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Research Article

In Vitro Antifungal Activity and Mode of Action of 2′,4′-Dihydroxychalcone against *Aspergillus fumigatus*

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Abstract 2′,4′-Dihydroxychalcone (2′,4′-DHC) was identified from a heat shock protein 90 (Hsp90)-targeting library as a compound with Hsp90 inhibitory and antifungal effects. In the presence of 2′,4′-DHC (8 μg/mL), radial growth of *Aspergillus fumigatus* was inhibited 20% compared to the control, and green pigmentation was completely blocked. The expression of the conidiation-associated genes *abaA*, *brlA*, and *wetA* was significantly decreased (approximately 3- to 5-fold) by treatment with 2′,4′-DHC. The expression of calcineurin signaling components, *cnaA* and *crzA*, was also significantly reduced. The inhibitory effects of 2′,4′-DHC on metabolic activity and mycelial growth were significantly enhanced by combination treatment with itraconazole and caspofungin. Docking studies indicated that 2′,4′-DHC bind to the ATPase domain of Hsp90. These results suggest that 2′,4′-DHC act as an Hsp90-calcinurin pathway inhibitor.

Keywords 2′,4′-Dihydroxychalcone, Antifungal activity, *Aspergillus fumigatus*, Hsp90 inhibitor

Invasive aspergillosis caused by *Aspergillus fumigatus* is a leading cause of infection and death in immunocompromised patients [1, 2]. Early detection and proper treatment can improve clinical outcomes [1]. Although *A. fumigatus* infections can be treated with triazole, polyene, or echinocandin drugs [3-5], their efficacy is limited as the mortality of invasive aspergillosis remains high. In addition, the clinical efficacy of the available drugs has decreased, due to the emergence of drug resistance. Therefore, there is a pressing need for new therapeutic strategies for life-threatening fungal infections. Heat shock protein 90 (Hsp90) in fungal pathogens has emerged as a promising target for new antifungals to improve the efficacy of existing antifungal drugs and to overcome drug resistance [6, 7].

Recently, we have launched a program to develop potent Hsp90 inhibitors against fungal pathogens. Our research on Hsp90 led to the development of target-focused compound libraries [8, 9]. A screening campaign using the target-focused libraries led to the discovery of 2′,4′-dihydroxychalcone (2′,4′-DHC), which exhibited antifungal activity against *A. fumigatus*.

Chalcones obtained by synthesis and isolated from *Zuccagnia punctate* (Fabaceae) exhibit a diverse range of pharmacological effects, including anticancer, antioxidant, and antibiotic activities [10-12]. 2′,4′-DHC showed moderate antifungal activities against the yeasts and strong antifungal activities against dermatophytic fungi [11]. 2′,4′-DHC would act by a different mechanism of action from the current clinical antifungal drugs, such as azoles or echinocandins, and the mode of action was yet to be elucidated. In the present paper, we suggest the mode of action of 2′,4′-DHC against *A. fumigatus*.

MATERIALS AND METHODS

Strain and chemicals. Antifungal agents were evaluated against wild-type *A. fumigatus* Af293 (Fungal Genetics Stock Center, Kansas City, MO, USA). Caspofungin (CSP) and itraconazole (ITC) were purchased from Sigma Chemical

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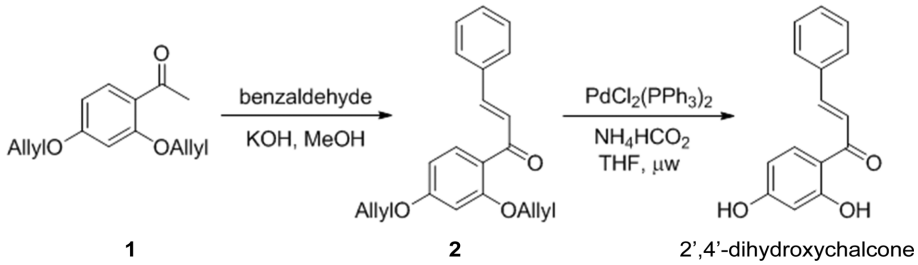

Fig. 1. Synthetic process for 2′,4′-dihydroxychalcone (2′,4′-DHC).

