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Original Article

The non-Geldanamycin Hsp90 inhibitors enhanced the antifungal activity of fluconazole

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Abstract: The molecular chaperone heat shock protein 90 (Hsp90) is highly conserved in eukaryotes and facilitates the correct folding, productive assembly and maturation of a diverse cellular proteins. In fungi, especially the most prevalent human fungal pathogen *Candida albicans*, Hsp90 influences development and modulates drug resistance. Here, we mainly explore the effect of non-Geldanamycin Hsp90 inhibitor HSP990 on the activity of fluconazole (FLC) against *Candida albicans* and investigate the underlying mechanism. We demonstrate that HSP990 has potent synergistic antifungal activity with FLC against FLC-resistant *C. albicans* through the checkerboard microdilution assay,agar diffusion tests and time-kill curves, and shows low cytotoxicity to human umbilical vein endothelial cells. Further study shows that the activity of FLC against *C. albicans* biofilm formation *in vitro* is significantly enhanced when used in combination with HSP990. In a murine model of disseminated candidiasis, the therapeutic efficacy of FLC is also enhanced by the pharmacological inhibition of *C. albicans* Hsp90 function with HSP990. Thus, the combined use of small molecule compound and existing antifungal drugs may provide a potential therapeutic strategy for fungal infectious disease.

Keywords: Hsp90 inhibitors, fluconazole, fungal pathogens, synergistic antifungal activity

Introduction

In recent years, there has been significant increase of invasive infections with human fungal pathogens, and *Candida albicans* is the most common pathogenic funguscausing a large proportion of invasive fungal infections or life-threatening diseases (such as systemic candidiasis) in humans, especially among individuals with disorder of the host's microbial flora or immune deficiency [1-6]. For treatment of invasive fungal infections, azole antifungal agent fluconazole (FLC) which shows fungistatic activity by inhibiting the biosynthesis of ergosterol is the most common agent used in clinic in virtue of its low price, few side effects and high effectiveness [7, 8]. However, with the long-time and wide use of FLC, FLC-resistant isolates are occurring rapidly [2, 9]. In addition, *C. albicans* biofilms formed on the surfaces of most medical devices, such as catheters, endoscopes and different types of stents, providing a reservoir for dissemination, show severe resistance to various types of clinical antifungal agents, further aggravating the use of FLC in clinic [10].

Heat shock protein 90 (Hsp90) is an abundant and essential eukaryotic molecular chaperone that modulates protein folding and governs the functions of key regulators in diverse cellular signaling [11, 12]. In fungi, Hsp90 acts as a biological transistor regulating the activity of fungal signaling networks, and has been demonstrated to mediate drug (such as azole) resistance and biofilm formation in diverse fungal species [13-17]. Therefore, the inhibition of Hsp90 function has emerged as a promising strategy to enhance the antifungal efficacy of azoles, and previous studies have also reported that classical Hsp90 inhibitors in clinic as anticancer agents such as geldanamycin (GdA) and

its derivatives docking at the ATP-binding site in the N-terminal domain of Hsp90 have the ability of significantly enhancing the efficacy of FLC against *C. albicans* [13, 18, 19]. Nonetheless, GdA and its derivatives have not been fully developed in clinic due to their physiochemical property limitation sand frequent clinicaltoxicities forliver and gastrointestinal tract, which have leaded to the generation and development of non-Geldanamycinsmall molecule Hsp90 inhibitors with improved safety profiles and highly potent preclinical efficacy [20-22].

In this study, we selected 4 Hsp90 inhibitors used as anticancer agents, NVP-AUY922 (AUY922), NMS-E973 (E973), NVP-HSP990 (HSP990) and Ganetespib STA-9090 (STA-9090), all of which are non-GdA Hsp90 inhibitors, but still bind to conserved ATP-binding pocket of Hsp90 [23-28]. To determine if the synergy that has been reported with GdA or its derivatives and azoles against FLC-resistant *C. albicans* previously could be also observed with these novel types of Hsp90 inhibitors, we tested their ability (especially for HSP990, an orally administrable drug) to enhance the antifungal efficacy of FLC *in vitro*against FLC-resistant *C. albicans*and *C. albicans* biofilm formation by different methods. Further, the therapeutic potential of HSP990 combined with FLC in fungal disease was explored using a murine model of disseminated candidiasis. Moreover, we also investigated thesynergistic mechanism of FLC and HSP990 against FLC-resistant *C. albicans* using a comprehensive comparative proteomic analysis.

Materials and methods

Strains and agents

Twenty clinical isolates of FLC-resistant *C. albicans*, one FLC-sensitive *C. albicans* SC5314, one *Cryptococcus neoformans* 56992, *Candida tropicalis* ATCC20026, *Candida parapsilosis* ATCC 22010, *Candida krusei* ATCC2340 and *Candida glabrata* ATCC1182 provided by professor Changzhong Wang (School of integrated traditional and western medicine, Anhui university of traditional Chinese medicine, Hefei, China) were used in this study. All strains were maintained on SDA agar (1% peptone, 4% dextrose, and 1.8% agar) plates and re-cultured at least monthly from -80°C stock. For use in the experiments, yeast-phase cells of the various strains were grown in YPD (1% yeast extract, 2% peptone and 2% dextrose) liquid medium overnight at 30°C in a rotary shaker.

Drugs prepared in dimethyl sulfoxide (DMSO) included FLC (Pfizer-Roerig Pharmaceuticals, New York, NY) and Hsp90 inhibitors AUY922, E973, HSP990 and STA9090 (Selleck Chemicals, USA), and their initial stored concentration was 6.4 mg/ml in DMSO [29].

Checkerboard microdilution assay

The MICs of compounds against all isolates were determined by the micro broth dilution-method according to the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) as described previously [29]. The beginning concentration of fungal suspension in RPMI 1640 medium was 10^3 CFU/ml, and the final concentrations ranged from 0.125 to 64 μg/mlfor FLC and 1 to 32 μg/ml for HSP990. The final concentration for FLC or HSP990 alone ranged from 0.125 to 64 μg/ml. 96-well flat-bottomed microtitration plates were incubated at 30°C for 24 h or 72 h. Optical density was measured at 630 nm. MIC_{80}s were determined as the lowest concentration of the drugs (alone or in combination) that inhibited growth by 80%, compared with that of drug-free wells. The fractional inhibitory concentration index (FICI) is defined as the sum of the MIC of each drug when used in combination divided by the MIC of the drug used alone. FICIwas calculated by the following equation: FICI=FIC A +FIC B, where FIC A is the MIC of the combination/the MIC of drug A alone, and FIC B is the MIC of the combination/the MIC of drug B alone. Synergy andantagonism were defined by FICIs of ≤0.5 and >4, respectively. An FICI result of >0.5 but ≤4 was considered indifferent [30, 31].

