Synergistic in vitro activity of sodium houttuynonate with fluconazole against clinical Candida albicans strains under planktonic growing conditions

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**ABSTRACT**

**Context:** Fluconazole resistance is an intractable problem of treating Candida albicans, calling for more antifungal agents to enhance the activity of fluconazole.

**Objective:** This work investigates the anti-C. albicans activities of sodium houttuynonate (SH) and/or fluconazole and the associated mechanism.

**Materials and methods:** The minimum inhibitory concentrations (MICs) of SH and fluconazole both ranging from 0.5 to 1024 \(\mu\)g/mL were determined by broth microdilution method in 19 C. albicans isolates, and their fractional inhibitory concentration index (FICI) was evaluated by checkerboard assay. After MIC\textsubscript{SH} and/or MIC\textsubscript{fluconazole} treatments, the expressions of IFD6, PHR1, ZAP1, ADH5, BGL2, XOG1 and FKS1 were analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in C. albicans 1601.

**Results and conclusion:** The MICs of SH alone ranged from 32 to 256 \(\mu\)g/mL and decreased 2–16-fold in combination. SH showed strong synergism with fluconazole with FICI < 0.13–0.5. In C. albicans 1601, we observed that (i) the expression of the seven genes increased notably in a range between 3.71- and 12.63-fold \((p < 0.05)\) when SH was used alone, (ii) the combined use of SH and fluconazole slightly inhibited the expression of IFD6 and PHR1 by 1.23- and 1.35-fold \((p > 0.05)\), but promoted evidently the expression of ZAP1, ADH5, XOG1 and FKS1 by 1.98-, 3.56-, 4.10- and 2.86-fold \((p < 0.05)\). The results suggested SH to be a potential synergist to enhance the antifungal activity of fluconazole in C. albicans resistant isolates, and the underlying mechanism may be associated with \(\beta\)-1,3-glucan synthesis and transportation.

**Introduction**

Recently, the incidence of invasive mycotic infection has increased significantly (Sardi et al. 2013). Among the infectious fungal pathogens, Candida albicans is the most frequently isolated opportunistic fungi from immunosuppressed individuals and patients implanted with types of catheters and artificial valves (Poulain 2013). Studies have shown that 75% of women were affected by vaginal candidosis at least once during their lifetime (Sobel 2007) and 90% of HIV-infected patients were suffered from oropharyngeal candidosis (de Repentigny et al. 2004). More importantly, invasive candidiasis was reportedly able to cause as high as 40–60% mortality rates (Tobudic et al. 2012).

Current antifungal agents include azoles, polyenes and echinocandins, among which azoles are the most common agent targeting the synthesis of fungal sterols (Zavrel & White 2015). As a consequence of increased abuse of traditional antifungal agents and antibiotics, the ever-increasing rate of resistance of C. albicans, especially to fluconazole poses a serious threat to antifungal therapy, calling for urgent need in search of novel antifungal drugs. To date, most of the reported chemicals claimed to possess potential antymycotic functions have relatively high minimum inhibitory concentrations (MICs); however, these antifungals usually had strong potential of resistance reversion in fluconazole-resistant C. albicans (Quan et al. 2006; Zhou et al. 2012; Letscher-Bru et al. 2013; Padmavathi et al. 2015). Therefore, finding new drugs capable of improving the antifungal activity of fluconazole can be taken into account as an alternative way to expand the antifungal bank (Guo et al. 2008).

Sodium houttuynonate (SH, \(\text{CH}_3(\text{CH}_2)_8\text{COCH}_2\text{CHOHSo}_4\text{Na}\)) is a chemical compound synthesized by houttuynin (\(\text{CH}_3(\text{CH}_2)_8\text{COCH}_2\text{CHO}\)) and sodium bisulfite (Shao et al. 2012). In previous reports, we observed antibacterial and antifungal potentials of SH against Pseudomonas aeruginosa, Staphylococcus epidermidis and C. albicans (Shao et al. 2013; Huang et al. 2015). Of interest, SH appeared to be more potent against C. albicans reference strain (MIC = 32–64 \(\mu\)g/mL) than P. aeruginosa reference strain (MIC = 256–512 \(\mu\)g/mL). To our knowledge, the antifungal effect of SH in combination with fluconazole and the underlying mode of action have not been reported.

The conventional mechanisms of resistance to fluconazole in C. albicans are usually attributed to overexpression/mutation of the target enzyme of azoles encoded by \textit{ERG11} and drug efflux pump controlled by Cdr1p, Cdr2p belonging to ATP-binding cassette superfamily (ABC transporter) and Mdr1p, a member of major facilitator superfamily (MFS) (Niimi et al. 2004; Holmes...
et al. 2008; Xiang et al. 2013; Prasad & Rawal 2014; Flowers et al. 2015). Nevertheless, a series of studies affirmed that sequestering fluconazole into cytoplasm caused by β-1,3-glucan, one of the main components in C. albicans cell wall and encoded by FKS1, accounted for fluconazole resistance in clinical C. albicans isolates (Mio et al. 1997; Nett et al. 2007; Zarnowskij et al. 2014). Furthermore, β-1,3-glucan can be secreted into the supernatant that constituted the complex three-dimensional structure of C. albicans biofilm, also responsible for conferring fluconazole resistance to biofilm phenotype (Nett et al. 2007; Zarnowskij et al. 2014). In this study, we employed 18 C. albicans clinical isolates as well as a standard one to test the antifungal effects of SH and/or fluconazole via broth microdilution method, scanning electron microscope (SEM) and the expression of seven genes associated with β-1,3-glucan synthesis and transportation by quantitative reverse transcription polymerase chain reaction (qRT-PCR).