Co. (St. Louis, MO, USA). 2′,4′-DHC was synthesized as follows. A mixture of compound **1** (0.30 g, 1.29 mmol), benzaldehyde (0.14 mL, 1.42 mmol), and KOH (0.6 g) in 12 mL methanol was stirred at room temperature for 4 days. The mixture was neutralized with 1 N HCl to pH 6, and then extracted with ethyl acetate. The organic layer was washed with a saturated sodium bicarbonate solution three times, dried over sodium sulfate, concentrated under reduced pressure, and purified by medium pressure liquid chromotography (MPLC; Biotage SNAP HP-Sil column; Biotage, Uppsala, Sweden) to produce compound **2** with a 73% yield. The resulting compound **2** was stirred under microwave irradiation (Biotage Initiator) for 30 min at 120°C in the presence of bis(triphenylphosphine) palladium (II) dichloride (13 mg) and ammonium formate (80 mg) in tetrahydrofuran (4 mL). The reaction mixture was diluted with ethyl acetate. The organic layer was washed with water, dried over sodium sulfate, concentrated under reduced pressure, and purified by MPLC to produce 2′,4′-DHC with a 39% yield: $R_f = 0.24$ (1 : 4 ethyl acetate: hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.41 (s, 1H), 7.88 (d, $J = 15.6$ Hz, 1H), 7.84 (d, $J = 9.2$ Hz, 1H), 7.66~7.63 (m, 2H), 7.57 (d, $J = 15.2$ Hz, 1H), 7.44~7.42 (m, 3H), 6.47 (d, $J = 2.4$ Hz, 1H), 6.45 (s, 1H); ESI MS (*m/e*) = 241 $[M+1]^+$ (Fig. 1).

Susceptibility assays. Antifungal susceptibility assay was performed by a previously described modification of the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method [13]. RPMI 1640 medium with L-glutamine and without sodium bicarbonate was buffered with 165 mM morpholinepropanesulfonic acid buffer (pH 7.0) and was used as the test medium. All test compounds were solubilized in dimethyl sulfoxide at 1.28 g/L. The compounds were serially diluted to final drug concentrations ranging from 0.06 to 256 μg/mL. Inoculum suspensions were prepared by a hemocytometric procedure and diluted in RPMI 1640 containing 0.2 mg of MTT per mL to obtain an inoculum ranging from 1×10^4 to 5×10^4 conidia per mL. The same volume (100 μL) of the inoculum and the compounds was then added to microplates, which resulted in a final MTT concentration of 0.1 mg/mL. The plates were then incubated at 37°C for 48 hr. After incubation, the formazan assay product was extracted, and the optical density at 540 nm was measured. Experiments were performed in triplicate independently.

Real-time reverse transcription-PCR (RT-PCR). To investigate the link between antifungal agents and conidiation, the expression of the *brlA*, *abaA*, and *wetA* genes, which regulate asexual development, was assessed. In addition, to assess the effect of the test drug on the calcineurin pathway, the expression of *cnaA* and *crzA* was analyzed. Conidial suspensions (5×10^5 conidia/mL) were inoculated in glucose minimal medium (MMG) medium [14] and grown for 48 hr at 37°C. RNA extraction, cDNA synthesis, and RT-PCR were performed as previously described [15]. The expression ratios were normalized to elongation factor 1α expression, and were calculated according to the DDCt method [16]. All experiments were performed in triplicate, and data were presented as the mean ± standard deviation (SD).

Microscopy. Micrographs were acquired using an Olympus Inverted Microscope IX50 equipped with a Lumenera Infinity camera (Olympus Corporation, Tokyo, Japan).

Statistical analyses. The unpaired Student's *t*-test was used for comparison of two separate sets of independent samples. A *p*-value less than 0.05 was considered statistically significant. The MTT conversion rates in the treatment groups and controls were compared using ANOVA followed by a *post hoc* Tukey comparison. Differences were considered significant when the *p*-value was less than 0.05. Statistical analyses were performed with IBM SPSS statistics ver. 21.0 (IBM, Armonk, NY, USA).