Agar diffusion test

C. albicans 103 (one FLC-resistant isolate with aMIC of 64 μg/ml for HSP990) was tested by agar diffusion assay as described previously [29]. A 4-ml aliquot of 10^6-CFU/ml suspension was spread uniformly onto the yeast extract-peptone-dextrose agar plate with or without 1 μg/ml FLC. Then, 6-mm paper disks impregnated with FLC and HSP990 alone or in combination were placed onto the agar surface. There was 5 μl of DMSO in control disks. Inhibition

zones were measured after incubation at 30°C for 48 h.

Time-kill test

C. albicans 103 in RPMI 1640 medium was prepared at the starting inoculum of 10^5 CFU/ml. The concentrations were 8 μg/ml of HSP990 and 4 μg/ml of FLC. DMSO comprised <1% of the total test volume. At predetermined time points (0, 4, 8, 12 and 24 h) after incubation in a shaking incubator with 200 rpmat 30°C, a 100-μl aliquot removed from every solution was serially diluted 10-fold in sterile water. The 100-μl aliquot from each dilution was spread on the SDA agar plate. Colony counts were determined after incubation at 30°C for 48 h. Synergism and antagonism were defined as a respective increase or decrease of ≥2 $\log_{10}$ CFU/ml in antifungal activity produced by the combination compared with that by the more active agent alone after 24 h, while a change of <2 $\log_{10}$ CFU/ml was considered indifferent [29].

Cytotoxicity evaluation of Hsp90 inhibitors using XTT assay

Human umbilical vein endothelial cell (HUVEC) was cultured in DMEM medium (HyClone) supplemented with 10% fetal bovine serum (HyClone). The cytotoxic effect of HSP990 on HUVEC viability was assessed by the XTT assay [32]. Briefly, cells (5×10^3 cells/well) were cultured in 96-well microtiter plates and treated with different concentrations of Hsp90 inhibitors for 24 h. At the end of incubation, 50 μL of PMS-XTT solution (final concentration, 50 μg of XTT and 0.38 μg of PMS per well) was added to each well and incubated at 37°C for 4 h. Absorbance at 450 nm was measured using the Eliza Plate Reader.

Biofilm formation and XTT reduction assay

The *in vitro* biofilm formation assay was performed according to the methods described previously [33] with some modifications. Briefly, the 100-μl aliquots of *C. albicans* cells (1.0×10^6 cells/ml) in RPMI 1640 medium were introduced into wells of 96-well tissue culture plates and were incubated statically at 37°C. After 1 h adhesion, the medium and non-adherent cells were removed, and the fresh RPMI 1640 was added. The plates were further incubated statically for 48 h at 37°C. A semiquantitative measure for the formation of biofilms was calculated using a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-terazolium-5-carboxanilide (XTT) reduction assay [34]. In order to investigate the effect of FLC in the absence or presence of HSP990 on the formation of *C. albicans* biofilms, different concentrations of the drugs wereadded to the fresh medium after 1 h adhesion. Following the incubation at 37°C for 48 h, biofilm cells were washed with phosphate-buffered saline (PBS) and incubated with 0.5 mg/ml XTT and 1 mM menadione in PBS at 37°C for 90 min. The optical density (OD) was measured at 490 nm using a microtitre plate reader.

Murine model of C. albicans infection

C. albicans 103 was cultured from -80°C stockon SDA agar platesand incubated at 30°C for 48 h. After grown in YPD broth for 14 h at 30°C at 200 rpm, cells were washed with PBS 3 times and diluted to the desired concentration (1×10^6 cells/ml) by hemacytometer counting. For murine exposure, on day 1, female C57BL/6 mice aged 6-8 weeks were infected via the tail vein with 100 μl of a 2×10^6 CFU/ml PBS suspension, and then randomly placed into three groups: untreated control group, FLC alone group and FLC+HSP990 group. At 1 h after infection, 0.5 mg/kg FLC was administeredintraperitoneally (i.p.) and 0.5 mg/kg HSP990 was administered by oral-gastric (OG) gavages, and then repeated on day 3. On day 4, mice were euthanized and the kidneys were removed aseptically and homogenized in 2 ml of PBS. Supernatants of kidney homogenates were serially diluted for measurement of kidney fungal burden. CFU values in kidneys were expressed as CFU/g of tissue.

Protein sample preparation and NanoLC-MS/MS

C. albicans 103 cells (OD_{600}=0.1) were treated or untreated with FLC (16 μg/ml) and/or HSP990 (64 μg/ml) at 30°C for 8 h in a shaking incubator with 200 rpm. Cells were washed with PBS (pH 7.4) 3 times, and then lysed in 5 ml of lysis buffer [50 mM Tris, 1.5 mM EDTA, 1% (v/v) Triton×100, 0.4% (w/v) SDS, pH 7.5] and 10 ml of 0.5 mm diameter glass beads (Biospec, Bartlesville, OK) by vortexing for 30 seconds and cooling on ice for 30 seconds

