Opposite Effects of Vitamin C and Vitamin E on the Antifungal Activity of Honokiol

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Introduction

Reactive oxygen species (ROS) are not only produced by normal cellular respiration processes, but also by compounds used as therapeutic drugs, which interfere with oxygen metabolism [1]. The over-accumulation of ROS can initiate the deleterious effects on cellular biomolecules such as protein, lipid, RNA, and DNA, and result in cell death [1]. Cells normally have the ability to protect themselves against ROS-mediated damage by intracellular antioxidant enzymes to maintain the homeostasis of ROS at a low concentration [2]. Antioxidants such as vitamin C (ascorbic acid, VC) and E (α-tocopherol, VE) can terminate free radicals by eliminating the unpaired condition of the radical [2, 3]. However, some researchers have put forward the paradox that VC and VE may have both pro- and antioxidant activities [4]. VC is soluble in water (hydrophilic) and VE in the lipids (lipophilic). They will directly react with or neutralize ROS such as hydrogen peroxide [4]. After that, VE itself becomes α-tocopheroxyl radicals while VC changes into a new radical (Asc·). Under these conditions, antioxidants exhibit pro-oxidant effects and can become harmful [2, 4]. VC itself is readily regenerated from Asc· with the help of NADH or NADPH-dependent reductases. In addition, the cytotoxicity of ascorbate in the presence of metal ions has been interpreted, from Fenton’s reaction, to occur via the generation of oxygen-derived free radicals [4, 5]. Previous studies have shown that VC inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and decreases the cellular glutathione level, leading to high ROS generation and oxidative stress [6].

Honokiol, a polyphenol compound present in the traditional Chinese medicine Magnolia officinalis Rehd. et Wils. bark, is an interesting small-molecule having various
pharmacological activities, including anti-tumor, antioxidation, anti-inflammatory, and anti-microbial activity as well as protection against neurotoxicity, hepatotoxicity, and thrombosis in preclinical experimental models [7–9]. Honokiol contains two phenolic groups that can exhibit antioxidant properties [7]. Previous mechanism studies, including those from our own laboratory, indicated that honokiol-induced apoptosis in C. albicans was associated with ROS production, which indicated the pro-oxidant activities of honokiol [10, 11]. In the present study, we investigated the effects of two well-known natural antioxidants, VC and VE, on the antifungal activity of honokiol against C. albicans.

Materials and Methods

Materials
Honokiol (5,5’-diallyl-2,4'-dihydroxybiphenyl) was purchased from Xi’an Yuquan Biological Technology Co., Ltd China and its purity is over 98%. VC and VE were obtained from Aladdin Bio-Chem Technology Co., LTD (China) and their purities are over 99% and 96%, respectively. VC and VE were dissolved in water and DMSO, respectively. DCFH-DA (2',7'-dichlorofluorescein diacetate), DPPP (diphenyl-1-pyrenylphosphine), DAPI, JC-1 (5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolocarbocyanine iodide), and other molecular grade chemicals were obtained from Sigma Chemicals (USA).

Microorganisms
The C. albicans strains SC5314, YEM30, and Tom70-GFP strain were cultured in YPD (yeast extract/peptone/dextrose) broth [10, 12]. For agar plates, 2% (w/v) bacto agar (Difco, BD Biosciences, USA) was added to the medium. 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.

Sensitivity Determination
The sensitivities of C. albicans to honokiol and antioxidants were tested by the broth microdilution method according to the CLSI standard M27-A3 with final inoculum of 0.5 × 10^6 to 2.5 × 10^6 cells/ml [13]. Quality control was performed with the antifungal agent fluconazole on each day of testing using the CLSI-recommended reference strain C. albicans ATCC 10231. The assay was performed with RPMI 1640 medium in 96-well microtiter plates (Costar,Corning, NY, USA). The minimum inhibitory concentrations (MICs) were determined by optical density measured at 600 nm with a BioTek Synergy 4 multi-detection microplate reader (Synergy HT; BioTek, USA) after incubation at 35°C for 48 h. The MICs were determined as the concentrations of drug that inhibited growth by 80% compared to that of cells grown in the control.