Analyses of domain structure and docking studies. The domain structure of Hsp90 was analyzed using the domain analysis site SMART (http://smart.embl-heidelberg.de), and protein alignment was performed using EMBOSS needle (ver. 6.6.0) (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). *In silico* docking of 2′,4′-DHC with yeast Hsp90 (PDB code: 2XX5) was accomplished using the AutoDock4.2 program downloaded from the Molecular Graphics Laboratory at Scripps Research Institute. The AutoDock4.2 program was chosen because it uses a genetic algorithm to generate the poses of the ligand inside a known or predicted binding site utilizing the Lamarckian version of the genetic algorithm, where the changes in conformations adopted by molecules after *in situ* optimization are used as subsequent poses for the offspring. In the docking experiments, Gasteiger charges

were placed on the X-ray structures of the N-terminal domain of Hsp90, along with 2′,4′-DHC, using tools from the AutoDock suite. A grid box centered on the N-terminal Hsp90 domain with definitions of 50_50_50 points and 0.375 Å spacing was chosen for the ligand docking experiments. The docking parameters consisted of setting the population size to 150, the number of generations to 27,000, and the number of evaluations to 25,000,000 while the number of docking runs was set to 100 with a cutoff of 1 Å for the root-mean-square tolerance of the grouping during each docking run. The docking model of yeast Hsp90 with 2′,4′-DHC is depicted, and rendering of the picture was generated using PyMol (DeLano Scientific, Palo Alto, CA, USA).

RESULTS

The antifungal activity of 2′,4′-DHC was tested, and the minimum inhibitory concentration (MIC) was determined. As shown in Fig. 2A, 2′,4′-DHC was effective against *A. fumigatus*. 2′,4′-DHC more than 64 μg/mL caused a significant decrease in MTT conversion activity. The MIC_{50} (MIC that inhibits 50% of growth) of 2′,4′-DHC was between 64 and 128 μg/mL. The inhibitory effect of 2′,4′-DHC was also examined using inverted microscopy. A clear visual difference in mycelia density and growth rate was observed at 64, 128, and 256 μg/mL (Fig. 2B). Treatment with 256 μg/mL of 2′,4′-DHC drastically decreased mycelial growth.

Radial growth of *A. fumigatus* in RPMI 1640 agar medium was inhibited by treatment with the test compound. 2′,4′-DHC (8 μg/mL) reduced the relative radial growth by 20% compared to the control. In addition, the treated colonies lacked green pigmentation, suggesting they formed few or no conidia (Fig. 3A).

These phenomena were clearly observed in liquid culture as well. As shown in Fig. 3B, green pigmentation was completely lacking after incubation with 2′,4′-DHC. Because of the strong inhibition of conidiation with drug treatment, we quantified the expression of *abaA*, *brlA*, and *wetA* genes encoding transcription factors that control asexual development (conidiation) in *Aspergillus* species [17, 18]. Compared to the control, the expression of all three genes was significantly decreased (about 3- to 5-fold) by treatment with 2′,4′-DHC (Fig. 3C).

The calcineurin signaling pathway is necessary for proper hyphal growth, and is activated in response to cell wall stress in *A. fumigatus* [19, 20]. To investigate the effects of 2′,4′-DHC in calcineurin signaling, we quantified the expression of two major signaling components, *cnaA* and *crzA*. While the expression of *cnaA* was decreased approximately 1.5-fold by 2′,4′-DHC, the expression of the zinc finger transcription factor, *crzA* (downstream of *cnaA*), was significantly reduced in the presence of 2′,4′-DHC (Fig. 4A). The extent of inhibition was greater for *crzA* than for *cnaA*.