**Materials and methods**

**Strains and cultivation**

*Candida albicans* SC5314 was kindly provided by Prof. YuanYing Jiang from College of Pharmacy, Second Military Medical University, Shanghai, China. The clinical *C. albicans* isolates were kindly provided by Prof. HuaiWei Lu, Clinical Laboratory, Anhui Provincial Hospital, Hefei, China. These isolates were preliminarily identified by germ tube production, carbohydrate assimilation and fermentation by commercial Yeast Identification Kit Systems (Tianhe, Hangzhou, China). Further identification was performed by PCR method as described previously (Miyakawa et al. 1993). All strains were stored in YPD medium (1% yeast extract, 2% peptone, 2% glucose; SHFENG, Shanghai, China) and 20% glycerol at −80°C. After subculturing on sabouraud dextrose broth (SDB, SHFENG, Shanghai, China) for 24 h at 37°C, a 1% cell suspension containing approximately 1 × 10⁶ CFU/mL was mixed with 8 × 10⁴ CFU/mL SH at 37°C for 24 h into a sterilized, flat-bottomed 24-well polystyrene microtiter plate (Corning, NY). The well with no agent was set as control. After centrifuging at 3000g for 5 min, the collected cell pellets were washed three times by sterilized PBS, and transferred into RNase-free screw-cap tubes. Total RNA was extracted by using MagExtractor-RNA kit (ToyoBo, Tokyo, Japan). Six microliters of the extracted total RNA was coincubated with 2 μL 4 × DNA Master: gDNA Remover and 2 μL 5RT-Master MixII. Then, the extracted RNA was reverse-transcribed into cDNA followed by: 65°C for 5 min, 4°C for 1 min; 50°C for 5 min, 98°C for 5 min and 4°C for 1 min, according to ReverTra Ace qPCR RT Master Mix with gDNA Remover kit (ToyoBo, Tokyo, Japan). The 10-fold diluted cDNA was used for all experiments. All primers were performed on ice. The primers of *IFD6, PHR1, ZAP1*, *ADH5, BGL2, XOG1, FKS1* and *ACT1* (Table 1) were synthesized by Sangon Biotech (Shanghai, China). Real-time PCR mixture (25 μL) was composed of 12.5 μL SYBR Green Realtime PCR, 0.5 μL cDNA and 10 μL ddH₂O. The reaction was run on ABI7000 fluorescent quantitative PCR system (Applied Biosystem, Shanghai, China) with conditions as follows: initial step at 95°C for 60 s, then 40 cycles at 95°C for 15 s, 55°C for 15 s, 72°C for 45 s. All data were normalized to housekeeping gene *ACT1* (the internal reference gene). The relative target-gene expression was calculated as a fold change of 2−ΔΔCt value, in which ΔΔCt = ΔCt_target gene − ΔCt_internal reference genes as previously described (Livak & Schmittgen 2001).

**SEM**

After the treatments of SH and/or fluconazole, the sample was fixed by 2.5% glutaraldehyde overnight, and dehydrated by 30, 50, 70 and 100% ethanol for 10 min each. After air drying, the sample was sputter coated with gold in a vacuum evaporator, and the morphological observation was performed by a scanning electron microscope (SEM, JSM-6700F, Japan).

**Statistical analysis**

All experiments were performed triplicate in three different occasions. The values were reported as mean ± standard deviation.

| Table 1. The primers for qRT-PCR. |
|----------------------------------|
| **Primer** | **Sequence (5’→3’)** |
| IFD 6-f | TGGGAGGATTTGGATCTCTGTGT |
| IFD 6-r | CGAGTGCAATGATTCCCTATAGTT |
| PHR 1-f | CGAACAACTGTCATAGGAGTAGAC |
| PHR 1-r | TGGATCCAGAAAGTAGATGGCAG |
| ZAP 1-f | CGACCTAACACACACCACGCCTAC |
| ZAP 1-r | CCCCCTGTGGCTTCTTTGG |
| ADH 5-f | GTGGCCCTGGTCATGCGAG |
| ADH 5-r | TACAAAGACCAACATCTTAGGG |
| BGL 2-f | CCACCGCTTCACTTCAAGG |
| BGL 2-r | TGGACTTTACGACATCTTTCAT |
| XOG 1-f | CAGTTGCCGAAATCTCATGCAAG |
| XOG 1-r | AAATCACAATGGTGAGTCAAG |
| FKS 1-f | TGCTGTCCTCAATGGAGTAGTT |
| FKS 1-r | TGAACATCCATGACCCCTAAAAC |
| ACT 1-f | AGCTTGGTCTGACAGATGATT |
| ACT 1-r | GGAGTTGAAATGTTTGGTCCA |
(SD) and calculated by SPSS 17.0 (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) was applied and \( p < 0.05 \) was considered as statistically significant.