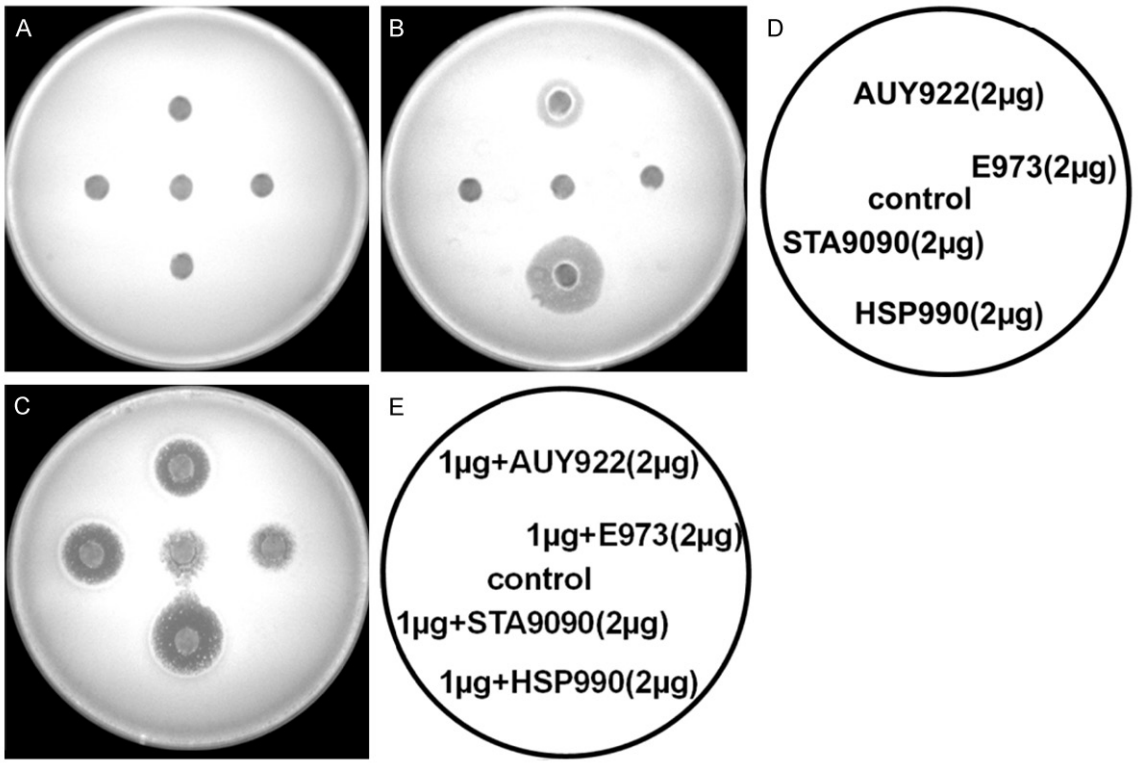


Figure 1. Agar disk diffusion assay of different Hsp90 inhibitors (AUY922, E973, HSP990, STA9090) combined with FLC against *C. albicans* 103. (A and C) show agar plates, and (B) shows an agar plate containing 1 μg/ml FLC. (D) describes the images for (A and B), containing 2 μg of different Hsp90 inhibitors and DMSO as control per disc. (E) describes the image for (C), the combination of different Hsp90 inhibitors (2 μg) with FLC (1 μg) or FLC (1 μg) alone as control were contained in each disc.

repeatedly in a Mini Bead-beater (Biospec) for 40 times. The clarified protein supernatant was determined using the RC DC Protein Assay Kit (Bio-Rad, Herclues, CA) after centrifugation at 4°C for 5 min at 13 000 g.

The identification of proteins with LC-MS/MS was performed asthe previously described method [35]. Briefly, protein abundance was calculated from all qualified corresponding peptides matched to that protein andfinal results were filtered using a 1% false discovery rate (FDR). Only proteins with at least one unique peptide and found in at least two replicates were considered identified.

Bioinformatics analysis

To investigate gene ontology (GO), the list of differential proteins with differential ratio of over ±1.2 and a nominal *p*-value of less than 0.05 by student's t test were in put into DAVID (the Database for Annotation, Visualization, and Integrated Discovery, http://david.abcc.ncifcrf.gov/) for functional annotation [36]. Briefly, the GO terms of "Biological Process", "Cellular Component" and "Molecular Function" were usedfor GO enrichment in DAVID with thedefault parameters on *C. albicans* gene. Protein-protein interaction (PPI) networks of the differential proteins were predicted and analyzed using STRING v9.1 (Search Tool for the Retrieval of Interacting Genes) database [37]. We constructed the PPI network based on confidence score, which implies that only interactions with the level of confidence were extracted from the database and considered as valid links, and used Cytoscape software for visual analysis of the constructed networks [38].

Statistical analysis

At least three biological replicates were performed for all experiments unless otherwise indicated. Analysis of variance (ANOVA) was used when multiple groups were analyzed and the two-tailed Student's t-test was used for analysis of two groups, with paired analysis when appropriate. Statistical significance was

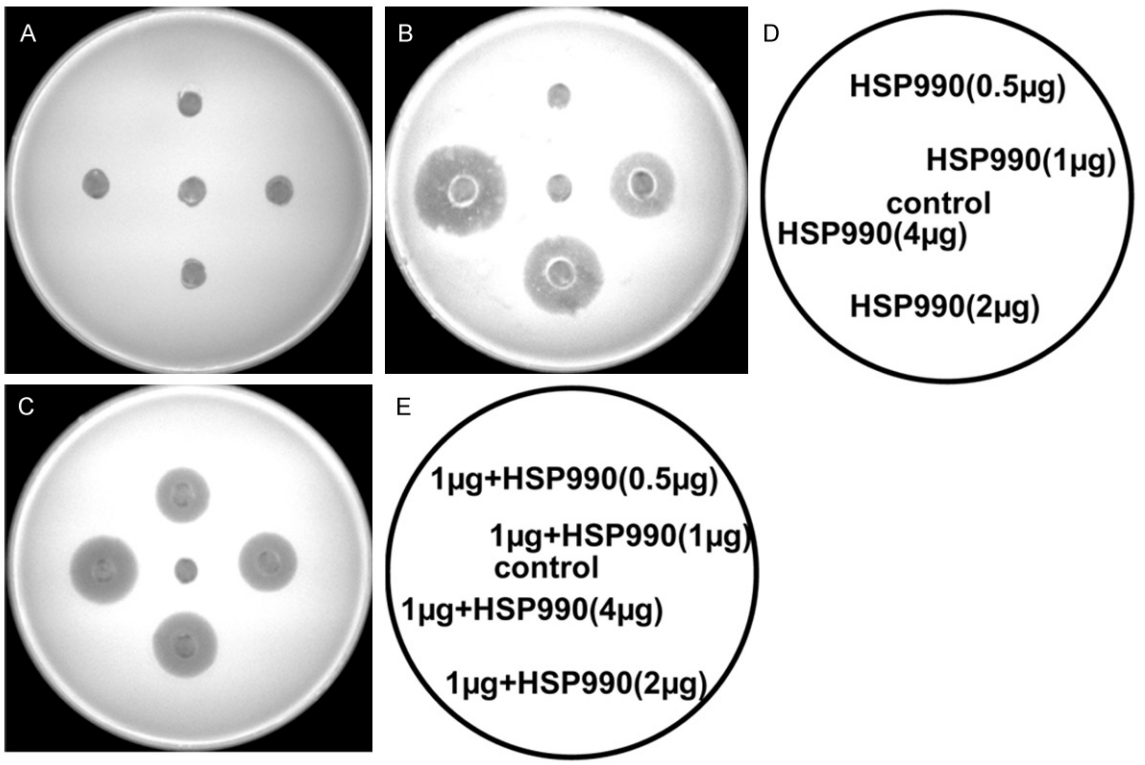


Figure 2. Agar disk diffusion assay of different concentrations of HSP990 combined with FLC against *C. albicans* 103. (A and C) show agar plates, and (B) shows an agar plate containing 1 μg/ml FLC. (D) Describes the images for (A and B), which contain 0.5, 1, 2, 4 μg of HSP990 and just DMSO as control per disc. (E) Describes the image for (C), the combination of HSP990 (0.5, 1, 2, 4 μg) with FLC (1 μg) and DMSO as control were contained in each disc.

set at a *p* value of 0.05, 0.01 or 0.001, indicated by *, **, ***, respectively.

