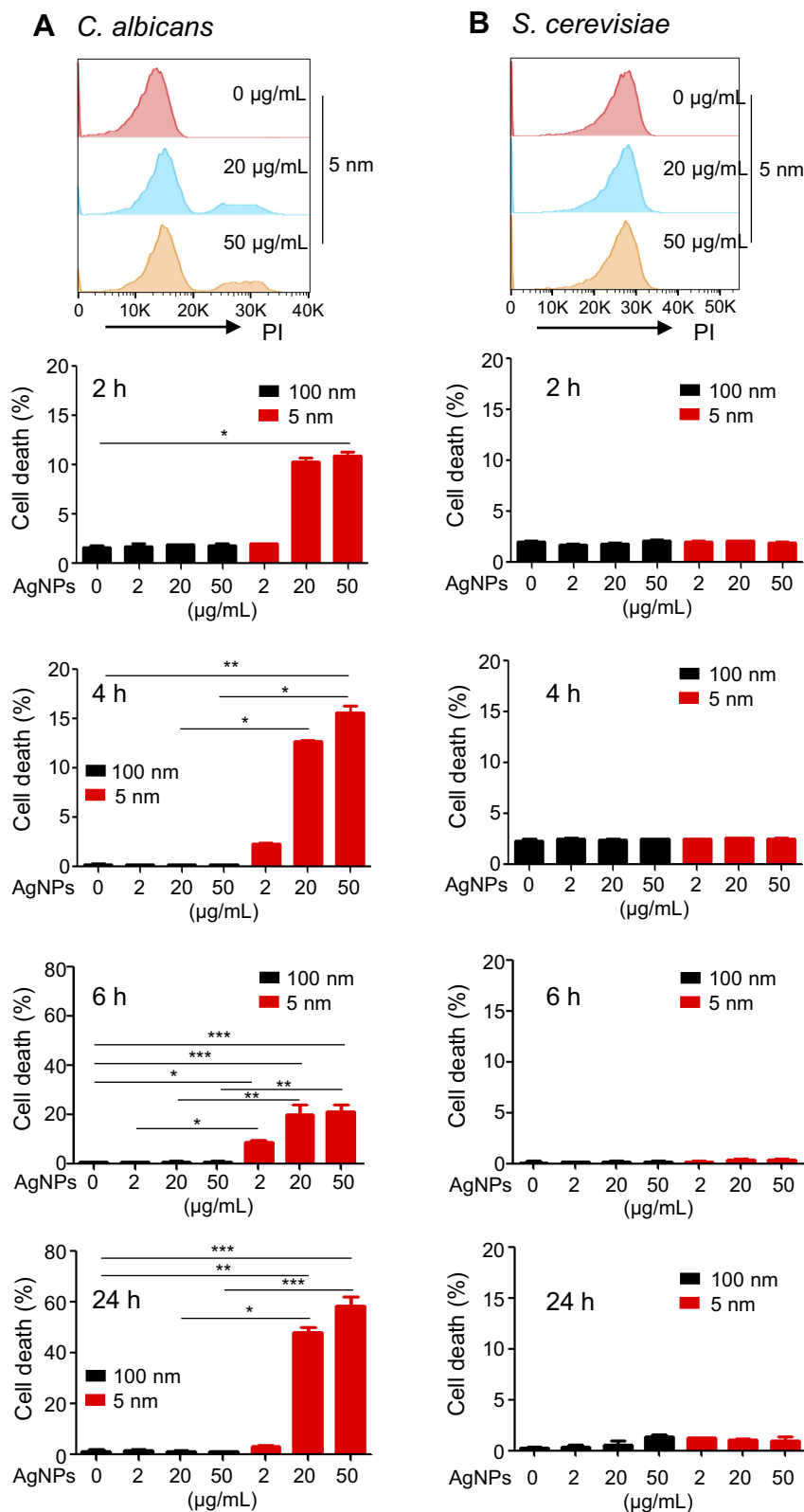


Figure 2 Enhanced cell death in *Candida albicans* but not in *Saccharomyces cerevisiae* in the presence of 5 nm AgNPs. Representative histograms of 6-hour culture and cell death frequency of (A) *C. albicans* and (B) *S. cerevisiae* in either the presence or absence of 5 nm or 100 nm AgNPs at 2, 4, 6, and 24 hours of culture. Data are presented as mean \pm SD from two independent experiments performed in triplicate. * P <0.05, ** P <0.005, *** P <0.001.

Abbreviations: AgNPs, silver nanoparticles; PI, propidium iodide.

of the control (Figure 2B). These results showed a differential effect of 5 nm AgNPs on cell death in *C. albicans* and *S. cerevisiae*, indicating that cell death could not account for the AgNP-mediated growth suppression in *S. cerevisiae*.

Treatment with 5 nm AgNPs delays cell cycle progression of both *C. albicans* and *S. cerevisiae* at G1 phase

We next evaluated the effect of AgNPs on the cell cycle progression. In this experiment, we included a positive control consisting of cells treated with nocodazole, which had an increased G2 phase cell frequency of *C. albicans* at 2 hours compared to the untreated control culture (Figure 3A). When *C. albicans* was exposed to 5 nm AgNPs, the cell cycle was arrested in G1 phase in a dose-dependent manner (Figure 3B and D). The G1 phase cell frequency of control culture of *C. albicans* (in the absence of AgNPs) was over 80% at the beginning of culture, which then decreased to approximately 60% and 40% at 0.5 and 1 hour, respectively. The time-dependent alteration in G1 phase cell frequency of the culture in the presence of 100 nm AgNPs was similar to that of control culture (Figure 3D). However, the G1 phase cell frequencies, in culture treated with 2 µg/mL of 5 nm AgNPs, were transiently but significantly increased after 30 minutes compared to control culture (Figure 3D) ($P < 0.05$). Further, the G1 phase cell frequencies in the presence of both 20 and 50 µg/mL of 5 nm AgNPs were significantly higher from 1 to 4 hour of culture compared to control, even though there was an exception that no statistical significance was observed at 2 hours after 20 µg/mL AgNP treatment (Figure 3D) ($P < 0.05$). Similarly, the G1 phase cell frequencies for *S. cerevisiae* were significantly higher in the presence of both 20 and 50 µg/mL of 5 nm AgNPs at 0.5, 1, 2, and 4 hours compared to control culture but were not different in the presence of 100 nm AgNPs (Figure 3C and E) ($P < 0.05$). These results demonstrate that treatment with 5 nm AgNPs may lead to G1 phase delay of *C. albicans* and *S. cerevisiae*.