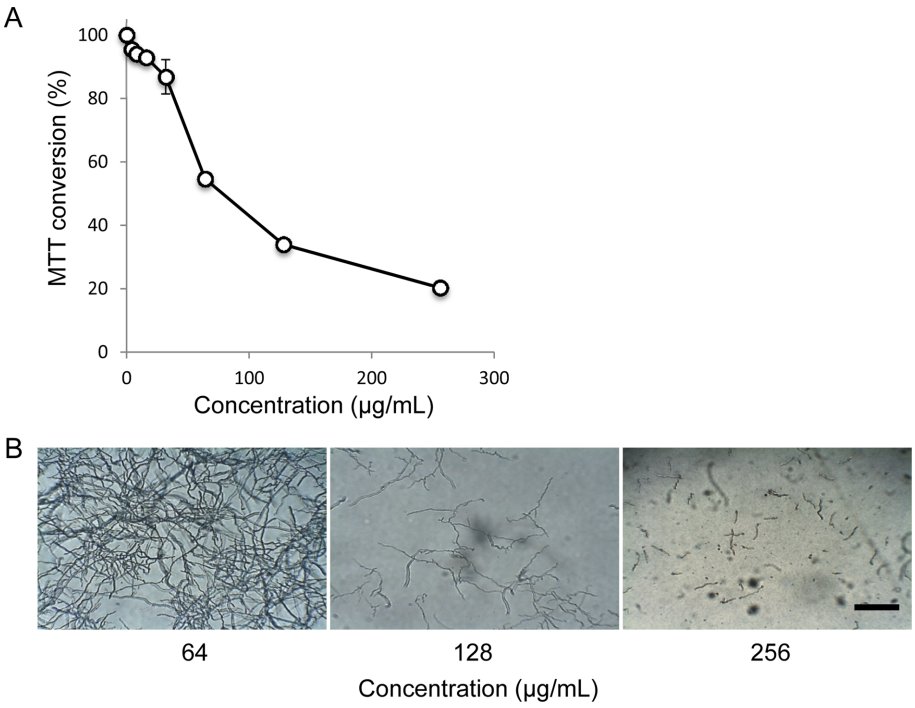

Fig. 2. A, Susceptibility of *Aspergillus fumigatus* Af293 to 2′,4′-dihydroxychalcone (2′,4′-DHC) in the modified 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Conidia suspensions were diluted in RPMI 1640 containing a final MTT concentration of 0.1 mg/L, and the plates were incubated for 48 hr. After incubation, the formazan reaction product was extracted and determined; B, Representative inverted microscopy images of *A. fumigatus* Af293. Visual differences in the mycelial growth are apparent at three different 2′,4′-DHC concentrations (64, 128, and 256 μg/mL) (scale bar = 200 μm).