Time-Kill Test
Time-kill tests were performed as described previously [14]. C. albicans SC5314 cells with starting inoculum of 10^7 cells/ml were exposed by different concentrations of antioxidants or honokiol. Samples were taken at 3, 6, 9, 12, and 24 h and the viabilities were determined by spot assay. Using a 20-μl sampling volume, the limit of detection was 50 CFU/ml. Synergy was defined as a ≥ 2 log_10 CFU/ml decrease after 24 h with the combination compared with honokiol alone; indifference was defined as a < 2 log_10 CFU/ml increase or decrease at 24 h with the combination compared with that of honokiol alone; and antagonism was a ≥ 2 log_10 CFU/ml increase after 24 h with the combination compared with that of honokiol alone [14]. Fungistatic effect was defined as a decrease < 3 log_10 CFU/ml compared to the initial inoculum and fungicidal as a decrease ≥ 3 log_10 CFU/ml [14].

ROS Determination
SC5314 cells were diluted to 1 × 10^7 cells/ml using YPD medium and exposed to VC (10 μM), VE (10 μM), honokiol (60 μM), the combinations, or the DMSO vehicle control at 35°C for 4 h. Intracellular ROS concentrations were determined using DCFH-DA staining method as previously described [10].

Cell Staining with DPPP
C. albicans SC5314 cells were diluted to 1×10^7 cells/ml with YPD medium and exposed to VC (10 μM), VE (10 μM), honokiol (60 μM), the combinations, or the DMSO vehicle control at 35°C for 4 h. The cell suspensions were stained with 10 μM DPPP at 37°C in the dark for 10 min [15]. The images were taken by a fluorescence microscope (Olympus IX71, Olympus Co., Japan) with a 340/380 nm excitation filter.

DAPI Staining
C. albicans SC5314 cells were adjusted to 1 × 10^7 cells/ml in YPD medium and exposed to VC (10 μM), VE (10 μM), honokiol (60 μM), the combinations, or the DMSO vehicle control at 35°C for 4 h. After that, cells were fixed with 70% ethanol for 10 min, and then stained with 5 μg/ml DAPI in the dark for 10 min. The images were taken by the fluorescence microscope with a 340/380 nm excitation filter.

JC-1 Assay
The membrane-permeant JC-1 dye is widely used to detect mitochondrial membrane potential (mVm). Polarized mitochondria are marked by punctate orange-red fluorescent staining [16]. The orange-red punctate staining is replaced by diffuse green monomer fluorescence during mitochondria depolarization [16]. SC5314 cells were diluted to 1 × 10^7 cells/ml with YPD medium and exposed to VC (10 μM), VE (10 μM), honokiol (60 μM), the combinations, or the DMSO vehicle control at 35°C for 4 h. C. albicans cells were stained by 5 μM JC-1 at 37°C for 30 min [17].

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Measurement of Intracellular ATP Levels

*C. albicans* strain SC5314 was diluted to 1×10⁷ cells/ml in YPD broth. After cell treatment with VC (10 mM) or VE (10 mM), honokiol (60 μM), the combinations, or the DMSO vehicle control for 4 h, the cells were washed twice with cold PBS. And then, the cells were suspended with ATP-releasing reagent provided by the ATP assay kit (Beyotime Institute of Biotechnology, China), mixed with glass beads (0.45–0.5 mm diameter), and broken by a FastPrep homogenizer (FastPrep FP120; Savant Instruments, USA). The homogenates were centrifuged at 10 000 g for 45 min to separate cell-free extracts. Intracellular ATP levels were determined by using a luciferase-based assay, according to the instructions of the manufacturer [18].

Measurement of Tdh3 Expression

*C. albicans* expressing GFP-TDH3 cells diluted to 1×10⁷ cells/ml in YPD broth were treated with VC (10 mM) or VE (10 mM), honokiol (60 μM), the combinations, or the DMSO vehicle control for 4 h [19]. The expression of Tdh3 was indicated by the fluorescence intensity of GFP measured by fluorescence microscope and flow cytometry [19].

Statistical Analysis

The data represent the average of three separate experiments and each was performed in triplicate. All data were presented as means ± standard error of mean (SEM). Graphs were generated using Microsoft Excel (Microsoft Corp., Redmond, WA, USA). Statistical analysis was performed using SPSS 12.0 (SPSS Inc., USA). Differences between groups were determined using analysis of variance (ANOVA). A p value of < 0.05 was considered statistically significant.

