Silver Nanoparticles Offer a Synergistic Effect with Fluconazole Against Fluconazole-Resistant *Candida Albicans* by Abrogating Drug Efflux Pumps and Increasing Endogenous ROS

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Research

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Abstract

Objectives: A frequent emergence of drug resistance has been observed and posed great threat to global public health recently. This work aimed to investigate the potential synergistic effect and the underlying mechanisms of AgNPs-fluconazole combination more extensively through 2 clinically isolated fluconazole-resistant *Candida albicans* (*C. albicans*) strains.

Methods: Antifungal properties of AgNPs and fluconazole alone or together against planktonic cells and biofilms were tested. Cellular and molecular targets associated with fluconazole resistance were monitored after AgNPs treatment. Antifungal potential of AgNPs-fluconazole combination was also explored in vivo using a mouse model of disseminated candidiasis. Tissue burden and survival rate were analyzed.

Results: The results indicated that AgNPs worked synergistically with fluconazole against both planktonic cells of fluconazole-resistant *C. albicans* and biofilms formed < 12 hours. AgNPs treatment down-regulated ERG1, ERG11, ERG25, and CDR2, decreased membrane ergosterol levels and membrane fluidity, reduced membrane content of Cdr1p, Cdr2p, and thus efflux pump activity. The elevated ROS production was also a likely cause of the synergistic effect. In vivo, AgNPs and fluconazole combination significantly decreased the fungal burden and improved the survival rate of infected mice.

Conclusion: In conclusion, these results further confirm that AgNPs-fluconazole combination is a hopeful strategy for the treatment of fluconazole-resistant fungal infections.

1. Introduction

The world-wide emergence of antifungal resistance among fungus (resistance towards conventional used antimicrobials) has posed great threat to global public health [1, 2]. With the growing incidence of immune-compromised diseases, as well as excessive use of broad spectrum antibiotics, infections caused by fungus, especially resistant fungus have caused great clinical problem, calling for improved therapies [3, 4]. Despite the great advance in the development of antifungal drugs, infections by *Candida* species including *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* still cause great morbidity and mortality [5]. Among these species, *C. albicans* constitutes the most problematic and prevalent one accounting for nearly 50% of candidiasis. This is mainly due to their striking ability in biofilm-forming and switching between different forms to adapt to host microenvironment [6, 7]. Currently, fluconazole is the most widely used antifungal drug for the treatment of fungal infections because of its low cost, low toxicity and high efficacy. However, extensive usage inevitably leads to resistance [8]. Indeed, various multi-drug resistant, especially fluconazole resistant *C. albicans* have been reported [9]. So, it is exigent to search for newer and more effective therapeutics [10]. In the last decade, much progress has been made in understanding the reasons responsible for antifungal resistance in *C. albicans*. Two major mechanisms have been identified (i) mutated ergosterol biosynthesis pathway (ii)

up-regulated efflux of antifungals [11–13]. Thus, design or discovery of new antifungal drugs that target these multiple cellular targets may offer us a chance to break drug resistance.

Metal nanoparticles, e.g. zinc oxide (ZnO), titanium oxide (TiO₂), silver, zinc, gold, have intrigued great interest in recent years, especially with the development of “green synthesis” method which makes it even more popular [14, 15]. A variety of nanoparticles with unique physiochemical attributes have been marketed for broad applications, including drug delivery, bio-detection and antimicrobial agents [16, 17]. Among these, silver nanoparticles (AgNPs), known for their high microbial toxicity and availability, represent the most prominent one [18–20]. AgNPs firstly demonstrated its antimicrobial effect against various bacterial, such as *E. coli*, *E. faecalis* [21, 22]. Recently, AgNPs was showing its antifungal value alone or in combinatory use [23, 24]. The Ag/AgCl nanoparticles synthesized from *Malva Sylvestris* leaf extracts exhibited antifungal, antibacterial, antibiofilm potential [25]. Silver nanoparticles conjugated with nystatin (NYT) or fluconazole (FLU) showed increased efficacy against *C. albicans* as compared to drug alone [26]. The synergistic effect between AgNPs and ketoconazole was reported, in which AgNPs greatly added to the fungicidal action of ketoconazole [27]. Silver nanoparticles also synthesized with *Lycopersicon esculentum* extracts against *Candida* species [28]. Previous studies have demonstrated the antifungal effect of AgNPs and fluconazole combination against the wild type or drug-resistant *C. albicans* strains [29, 30]. In these studies, AgNPs was showed to reduce the MICs of fluconazole significantly. Although AgNPs alone exhibited no inhibitory effect on biofilm cells, the combination with fluconazole caused a significant dose-dependent inhibition on biofilm cell, while no cytotoxic effect was observed on mammal cells [30]. The synergistic effect was further linked to the disruption of cell membrane integrity and budding process caused by AgNPs. Moreover, defects in biosynthetic pathway and efflux pump functions were also detected, as the expressions of some related genes *ERG5*, *ERG1*, *ERG25*, *ERG11*, *MDR1*, and *CDR2* genes were disregulated and membrane ergosterol level was reduced [29], but the underlying mechanisms were not much elucidated. In wild-type *C. albicans* strain SC5314, AgNPs exerted antifungal activity by altering membrane microenvironment, predominantly ergosterol content and fatty acid composition [31]. AgNPs could also induce intracellular ROS production [31]. These factors are essential for drug resistance in the fungal cells [32, 33] Hence, in this study, we aimed to study the antifungal activity of AgNPs-fluconazole combination more extensively, both *in vitro* and *in vivo* and tried to investigate if AgNPs treatment could sensitize resistant *C. albicans* to fluconazole by targeting these cellular molecules and events related to drug resistance. Our study opens up a new therapeutic alternative and further confirms the therapeutic value of AgNPs-fluconazole combination against resistant fungus.