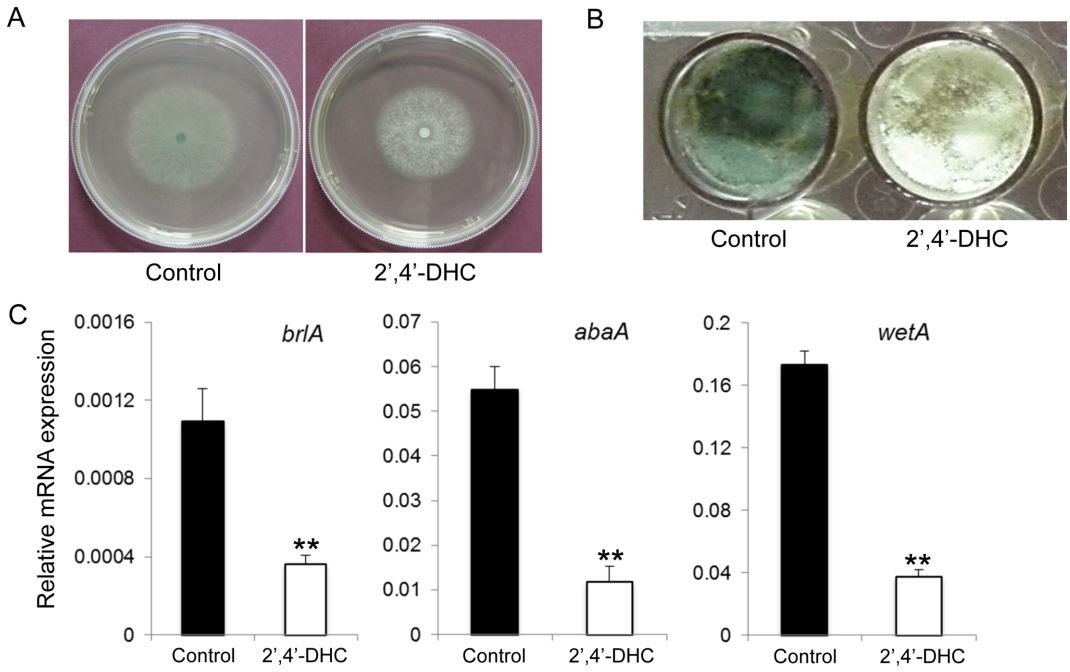

Fig. 3. *In vitro* antifungal activity of 2′,4′-dihydroxychalcone (2′,4′-DHC; 8 μg/mL) against *Aspergillus fumigatus* Af293. Hyphal growth and pigment formation defects after 2′,4′-DHC treatment in solid RPMI 1640 media (A) and liquid glucose minimal medium (B). C, Quantification of conidiation-specific gene transcription (*abaA*, *brlA*, and *wetA*) by real-time PCR. Data are presented as the mean ± SD, $^{**}p < 0.01$.

It has been reported that CrzA plays a partial role in azole and echinocandin tolerance in yeast [21]. We assessed the activity of 2′,4′-DHC when tested in combination with ITC (azole, 0.2 μg/mL) and CSP (echinocandin, 2.0 μg/mL). While ITC alone showed minimal inhibitory activity, combined treatment of 2′,4′-DHC (64 μg/mL or greater) with ITC significantly enhanced the inhibitory activity (Fig. 4B). Furthermore, combination of CSP and increasing concentrations of 2′,4′-DHC progressively inhibited *A. fumigatus*, and completely inhibited growth at a concentration of 256 μg/mL (Fig. 4B). The inhibitory effect of the antifungals ITC and CSP in combination with 2′,4′-DHC was also examined using light microscopy. Mycelial growth was substantially reduced in combined treatments. Interestingly, the germination rate was also decreased when 2′,4′-DHC was used in combination with ITC (Fig. 4C).

A. fumigatus Hsp90 (AFUA_5G04170) is composed of 706 amino acid residues, and contains an N-terminal HATPase c domain (AA 28-177) and an Hsp90 domain (AA 183-706). Hsp90 proteins from *A. fumigatus* and *Saccharomyces cerevisiae* are highly conserved (Fig. 5A), showing 75.3% identity and 85.8% similarity each other, and the possible 2′,4′-DHC interacting amino acids completely coincide (Fig. 5B). To delineate the biding mode of 2′,4′-DHC in the ATP-binding site of Hsp90, docking studies were performed using the *S. cerevisiae* Hsp90 crystal structure (PDB code: 2XX5), its native ligand 13N, and 2′,4′-DHC. 2′,4′-DHC was docked with the 3D coordinates of the Hsp90 N-terminal domain using AutoDock4.2 program. Comparison of 2′,4′-DHC and 13N in the ATP-binding site of Hsp90 revealed that 2′,4′-DHC bound to Hsp90 in a similar manner to its native ligand 13N (Fig. 5C, upper panel). The resorcinol ring and the phenyl ring of 2′,4′-DHC superimposed with the resorcinol ring and the phenyl ring of 13N in the ATP-binding site of Hsp90, while the enone moiety of 2′,4′-DHC adopted a different conformation from the macrolactam ring of 13N. In particular, two hydroxyl groups of the resorcinol ring and the carbonyl oxygen atom of 2′,4′-DHC formed hydrogen bonds with Asp79, Asn37, and a conserved water molecule in the hydrophilic region of the pocket. Meanwhile, the phenyl ring of 2′,4′-DHC projected into the hydrophobic region lined by amino acid residues Met84, Leu89, Phe124, Val136, and Trp148, and made close lipophilic contacts to Met84, Leu89, Phe124, Val136, and Trp148 residues (Fig. 5C). Collectively, the hydrogen-bonding and hydrophobic interactions of the resorcinol ring, the carbonyl oxygen atom, and the phenyl ring of 2′,4′-DHC contributed to the binding of 2′,4′-DHC to yeast Hsp90, and estimated binding energy (ΔGb) and inhibition constants (Ki) using the Lamarckian genetic algorithm were −7.65 kcal/mol and 2.46 μM, respectively.

