Title: The Roles of Hsp90–calcineurin pathway in the antifungal activity of honokiol

Article Type: Research article

Keywords: Candida albicans, honokiol, HSP90, calcineurin, cyclosporin A, mitochondria
The Roles of Hsp90-calcineurin pathway in the antifungal activity of honokiol

Kai Liao ¹, Lingmei Sun ²*

Affiliation

¹Department of Pathology and Pathophysiology, Medical School of Southeast University, Nanjing, China.
²Department of Pharmacology, Medical School of Southeast University, Nanjing, China.

Running title: Hsp90-calcineurin pathway and honokiol

* Correspondence
Dr. Lingmei Sun, Department of Pharmacology, Medical School of Southeast University, Nanjing, China. E-mail: sunlingmei@seu.edu.cn Tel.: +86-25-83272525.
Abstract

Honokiol, a bioactive compound isolated from the cone and bark of *Magnolia officinalis*, has been shown to have various activities including the inhibition of the growth of *Candida albicans*. We here investigated the roles of Hsp90-calcineurin pathway in the antifungal activity of honokiol. The pharmacologic tool was employed to evaluate the effect of Hsp90 and calcineurin in the antifungal activity of honokiol. We also evaluated the protective effects of calcineurin inhibitor cyclosporin A (CsA) on honokiol-induced mitochondrial dysfunction by fluorescence staining method. Hsp90 inhibitor potentiated the antifungal activity of honokiol. Strain deleted for calcineurin gene displayed enhanced sensibility of *C. albicans* to honokiol. However, co-treatment with calcineurin inhibitor CsA attenuated the cytotoxic activity of honokiol due to its protective effect on mitochondria. Our results provide insight into the understanding of the action mechanism of honokiol.

Keywords: *Candida albicans*; honokiol; HSP90; calcineurin; cyclosporin A; mitochondria
Introduction

The heat shock response is considered to be among the most fundamentally important and ubiquitous stress responses in nature [1]. Many heat shock proteins (HSPs) are molecular chaperones that play an important role in promoting the folding, assembly, or cellular localization of client proteins [1]. Hsp90, a specialized chaperone that regulates the form and function of many key signal transducers, enables the emergence and maintenance of drug resistance in diverse fungal species [2]. Inhibition of Hsp90 reverses drug resistance in diverse human fungal pathogens, rendering resistant infections responsive to antifungal treatment. Hsp90 function can be abrogated by natural products, such as geldanamycin (GdA) and radicicol, as well as by diverse chemical scaffolds that have been developed to target Hsp90’s key role in enabling malignant transformation [3]. Hsp90 regulates drug resistance by stabilizing the protein phosphatase calcineurin, which is the target of the natural products and immunosuppressants tacrolimus (FK506) and cyclosporine A (CsA) [3].

Calcineurin is a heterodimer comprised of a catalytic and regulatory subunit [4,5]. It is also known as protein phosphatase 2B and is activated through the binding of Ca\(^{2+}\)-calmodulin (CaM) [4]. Among the known serine/threonine protein phosphatases, calcineurin is the only phosphatase that requires Ca\(^{2+}\) and CaM for its enzymatic activity and exhibits restricted substrate specificity [4,5]. Calcineurin is a target of immunosuppressive agents, FK506 and CsA, mediated via their respective immunophilins, FK binding protein 12 (FKBP12), and cyclophilin A [4,5].

Calcineurin inhibitors are attractive as new antifungal agents due to their specific...
mode of action from other antifungal classes (polyenes, triazoles, and echinocandins) that would target the top of a critical cell signaling pathway, efficacy against emerging azole- and echinocandin-resistant strains, and synergistic nature with existing antifungals such as the echinocandin antifungal caspofungin [6-8].

Honokiol, a natural product found in the cone and bark of *Magnolia officinalis*, has various activities and has been used in traditional medicine to treat anxiety, thrombosis, and emesis [9]. Honokiol has been found to target on mitochondrial respiratory chain complex I (CI), inducing reactive oxygen species (ROS) accumulation, disruption of intracellular redox homeostasis, irreversible oxidative modifications of lipid, protein or DNA, and activation of autophagy or apoptosis signaling pathway [10,11]. In the present work, we investigated the roles of Hsp90-calcineurin pathway in the antifungal activity of honokiol. Findings from this study will be helpful to understand the action mechanism of honokiol against *Candida albicans*.