Results

Hsp90 inhibitor HSP990 has powerful synergistic antifungal activity with FLC against FLC-resistant C. albicans

To determine if the synergy that has been reported with GdA or its derivatives and azoles previously [13, 19] was also observed with these non-Geldanamycin Hsp90 inhibitors (AUY922, E973, HSP990 and STA9090), we tested their effect on FLC-resistant *C. albicans* 103 when combined with FLC or not by agar diffusion tests (**Figure 1**). Four non-Geldanamycin Hsp90 inhibitors have no antifungal activity in small amounts at 2 μg alone (**Figure 1A**). AUY922, E973 and STA9090 showed weak synergistic antifungal effects when combined with FLC. In contrast, HSP990 showed potent synergistic antifungal activity (**Figure 1B**, **1C**). The mean diameters of the inhibitory zones on the agar plate with 1 μg/ml FLC were 9.0, 0, 18.5 and 0 mm for AUY922, E973, HSP990 and STA9090, respectively (**Figure 1B**). In addition, as shown in **Figure 1C**, the sizes of inhibitory zones were 13.0, 9.6, 16.5 and 14.3 mm around disks impregnated with 1 μg FLC plus Hsp90 inhibitors (AUY922, E973, HSP990 and STA9090), respectively. Thus, in small amounts (2 μg), HSP990 exhibited much more powerful synergistic antifungal activity with azoles on rich medium (YPD), when compared with other three Hsp90 inhibitors (AUY922, E973 and STA9090).

HSP990 creates a fungicidal combination with FLC at a low concentration

To investigate if the combination of FLC and HSP990 in lower dosage still exhibits the synergism, different concentrations of HSP990, FLC and the combination of HSP990 and FLC (1 μg) were analyzed by agar disk diffusion assay. HSP990 alone at 4, 2, 1, 0.5 μg per disc had no antifungal activity against the FLC-resistant *C. albicans* 103 (**Figure 2A**). HSP990 at 1 μg still showed synergistic antifungal effect on the

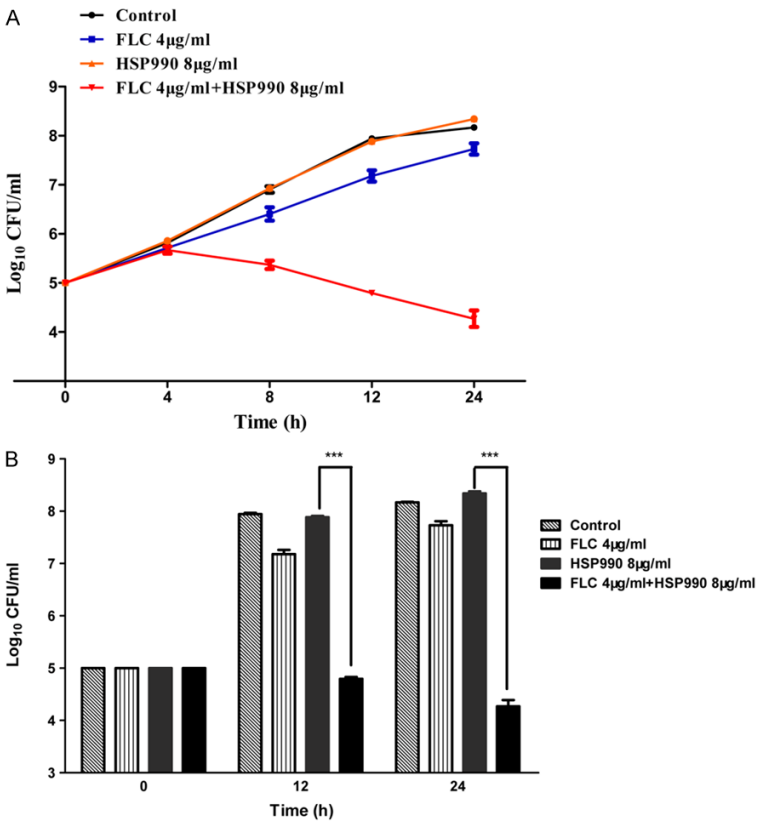


Figure 3. Time killing curves of *C. albicans* 103 treated with HSP990 and FLC. FLC-resistant *C. albicans* 103 were treated with FLC (4 μg/ml), HSP990 (8 μg/ml and FLC+HSP990 (4+8) μg/ml by using initial inoculums of 10^5 CFU/ml. Aliquots were obtained at the indicated time points and serially dilutions were spreaded on SDA agar plates. Colony counts were determined after 48 h incubation.

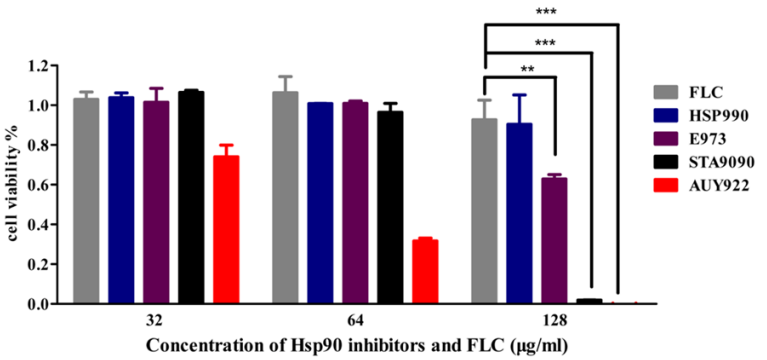


Figure 4. The in vitro cytotoxicity evaluation of Hsp90 inhibitors using the XTT assay.The cytotoxic effect of Hsp90 inhibitors (AUY922, E973, HSP990 and STA9090) towards HUVEC viability was assessed by an XTT test following a 4-h treatment, when compared to that of FLC. **, p<0.01; ***, p<0.001.

agar plate containing 1 μg/ml FLC (**Figure 2B**). The mean diameters of the inhibitory zones were 0, 15.5, 18.5 and 22.0 mm around disks in an agar plate containing 1 μg/ml FLC for 0.5, 1, 2 and 4 μg HSP990, respectively. Further, as shown in **Figure 2C**, the clear halos surrounding the disc were observed when different amounts of HSP990 (0.5, 1, 2 and 4 μg) was combined with FLC (1 μg), and the mean diameters of these zones were 12.0, 13.5, 15.5 and 16.5 mm, respectively.Thus, the non-Geldanamycin Hsp90 inhibitor HSP990 at lower concentration can reduce FLC-resistant *C. albicans* azole resistance in culture.