DISCUSSION

Invasive aspergillosis is one of the most frequent causes of infection and death in immunocompromised patients [1, 2]. In the expanding population of immunocompromised patients, treatment of *Aspergillus* infections is a challenge,

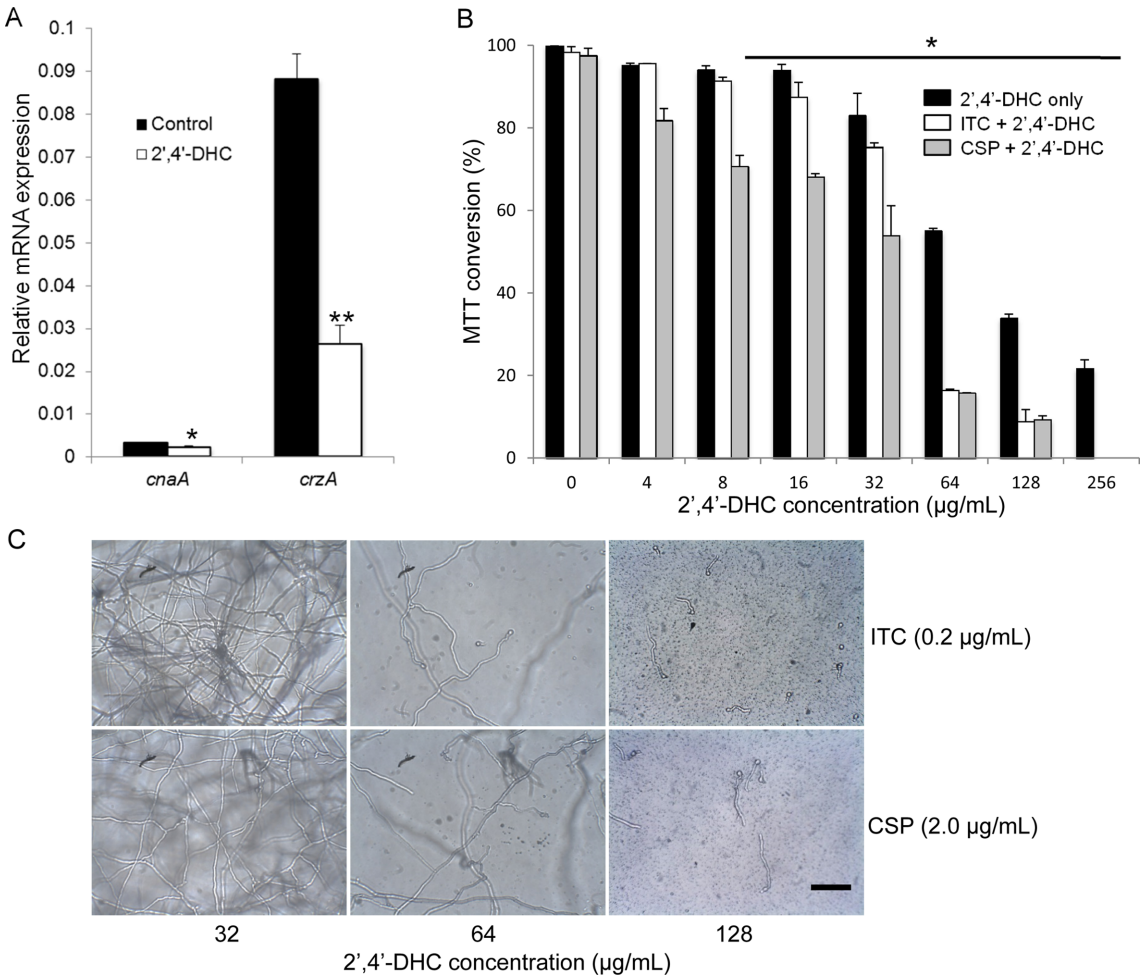

Fig. 4. A, Quantification of the transcription of calcineurin (*cnaA*) and the transcription factor *crzA* by real-time PCR in the presence of 2′,4′-dihydroxychalcone (2′,4′-DHC; 8 μg/mL). Data are presented as the mean ± SD, $^{**}p < 0.01$ and $^{*}p < 0.05$; B, *In vitro* effect of different concentrations of 2′,4′-DHC (0 to 256 μg/mL) combined with a constant concentration of itraconazole (ITC; 0.2 μg/mL) and caspofungin (CSP; 2.0 μg/mL). Graphs represent the mean ± SD, $^{*}p < 0.05$; C, Representative inverted microscopy images of *Aspergillus fumigatus* Af293. Visual differences in the mycelial growth are apparent at three different 2′,4′-DHC concentrations (32, 64, and 128 μg/mL) (scale bar = 50 μm).

and there is a need for new antifungal agents with broad-spectrum activity, little or no drug resistance, and reduced adverse effects compared to currently available drugs.