Treatment with 5 nm AgNPs decreases glucose uptake in *C. albicans* and *S. cerevisiae*

We then examined whether 5 nm AgNPs alter the glucose uptake of *C. albicans* and *S. cerevisiae* (Figure 4). As we had earlier observed a 5 nm AgNP-mediated cell cycle delay of 30 minutes, we assessed glucose uptake for 30

minutes after AgNP treatment. Although the G1 cell cycle delay observed 30 minutes after treatment was similar for cultures treated with 2, 20, and 50 µg/mL 5 nm AgNPs, glucose uptake was significantly reduced only in the presence of 50 µg/mL 5 nm AgNPs compared to the control ($P < 0.05$). Although we could not determine whether the alteration in glucose uptake was the cause or the consequence of AgNP-induced G1 cell cycle delay, these results showed similar effects of 5 nm AgNPs on the glucose uptake in *C. albicans* and *S. cerevisiae*.

Treatment with 5 nm AgNPs increases ROS production in *C. albicans* but not in *S. cerevisiae*

We then investigated the ROS production in *C. albicans* and *S. cerevisiae* in the presence of 5 nm AgNPs. We first assessed intracellular ROS levels using DCFDA labeling (Figure 5). Compared to control, gMFI, representing ROS levels, was significantly increased in *C. albicans* in the presence of 2, 20, and 50 µg/mL AgNPs at 30 minutes (Figure 5A and C) ($P < 0.05$) and was downregulated by the co-presence of AgNPs and an ROS scavenger, NAC (Figure 5E) ($P < 0.05$). However, gMFI was not increased in *S. cerevisiae* in the presence of 5 nm AgNPs (Figure 5B and D). Moreover, gMFI was similar for the untreated and NAC-treated *S. cerevisiae* (Figure 5F). We next examined mitochondrial ROS production by MitoSOX Red labeling. Increased gMFI was observed in *C. albicans* cultured for 30 minutes in the presence of 20 and 50 µg/mL AgNPs (Figure 6A and C) ($P < 0.05$), whereas gMFI was decreased in the co-presence of AgNPs and NAC (Figure 6E) ($P < 0.005$). In contrast, gMFI in *S. cerevisiae* was not increased in the presence of AgNPs (Figure 6B and D), and it was decreased when NAC was also present at 2 or 20 µg/mL of AgNPs (Figure 6F). These results suggest that treatment with 5 nm AgNPs induced ROS production in *C. albicans* but not in *S. cerevisiae*, at least during the initial 30 minutes of culture time.

Inhibitory effect of 5 nm AgNPs on cell growth is similar between *S. cerevisiae* wild type and *S. cerevisiae* mutants harboring deletion of oxidative or cell wall stress response genes

To further elucidate whether ROS are involved in the suppressive effect of 5 nm AgNPs on *S. cerevisiae* growth, we