Their synergism was also confirmed by time-killing test (**Figure 3**). FLC (4 μg/ml) alonehad weak effect on the growth of *C. albicans* 103, while HSP990 (8 μg/ml) alone had no impact on viability of the clinical *C. albicans* 103 compared with control after 24 h (**Figure 3A**). However, the combination of HSP990 and FLC yielded 3.46-$\log_{10}$-CFU/ml decrease ($p<0.001$) compared with the group treated by FLC alone at 24 h (**Figure 3B**), exhibiting their powerful synergistic activity. Notably, relative to the untreated control with the beginning inoculum of 10^5 CFU/ml, the combination of HSP990 and FLC even blocked the growth of *C. albicans* 103 and yielded 0.73-$\log_{10}$-CFU/ml decrease (**Figure 3**), indicating the antifungal activity of FLC was converted into a fungicidal activity by the inhibition of Hsp90 with HSP990.

HSP990 has lower cytotoxicity when compared to other three Hsp90 inhibitors

It is necessary to evaluate the toxicity of Hsp90 inhibitors because of the clinic use. In the study, the *in vitro* cytotoxic effect of HSP990 against HUVEC was performed using an XTT

Table 1. MICs of HSP990 alone and in combination with FLC against 20 clinical FLC-resistant *C. albicans*

	MIC80 (μg/ml)	
	median	range
FLC	>64	>64
HSP990	32	32->64
FLC/HSP990[a]	0.5/1	0.25-0.5/1-1
FIC index	0.033	0.012-0.035
interaction effect (n/%)[b]	Syn (20/100)	

[a]MIC in combination expressed as [FLC]/[HSP990]. [b]Syn, synergism. The number of strains and percentage for the interaction effect were shown

Table 2. MICs of HSP990 alone and in combination with FLC against varied yeast strains

Yeast strains	MIC80 (μg/ml)			FICI
	FLC	HSP990	FLC/HSP990	
C. albicans SC5314	1	>64	1/1	1.008
C. neoformans 56992	4	>64	0.125/16	0.156
C. tropicalis ATCC20026	>64	>64	1/1	0.016
C. parapsilosis ATCC 22010	2	>64	2/1	1.008
C. krusei ATCC2340	64	>64	32/32	0.750
C. glabrata ATCC1182	8	>64	64/1	1.008

assay. As shown in **Figure 4**, HSP990 had no significant toxic effect on HUVEC at the concentration of 128 μg/ml, while the other three Hsp90 inhibitors significantly ($p<0.01$) reduced cell viability at the same concentration. Especially for AUY922, it reduced cell viability to less than 50% ($p<0.001$) even at the concentration of 64 μg/ml and almost completely blocked the growth of HUVEC (cell viability <0.1%, $p<0.001$) at the concentration of 128 μg/ml (**Figure 4**). The results indicated that HSP990 showed much lower toxicity when compared to other three Hsp90 inhibitors. Therefore, we used HSP990 as a promising and representativenon-Geldanamycin Hsp90 inhibitor to conduct all subsequent studies.

The combination of FLC and HSP990 against clinical FLC-resistant C. albicans and other-yeast strains

Due to the above potent synergism in combination with FLC when against FLC-resistant *C. albicans* 103, we further tested the synergistic activity of HSP990 in combination with FLC against 20 clinical strains of FLC-resistant *C. albicans* by Checkerboard microdilution assay. The MIC results of HSP990 alone or in combination with FLC against 20 clinical FLC-resistant *C. albicans* were summarized in **Table 1**. The MICs of FLC alone against all tested strains were >64 μg/ml, while the MICs of HSP990 alone ranged from 32 μg/ml to >64 μg/ml. The combination of FLC and HSP990 markedly reduced the MIC_{80}s, and the median FICI was 0.033 (range, 0.012 to 0.035). The powerful synergism were observed in all 20 FLC-resistant *C. albicans* (100%) tested, according to the analysis of FICI method.

In addition, we also confirmed the antifungal activity of HSP990 alone or in combination with FLC in other yeast strains (FLC-sensitive *C. albicans, C. neoformans, C. tropicalis*, *C. krusei*, *C. parapsilosis* and *C. glabratas*) (**Table 2**). The MICs of HSP990 alone against all these strains tested were all >64 μg/ml, and ranged from 1 μg/ml to 32 μg/ml, when in combination with FLC. In terms of MIC_{80}s, the synergistic activity was not observed in *C. parapsilosis*, *C. glabratas*and *C. krusei*. However, in *C. tropicalis*, the MIC of FLC was reduced from >64 μg/ml to 1 μg/ml when in combination with HSP990. Interestingly, no synergism was shown when the combination of FLC and HSP990 against FLC-susceptible*C. albicans* SC5314.

The combination of FLC and HSP990 inhibits the formation of C. albicans biofilms in vitro

The effect of FLC combined with HSP990 on *C. albicans* biofilm formation was assessed by XTT reduction assay (**Figure 5**). The result showed that addition of HSP990 alone to *C. albicans* cells after 1-h adhesion did not inhibit biofilm formation and had no significant effects on *C. albicans* biofilm viability, which was also observed with other Hsp90 inhibitors in previous report [15]. However, when combined with 16 μg/ml FLC, which tested alone has no significant effect on the viability of biofilm cells, HSP990 could enhance the activity of FLC against *C. albicans* biofilm formation in a dose-dependent manner as shown in **Figure 5**. Notably, when at the concentration of 8 μg/ml, HSP990 in combination with 16 μg/ml FLC