After screening a number of compounds from our chemical library, we selected a new Hsp90 inhibitor, 2′,4′-DHC, and examined its potential activity against *A. fumigatus*. 2′,4′-DHC had moderate levels of antifungal activity (Fig. 2A). In microscopic observations, 2′,4′-DHC showed inhibitory effects on mycelial growth (Fig. 2B).

The radial growth in solid media was also inhibited by 2′,4′-DHC (Fig. 3A), suggesting that 2′,4′-DHC may affect cell wall integrity and Hsp90 activity. Hsp90 is possibly linked with the β-1,3-glucan synthesis axis, and may play an important role in *A. fumigatus* growth and the maintenance of cell wall integrity [22]. In addition, pigmentation was completely blocked (Fig. 3B), and the expression of the conidiation-specific genes *abaA*, *brlA*, and *wetA* was decreased significantly in the presence of 2′,4′-DHC (Fig. 3C). The *brlA* gene is the first key transcription factor activated during conidiation, which then activates *abaA* in the middle stage of conidiation [18]. Thereafter, *wetA*, is sequentially activated by *abaA* in the late phase of conidiation [18]. These genes were also involved in the synthesis of conidial wall pigment, through regulation of *velvet* complex (*veA*, *velB*, and *velC*) expression [18]. Recently, Hsp90 repression was associated with decreased conidia formation and a significant decrease in the expression of the *abaA*, *brlA*, and *wetA* in *A. fumigatus* [22].

In previous studies with *A. fumigatus* and *Candida albicans*, calcineurin regulated the response to azole-induced cell membrane stress and echinocandin-induced cell wall stress [7, 21]. Hsp90 interacts with the catalytic subunit of calcineurin, maintaining it in a stable conformation that is poised for activation, and activated calcineurin plays a role in azole and echinocandin tolerance [6, 21, 22]. We investigated repression of the calcineurin pathway by 2′,4′-DHC, and