**Materials and methods**

**Materials**

Honokiol (5,5’-diallyl-2,4’-dihydroxybiphenyl) was obtained from Xi’an Yuquan Biological Technology Co., Ltd and its purity is over 98% analyzed by high-performance liquid chromatography. GdA and CsA were purchased from Shanghai Aladdin Bio-Chem Technology Co., LTD and their purities are over 98%. Glusulase, lyticase, DCFH-DA (2’,7’-dichlorofluorescein diacetate), JC-1 (5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolocarbocyanine iodide), and
other molecular grade chemicals were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.).

**Microorganisms**

DAY364 (Δcnb1/Δcnb1-mutant strain lacking calcineurin B regulatory subunit) and MCC85 (Δcnb1/Δcnb1+CNB1-mutant strain with a constitutive calcineurin B) *C. albicans* strains were cultured in YPD (yeast extract/peptone/dextrose) broth [4]. YPD-uracil (100ml YPD plus 400μl of 0.1 mol/l uracil) was used as an agar (2%) or liquid medium for culturing wild-type strain CAI4. The strains were stored as frozen stock with 15% (v/v) glycerol at –80°C. Before each experiment, cells were freshly revived on YPD plate from the stock.

**Quantification analysis by real-time reverse transcription-polymerase chain reaction (qRT-PCR)**

*C. albicans* wild-type strain CAI4 was grown overnight in YPD medium and diluted to a cell density of $1.0 \times 10^7$. Yeasts were incubated at 30°C for 4 h followed by centrifugation at 4°C. The total RNAs were isolated using the hot phenol method [10]. Approximately 1 μg of total RNA was used to synthesize cDNA using AMV reverse transcriptase (Promega, WI, USA). Primer sequences for the house keeping gene *18S rRNA* and *HSP90* are shown in supplementary table S1. The qRT-PCR and data analysis were performed as previously described [10].

**Sensitivity determination**

The sensitivities of compounds against *C. albicans* were tested by the broth
microdilution method according to CLSI standard M27-A3 [12].

**Measurement of ROS generation**

*C. albicans* wild-type strain CAI4 was grown overnight in YPD medium and diluted to a cell density of $1.0 \times 10^7$. Yeasts were incubated at 30°C for 4 h. Cellular ROS generations were evaluated by a flow cytometry (Becton-Dickinson Immunocytometry Systems, San Jose, CA) with DCFH-DA staining as described previously [13,14].

**Analysis of early marker of apoptosis**

*C. albicans* CAI4 cells were treated by honokiol (60 μM) with or without CsA (10 μM) for 4 h. The cells were harvested and washed with sorbitol buffer (1.2 M sorbitol, 0.5 mM MgCl$_2$, 35 mM potassium phosphate, pH 6.8), digested with 2% gluulase and 15 U/ml lyticase in sorbitol buffer for 2 h at 28°C. Protoplasts were harvested by centrifuge at 1000 g for 10 min. Protoplasts of *C. albicans* were stained with the Annexin V FITC apoptosis detection kit (Beyotime Biotechnology, Shanghai, China) to assess cellular integrity and the externalization of phosphatidylserine as described earlier [10].

**Mitochondrial membrane potential (mtΔψ)**

MtΔψ is an important parameter of mitochondrial function and an indicator of cell health. For determinations of mtΔψ, the fluorescent dye JC-1 was used and the method was performed as described previously [10].

**Cytochrome c (Cyt c) release**

Isolation of mitochondria was performed according to the published literature [15].
Cell grown in YPD broth at 30°C to early stationary phase were cultured, diluted to 1×10^7 cells/ml with fresh YPD broth with different concentrations of compounds for 4 h. After centrifugation, the pellet was resuspended in homogenization buffer (50 mM Tris, pH7.5, 2 mM EDTA, and 1 mM phenylmethysulfonyl fluoride). Glass beads (0.45 ± 0.5 mm diameter) were added to this suspension, and then homogenized using a FastPrep homogenizer (Fastprep FP120; Savant Instruments, NewYork, USA). After that, the homogenization was supplemented with 2% glucose and centrifugation at 2000 ×g at 4°C for 5 min to remove unbroken cells and glass beads. The supernatants were collected and centrifuged at 30,000 ×g for 45 min, and then supernatant was used for assay of Cyt c released from mitochondria to cytoplasm. The pellet was used for determination of Cyt c remaining in mitochondria. Protein quantity was estimated by BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). Mitochondria were suspended at 2 mg/ml. After being reduced by 500 mg/ml ascorbic acid at room temperature for 5 min, the quantities of Cyt c in supernatants and mitochondria were determined by measuring absorbance at 550 nm with the BioTek Synergy 4 microplate reader (BioTek Instruments Inc., USA)