Results

Effects of VC and VE on the Antifungal Activity of Honokiol against *C. albicans*

The MICs for honokiol, VC, and VE against *C. albicans* SC5314 were 120 μM, > 50 mM, and > 50 mM, respectively. Previous studies indicated that honokiol could induce apoptosis in *C. albicans*, associated with production of ROS [10]. To test if antioxidants like VC and VE could protect *C. albicans* from oxidative damage after honokiol exposure, SC5314 cells were incubated with VC (10 mM) and VE (10 mM) alone or in combination with honokiol (30, 60, and 120 μM). VC did not reduce, in fact enhanced, the honokiol-induced cell death in *C. albicans* cells (Fig. 1A). Given the initial inoculum of 10⁷ cells/ml, honokiol as a fungicide compound, exhibited no growth at MIC (120 μM). While honokiol alone had no antifungal effect at 30 μM, when administered in combination with 10 mM VC, it induced a 1.35 log₈ CFU/ml decrease, compared with honokiol administrated alone at 24 h. Honokiol (60 μM)-VC (10 mM) combination exhibited even better fungicidal activity (> 3log₈ CFU/ml decrease compared with starting inoculum) by 24 h (Fig. 1A). In addition, a synergistic interaction between honokiol (60 μM) and VC (10 mM) was also confirmed according to the definition of synergistic interaction assessed by time-kill assay. After 24 h, the growth of the honokiol-vitamin C combination group was accelerated but the log₁₀ CFU/ml were still lower than when the individual compounds were used alone (data not shown). In contrast, VE could effectively protect *C. albicans* from honokiol-induced cell death, even when combined with the fungicidal concentration of 120 μM of honokiol (Fig. 1B). Hence the antagonism was observed when honokiol (120 μM) was combined with VE (10 mM). The same phenomenon has been observed in another *C. albicans* strain YEM30 (Fig. S1). We next investigated whether a 1-h pretreatment with VC and VE could protect *C. albicans* from honokiol-induced cell death. Pretreatment with VC (10 mM) could not attenuate the antifungal activity of honokiol while that with VE (10 mM) still hindered the efficacy of honokiol treatment (Fig. 1C). In order to further verify the opposite effects of VC and VE on the antifungal activity of honokiol against *C. albicans*, different concentrations (lower than MICs) of both vitamins, ranging from 0.3125 to 40 mM, were tested. Even at the lowest concentration of VC (0.3125 mM), the antifungal activities of honokiol (at 30 or 60 μM) were significantly enhanced, compared to the situation when the individual compounds were used alone (Fig. 1D). The fungicidal activity of honokiol (60 μM) was noted with VC (≥ 10 mM) in a concentration-dependent manner. In addition, there is a dose-dependent protective effect on *C. albicans* when honokiol is used in combination with VE (Fig. 1E).

Effects of VC and VE on ROS Generation and Mitochondrial Lipid Peroxidation after Honokiol Exposure

VC and VE are perhaps the most well-known antioxidants because of their ability to scavenge ROS [4]. The antifungal mechanism of honokiol was associated with mitochondrial dysfunction accompanied with ROS production [10]. We examined the effects of antioxidants, VC and VE (both at
Honokiol Combined with Vitamin C or E

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Honokiol (60 μM) induced DCF-positive staining in 36.0% ± 10.0% cells while VC supplement resulted in a 1.8-fold increase of ROS positive cells compared to that in honokiol alone (Fig. 2B). When supplemented with VE, honokiol-induced ROS generation was decreased 6.5-fold, indicating that VE could effectively defend the cells against honokiol-induced ROS generation (Fig. 2B). Mitochondrion is the primary source of ROS, such as the superoxide anion radicals, so it might also be a primary target for the damaging effects of ROS, like mitochondrial membrane lipid peroxidation. DPPP is an agent that reacts with lipid peroxidations to generate the fluorescent product DPPP oxide [20]. To elucidate the lipid peroxidation on the mitochondrial membrane, C. albicans expressing Tom70 (a constitutive protein of mitochondria)-GFP were analyzed in this study. Tom70-GFP strain cells were stained with DPPP after honokiol exposure. We observed that there was a co-localization of the fluorescent product, DPPP oxide, on the mitochondria, hence suggesting honokiol-induced ROS generation and stimulation of lipid peroxidation in mitochondria (Fig. S2). Compared to the honokiol exposure alone, VC-supplemented honokiol induced a 1.5-fold...
increase in the percentage of DPPP positive cells (Figs. 2C and 2D). However, when supplemented with VE, honokiol-induced lipid peroxidation was almost completely reversed (Figs. 2C and 2D).