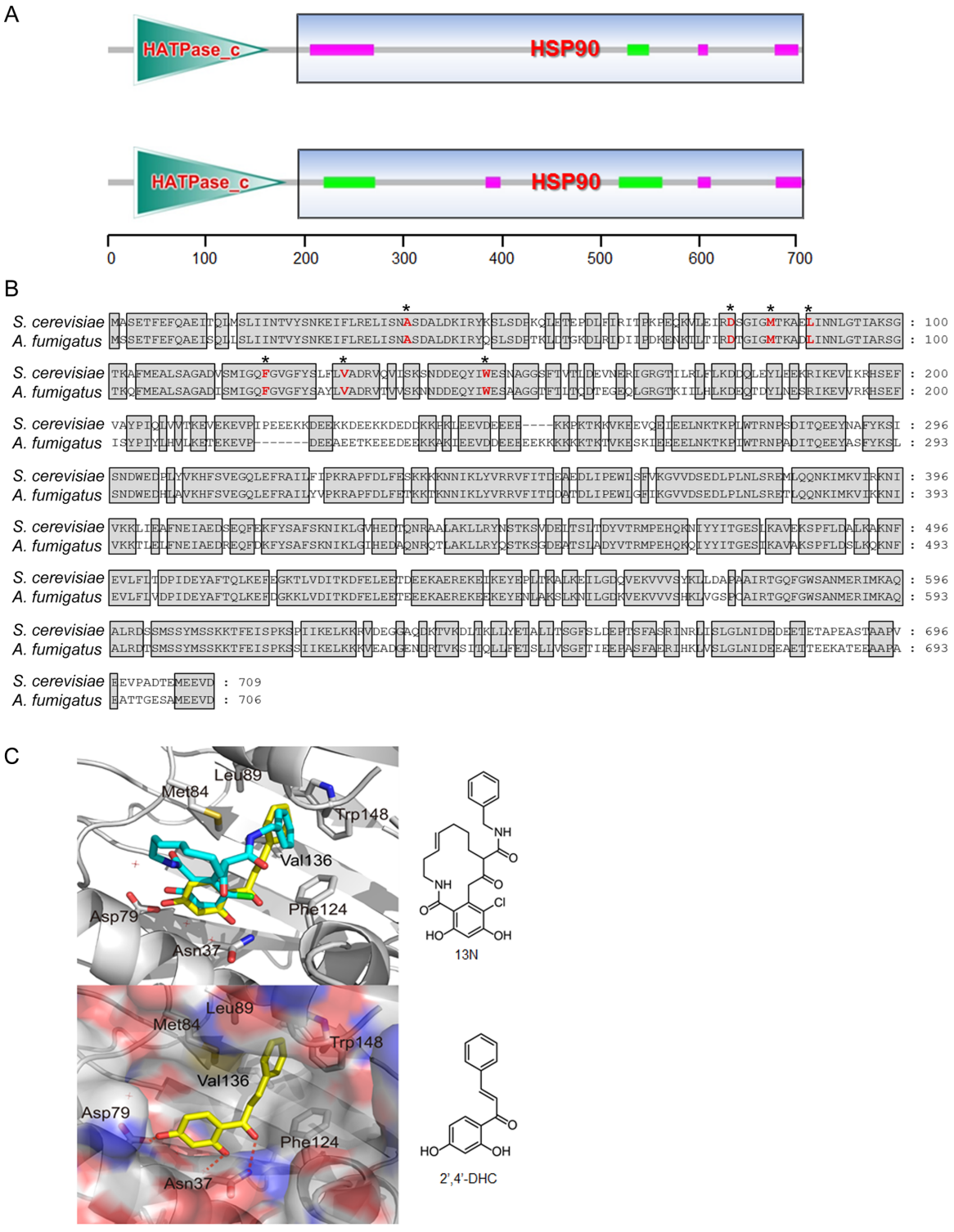

Fig. 5. A, Domain structure *Aspergillus fumigatus* heat shock protein 90 (Hsp90) (bottom) compared with that of *Saccharomyces cerevisiae* (upper). The two Hsp90 proteins were highly conserved and consisted of HATPase and Hsp90 domains. Pink regions are low complexity regions and green regions are coiled coil regions; B, Alignment of the amino acid sequences of two Hsp90 proteins. Asterisks indicate possible 2′,4′-dihydroxychalcone (2′,4′-DHC) interacting amino acids; C, Analysis of 2′,4′-DHC binding to yeast Hsp90. Comparative docking poses of yeast Hsp90 (PDB code: 2XX5) with ligand-13N and SY-032 (upper panel). Docking model of 2′,4′-DHC in ATP-binding pocket of yeast Hsp90 (lower panel). The carbon atoms of 13N and 2′,4′-DHC are shown in cyan and yellow, respectively. The oxygen, nitrogen, and chlorine atoms of 13N and 2′,4′-DHC are shown in red, blue, and green, respectively. The side chains of ATP-binding site of Hsp90 are colored by atom types (carbon, gray; oxygen, blue; nitrogen, red; sulfur, yellow) and labeled with their residue names. Hydrogen bonds are shown in dashed red lines.

observed that the expression of two major components of calcineurin pathway, *cnaA* and *crzA*, was significantly reduced (Fig. 4A). Further, combination of 2′,4′-DHC with ITC and CSP enhanced the effect of 2′,4′-DHC (Fig. 4B and 4C), which may due to the repression of calcineurin pathway.

To assess the mode of 2′,4′-DHC action in Hsp90 inhibition, we performed docking experiments using *S. cerevisiae* Hsp90, because the structure of *A. fumigatus* Hsp90 has not been solved. The domain structure and amino acid sequence of *A. fumigatus* and *S. cerevisiae* Hsp90 are highly conserved, and the possible 2′,4′-DHC binding amino acids are completely conserved (Fig. 5A and 5B). Comparison with 13N in the ATP-binding site of Hsp90 revealed that 2′,4′-DHC binds Hsp90 in a similar manner to its native ligand, 13N (Fig. 5C), and blocked the binding of nucleotides to Hsp90. From these results, we concluded that 2′,4′-DHC may bind to the ATP-binding pocket in the N-terminal domain of Hsp90, thereby acting as an Hsp90-calcineurin pathway inhibitor.

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