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Synergistic effect of caffeic acid phenethyl ester with caspofungin against *Candida albicans* is mediated by disrupting iron homeostasis

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1. Introduction

The incidence of invasive fungal infections has significantly increased over the recent years causing morbidity and mortality of immunocompromised patients (Yang et al., 2012; Crunkhorn, 2016). One way to enhance treatment is to combine antifungal agents. Antifungal agents given in combination may improve efficacy due to synergism, and because the dose can possibly be lowered, side effects could be reduced. Another advantage of combination therapy is the reduction of the development of resistance and possibly a shorter duration of therapy (Baddley and Pappas, 2005). Caspofungin (CAS), the first member of the echinocandin class of antifungal drugs to be licensed for clinical use, inhibits the synthesis of the fungal cell wall component β-1,3-glucan, resulting in osmotic disruption of the fungal cell (DiNubile et al., 2004). CAS has been effective in treating fungal infections caused by *Aspergillus* and *Candida* species. However, due to the emergence of CAS-resistant *Candida* species and the higher economic burden of fungal infections treated with CAS compared with the other older antifungals, the combination of natural products with CAS is an important therapeutic strategy for fungal infections (Bruynesteyn et al., 2007; Fuentefria et al., 2018).

Polyphenols are widely used in food and cosmetics to prevent undesirable oxidation by free radical scavenging (Iwasaki et al., 2011). However, compounds with antioxidant activity may exhibit prooxidant behavior under certain conditions (Fukumoto and Mazza, 2000). Phenolic compounds have been identified as potent inhibitors of iron absorption presumably by forming insoluble complexes with iron ions (Brune et al., 1989). If iron is complexed with phenolic hydroxyl groups, less iron may be available for heme synthesis leading to deficiencies in cytochromes and thus respiratory deficits, as well as decreases in the activities of the specific Fe-S cluster enzymes affected by excess superoxide (De Freitas et al., 2000; Seyoum et al., 2016). Superoxide radical has been shown to inactivate certain [4Fe-4S] cluster-containing enzymes by oxidizing one iron, causing its release from the cluster (De Freitas et al., 2000). This process leads to both enzyme inactivation and further oxidative damage of other cellular components, as “free” iron can promote, via the Fenton reaction, the formation of hydroxyl radicals (De Freitas et al., 2000).

Caffeic acid phenethyl ester (CAPE, Fig. 1), a polyphenol with hydroxyl groups within the catechol ring, is one of the most extensively investigated active components of propolis which possess many biological activities, especially its anti-oxidant activity (Tolba et al., 2016). Furthermore, CAPE has antifungal activity against *Candida albicans* with the minimum inhibitory concentration (MIC) at 32-64 μg/ml (Coleman et al., 2016; Breger et al., 2007). In this study, we assessed the combined effect of CAPE and CAS against *C. albicans* and also investigated the potential molecular mechanism related to prooxidant behavior of CAPE via disrupting iron homeostasis.

2. Materials and methods

2.1. Materials

CAPE was obtained from Shanghai Aladdin Bio-Chem Technology Co., LTD and its purity is over 97%. DCFH-DA (2′,7′-dichlorofluorescein diacetate), dihydroethidium (DHE), 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), and other molecular grade chemicals were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.).

2.2. Microorganisms

The *C. albicans* strain YEM30 was cultured in YPD (yeast extract/peptone/dextrose) broth (Sun et al., 2017a). The strain was 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.

2.3. Sensitivity determination

The sensitivities of CAPE and CAS against *C. albicans* YEM30 was tested by the broth microdilution method as previously described (Sun et al., 2009). The MICs were defined as the concentrations of drug that reduced growth by 90%.

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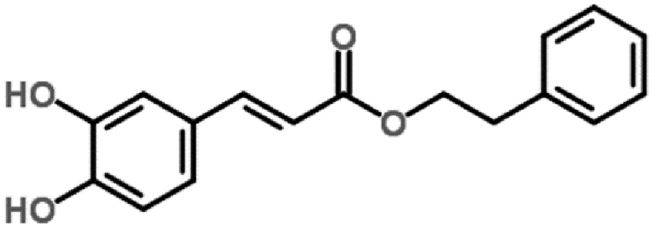

Fig. 1. The structure of CAPE. Molecular formula of CAPE is $C_{17}H_{16}O_4$. Molecular weight of CAPE is 284.31.

2.4. Interactions between CAPE and CAS

Drug interactions were assessed by broth microdilution checkboard assays (Sun et al., 2009). The data obtained from the checkerboard tests were analyzed by the non-parametric approach of fractional inhibitory concentration index (FICI) which is expressed as: $\Sigma FIC = FIC_A + FIC_B = MIC_{AB}/MIC_A + MIC_{BA}/MIC_B$, where MIC_A and MIC_B are the MICs of drugs A and B when acting alone and MIC_{AB} and MIC_{BA} are MICs of drugs A and B when acting in combination, respectively. Synergy was defined as when FICI of ≤ 0.5, while antagonism was defined when the value of FICI > 4. An FICI result between 0.5 and 4 ($0.5 < FICI \leq 4$) was considered indifferent (Odds, 2003). In addition, isobologram was plotted from results of checkerboard test in combination with CAPE and CAS. For a synergistic drug combination, the isobole is concave; if the combination is antagonistic, the isobole is convex (Haynes et al., 1994).