**Statistical analysis**

All data were presented as means ± standard error of the mean (S.E.M.). Graphs were generated using Microsoft Excel (Microsoft Corp., Redmond, WA). Statistical analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, USA). Differences between groups were determined using analysis of variance (ANOVA). A p value < 0.05 was considered statistically significant.
Results

Hsp90 plays a crucial role in honokiol tolerance of C. albicans

Under normal conditions, molecular chaperones such as HSP90 have numerous roles in cellular processes, including normal protein folding during translation, refolding of accumulated proteins and the regulation of protein degradation. In response to various environmental stresses, HSP90 is upregulated to refold damaged molecules and/or to prevent their irreversible aggregation with other proteins. Given Hsp90’s role in azole and echinocandins resistance, we postulated that Hsp90 might be also required for the basal tolerance of C. albicans to honokiol. In this study, we observed elevated expressions of HSP90 in response to honokiol exposure in a concentration-dependent manner (Fig. 1A). Inhibitors of Hsp90 have been shown to diminish calcineurin signaling in S. cerevisiae and to synergistically inhibit growth in combination with azoles. To determine the impact of compromising Hsp90 function on tolerance to honokiol, we used an inhibitor GdA that bind with high affinity to Hsp90’s unusual adenosine triphosphate (ATP) binding pocket and inhibit ATP-dependent chaperone function. We used the concentration of GdA (1µM) that abrogate resistance to honokiol, but have no impact on growth on its own. It slightly suppressed cell growth when GdA was used at 2 or 4 µM. As shown in Fig. 1B, pharmacological impairment of Hsp90 by increasing concentration of GdA increased the antifungal activity of honokiol as expected. The findings suggest that Hsp90 plays a crucial role in honokiol tolerance of C. albicans.

Calcineurin mutant is sensitive to honokiol
Hsp90 regulates drug resistance by stabilizing the protein phosphatase calcineurin. Compromising calcineurin function phenocopied compromising Hsp90 function. To test if calcineurin is involved in toxic action of honokiol, we tested calcineurin mutant (Δcnb1/Δcnb1) and calcineurin-reconstituted (Δcnb1/Δcnb1+CNB1) strains. The calcineurin mutant was more sensitive to honokiol than WT or calcineurin-reconstituted strain (Fig. 2). The MIC$\text{}_{50}$ value of honokiol for cnb1/cnb1 was 15 µM; whereas for WT or cnb1/cnb1+ CNB1 strain it was 60 µM. These results suggested that the key mediator of Hsp90-dependent honokiol resistance is calcineurin.

**Inhibitor of calcineurin attenuates the antifungal activity of honokiol**

CsA is natural products of bacteria and fungi, respectively, with potent immunosuppressive and antimicrobial activity. Despite differing chemical structures, their mechanisms of action and cellular effects are very similar, resulting in the inhibition of the protein phosphatase calcineurin. We next examine whether CsA potentiates the antifungal activity of honokiol by targeting calcineurin. CsA exhibited no activity against *C. albicans* when grown under tested concentration, which was consistent with data reported in the literature [16]. Contrary to expectations, increasing immunosuppressive drug CsA did not increase death of *C. albicans* cells treated with honokiol and instead prevented the death of *C. albicans* cells (Fig. 3).

**CsA reduces honokiol-induced apoptosis in *C. albicans***

It is believed that honokiol treatment results in higher levels of apoptosis in *C. albicans* [10]. We next examine whether CsA attenuates the antifungal activity of
honokiol by inhibiting apoptosis using FITC Annexin V and PI staining. In apoptotic
cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the
cytosolic side of the plasma membrane to the cellular surface [17]. Annexin V binds
to exposed apoptotic cell surface PS with high affinity, which can be used as a marker
of early apoptosis [17]. Vehicle (DMSO) and CsA treatment group cells were almost
hardly stained by FITC Annexin V and PI evaluated by flow cytometric analysis.
Honokiol (60 µM) treatment induced externalization of PS after 4 h incubation,
whereas co-treatment with CsA significantly decreased early apoptotic cells from 27.9%
± 5.9% to 3.6% ± 0.8%, indicating CsA prevented honokiol-induced apoptosis (Fig.
4).