**Effects of VC and VE on mtDNA and mtΔψ after Honokiol Exposure**

Because of the lack of protective histones and the close proximity to the electron transport chain, the major locus for free radical production, mtDNA is especially sensitive to ROS attack [1]. DAPI stain is effective for visualization of nuclear DNA and mtDNA in a variety of organisms [21]. Staining with DAPI revealed small fluorescent spots in the cytoplasm for mtDNA aggregates in the control group, when observed under fluorescence microscope (Fig. 3A). VC or VE administered alone had almost no effect on mtDNA in *C. albicans*. VC-supplemented honokiol, however, showed extremely condensed nuclei with cytoplasm completely devoid of mtDNA while VE-supplement could prevent the honokiol-induced damage to mtDNA (Fig. 3A).

In addition, we used JC-1 dye to monitor mitochondrial health. The depolarization of mitochondria is indicated by a decrease in the red/green fluorescence intensity ratio [16]. As expected, consistent with a mitochondrial localization, the red fluorescence was mostly found throughout the cytoplasm in the control, VC- and VE-treated group (Fig. 3B). Treatment with honokiol showed some diffused red fluorescence and increased green fluorescence. Honokiol supplemented with VC showed a noticeable increase in the green fluorescence and changed the distribution of red fluorescence which was dispersed in the cytoplasm, suggesting that the mitochondrial membrane was depolarized. However, VE supplement repressed the honokiol-induced changes of mtΔψ, as implicated by a reduction of green fluorescence and restoration of red fluorescence (Fig. 3B). The effect of VC and VE on mtΔψ in *C. albicans* after honokiol exposure was further confirmed by flow cytometry. As shown in Figs. 3C and 3D, honokiol,

![Fig. 2. Effects of VC and VE on the ROS production and oxidation of lipids after honokiol exposure.](image-url)
supplemented with VC, showed a significant fall in mtΔψ compared to that when administered alone, whereas honokiol supplemented with VE attenuated the honokiol-induced collapse of mtΔψ in *C. albicans*.

**Effects of VC and VE on Tdh3 Expression and ATP Level in *C. albicans* after Honokiol Exposure**

Glycolysis is important for maintaining the tricarboxylic acid (TCA) cycle, oxidative phosphorylation, and the mitochondrial function [22]. In *C. albicans*, TDH3 encodes an NAD-linked glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme of the glycolysis pathway. VC could induce oxidative stress and inhibits the activity of GAPDH [22]. We found that the expression of Tdh3 significantly increased after honokiol exposure (Figs. 4A and 4B). When supplemented with VC, the expression of Tdh3 was 1.6-fold lower in honokiol-treated cells compared to vehicle-treated ones (Fig. 4B). However, there was almost no change in the expression of Tdh3 between vitamin E-honokiol-treated cells and vehicle-treated cells. ATP is one of the end products of glycolysis and TCA cycle/photophosphorylation. There was no effect on the ATP

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**Fig. 3.** Effects of VC and VE on mtDNA and mtΔψ in *C. albicans* after honokiol exposure.

(A) Fluorescent images of *C. albicans* SC5314 cells stained with DAPI. N, nucleus; M, mtDNA. (B) Fluorescent images of *C. albicans* SC5314 cells stained with JC-1. (C and D) The mtΔψ was measured by flow cytometry—High mtΔψ is identified as the % of cells emitting fluorescence signals in the UR quadrant and low mtΔψ is identified as the % of cells emitting fluorescence signals in the LR quadrant. **, p < 0.01 vs. honokiol exposure alone.
Honokiol treatment, however, caused a significant decrease in ATP level and the drop of ATP level was increased when supplemented with VC (Fig. 4C). On the contrary, VE abolished the ability of honokiol to decrease the ATP level (Fig. 4C).

Effect of Glycolysis Inhibitor 2-Deoxy-Glucose (2-DG) on the Antifungal Activity of Honokiol

2-DG, a glucose analog, has been known to be used as a classical glycolysis inhibitor by targeting hexokinase [23]. 2-DG exhibits a cytotoxic effect in cancer cells, especially those with mitochondrial respiratory defects, or cells in a hypoxic environment [23]. Given the starting inoculum of $10^5$ cells/ml and then incubation at 35°C for 24 h, 2-DG alone had almost no toxic effect against C. albicans SC5314, but it significantly enhanced the antifungal activity of honokiol (60 μM) (Fig. 5).