2.5. Time-killing test

C. albicans YEM30 cells with starting inoculum of 10^5 cells/ml were exposed by different concentrations of CAS or CAPE. Samples were taken at 3, 6, 9, 12, and 24 h, and then the viabilities were determined by spot test using 10-fold dilutions plated on YPD plates. The limit of quantitation was 100 CFU/ml. All experiments were performed in triplicate and repeated on different days. Synergy was defined as a ≥ 2 $\log_{10}$ decrease in colony count after 24 h with the combination compared with the most active single agent alone; indifference was defined as a < 2 $\log_{10}$ increase or decrease in colony count at 24 h with the combination compared with that of the most active single agent alone; and antagonism was a ≥ 2 $\log_{10}$ increase in colony count after 24 h with the combination compared with of the most active single agent alone (Klepser et al., 1998).

2.6. Intracellular free Fe^{2+} quantification

Iron in the *C. albicans* was determined using the fluorescent, cell-permeable indicator for heavy metals Phen green FL (Molecular Probes, Invitrogen), which can be used to detect a broad range of ions. Fluorescence of Phen green FL disappears after binding of free Fe^{2+}. Therefore, the strong fluorescence of Phen green FL indicates free Fe^{2+} deprivation. After treatment with CAPE, *C. albicans* cells suspensions (1×10^7 cells/ml) were incubated with Phen green FL (5 μg/ml) for 30 min in the dark. The images were taken by a fluorescence microscopy with FITC filter (Olympus IX71, Olympus Co.,Tokyo, Japan) and the fluorescence was quantified by flow cytometry (Becton-Dickinson Immunocytometry Systems, San Jose, CA) monitoring the emission fluorescence in channel FL1.

2.7. ROS determination

C. albicans cells were adjusted to 1×10^7 cells/ml in YPD medium and exposed to different compounds at 35 °C for 4 h. Intracellular ROS concentrations were determined using DCFH-DA and DHE staining methods as previously described by our group (Sun et al., 2017b).

2.8. Determination of mitochondrial respiratory chain complex I (C I) activity

Extraction of mitochondrial proteins was performed as previously described by our group (Sun et al., 2017b).

2.9. Respiratory activity

The tetrazolium salt CTC is frequently used as indicator of microorganisms' respiration (Kobayashi et al., 2012). The CTC staining assay was performed as previously described by our group (Sun et al., 2017b).

2.10. Measurement of intracellular ATP levels

Intracellular ATP levels were determined using an ATP assay kit as previously described (Sun et al., 2011).

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

C. albicnas wild-type strain YEM30 was grown overnight in YPD medium and diluted to a cell density of 1.0×10^7. Yeasts were treated by 64 μg/ml CAPE at 30 °C for 4 h. The total RNAs were isolated using the hot phenol method (Sun et al., 2017a). Primer sequences are shown in Supplementary Table S1. The qRT-PCR and data analysis were performed as previously described (Sun et al., 2017a).

2.12. 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., Chicage, USA). Differences between groups were determined using analysis of variance (ANOVA). A p value < 0.05 was considered statistically significant.

3. Results

3.1. CAPE displays synergism with CAS against C. albicans

The MICs of individual drugs are 64 μg/ml for CAPE and 0.125 μg/ml for CAS against *C. albicans* YEM30. However, when used in combination, the MICs of CAPE and CAS were significantly decreased 16-fold when compared to individual drugs. The FICs of CAPE and CAS against *C. albicans* YEM30 strain are shown in Fig. 2A. Among all of the ΣFIC values calculated for each data set, the FICI was determined as ΣFIC_{min} (the lowest ΣFIC) when ΣFIC_{max} (the highest ΣFIC) was less than 4; therefore, the $FICI_{min}$ (0.12) was reported as FICI, indicating synergistic effect between CAPE and CAS. The isobologram for the interaction between CAPE and CAS also yielded a highly concave curve, characteristic of strong drug synergism (Fig. 2A). Next, time kill experiments were performed to confirm drug synergism and to quantify the killing produced by combinations of CAPE and CAS. Cells were exposed to CAPE, CAS or a combination of both agents. The concentration of each agent was based on the checkerboards. At 3, 6, 9, 12, and 24 h, samples from each treatment were removed, washed and plated for colony counts. With starting inoculum of 10^5 CFU/ml, exposure to CAPE or CAS alone did not reduce colony counts, but combinations of the two were found to be lethal, which induced a 2.0, 2.1, and 5.3 $\log_{10}$ CFU/ml decrease as compared with CAS alone at 0.03125, 0.0625, and 0.125 μg/ml respectively (Fig. 2B). These results indicated the synergy effect between CAPE and CAS against *C. albicans*.