2. Results

2.1. Antifungal interactions of AgNPs with fluconazole in combination treatment

Antifungal property of AgNPs and fluconazole against fluconazole resistant *C. albicans* strains were determined by the microdilution method (Table 1). Consistent with their intrinsic resistance property, High MIC (128 µg/ml) values for fluconazole were observed for both CA-R15 and CA-R21. The MICs of AgNPs against both CA-R15 and CA-R21 were 32 µg/ml. The MICs for fluconazole decreased to 0.5 µg/ml (CA-R15) and 1 µg/ml (CA-R21) when used in combination with AgNPs. Interestingly, we also observed a great reduction in the MICs for AgNPs. Based on fractional inhibitory concentration index (FICI) model, an evident synergistic effect ($FICI \leq 0.5$) between AgNPs and fluconazole against resistant strains (Table 1) were observed. The MIC of fluconazole against quality control strain SC5314 (0.5 µg/ml) was within the reference range. There was no difference between the MICs of AgNPs against resistant and susceptible *C. albicans*. A little reduction for MICs for AgNPs and fluconazole against susceptible strain SC5314 was also observed. However, it doesn't result in synergistic effect.

Table 1
Drug interactions of AgNPs and fluconazole against 2 resistant *C. albicans*. *in vitro*.

<i>C. albicans</i> strain	MIC (µg/mL)		FICI model			
	MIC _{FLC}	MIC _{AgNPs}	C _{FLC}	C _{AgNPs}	FICI	Interpretation
SC5314	0.5	32	0.25	0.5	0.52	additivity
CA-R15	128	32	0.5	0.5	0.02	synergism
CA-R21	128	32	1	0.5	0.02	synergism
FLC, fluconazole; FICI, fractional inhibitory concentration index. MIC, minimum inhibitory concentration of drug that inhibited fungal growth by 80% compared with the growth control. MIC _{FLC} and MIC _{AgNPs} , MICs of fluconazole and AgNPs when they were used alone. C _{FLC} and C _{AgNPs} , MICs of fluconazole and AgNPs when they were used in combination.						

2.2. Synergistic effect of AgNPs and fluconazole on biofilm formation activity of *C. albicans*

We further tested the antibiofilm activity of AgNPs combined with fluconazole on resistant *C. albicans*. The results were also interpreted by FICI model. The results were shown in Table 2. When the formation of biofilms was at its early stage (6hr), fluconazole could inhibit their viability with MICs of 128 µg/ml for both CA-R15 and CA-R21. However, when the formation of biofilms got into its late stage (12hr) and finally to early mature stage (24hr), fluconazole became invalid. Fluconazole could effectively inhibit the activity of SC5314 biofilms until their early mature stage (24hr). AgNPs had the similar activity against the biofilms of fluconazole-resistant and sensitive strains. Synergies existed for the AgNPs and fluconazole combinations against 6-hour and 12-hour biofilms of fluconazole-resistant strains, with all FICIs < 0.5, but not for biofilms formed for 24 hours and biofilms of fluconazole-sensitive strains SC5314.

Table 2

Synergistic effect of AgNPs and fluconazole on biofilm formation activity of resistant *C. albicans*

<i>C. albicans</i> strain	Time/hours	sMIC ($\mu\text{g/mL}$)				FICI model	
		sMIC _{FLC}	sMIC _{AgNPs}	C _{FLC}	C _{AgNPs}	FICI	Interpretation
SC5314	6	4	64	2	8	0.51	additivity
	12	8	64	2	16	0.5	additivity
	24	256	128	256	128	> 0.5	Indifference
CA-R15	6	128	128	4	8	0.16	synergism
	12	256	128	4	16	0.14	synergism
	24	256	128	128	128	> 0.5	Indifference
CA-R21	6	128	128	4	16	0.16	synergism
	12	256	128	8	16	0.16	synergism
	24	256	128	256	128	> 0.5	Indifference

FLC, fluconazole; FICI, fractional inhibitory concentration index. sMIC, sessile minimum inhibitory concentration of drug that reduce biofilms by 50% compared with the growth control. sMIC_{FLC} and sMIC_{AgNPs}, sMICs of fluconazole and AgNPs when they were used alone. C_{FLC} and C_{AgNPs}, sMICs when fluconazole and AgNPs were used in combination.