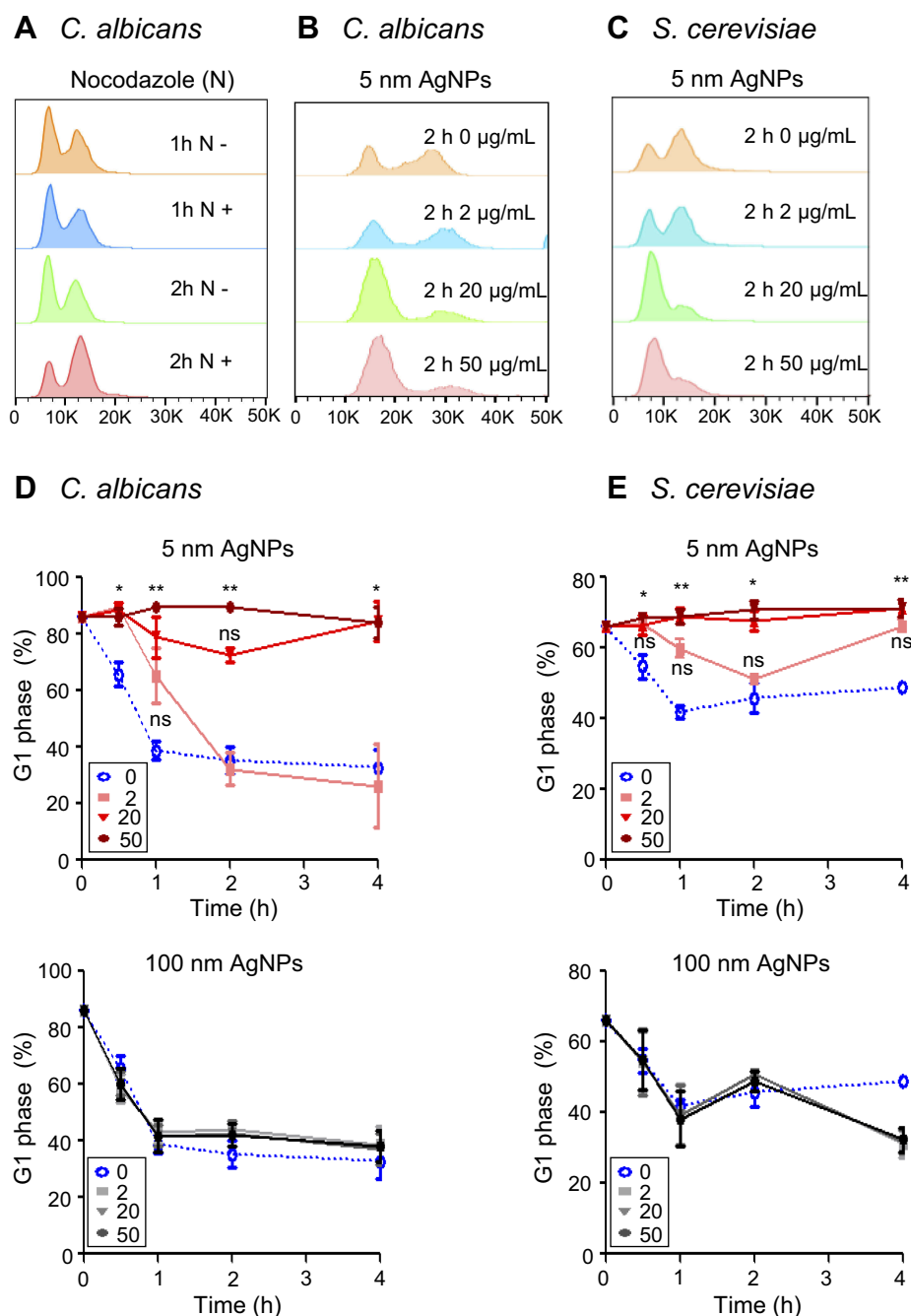


Figure 3 G1 phase delay in *Candida albicans* and *Saccharomyces cerevisiae* in the presence of 5 nm AgNPs. (A) Representative cell cycle analysis histogram of *C. albicans* either treated (N +) or not treated (N -) with 15 µg/mL nocodazole at the indicated time-points. Representative histograms of (B) *C. albicans* and (C) *S. cerevisiae* treated with 0, 2, 20, and 50 µg/mL 5 nm AgNPs for 2 hours and then analyzed by propidium iodide labeling. The left peak represents G1 phase and a single copy of DNA per cell, and the right peak represents G2 phase and two copies of DNA. G1 phase cell proportion of (D) *C. albicans* and (E) *S. cerevisiae* in the presence of 5 nm or 100 nm AgNPs at the indicated time-points. Data are presented as mean ± SD from two independent experiments performed in triplicate. * $P < 0.05$, ** $P < 0.005$.

Abbreviations: AgNPs, silver nanoparticles; ns, not significant.

investigated the proliferation of *S. cerevisiae* wild-type BY4741 and deletion mutants with mutations in one or two genes involved in the oxidative or cell wall damage response in the presence of 5 nm AgNPs. If ROS production were important for the AgNP effect, the AgNP-mediated growth inhibition would have been greater in the mutants

than in the wild-type strain. Compared to the control (without AgNPs), the growth of the deletion mutants *SKN7*, *YAP1*, *HOG1*, and *SLT2*, as well as wild-type strain BY4741, was significantly suppressed in the presence of 10, 20, and 50 µg/mL of 5 nm AgNPs, but not in the presence of 2 µg/mL of 5 nm or 2 to 50 µg/mL of 100 nm

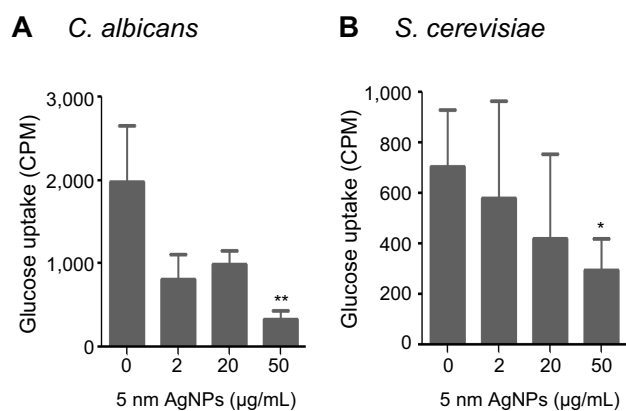


Figure 4 Decrease in glucose uptake of *Candida albicans* and *Saccharomyces cerevisiae* in the presence of 5 nm AgNPs. Glucose uptake of (A) *C. albicans* and (B) *S. cerevisiae* treated with 5 nm AgNPs for 30 minutes. Data are presented as mean \pm SD from two independent experiments performed in triplicate. * $P < 0.05$, ** $P < 0.005$.

Abbreviation: AgNPs, silver nanoparticles.

AgNPs (Figure 7A) ($P < 0.005$). The growth kinetics as well as the spot assay results showed that the inhibitory effects of 5 nm AgNPs on the deletion mutants *SKN7*, *YAP1*, *HOG1*, and *SLT2* were similar to the effects on the wild-type strain BY4741 (Figure 7A and B). These results suggest that the expression of *SKN7*, *YAP1*, *HOG1*, or *SLT2* genes, as well as ROS production, may not be pivotal for the 5 nm AgNP-mediated growth suppression in *S. cerevisiae*.

Treatment with ROS scavenger reduces G1 phase cell frequency in *C. albicans* exposed to 5 nm AgNPs but not in *S. cerevisiae*

As there was a significant increase in ROS production in 5 nm AgNP-treated *C. albicans* but not in *S. cerevisiae*, we speculated that an ROS scavenger could restore cell cycle progression in *C. albicans* but not in *S. cerevisiae*. Thus, we examined the cell cycles of *C. albicans* and *S. cerevisiae* pretreated with 5 mM NAC, and then treated with 5 nm AgNPs (Figure 8). As expected, G1 phase cell frequency, which was increased in the presence of 5 nm AgNPs alone, was significantly reduced after treatment with NAC in *C. albicans* (Figure 8A) ($P < 0.005$). However, NAC treatment did not decrease G1 phase cell frequency in *S. cerevisiae* treated with 5 nm AgNPs (Figure 8B). These results support our presumption that 5 nm AgNP-induced ROS production leads to G1 phase delay in *C. albicans* and that ROS production is not involved in AgNP-mediated G1 phase delay in *S. cerevisiae*.

Treatment with ROS scavenger restores glucose uptake in 5 nm AgNP-treated *C. albicans*

We next investigated whether an ROS scavenger could restore glucose uptake in AgNP-treated *C. albicans* and *S. cerevisiae*. We assessed the glucose uptake of *C. albicans* and *S. cerevisiae* pretreated with 5 mM NAC for 1 hour, and then treated with 5 nm AgNPs for 30 minutes. Glucose uptake was significantly increased in *C. albicans* in the co-presence of NAC and AgNPs compared to that in the presence of AgNPs alone (Figure 9A) ($P < 0.005$). However, glucose uptake in *S. cerevisiae* co-treated with NAC and AgNPs was not significantly increased compared to that in *S. cerevisiae* treated with AgNPs alone (Figure 9B). These results indicate that AgNP-induced ROS production may be responsible for the reduction of glucose uptake in *C. albicans* but not in *S. cerevisiae*.

Treatment with 5 nm AgNPs and a glycolysis inhibitor, bromopyruvate, synergistically enhances cell death in *C. albicans* but not in *S. cerevisiae*

As treatment with 5 nm AgNPs reduced glucose uptake in both *C. albicans* and *S. cerevisiae*, we investigated whether inhibition of glucose consumption enhanced 5 nm AgNP-mediated cell death in *C. albicans* and *S. cerevisiae*. Treatment with the glycolysis inhibitor BrPA alone did not enhance cell death in *C. albicans* or *S. cerevisiae*, compared to that of the untreated control (Figure 10A and B). As expected, 200 μ M BrPA treatment with 2 or 20 μ g/mL of 5 nm AgNPs significantly increased cell death in *C. albicans* at 6 and 24 hours of culture time (Figure 10A) ($P < 0.05$). In *C. albicans* treated with 80 μ M BrPA, cell death frequency was significantly elevated in the presence of 20 μ g/mL of 5 nm AgNPs but not in the presence of 2 μ g/mL (Figure 10A) ($P < 0.05$), indicating that BrPA treatment upregulated AgNP-induced cell death in *C. albicans* in a dose-dependent manner. However, the synergistic cytotoxic effect of BrPA and 5 nm AgNPs was not observed in *S. cerevisiae* (Figure 10B). Taken together, these results show that 5 nm AgNPs differentially regulated the growth and survival of *C. albicans* and *S. cerevisiae*.