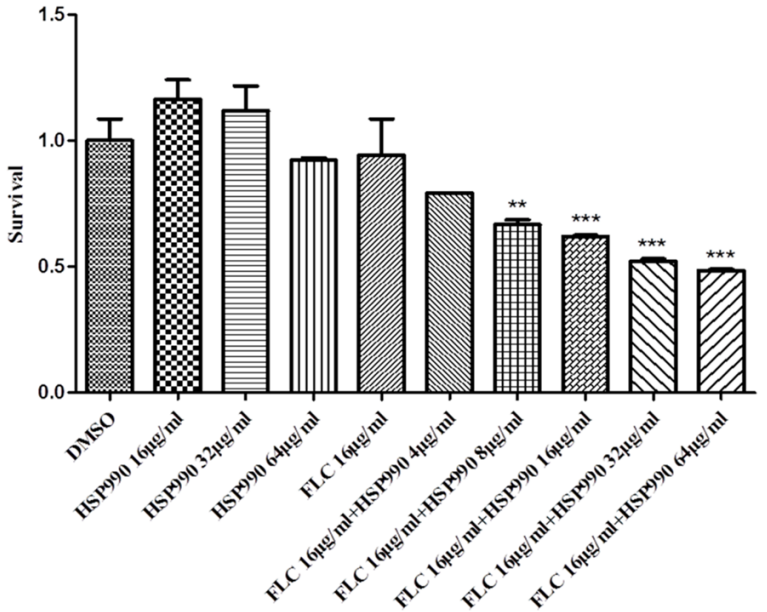


Figure 5. The combination of Hsp90 inhibitor HSP990 and FLC inhibits *C. albicans* biofilm formation in vitro. Effects of different concentrations of HSP990 alone and in combination with 16 μg/ml FLC on *C. albicans* biofilm formation were evaluated by the XTT reduction assay through calculating the percent of viable *C. albicans* cell relative the control cells without drug treatment. Data are shown as means ± standard deviation. **, p<0.01; ***, p<0.001 compared with the value of the group treated with FLC alone.

exhibited significant inhibition of biofilm formation (P<0.01), and the anti-biofilm effect increased with the increase of HSP990 concentrations. The combination of 16 μg/ml FLC and 64 μg/ml HSP990 dramatically compromised biofilm formation (P<0.001), resulting in less than 50% viable biofilm cells when compared with the group treated with FLC alone.

Combination therapy with HSP990 enhanced the therapeutic efficacy of FLC

To detect antifungal drug efficacy and evaluate the benefits of combination therapy with FLC and HSP990, we used a well-established murine model in which fungal inoculum is delivered by tail-vein injection. Treatment of mice with FLC significantly (p<0.001, Nonparametric test) reduced fungal burden compared to the untreated counterparts (**Figure 6**). Notably, the combination therapy with FLC and HSP990 further enhanced the *in vivo* efficacy of FLC alone, and resulted in significant (p<0.05, Nonparametric test) difference in fungal burden compared to treatment with FLC on its own (**Figure 6**). The findings provide an attractive compound which would abrogate drug resistance and enhance the antifungal efficacy of existing drugs, and a new therapeutic clue for fungal disease.

The synergism of FLC and HSP990 promotes degradation of Hsp90 client proteins and interactors investigated by proteomic analysis

Hsp90 interacts with about 10% of the proteome in yeast and functions in drug resistance to azoles inhibiting ergosterol biosynthesis through the regulation of client proteins relative to azole resistancein *C. albicans* [13, 19, 39]. To gain an further insight into the possible synergistic mechanism of FLC and HSP990 against FLC-resistant *C. albicans*, we carried out a comprehensive comparative proteomic analysis among FLC-resistant *C. albicans* 103 cells treated or untreated with FLC (16 μg/mL) and/or HSP990 (64 μg/mL) using nano LC-MS/MS (**Figure 7A**, **7B**), and focused on differential proteins from comparison 3 (FLC+HSP990-treated cells versus FLC-treated cells) to perform GO category enrichment and network analysis using DAVID and STRING.

The GO enrichment analyses showed that the combination of FLC and HSP990 significantly influenced some key GO categories, such as translation factors, Heat shock protein family chaperones, and diverse enzymes (**Figure 7C**). Compared with cells treated with FLC alone, proteins interacting with Hsp90 were remarkably decreased in cells treated with FLC+HSP990 (**Figure 7C**, **7D**), indicating the inhibition of Hsp90 could promote the dramatic degradation of all these Hsp90 interactors,such as translation elongation factors, diverse enzymes relevant to protein processing, and other chaperones. Particularly, CaMsi3, an essential HSP70 family protein up-regulated by FLC exposure and required for calcineurin-dependent transcription [40], was significantly

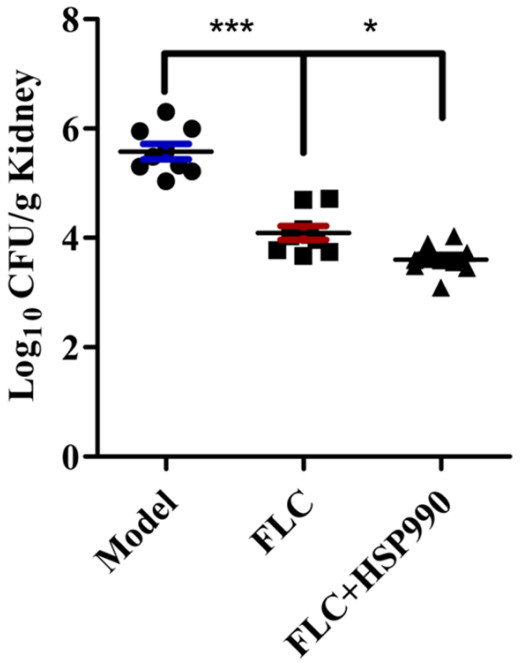


Figure 6. Pharmacological inhibition of Hsp90 inhibitor HSP990 enhances the therapeutic efficacy of FLC in a murine model of disseminated disease. C57BL/6 mice were infected with an inoculum of 200 μl of a 1×106 CFU/ml of FLC-resistant *C. albicans* 103. 0.5 mg/kg FLC was administered IP and 0.5 mg/kg HSP990 was administered by oral gavages at 1 h after infection (day 1) and then repeated on day 3.

down-regulated in FLC+HSP990-treated cells (**Figure 7D**). Notably, the combination treatment of FLC and HSP990, compared to FLC alone, leaded to depletion of the Ca++ binding protein CaCmd1 (**Figure 7D**), regulating Ca++ dependent processes and targeting the protein phosphatase calcineurin known as a criticalmediator of Hsp90-dependent drug resistance [41, 42].