2.3. AgNPs treatment decreased membrane ergosterol levels

The effect of AgNps on membrane ergosterol content was tested in CA-R15 and CA-R21. As depicted in Fig. 1A, a gradual reduction in ergosterol content was observed with the increasing concentration of AgNps in both strains. At the concentration of 30 $\mu\text{g/mL}$, there was the maximum reduction of ergosterol by 24.7% (CA-R15) and 36.5% (CA-R21). The technology of qRT-PCR was conducted to test the expression pattern of genes related to ergosterol biosynthesis. *ERG1*, *ERG3*, *ERG11* and *ERG25* were examined. AgNPs treatment greatly inhibited the expression of *ERG1*, *ERG11* and *ERG25*, while for *ERG3*, no significant changes were observed (Fig. 1B, C). These results indicate that AgNps may impair membrane structure by down-regulation of ergosterol biosynthesis genes and depletion of ergosterol.

2.4. AgNPs treatment inhibited efflux pump activity

Ergosterol constitutes an important part in membrane microdomains. Depletion of ergosterol could affect the membrane dynamics and compromise the membrane integrity [35, 37]. We then used fluorochrome, DPH to examine the changes in the organizational dynamics of membrane (fluidity) after interruption of ergosterol biosynthesis by AgNPs treatment. Both CA-R15 and CA-R21 showed a dose-dependent increase in fluorescence polarization, thus implying a gradually decreased fluidity of the membranes of

these two strains (Fig. 2A). Since the fluidity and ergosterol content of the membrane could affect the localization and function of Cdr1p and Cdr2p which were major efflux pumps related to azoles resistance [8], so we further tested the localization of Cdr1p and Cdr2p on membranes in AgNPs-treated *C. albicans*. As expected, increased membrane content of Cdr1p and Cdr2p was observed in resistant strains CA-R15 and CA-R21 compared with azole-sensitive strain SC5314. However, AgNPs-treatment resulted in significantly decreased surface localization of Cdr1p and Cdr2p Fig. 2B-D. The total expression of Cdr1p was barely affected, whereas for Cdr2p, it showed a great decrease, as indicated by the western blotting results in Fig. 2E,F. The expression level of gene *CDR1* and *CDR2* were also tested (Fig. 2G,H). AgNPs did not affect the expression of genes *CDR1*, while the expression of gene *CDR2* decreased with AgNPs treatment.

We used R6G to determine whether co-treatment of AgNPs affected activity of efflux pumps. As was shown by the results in Fig. 2I, the fluorescence intensity of R6G in the AgNPs-treated group was significantly higher than that in the control group, which meant that AgNPs could inhibit the efflux of fluconazole. Taken together, these results demonstrate that the disruption of membrane ergosterol by AgNPs results in reduced efflux pump activity. Considering the great roles ergosterol and efflux pump activity played in drug resistance, we speculate that AgNPs may sensitize resistant strains to fluconazole.

In order to investigate the inhibitory effect of AgNPs more closely, ergosterol was supplemented into the growth media of CA-R15 and CA-R21 (Fig. 3). Figure 3A showed enhanced ergosterol content after outward ergosterol supplementation. The localization of Cdr1p and Cdr2p on membrane was partially restored with Cdr1p to a much more extent, which probably meant Cdr1p was more sensitive to the lipid environment of the membrane (Fig. 3B, C). At the same time, decreased accumulation of R6G was observed when compared with the AgNPs alone treated cells (Fig. 3D). All these results further demonstrated that AgNPs facilitate the accumulation of fluconazole in *C. albicans* cells by targeting ergosterol biosynthesis pathway and changing membrane environment.

2.5. Production of ROS is upregulated by AgNPs and fluconazole co-treatment

Augmentation of ROS release is an important mediator of antifungal effects exerted by fluconazole [38–41]. Inversely, the increasing clean-up of ROS may be linked to the resistance of fluconazole [42, 43]. ROS promotion contributes to combatting fluconazole-resistance [33]. It is reported that the antifungal effect of AgNPs was partially attributed to its induction of oxidative stress via ROS generation [8]. So, we tried to assess this ROS promotion effect of AgNPs in CA-R15 and CA-R21. In view of the above mentioned fact that AgNPs could facilitate the accumulation of fluconazole, we also designed the AgNPs and fluconazole co-treatment group. The results were demonstrated in Fig. 4. We found that in the fluconazole-treated group, ROS was barely induced when compared with the basal ROS levels in control group. AgNPs alone could also induce significant accumulation of ROS. However, the largest production of cellular ROS was observed in the combination group, much higher than that of the AgNPs group. A similar phenomenon was also observed in CA-R15 (data not shown). These results suggest that AgNPs

may promote the generation of ROS to overcome the resistance of *C. albicans* against fluconazole. In addition, the facilitated accumulation of fluconazole may further exacerbate the production of ROS, which means AgNPs and fluconazole synergistically promote the production of ROS.