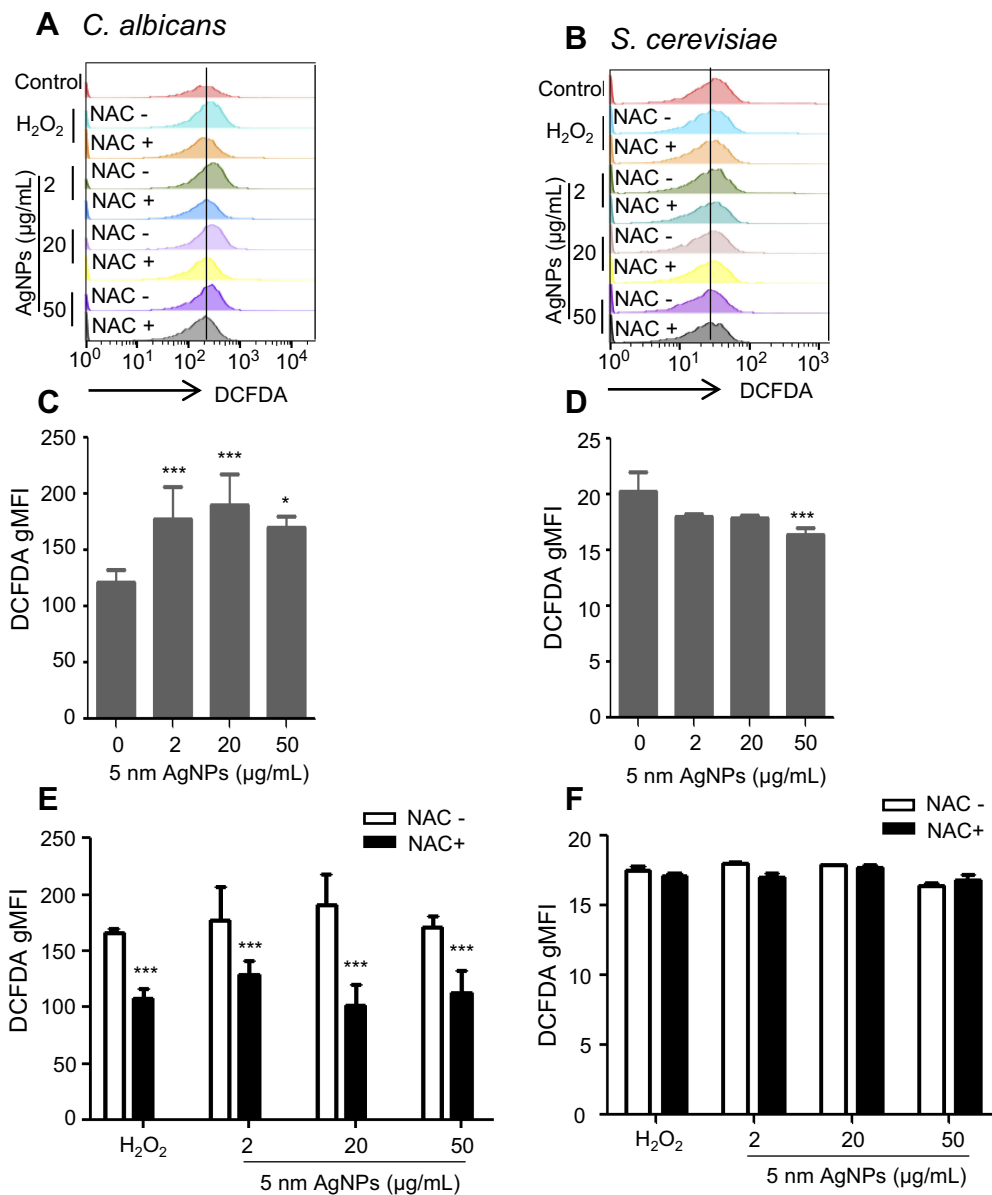


Figure 5 Increased cellular ROS production in *Candida albicans* in the presence of 5 nm AgNPs. *Candida albicans* (A, C, E) and *Saccharomyces cerevisiae* (B, D, F) were cultured in the presence of 5 nm AgNPs for 30 minutes. Then, ROS levels were assessed using DCFDA labeling. NAC was added to the culture 1 hour prior to the addition of AgNPs or H₂O₂ (100 μM for *C. albicans* and 580 μM for *S. cerevisiae*). Control consists of yeast cells not treated with AgNPs. Data are presented as mean ± SD from two independent experiments performed in triplicate. **P*<0.05, ****P*<0.001.

Abbreviations: AgNPs, silver nanoparticles; DCFDA, 2,2'-dichlorofluorescein diacetate; gMFI, geometric mean fluorescence intensity; NAC, N-acetylcysteine; ROS, reactive oxygen species.

Discussion

Previously, the growth suppression effect of AgNPs has been consistently observed across diverse fungal species, but the underlying mechanisms of action have varied. In this study, we showed common and differential effects of 5 nm AgNPs in *C. albicans* and *S. cerevisiae*; growth suppression, cell cycle delay, and glucose uptake reduction were commonly observed in both fungi, whereas the increase in ROS production and

cell death, as well as the synergic effect of 5 nm AgNPs with BrPA, were observed only in *C. albicans*. Our results also indicate that an ROS scavenger restored the cell cycle progression and glucose uptake in *C. albicans* treated with 5 nm AgNPs but not in *S. cerevisiae*, suggesting different antifungal mechanisms of 5 nm AgNPs in the two species. Considering this possibility, three main points raised from our results are discussed.