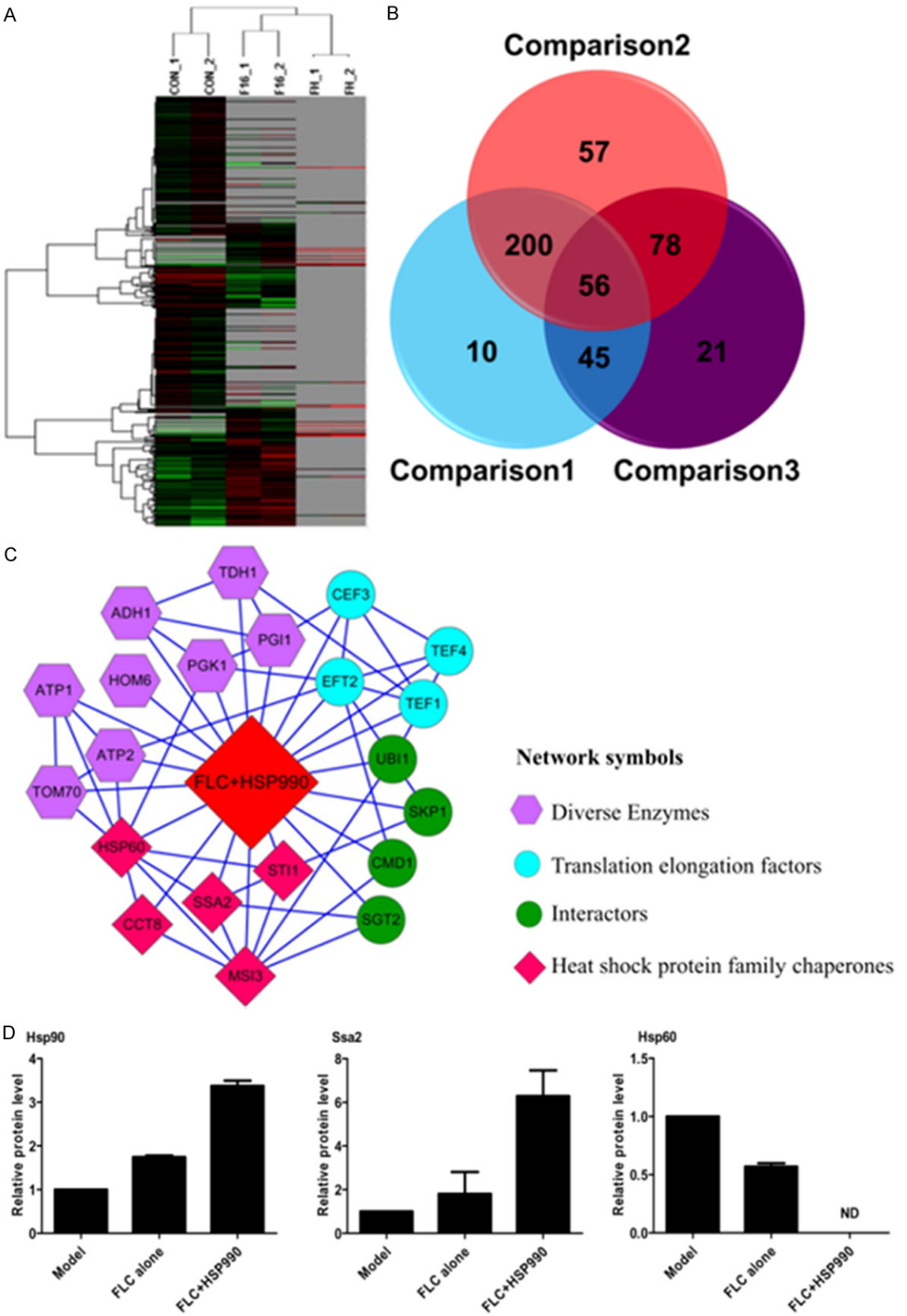
Discussion

Invasive fungal infections are a leading cause of human mortality, especially among immunocompromised individuals, and *C. albicans* is the most common cause of hospital-acquired infectious disease and systemic candidiasis [2, 4, 43]. Moreover, there are the frequent emergence and maintenance of drug resistance in diverse fungi, and large part of antifungal drugs with high toxicity to human because of the close relation between fungi and their human hosts, resulting in the development of new therapeutic strategies for fungal infectious disease.

In recent years, studies of Hsp90's role in drug resistance have been carried out most widely in *Candida* species. A vast number of reports have established that Hsp90 mediates drug resistance in *C. albicans*, and targeting fungal Hsp90 has been a promising strategy for the treatment of fungal infections [44, 45]. Hsp90 regulates drug resistance through its downstream effectors. The client protein phosphatase calcineurin and Mkc1 which is the terminal kinase of the Pkc1-regulated MAPK cascade are two key mediators of Hsp90-dependent drug resistance to azole in *C. albicans* planktonic cells [46]. Calcineurinis part of a membrane stress survival pathway and regulates crucial cellular responses to the membrane stress induced by azole in *C. albicans* [41, 42]. Hsp90 stabilizes calcineurin and Mkc1 by directly interacting with them, so the reduction of Hsp90 level or the inhibition of Hsp90 functions leads to the destabilization or depletion of client proteins calcineurin and Mkc1 in *C. albicans* planktonic cells [44-46].

In *C. albicans*, previous documents have showed that pharmacological inhibition of Hsp90 blocks the rapid evolution of azole resistance [13, 19]. Inhibition of Hsp90 function with Hsp90 inhibitors such as GdA or its derivatives creates a fungicidal combination with azoles [13, 18, 19]. However, considerable toxicity has been also observed with Hsp90 inhibitors GdA or its derivatives in a murine model infected with *C. albicans*, suggesting the challenge that further development of this combination therapy requires the identification of fungal selective Hsp90 inhibitors based on divergence between human hosts' and fungal Hsp90 orthologues in terms of sequence or conformational states in order to ameliorate host toxicity problems.