2.6. In vivo response of AgNPs and fluconazole co-treatment

We further investigated the *in vivo* susceptibility of *C. albicans* to AgNPs and fluconazole combinatory treatment. Firstly, we determined the influence of this drug combination on tissue fungal burden of infected mice. In mice infected with isolates CA-R15, CA-R21, fluconazole alone showed no effect in reducing the fungal loads in both livers and kidneys. AgNPs alone at a dose of 3 mg/kg was effective at reducing the fungal burden compared against the controls (liver: $p < 0.05$ for both CA-R15 and CA-R21; kidney: $p < 0.01$ for both CA-R15 and CA-R21). However, the most prominent effect was observed when AgNPs and fluconazole were administrated in combination (Fig. 5A-D). Besides, Kaplan-Meier survival plots were analyzed by a log-rank test. Consistent with their resistant property, in mice infected by CA-R15 or CA-R21, fluconazole exerted no effect on their survival. Monotherapy with AgNPs prolonged the overall survival of the infected mice. But once AgNPs were discontinued, the survival rates declined. Combination treatment with AgNPs and fluconazole significantly improved the survival rate of infected mice compared with that of mice treated with AgNPs or fluconazole monotherapy (Fig. 5E-F).

3. Discussion

C. albicans is the most common opportunistic fungal pathogen causing great threat to human beings, especially immunocompromised patients. For a long time, people have been searching for effective methods treating the resistant fungus. Combination therapy offers an attractive and valid strategy which can increase drug potency and decrease toxicity, in the meantime, overcome resistance of *C. albicans* [44]. AgNPs, has demonstrated its antifungal effects for a long time. Various mechanisms contributed its fungicidal effect, including ROS promotion, blocking the synthesis of ATP, changing surface morphology, cellular ultrastructure, membrane microenvironment, membrane fluidity, ergosterol content, and fatty acid composition [45]. Many of these factors are also crucial for drug resistance and pathogenicity of *C. albicans*. However, high dose of AgNPs might also exert cytotoxic effects on mammalian cells [8, 46]. Under these circumstances, nano silver-based drug combination therapy may offer as an ideal candidate in addressing the challenges of drug resistance. The synergistic effect between AgNPs and azoles against fluconazole-resistant *C. albicans* has been reported earlier [29, 30]. But they were more focused on the disruption of cell membrane integrity, while the intrinsic events were not quite elucidated. Here, in the present study, we tried to explore the effect of AgNPs on fluconazole-resistant *C. albicans* and the synergism between AgNPs and fluconazole more thoroughly and extensively. We tried to understand whether AgNPs can exert the same effect on fluconazole-resistant *C. albicans*, and thus, sensitize the strains to fluconazole treatment.

Synergistic antifungal effects of AgNPs with fluconazole on resistant *C. albicans* strains were found in planktonic cell. In contrast to previous report, we also observed synergy for AgNPs and fluconazole against biofilms formed for 6 and 12 hours, but not for 24 hours [29]. This indicates that this combination may be more viable in the early treatment of biofilm-dependent *C. albicans* infections. The controversy may on the other hand, demonstrated the complexity drug resistance. These observations made the application of AgNPs with fluconazole combination a potential therapy in the treatment of resistant *C. albicans* infections. Besides, we found that the MICs of AgNPs was also greatly reduced. The large reduction in the AgNPs dose would also significantly restrict its side effects.