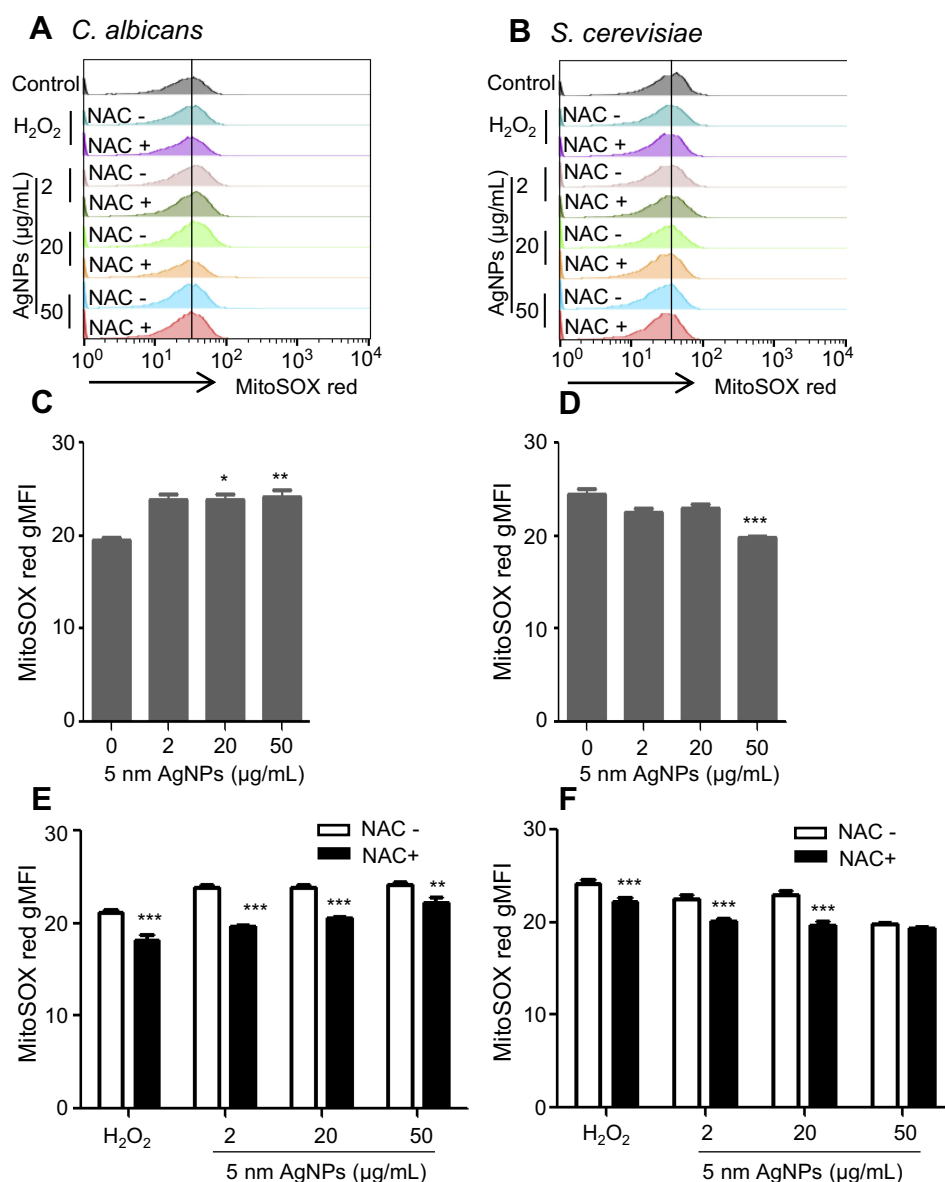


Figure 6 Increased mitochondrial ROS production in *Candida albicans* in the presence of 5 nm AgNPs. *Candida albicans* (A, C, E) and *Saccharomyces cerevisiae* (B, D, F) were cultured in the presence of 5 nm AgNPs for 30 minutes. Then, ROS levels were assessed using MitoSOX Red labeling. NAC was added to the culture 1 hour prior to the addition of AgNPs or H₂O₂ (100 µM for *C. albicans* and 580 µM for *S. cerevisiae*). Control consists of yeast cells not treated with AgNPs. Data are presented as mean ± SD from two independent experiments performed in triplicate. *P<0.05, **P<0.005, ***P<0.001.

Abbreviations: AgNPs, silver nanoparticles; gMFI, geometric mean fluorescence intensity; NAC, N-acetylcysteine; ROS, reactive oxygen species.

The first discussion point is that 5 nm but not 100 nm AgNPs inhibit the growth and cell cycle progression in both *C. albicans* and *S. cerevisiae*. These results are concordant with previous reports in that the size of AgNPs influences biological effects such as cytotoxicity.²³ In human cells, smaller AgNPs exhibit greater toxicity.²³ In addition, small-size AgNPs in the range of 1–10 nm in diameter have the advantage of binding to bacteria and viruses.^{24,25} We could not find a report that directly compared the impact of AgNP size on the antifungal activity. However, the growth-

inhibition effect of AgNPs with sizes ranging from 3 nm to 85 nm has been clearly demonstrated in various fungi, including *C. albicans* and *S. cerevisiae*.^{14,16,26–28}

Our findings that 5 nm AgNPs induced cell cycle arrest in both *C. albicans* and *S. cerevisiae* corroborate the previous observation that AgNPs lead to cell cycle arrest in human cancer cells as well as fungi.^{12–14,29} However, the phase at which cells are arrested by treatment with AgNPs is different depending on the study. In line with our results, G1 phase arrest was demonstrated in A549 cells after