In this study, we revealed the potent synergistic activity of non-Geldanamycin Hsp90 inhibitor HSP990 in combination with FLC against FLC-resistant *C. albicans* and *C. albicans* biofilms. In addition, we investigated the possible synergistic mechanism of FLC and HSP990 through proteomic analysis (**Figure 8**). Further studies are required toconfirm these hypotheses. In the near future, we plan to further identify the detail synergisticmechanisms and client proteins targeted by Msi3 and Cmd1 in *C. albicans*.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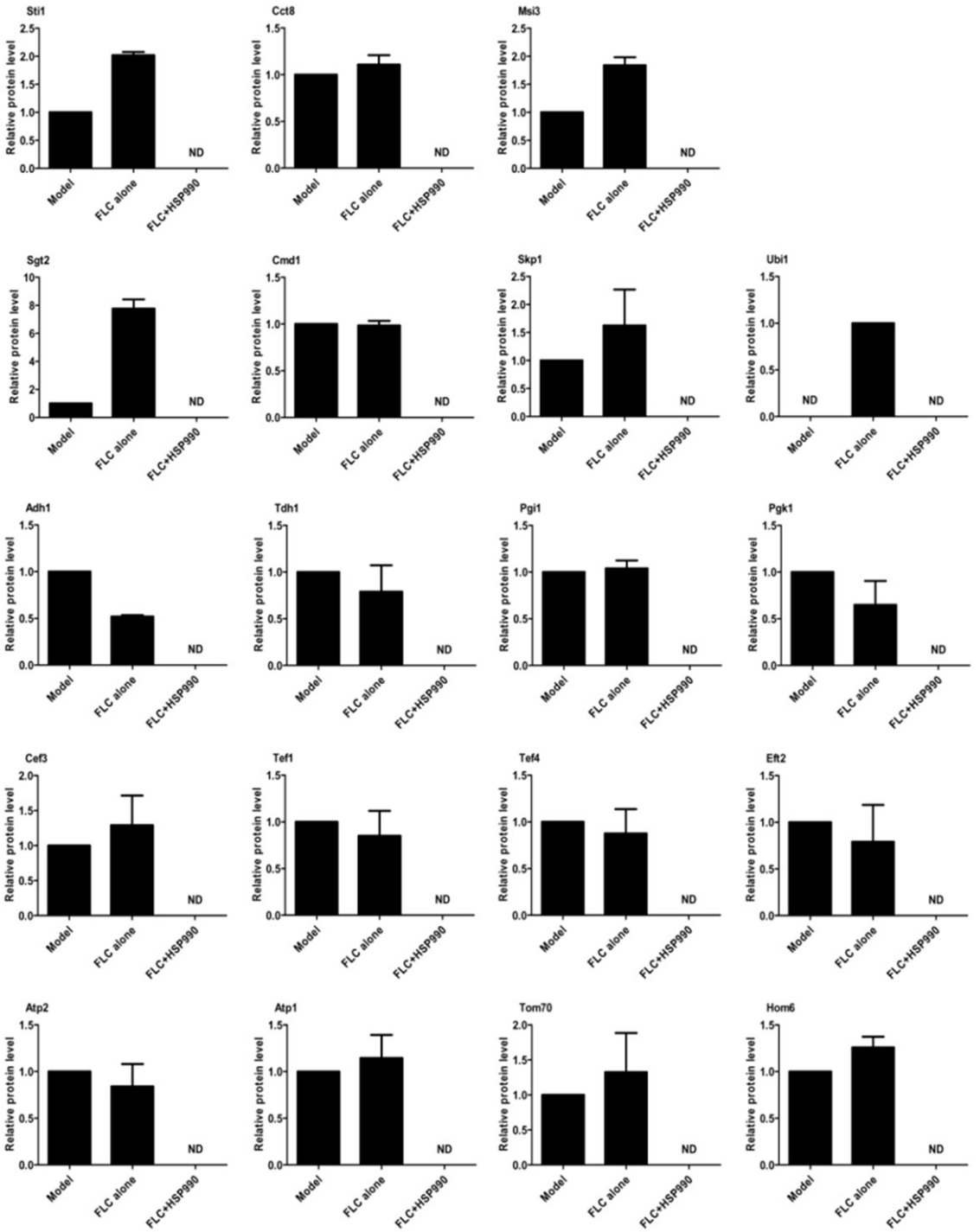

Figure 7. The proteomics of *C. albicans 103* treated with Hsp90 inhibitor HSP990 and FLC using LC-MS/MS. (A) Cluster analysis of protein level based on LC-MS/MS data, depicting the differentiation of protein level among samples treated by different drugs (Con: the untreated Control; F16: samples treated by 16 μg/ml FLC; FH: samples treated by 16 μg/ml FLC and 64 μg/ml HSP990). (B) Venn diagram for differential proteins in paired comparisons. Comparison 1: differential proteins identified in FLC-treated sample versus Control sample; Comparison 2: differential proteins identified in FLC+HSP990-treated sample versus Control sample; Comparison 3: differential proteins identified in FLC+HSP990-treated sample versus FLC-treated sample. (C) The interaction network of differential proteins associated with C. albicans Hsp90. Network symbols indicate the classification of these differential proteins from Comparison 3 (FLC+HSP990-treated sample versus FLC-treated sample). (D) The relative protein level of differential proteins compared to Control, corresponding to proteins shown in (C).

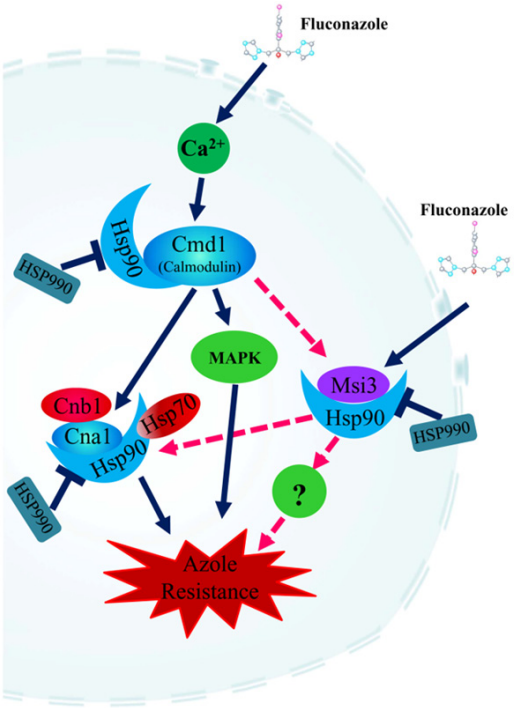

Figure 8. The scheme of a proposed model of fluconazole resistance by the activation of diverse signaling pathways. Solid and dashed arrow lines indicate known pathways and putative pathways predicted in the present study, respectively. Under fluconazole exposure, upregulation of CaMsi3 level might have activated the calcineurin signaling pathway or other unknown pathways required for azole resistance. The inhibition of Hsp90 function by HSP990 leaded to significantly reduced levels of CaMsi3 and CaCmd1 which is a component of the calcineurin signaling pathway. we propose that Msi3 functions in azole resistance cooperatively with Hsp90 as a cochaperone through the activation of the calcineurin signaling pathway or some unknown mechanisms.

In a summary, the non-Geldanamycin Hsp90 inhibitor HSP990 has powerful synergistic antifungal activity with FLC against FLC-resistant *C. albicans* and *C. albicans* biofilm formation. Further, the combination of FLC and HSP990 shows the fungicidal activity. Notably, combination therapy with Hsp90 inhibitor HSP990 and FLC exhibits better therapeutic efficacy in a murine model of disseminated *C. albicans*disease compared with FLC alone. At last, we have investigated that some pathways mediated by *CaCMD1* and *CaMSI3* played an essential role in enhancing the antifungal efficacy of FLC by Hsp90 inhibitors, which provides useful information for the development of new strategies to enhance the activity of antifungal agents.

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Disclosure of conflict of interest

None declared.

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