The synergism between AgNPs with fluconazole indicates that AgNPs can overcome fluconazole resistance. We next examined whether AgNPs also target the multiple cellular targets in resistant *C. albicans* as in susceptible ones, especially the molecules associated with the two major mechanisms mediating fluconazole-resistance. Consistent with previous report, our data also suggested that AgNPs treatment decreased membrane ergosterol levels and inhibited the expression of *ERG1*, *ERG11* and *ERG25*. The ergosterol biosynthetic pathway, regulated by various *ERG* genes is a target for many antifungals, such as fluconazole [13]. So the decreased ergosterol indicates that some other mechanisms may participate in the enhanced fungicidal effect of fluconazole when used in combination with AgNPs. Up-regulation of sterol-rich microdomains has been reported to be linked to drug-resistant in cancer cell lines through subsequent increased export of cytotoxic drugs [47, 48]. Lack of ergosterol could lead to reduced plasma membrane (PM) fluidity, resulting in reduced intracellular ATP level and delocalization of Cdr1p efflux pump from membrane [37]. So we then tested the efflux pump activity after AgNPs treatment. As previously reported [31], we also noted decreased membrane fluidity after AgNPs treatment. Treating cell with AgNPs caused greatly reduced membrane content of Cdr1p and Cdr2p. To determine if the reduced content was attributed to delocalization or downregulation, we checked the total level of these two proteins. While Cdr1p was not affected, Cdr2p was quite decreased, which is in accordance with the changes in gene level. Commonly, intracellular accumulation of fluconazole was greatly reduced because of the overexpression of drug transport pumps [49]. So we speculated that the depletion of ergosterol by AgNPs, and the subsequent decreased efflux pumps on membrane would result in increased accumulation of fluconazole. In this study, using R6G as a surrogate, we found that AgNPs could significantly inhibit the efflux and promote the accumulation of fluconazole. The participation of ergosterol in the effect of AgNPs on efflux pumps was further confirmed by ergosterol supplementation experiment. We found that supplemented ergosterol could partially reverse the effect of AgNPs.

Our data also suggested that the increased production of ROS is a likely cause for the synergy between AgNPs and fluconazole. In this study, we found that the largest production of cellular ROS was observed in the combination group, much higher than the AgNPs group. This result suggested that AgNPs may promote the accumulation of ROS to overcome the resistance of *C. albicans* against fluconazole. Besides, the aforementioned promoted accumulation of fluconazole can further enhance the production of ROS. This may finally result in the synergistic effect between AgNPs and fluconazole. However, the ROS-inductive ability could also trigger mammal cell death processes, including apoptosis and necrosis, and thus cause toxicity on treated mammals. It was reported that AgNPs could cause hazardous effects

on mouse oocyte maturation, *in vitro* fertilization (IVF), and subsequent preimplantation and postimplantation development *in vitro* and *in vivo* [50]. The maximum toxicity dose and regulatory mechanisms contributed to AgNP toxicity also needs further exploration.

We also tested the antifungal effects of AgNPs-fluconazole on a murine model of systemic candidiasis. AgNPs and fluconazole combination demonstrated to be most effective in reducing fungal burden. Besides, combination treatment with AgNPs and fluconazole showed the greatest effect in improving the survival rate of infected mice.

In conclusion, the results of this study demonstrate AgNPs could promote the accumulation of fluconazole in resistant *C. albicans*, and synergize with fluconazole in ROS induction. All these contribute to the synergism of AgNPs and fluconazole in combatting in fluconazole-resistant *C. albicans*.

4. Materials And Methods

4.1. Isolates

Control organisms *C. albicans* SC5314 and additional 2 clinical isolates of *C. albicans* CA-R15 and CA-R21 were investigated in this study. *C. albicans* SC5314 was obtained from American Type Culture Collection. The 2 clinical isolates were obtained from the intensive care unit of Qingpu Branch of Zhongshan Hospital, Shanghai, China. All clinical isolates were recovered from oral, thigh and each represented a unique isolate from a patient. Yeasts were identified at the species level by CHROMagar (KANTO CHEMICAL) and Rapid Tm Yeast plug system (REMEL Inc) and stored at -70 °C in 10% glycerol. Before the initiation of the study, yeast isolates were sub-cultured on antimicrobial agent-free medium to ensure viability and purity.

4.2. Antifungal Agents

Fluconazole was purchased from Sigma-Aldrich (St. Louis, MO). 10 nm poly-vinylpyrrolidone (PVP) coated biopure silver nanoparticles were purchased from Nano Composix Inc. (San Diego, CA). The mass concentration of the AgNPs used in this study was 1 mg/ml. The size distribution is 8–12 nm.

4.3. In vitro antifungal assays

Minimum inhibitory concentrations of AgNPs and fluconazole were tested by the microdilution method as outlined in Clinical and Laboratory Standards Institute document M27-A3 and as previously described using 96-well microtiter plates [34]. The concentrations for fluconazole were 0.125 µg/ml to 128 µg/ml. For AgNPs, the concentrations were set from 0.25-64 µg/ml. 100 µl of the inoculum suspension with a concentration of 2×10^3 cells/ml in RPMI 1640 medium without phenol red and sodium bicarbonate (Sigma-Aldrich. St. Louis, MO), was incubated with these various concentrations of antifungals in 96-well plates and cultured at 35°C for 48 h. After incubation, the cell growth was detected by XTT reduction assay. Absorption at 492 nm was detected by the microplate reader. The MICs were defined as the lowest concentration of drugs that caused > 80% inhibition of *C. albicans*. When the MICs were higher than the

top concentration, then the top concentration was regarded as the MICs. The types of interaction between AgNPs and fluconazole were analyzed with the checkerboard method. Briefly, various concentrations of AgNPs and fluconazole were combined to provide 80 combinations and incubated with *C. albicans* cells. The interaction of AgNPs and fluconazole was interpreted using fractional inhibitory concentration index (FICI) model: $FICI = FIC_{AgNPs} + FIC_{FLC} = (\text{MIC of AgNPs in combination} / \text{MIC of AgNPs alone}) + (\text{MIC of fluconazole in combination} / \text{MIC of fluconazole alone})$. FICI values ≤ 0.5 synergy, $0.5 < FICI \leq 1$ additivity, $1 < FICI \leq 4$ indifference, > 4 antagonism. Each assay was performed in triplicate.