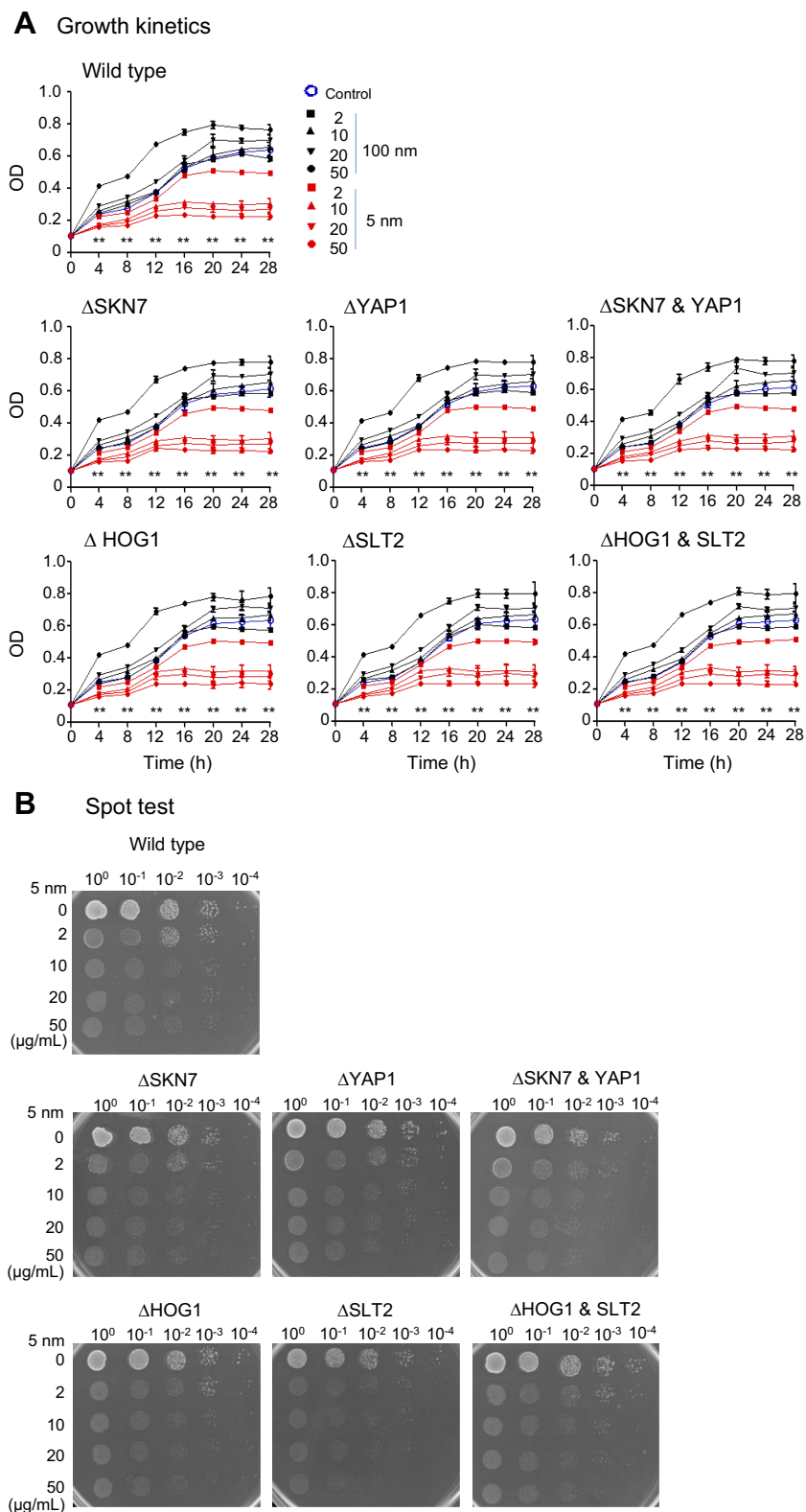


Figure 7 Growth inhibition in *Saccharomyces cerevisiae* deletional mutants treated with 5 nm AgNPs. **(A)** Growth kinetics and **(B)** spot assay results of *S. cerevisiae* wild-type BY4741 and deletional mutants of the indicated genes in the presence or absence of AgNPs. Cell growth was analyzed by measuring OD_{600} . Spot assay was performed by inoculation of serial dilutions of 28-hour culture on the YPD agar plates. Control consists of yeast cells not treated with AgNPs. Data are presented as mean \pm SD from two independent experiments performed in triplicate. ** $P < 0.005$.

Abbreviations: AgNPs, silver nanoparticles; YPD, 1% yeast extract, 2% peptone, and 2% dextrose.

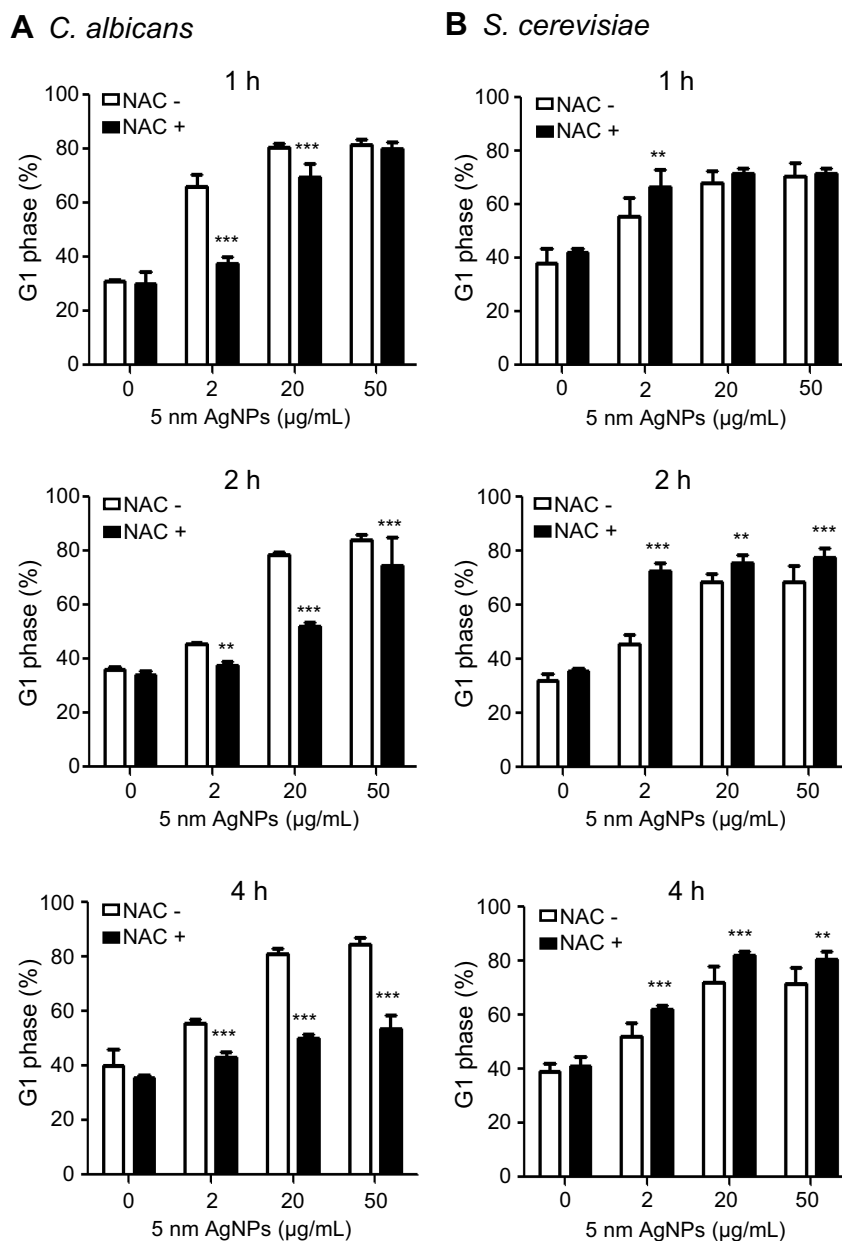


Figure 8 An ROS scavenger restored AgNP-induced G1 phase delay in *Candida albicans* but not in *Saccharomyces cerevisiae*. G1 phase cell percentage was analyzed by flow cytometry in (A) *C. albicans* and (B) *S. cerevisiae* cultured in either the presence or absence of 5 nm AgNPs at the indicated time-points. NAC was added to the culture 1 hour prior to addition of AgNPs. Data are presented as mean \pm SD from two independent experiments performed in triplicate. ** $P < 0.005$, *** $P < 0.001$.