**Fig. 4.** Effects of VC and VE on Tdh3 expression and ATP level in C. albicans after honokiol exposure. (A) Fluorescent images of C. albicans TDH3-GFP-CAI4 cells. (B) Relative GFP-Tdh3 level in C. albicans. (C) ATP level in C. albicans SC5314. **, p < 0.01.

**Fig. 5.** Comparisons of the effects of 2-DG, honokiol, and the combination of 2-DG and honokiol on the viability of C. albicans SC5314. ***, p < 0.01.
Discussion

C. albicans, an opportunistic pathogen, can lead to infections ranging from mild mucosal lesions to life-threatening systemic diseases depending on the physiological or immunological status of the host [24]. Because of the limited numbers of antifungal drugs, undesirable toxic side effects, and the emergence of resistant strains, the treatment of candidiasis faces an enormous clinical challenge [25]. However, some natural molecules showing promising antifungal properties could be considered as an important source for development of novel anti-Candida therapy [26–28]. During the last decade, honokiol has been reported to have potent anticancer and antimicrobial activities, including anti-Candida activities [12]. In our previous research, the action of honokiol against C. albicans was shown to be associated with mitochondrial dysfunction accompanied with ROS production [10]. A supplement with the inhibitor of superoxide dismutases increased efficacy of honokiol against C. albicans [10].VC and VE, two well-known antioxidant nutrients, can protect cells from oxidative stress [4]. Therefore, some published studies support the idea that antioxidants VC or VE hinder the efficacy of chemotherapy treatment by disturbing the oxidative mechanisms of action [29]. However, it is very interesting that researchers, now, believe that antioxidant supplementation can actually potentiate the therapeutic index of amphotericin B [30].

In this study, we investigated the effect of VC and VE on the antifungal activity of honokiol against C. albicans. Interestingly, we observed the two vitamins to have opposite effects on the antifungal activity of honokiol. Our results showed VC supplement effectively enhanced the efficacy of honokiol against C. albicans, whereas in combination with VE, we observed a protective effect against honokiol-induced oxidative stress in C. albicans (Fig. 1). The effects of pretreatment with VC on honokiol activity against C. albicans are the same as that of concomitant administration (Fig. 1). In this study, a wide range of concentrations of VC and VE were tested. We observed that even a lower concentration (0.3125 mM) of VC was able to potentiate the antifungal activity of honokiol after 24 h-incubation (Fig. 1). It has been reported that plasma VC concentrations, higher than 10 mM, could be easily achieved in humans and in the murine pharmacokinetic study without significant toxicity [22]. Our results provide a possible approach to explore the therapeutic use of VC-honokiol combination against Candida infection.

ROS being generated continuously by the mitochondrial respiratory chain causes the mitochondria to be particularly susceptible to ROS-induced damage [1]. Supplement with VC exacerbated the honokiol-induced disruption of mitochondrial function, as evaluated by ROS generation (Fig. 2), mitochondrial lipid peroxidation (Figs. S2 and 2), loss of mtDNA (Fig. 3), and 

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mitochondrial membrane, indicating the better ability of lipid-soluble VE to prevent lipid peroxidation of the mitochondrial membrane compared to that of water-soluble VC (Figs. S2 and 2).

It is reported that VC not only induces high ROS production and oxidative stress but also interferes with glycolytic metabolism by inhibiting GAPDH glycolytic enzyme activity [6, 38]. Our previous data demonstrated that honokiol treatment suppresses mitochondrial respiration and oxidative phosphorylation, and increases ROS generation in the mitochondria, leading to the induction of apoptosis in C. albicans cells [24]. Due to the impaired mitochondrial function induced by honokiol exposure, C. albicans cells may be highly sensitive to glycolytic inhibitors such as VC and 2-DG (Figs. 4 and 5). Therefore, VC-supplemented honokiol will more severely target cell inhibitors such as VC and 2-DG (Figs. 4 and 5). Therefore, VC-supplemented honokiol could be used effectively to fight against C. albicans.

In summary, our study showed that VC potentiated the antifungal activities of honokiol whereas VE hindered the efficacy of honokiol treatment by exerting protective effects on mitochondria. Therefore, VC-supplemented honokiol could be used effectively to fight against C. albicans.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

Reference