4.4. Biofilm studies

50 μl of *C. albicans* cells diluted in RPMI-1640 with a concentration of 1.0×10^6 cells/ml were added to 96-well plates to a final volume of 200 μl and cultured under static conditions at 35°C for 6, 12 or 24 hours to form biofilm. RPMI-1640 without cells was used as a negative control. After that, the wells were carefully aspirated and gently washed three times with 200 μL of sterile phosphate-buffered saline (PBS) to remove any remaining suspended cells. Then, an equal volume of fresh RPMI-1640 with various concentrations of antifungal fluconazole (2–256 $\mu\text{g}/\text{ml}$) and AgNPs (8–512 $\mu\text{g}/\text{ml}$) were added. After incubation for another 48 h at 35°C, XTT reduction assay was used to measure the viability of biofilms. And absorption at 492 nm was detected by the microplate reader. The MICs were defined as the lowest concentration of drugs that reduces the absorption value by 50% relative to control. Each assay was performed in triplicate. The interaction of AgNPs and fluconazole was also interpreted using fractional inhibitory concentration index (FICI) model: $FICI = FIC_{AgNPs} + FIC_{FLC} = (\text{MIC of AgNPs in combination} / \text{MIC of AgNPs alone}) + (\text{MIC of fluconazole in combination} / \text{MIC of fluconazole alone})$. FICI values ≤ 0.5 synergy, $0.5 < FICI \leq 1$ additivity, $1 < FICI \leq 4$ indifference, > 4 antagonism.

4.5. Ergosterol measurement

Sterols were extracted and analyzed as described in [35]. Briefly, cells, after grown overnight in YPD medium in the presence of AgNPs (0, 15, 30 $\mu\text{g}/\text{ml}$), were harvested and boiled in 25% alcoholic KOH for 1 h. Then sterols were extracted from the boiled cells with petroleum ether. Ergosterol content was determined spectrophotometrically by analyzing the specific pattern of absorbance between 200 and 320 nm.

4.6. Quantification Analysis by Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The RNA isolation and cDNA synthesis were performed as previously described[29]. 18S ribosomal RNA (18S rRNA) was used as internal control.

4.7. Total protein extractions, Plasma Membranes isolation and western blotting

Cells were cultured in YPD liquid medium overnight in the presence of AgNPs (0, 15, 30 $\mu\text{g}/\text{ml}$) and harvested. Then cells were washed twice in PBS and resuspended in homogenization medium containing

50 mM Tris (pH 7.5), 2 mM EDTA, and protease inhibitor cocktail (Roche Diagnostics, Germany). After incubation at 37°C for 30 min, cells were disrupted by Braun cell homogenizer. Cell lysate was centrifuged at 2,000 × g for 10 min. The supernatant were collected to obtain total proteins. For plasma membrane isolation, the supernatant was ultra-centrifuged at 30,000 × g for 45 min to isolate crude membrane fraction. A micro-BCA protein assay kit was used to determine protein concentrations (Pierce, Rockford, IL). A total of 30 µg protein was separated on 6% or 8% SDS-PAGE gel. After that, the gel was transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) with Trans Blot SD Semi-Dry transfer cell (Bio-Rad). Ponceau S reagent (0.1% Ponceau S in 5% acetic acid) staining was performed to visualize the transferred proteins on membranes. Subsequently, the membranes were incubated with anti-Cdr1p (1:1000) or anti-Cdr2p (1:1000) polyclonal antibodies, then with secondary anti-rabbit IgG peroxidase (1:5000, Santa Cruz Biotechnology, TX). The anti-Cdr1p and anti-Cdr2p polyclonal antibodies were generated in rabbits as described in [8].

4.8. Membrane Fluidity Assessment

The fluorescence polarization measurements were carried out to assess the physical state (fluidity) of the membrane. After corresponding treatment, cells were harvested and incubated with lyticase (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's introduction to get spheroplasts. Then the prepared spheroplasts were labeled with 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) (Aladdin Industrial Corporation, Los Angeles, CA). Fluorescence polarization (p value) was measured with spectrofluorometer Hitachi F-7000 (Hitachi, Tokyo, Japan) at excitation and emission wavelengths of 360 nm and 450 nm respectively.