Abbreviations: AgNPs, silver nanoparticles; NAC, N-acetylcysteine; ROS, reactiveoxygen species.

treatment with AgNPs.³⁰ However, the same A549 cells were arrested at the G2/M phase by treatment with AgNPs in a different study.³¹ In contrast to our results, Kim et al reported that 3 nm AgNPs increased the G2/M phase cell frequency in *C. albicans*.¹² We do not know what determines the phase in cells treated with AgNPs; however, there are several differences between the experimental conditions in our study and those of Kim et al: PVP-coated AgNPs vs uncoated AgNPs, culture at 37°C vs at

28°C, and cell cycle analysis time-points at 4 hours vs at 8 hours, respectively.¹² To support our findings, we demonstrated that nocodazole treatment increased G2/M phase cell frequency in *C. albicans* and *S. cerevisiae* (Figure 3A, and data not shown). Furthermore, we could restore AgNP-induced G1 phase delay using an ROS scavenger, NAC, and these results are in line with a report by Correia et al showing that the ROS-inducing molecule H₂O₂ led to a G1 phase delay in *C. albicans*.³²

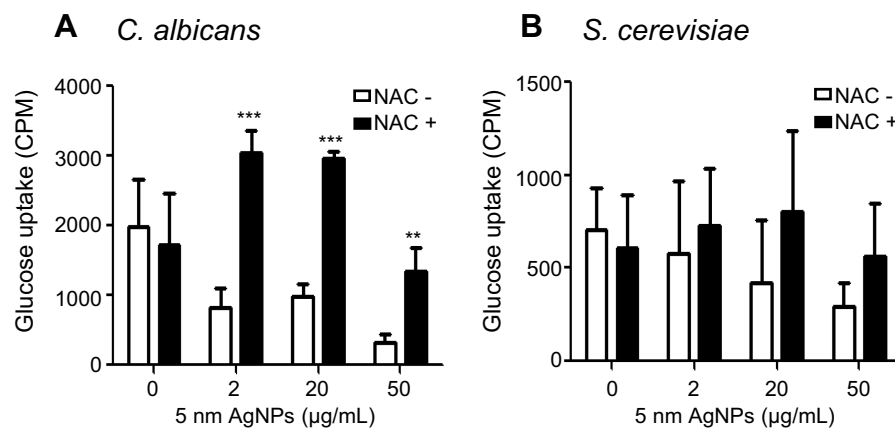


Figure 9 An ROS scavenger restored AgNP-mediated glucose uptake reduction in *Candida albicans*. Glucose uptake of (A) *C. albicans* and (B) *Saccharomyces cerevisiae* after culturing for 30 minutes in either the presence or absence of AgNPs. NAC was added to the culture 1 hour prior to addition of AgNPs. Data are presented as mean \pm SD from two independent experiments performed in triplicate. ** $P < 0.005$, *** $P < 0.001$.

Abbreviations: AgNPs, silver nanoparticles; NAC, N-acetylcysteine; ROS, reactive oxygen species.

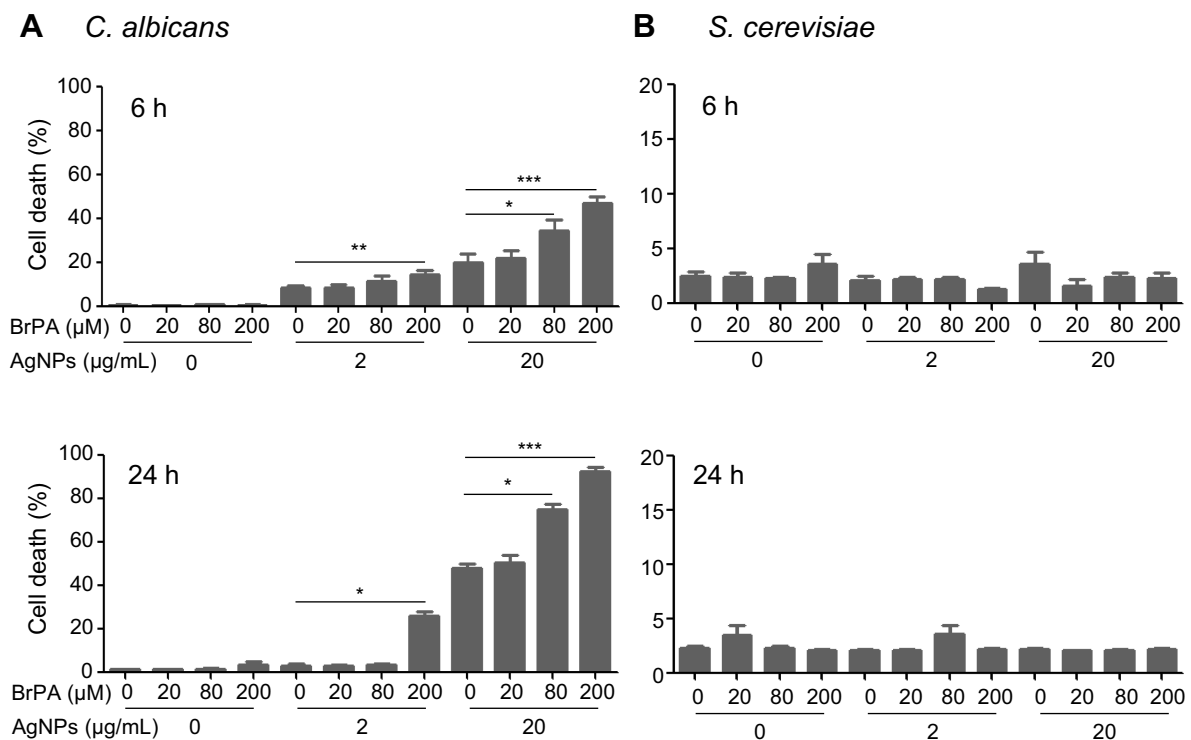


Figure 10 Synergistic cytotoxicity of AgNPs with BrPA in *Candida albicans*. Cell death in (A) *C. albicans* and (B) *Saccharomyces cerevisiae* in the presence of BrPA, 5 nm AgNPs, or a combination of both was analyzed using propidium iodide labeling at 6 and 24 hours. Data are presented as mean \pm SD from two independent experiments performed in triplicate. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$.