CsA impeded honokiol-induced mitochondrial dysfunction

ROS generation is an important factor responsible for the fungicidal activity of
honokiol [10]. Mitochondrion is the major source of ROS, especially superoxide
anions, which are highly involved in mitochondrial dysfunction. We further evaluated
whether treatment with CsA could decrease ROS-generation in *C. albicans* induced
by honokiol. As expected, treatment with honokiol induced ROS generation in a
concentration-dependent manner, whereas co-treatment with CsA (10 µM) largely
suppressed honokiol-induced ROS generation (Fig. 5A and B).

It is reported that CsA is a potent inhibitor of mitochondria permeability transition
pore (mPTP), a protein that is formed in the inner membrane of the mitochondria
[18,19]. Induction of mPTP can lead to mitochondrial swelling and cell death through
apoptosis or necrosis [19]. Previous reports have shown that honokiol induced
mitochondrial dysfunction was related to apoptosis [10,11]. Next, we examined
whether the protective effect of CsA on C. albicans was dependent on mitochondrial
functions. JC-1 staining showed that CsA treatment inhibited honokiol-induced
decrease in mtΔψ (Fig. 6A). Cyt c, a component of the electron transport chain, is a
water-soluble mitochondrial intermembrane-space protein loosely attached to the
inner mitochondrial membrane. The release of Cyt c from mitochondria to the cytosol,
where it activates the caspase family of proteases is believed to be primary trigger
leading to the onset of apoptosis [20]. Measuring the amount of Cyt c leaking from
mitochondria to cytosol, or out of the cell to culture medium, is a sensitive method to
monitor the degree of apoptosis [21]. Further studies showed that there was a
significant accumulation of Cyt c in the cytosol after incubation of cells with honokiol
(60 µM), whereas mitochondrial Cyt c was reduced by about 56% (p < 0.01) (Fig. 6B).
Next, we evaluated whether CsA (10 µM) prevents mitochondrial Cyt c release
induced by honokiol (60 µM). As expected, treatment with CsA inhibited the increase
of Cyt c (cytosolic fraction) induced by honokiol (Fig. 6B).

Discussion

C. albicans is the most frequently encountered Candida species in the clinic and is
the fourth most common cause of hospital acquired infectious disease with mortality
rates approaching 50 % [22]. Treatment of invasive fungal infections remains
notoriously challenging, due in large part to the limited availability of clinically useful
antifungal drugs [1].

Natural products have provided an unparalleled source of therapeutic agents that
have revolutionized modern medicine [23]. Honokiol, a natural product found in the cone and bark of *M. officinalis*, has various activities and has been used in traditional medicine to treat anxiety, thrombosis, and emesis [24,25]. Honokiol has been found to have a remarkable inhibitory effect on *C. albicans* via targeting on mitochondrial respiratory chain CI, resulting in ROS accumulation and mitochondrial dysfunction [10,11]. Hsp90 is a molecular chaperone that is induced by stress in eukaryotes and regulates the folding and transport of client proteins [2]. In fungi, Hsp90 has been demonstrated to mediate drug (such as azole) resistance and biofilm formation in diverse fungal species [22]. In the present study, honokiol treatment induced a substantial increase in the expression of HSP90 and Hsp90 inhibitor enhanced the antifungal efficacy of honokiol against *C. albicans*, indicating Hsp90 mediated honokiol tolerance (Fig. 1). Hsp90 regulates drug resistance by stabilizing the protein phosphatase calcineurin, which is the target of the immunosuppressant CsA [1]. Calcineurin B is encoded by a single gene (*CNB1*) in *C. albicans* and is known to be essential for calcineurin activity [26]. As expected, the *cnb1/cnb1* mutant lacking calcineurin was hypersensitive to honokiol as compared with WT and calcineurin-reconstituted strains (Fig. 2).