4.9. Rhodamine 6G (R6G) efflux assay

Rhodamine 6G (R6G) purchased from Shanghai Macklin Biochemical Co. was used as a surrogate to investigate the uptake and efflux of azoles as described previously [36]. The *C. albicans* cells were inoculated in SDA medium and cultured at 35°C by at 200 rpm for 16 hours. After reaching exponential phase, cells were harvested by centrifugation at 3000 rpm and 4°C for 5 min. Then wash the cells three times with precooling PBS. After that, cells were re-suspended in glucose-free PBS (pH = 7.0) and adjusted to 1 × 10⁸ cells /ml.

R6G efflux assay was performed as follows: After adjusting to 1 × 10⁸ cells /ml in glucose-free PBS and de-energized for 2 h, cells were treated with R6G (10 µM) at 35°C for 60 minutes to preload the cells with R6G. Cells were then treated with precooling PBS to stop the uptake of R6G, and were centrifuged at 3000 rpm and 4°C for 10minutes. After washed twice in PBS to remove the remaining extracellular R6G, cells were resuspended in PBS with 0.1 M glucose and AgNPs (0 µg/ml, 15 µg/ml, and 30 µg/ml). Cells were collected at specific time intervals. Mean fluorescence intensity of the intracellular R6G was detected by CytoFLEX (Beckman Coulter, USA) at excitation and emission wavelengths of 488 and 530 nm respectively. Each assay was performed in triplicate.

4.10. Ergosterol supplementation experiments

Ergosterol was dissolved in petroleum ether and added directly and concomitantly with AgNPs into the medium during inoculation to a final concentration of 10 µg/ml.

4.11. ROS production assay

CA-R21 cells were grown until mid-exponential phase. After that, cells were harvested and washed twice with PBS. Then, cells (1×10^8 cells/mL) were treated with fluconazole 0.5 µg/ml for another 4 hours, together with AgNPs 0 µg/ml, 0.5 µg/ml, and 1 µg/mL. After incubation, cells were centrifuged at 5,000 rpm for 10 minutes at 4 °C to harvested, followed by washing twice with PBS buffer to remove the media. Then cells were resuspended in PBS buffer and the fluorescent probe DCFH-DA was added to a final concentration of 10 µM. After incubation in the dark for 30 minutes, cells were washed in PBS for three times to remove the residual dye. Mean fluorescence intensity was detected by CytoFLEX (Beckman Coulter, USA) at excitation and emission wavelengths of 488 and 540 nm respectively.

4.12. In vivo experiment

Female, 5-week-old, BALB/c mice (CLEA Japan.Inc.) ,15 to 18 g, were used in the experiments (n = 18/group),and maintained in micro-isolator boxes with a standard rodent diet according to National Institutes of Health guidelines for animal care and in fulfillment of American Association for Accreditation of Laboratory Animal Care criteria. Immune-compromised mice were obtained by administrating 200 mg/kg cyclophosphamide (Sigma-Aldrich, St. Louis, MO) and 250 mg/kg cortisone acetate (Sigma-Aldrich, St. Louis, MO) intraperitoneally 2 days prior to yeast challenge and continued until the day of challenge. Clinically isolated *C. albicans* were sub-cultured overnight in YPD broth (Becton, Dickison and company) at 30 °C 1 day prior to challenge. On the day of challenge, the subculture was pelleted, and exponential-phase cells were harvested, washed, and resuspended in sterile saline. The concentration of blastospores was counted with an absorbance detector, and the blastospore suspension was adjusted to give 2×10^8 blastospores/ml for the *in vivo* experiments. Actual CFU in the inocula were determined by culturing serial dilutions of each preparation onto YPD plates. Murine models of systemic candidiasis were established by infecting immune-compromised mice intravenously with 0.2 ml of *Candida* cell suspension of these strains through the lateral vein. After that, mice were further divided into two sub-groups with one sub-group for tissue burden analysis (8 mice) and the other one for survival analysis (10 mice). Therapy began four hours after challenge and continued for 12 days. A dose of 10 mg/kg fluconazole was given intravenously once daily. AgNPs were administrated intravenously at doses of 1 mg/kg and 3 mg/kg separately. After 7 days of consecutive therapy, mice for tissue burden analysis were euthanized. Kidneys and livers were aseptically removed, and the entire organs were homogenized (Shake master, NEO bio medical science) in 1 ml of sterile saline. Serial 10-fold dilutions of the homogenates were plated on YPD agar (Becton, Dickison and company), incubated at 35 °C, and examined the number of CFU per organ of tissue. The mice for survival analysis were kept and monitored daily for mortalities in the next two weeks.

4.13. Statistical analysis

A one-way analysis of variance (ANOVA) was applied for analysis and where differences occurred, a two-tailed Tukey's multiple-comparison test was done between paired groups. A p value of < 0.05 was considered significant. Kaplan-Meier survival plots were analyzed by a log-rank (Mantel-Cox) test. All statistical analysis was performed using GraphPad Prism, version 7.03 (GraphPad Software, Inc., San Diego, CA).