Abbreviations: AgNPs, silver nanoparticles; BrPA, 3-bromopyruvate.

The second discussion point is that different mechanisms of action of AgNPs may work in *C. albicans* and *S. cerevisiae* BY4741. We revealed that ROS may play a role in AgNP-mediated growth suppression of *C. albicans* but not of *S. cerevisiae*. This is supported by three findings. First, there is increased ROS production following treatment with 5 nm AgNPs in *C. albicans* but

not in *S. cerevisiae* (Figures 5 and 6). Second, cell cycle progression and glucose uptake are restored by NAC co-treatment with 5 nm AgNPs in *C. albicans* but not in *S. cerevisiae* (Figures 8 and 9). Third, there is no increased sensitivity to 5 nm AgNPs in *S. cerevisiae* mutants with deletions in genes associated with ROS stress response, such as *HOG1*, *SKN7*, *YAP1*, and *SLT2*, compared with

wild-type *S. cerevisiae* BY4741 (Figure 7). Concordantly, Hwang et al reported that treatment with 3 nm AgNPs increased hydroxyl radical production, leading to reduced mitochondrial membrane potential and to cell death in *C. albicans*.¹⁴ Radhakrishnan et al speculated that both ROS-dependent and ROS-independent pathways are involved in the induction of cell death in *C. albicans* when exposed to 10–30 nm citrate-reduced AgNPs.^{15,33} In addition, in fission yeast, the toxic effects of AgNPs are attributed to the generation of ROS through the release of Ag ions inside the cells internalizing AgNPs.³⁴ Consistent with our results, no increase in ROS production in *S. cerevisiae* BY4741 treated with 4–100 µg/mL PVP-coated AgNPs,¹⁵ and a similar effect of AgNPs on the growth of wild-type and single-gene deletion mutants of *S. cerevisiae* defective in oxidative stress defense (*yap1Δ*, *sod1Δ*, or *sod2Δ*) have been reported.¹⁵ However, Sillapawattana et al observed increased ROS production in *S. cerevisiae* BY4742 and more prominent growth suppression in *ctl1*, *sod2*, or *gsh2* deletion mutants compared to wild type when treated with AgNPs.³⁵ Of note, the discordance of our results with those of Sillapawattana et al may be related to the difference in *S. cerevisiae* strains and culture conditions. We used *S. cerevisiae* BY4741, which is characterized by the MATa and *met15Δ* phenotype, and cultured in YPD media containing 2% glucose at 30°C, while Sillapawattana et al used *S. cerevisiae* BY4742, which is characterized by the MATa and *lys2Δ* phenotype, and cultured in complete synthetic dropout medium containing 4% glucose at 28°C.^{35,36} More studies are required to further elucidate the apparently inconsistent results obtained from *S. cerevisiae*. Still, our results suggest ROS-dependent and ROS-independent antifungal mechanisms of AgNPs, according to the species and strain of fungi.

The third discussion point is the synergistic effect of a glycolysis inhibitor, BrPA, on AgNP-induced cell death in *C. albicans*. To the best of our knowledge, the combinatorial effect of BrPA and AgNPs against fungi has not been reported previously. Although BrPA was reported as a frequently suppressive drug in the antifungal drug pairs against *S. cerevisiae*, in that study AgNPs were not included.³⁷ Compared to previous reports evaluating the effect of BrPA alone on various fungi, we used a relatively low concentration of BrPA.^{38,39} The minimal inhibitory concentrations (MICs) for the clinically isolated *C. albicans* were reported to be within 2.4–3.6 mM and the MIC for *S. cerevisiae* was 1.8 mM.^{38,39} We used much lower amounts of BrPA

(20, 80, and 200 µM), as we wanted to examine the therapeutic potential of BrPA against *Candida* infection at the concentration that minimized human cell death. Our previous study found slightly decreased cell survival of peripheral blood mononuclear cells at 80 µM of BrPA.⁴⁰ In this study, we demonstrated that both 80 and 200 µM of BrPA synergistically enhanced cell death in *C. albicans* (Figure 10), thus revealing the potential of the combination of AgNPs and BrPA as an antifungal agent against *C. albicans*.

The synergic effect of BrPA with AgNPs may be linked with the increased ROS production and decreased glucose uptake in *C. albicans* by treatment with AgNPs. Previously, the main toxic effect of BrPA was suggested to be intracellular ATP depletion, and BrPA efficacy was inversely correlated with intracellular levels of reduced glutathione.^{39,41} Glucose is required for the production of reduced glutathione through the pentose phosphate pathway, and increased ROS production leads to the consumption of reduced glutathione.^{42,43} It is conceivable that the level of glutathione is reduced in AgNP-treated *C. albicans* as a result of increased ROS production, and thus, a synergic effect of BrPA and AgNPs was observed in *C. albicans*. However, ROS production was not increased after treatment of *S. cerevisiae* with AgNPs, and, thus, no synergic effect of BrPA and the AgNPs was observed in this yeast.

Conclusion

Our results demonstrate that AgNPs exert similar growth-inhibition effects in *C. albicans* and *S. cerevisiae*, even though the underlying mechanisms may be different. In addition, we suggest that the antifungal activity of AgNPs may be enhanced by combination with an inhibitor of glucose metabolism.

Abbreviation list

AgNPs, silver nanoparticles; BrPA, 3-bromopyruvate; DCFDA, dichlorofluorescein diacetate; 2-DG, deoxyglucose; gMFI, geometric mean fluorescence intensity; MAPK, mitogen-activated protein kinase; NAC, N-acetylcysteine; PBS, phosphate-buffered saline; PVP, polyvinylpyrrolidone; ROS, reactive oxygen species; YPD, 1% yeast extract, 2% peptone, and 2% dextrose.

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Disclosure

The authors report no conflicts of interest in this work.

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