However, pharmacological blockade of calcineurin activity by CsA attenuates the antifungal activity of honokiol (Fig. 3). Annexin V and PI staining showed the declined cytotoxicity of honokiol induced by CsA was due to inhibition of apoptosis in *C. albicans* (Fig. 4). CsA binds to the cytosolic protein cyclophilin A (CyPA) to form CsA-CyPA complex which inhibits a calcium/calmodulin-dependent
phosphatase, calcineurin (Fig. 7). CsA also binds to Cyclophilin D (CyPD) located in the matrix of mitochondria and is thought to regulate the opening of mPTP, and then inhibits the mPTP opening [18]. Co-treatment with CsA, the antifungal sensitivity of honokiol should be increased by formation CsA-CyPA complex and inhibiting calcineurin (Fig. 7). However, the antifungal mechanism of honokiol was related with mitochondrial dysfunction, accompanied by increased cellular superoxide anion and collapse of the mtΔψ [11]. It is reported that the affinity of the mitochondrial site of action for CsA is probably higher than the affinities of other binding sites [18]. Co-treatment with CsA, the formation CsA-CyPD complex prevents the mPTP from opening, which may result in the decrease of ROS generation, collapse of the mtΔψ, and Cyt c release from mitochondria induced by honokiol treatment (Fig. 5 and 6). We also test the other immunosuppressive drug, FK506, which binds to a small soluble protein FKBP12, inhibits calcineurin [27]. Treatment with FK506 also attenuated the cytotoxic activity by blocking apoptosis induced by honokiol (data not shown). FK506 is reported to show a protective effect against mitochondrial dysfunction and could reduce mitochondrial-dependent apoptotic cell death induced by 3-nitropropionic acid in neuronal cultures [28].

In conclusion, though Hsp90 has been proposed to promote drug resistance by simulating calcineurin function, inhibitors of calcineurin did not mimic inhibitor of HSP90 in the ability to increase the antifungal sensitivity of honokiol against C. albicans. Instead, inhibitors of calcineurin, such as CsA, hindered the efficacy of honokiol treatment by protective effects to mitochondria.
Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 81302814 and 81703574), Jiangsu Province Natural Science Foundation (No. BK20130640 and BK20140624), and the Fundamental Research Funds for the Central Universities (No. 2242017K40096 and 2242017K40093).

Conflict of Interest

I declare that there are no conflicts of interest.

Reference


Figure legends

Fig. 1. Hsp90 plays a crucial role in honokiol tolerance of C. albicans. (A) HSP90 transcript levels increase following honokiol exposure. (B) GdA increased the sensitivity of C. albicans to honokiol. **p < 0.01.

Fig. 2. Mutation of calcineurin potentiates the antifungal activity of honokiol. Test strains: (■) CAI4, (▲) DAY364, Δcnb1/Δcnb1, (●) MCC85, Δcnb1/Δcnb1+CNB1. **p < 0.01.

Fig. 3. Attenuation of honokiol-induced cytotoxicity by CsA against C. albicans CAI4. Test concentration: (■) control, (□) 30 μM honokiol, (△) 60 μM honokiol, (▲) 120 μM honokiol.

Fig. 4. Attenuation of honokiol-induced apoptosis by CsA against C. albicans CAI4. A. Cell apoptosis were analysed by flow cytometry. B. The percentage of necrotic, early and late apoptotic cells. C. albicans CAI4 were treated with 10 μM CsA, 60 μM honokiol or their combination for 4 h, and then stained with annexin V-FITC and PI. Annexin positive: early apoptosis; annexin and PI positive: late apoptosis; PI positive: necrosis. **p < 0.01.

Fig. 5. Treatment with CsA (10 μM) decreased ROS-generation induced by honokiol in C. albicans CAI4. A. The percentage of cells that produce ROS in honokiol-treated C. albicans measured by flow cytometry. B. ROS induction in C. albicans cells treated with honokiol (60 μM) or CsA (10 μM) observed by fluorescence microscopy.
**p < 0.01.

**Fig. 6.** Treatment with CsA prevented mitochondrial injury induced by honokiol in *C. albicans* CAI4. A. The effect of CsA (10 μM) on honokiol (60 μM)-induced collapse of mtΔψ in *C. albicans*. B. The effect of CsA (10 μM) on honokiol (60 μM)-induced Cyt c release from mitochondria in *C. albicans*. **p < 0.01.

**Fig. 7.** A model of Hsp90-calcineurin pathway in the antifungal activity of honokiol. Honokiol treatment induced a substantial increase in the expression of HSP90 and Hsp90 inhibitor enhanced the antifungal efficacy of honokiol. Hsp90 regulates drug resistance by stabilizing the protein phosphatase calcineurin. CsA binds to the cytosolic protein cyclophilin A (CyPA) to form CsA-CyPA complex which inhibits a calcium/calmodulin-dependent phosphatase, calcineurin. CsA also binds to Cyclophilin D (CyPD) located in the matrix of mitochondria. Due to the formation of CsA-CyPD complex prevents the mPTP from opening, CsA attenuates the antifungal activity of honokiol. ETC: electron transfer chain.

**Supplementary Information:**

Table S1. Gene-specific primers used for qRT-PCR.
Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.
Fig. 5.
Fig. 6.
Fig. 7.