Declarations

Author Contributions: Conceptualization, resources, writing—review & editing, supervision, Dogmei Jia. Methodology, formal analysis, investigation, validation, writing—original draft, Wenping Sun. All authors have read and agreed to the published version of the manuscript.

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Figures

Figure 1

Inhibitive effect of ergosterol biosynthesis by AgNPs in resistant *C. albicans* CA-R15 and CA-R21. (A) Membrane ergosterol was extracted from cells treated with different concentrations of AgNPs (0, 15, 30µg/ml) and measured. AgNPs treatment decreased membrane ergosterol content in a concentration-dependent manner. Bars represent means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 versus AgNPs 0µg/ml group. (B) The expression pattern of ergosterol biosynthesis related genes ERG1, ERG3, ERG11 and ERG25 in response to AgNPs treatment were examined by qRT-PCR. AgNPs treatment greatly inhibited the expression of ERG1, ERG11 and ERG25, while for ERG3, no significant changes were observed. Data represent means ± SD. *P < 0.01, ***P < 0.001 versus control (AgNPs 0µg/ml group) group. Experiments were conducted in triplicate.

Figure 2

Membrane fluidity and efflux pump activity after AgNPs treatment (0, 15, 30µg/ml). (A) Membrane fluidity was assessed by steady-state fluorescence polarization measurements (p value). Both CA-R15 and CA-R21 showed a dose-dependent increase in fluorescence polarization (p value), which implied a gradually decreased fluidity of the membranes of these two strains. (B-D) Immunoblot analysis and quantification of membrane Cdr1p and Cdr2p level were performed. Ponceau S staining was used as the loading control. Resistant *C. albicans* CA-R15 and CA-R21 showed higher membrane Cdr1p and Cdr2p level compared with susceptible strain SC5314. However, AgNPs-treatment resulted in significantly decreased surface level of Cdr1p and Cdr2p. (E-G) Immunoblot analysis and quantification of total Cdr1p and Cdr2p level were performed. Ponceau S staining was used as the loading control. AgNPs-treatment decreased Cdr2p level, while showed no effect on Cdr1p. (H) The expression pattern of CDR1 and CDR2 were determined by qRT-PCR. AgNPs-treatment decreased CDR2 level, while showed no effect on CDR1. (I) Efflux pump activity was assessed by R6G efflux assay. *C. albicans* were treated with different concentrations of AgNPs or not. Mean fluorescence intensity (MFI) of the intracellular R6G in *C. albicans* cells was determined by flow cytometry. M, membrane protein. T, total protein. Data represent means $\pm$ SD. *P < 0.01, **P < 0.05, ***P < 0.001 versus control (AgNPs 0µg/ml group) group. Experiments were conducted in triplicate.

Figure 3

Ergosterol supplementation partially reversed the inhibitive effect of AgNPs on efflux pumps. (B) The cells were supplemented with 10µg/ml of ergosterol together with 30µg/ml AgNPs and grown for 48 h at 35°C before lipid extraction. An increase was observed in membrane ergosterol content. (C-D) The localization of Cdr1p and Cdr2p on membrane was partially restored. (E) A final concentration of 50µg/ml ergosterol was supplemented concomitantly with AgNPs treatment. Ergosterol supplementation resulted in decreased accumulation of R6G. Data represent means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group. &P < 0.05, &&P < 0.01 versus AgNPs group. Experiments were conducted in triplicate.

Figure 4

AgNPs supplementation promoted the ROS production in *C. albicans*. CA-R21 was treated with sub-inhibitive dose of fluconazole combined with of AgNPs (0, 0.5, 1µg/ml) for 4 hours. The ROS production was detected by flow cytometry. FLC, fluconazole. Data represent means ± SD. * P < 0.05, ** P < 0.01, *** P < 0.001 versus control. &&& P < 0.001 of combinatory use versus AgNPs used alone. Experiments were conducted in triplicate.

Figure 5

In vivo response of AgNPs and fluconazole co-treatment. Effects of the antifungal treatments on fungal load of the *C. albicans*. CA-R15 (A, B) and CA-R21 (C, D) in livers and kidneys of mice. Immune-compromised mice were infected with CA-R15 or CA-R21 strains. Then the infected mice were treated with AgNPs intravenously at doses of 1mg/kg or 3mg/kg together with fluconazole at a dose of 10mg/kg or not. After 7 days of consecutive therapy, the mice were euthanized for tissue burden analysis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (E, F) Effects of the antifungal treatments on the survival rates of immune-compromised mice infected with CA-R15 (E) and CA-R21 (F). FLC, fluconazole. ** $P < 0.01$, *** $P < 0.001$ versus control.