**Results**

**Antifungal activity of SH and/or fluconazole**

We employed 18 clinical *C. albicans* isolates as well as a reference strain *C. albicans* SC5314 to survey the antifungal effects of SH and/or fluconazole. It could be observed that the MICs of SH ranged from 32 to 256 \( \mu \)g/mL when SH was used alone, while decreased in a range of 8–64 \( \mu \)g/mL when used in combination with fluconazole. Compared with fluconazole used alone, the MICs of fluconazole were reduced 2–256-fold in concomitant use with SH. According to the FICI calculated, we found that SH was readily inclined to display synergism with fluconazole against fluconazole-resistant *C. albicans* isolates (MIC >64 \( \mu \)g/mL) in comparison with fluconazole-sensitive isolates (MIC <64 \( \mu \)g/mL, Table 2). Subsequently, the morphology was inspected by SEM in *C. albicans* 1601 at their synergistic MIC (8 \( \mu \)g/mL fluconazole and 16 \( \mu \)g/mL SH). Compared with the control, it was clear that the fungal cells were dramatically reduced and only yeast-form cells remained (Figure 1).

**Impacts of SH and/or fluconazole on gene expressions**

Compared with the reference gene ACT1, the expression of ZAP1 and ADH5 was kept constant, while IFD6 and PHR1 was downregulated by 5-fold and 2-fold, respectively (\( p < 0.05 \)), BGL2, XOG1 and FKS1 were upregulated by 1.82-, 1.92- and 1.47-fold after 8 \( \mu \)g/mL fluconazole treatment (\( p < 0.05 \), Figure 2). When exposed to 16 \( \mu \)g/mL SH, the expression of all tested genes exhibited notable increase in a range between 3.71- and 12.63-fold (\( p < 0.05 \), Figure 2). Under the combined application (8 \( \mu \)g/mL fluconazole +16 \( \mu \)g/mL SH), it could be observed that (i) BGL2 was not affected, (ii) the expression of IFD6 and PHR1 was slightly inhibited by 1.23- and 1.35-fold respectively with no significant differences (\( p > 0.05 \)), (iii) the expression of ZAP1, ADH5, XOG1 and FKS1 increased evidently ranging between 1.98- and 4.10-fold (\( p < 0.05 \), Figure 2). A simple illustration was presented to describe the effect of SH in combination with fluconazole on the gene expression associated with \( \beta \)-1,3-glucan transportation and biofilm maturation (Figure 3).

**Discussion**

*Candida albicans* is currently the main cause for invasive fungal infections due to the recalcitrant resistance to traditional antifungal agents, such as fluconazole. Recruiting more drug-assisting fluconazole from the existent antibacterial and anti-inflammatory agents is a favourable option. SH was reported to possess mild antimicrobial activity against *P. aeruginosa* and *S. epidermidis*, and also show a certain inhibition on *C. albicans*. Due to limited isolates and experimental conditions adopted, however, we did not observe consistent MICs of SH against *C. albicans* (Shao et al. 2013; Huang et al. 2015). Herein, we expanded the *C. albicans* isolates and made a rigorous test on the antifungal activity of SH and/or fluconazole. SH alone displayed more efficient anti-*C. albicans* effect compared with its effect on pathogenic bacteria (Table 2). The synergism of SH with fluconazole against fluconazole-resistant *C. albicans* indicated the strong potential of SH to
promote the therapy of fluconazole (Table 2). In addition, the haemolysis rate was less than 15% when the used concentration of SH alone reached to 256 μg/mL in a previous study of our group (Huang et al. 2015). As for C. albicans 1601, the cytotoxicity caused by the combined concentration of SH (=16 μg/mL) can be negligible. Actually, we have injected 500 mg/kg SH (much higher dosage than that for clinical use) into 15 BALB/c mice, fed them for 90 days to evaluate their tolerance, and observed no death (Huang et al. 2015). However, we are trying an in vivo test to further evaluate whether SH can be a promising synergist in the treatment of fluconazole-resistant C. albicans.

As described previously, β-1,3-glucan in cell wall could nonspecifically interact with fluconazole to prevent from penetration into fungal cell (Nett et al. 2007), conferring mostly a resistance to fluconazole in C. albicans. In this study, a group of genes associated with β-1,3-glucan synthesis and transportation were analyzed by qRT-PCR. As demonstrated previously, ADH5 and IFD6, both of which are predicted to encode alcohol dehydrogenases, receive respectively negative and positive regulations of ZAP1 encoding the zinc-response transcription factor Zap1 (Nobile et al. 2009). ZAP1 mutant strain could promote the production of β-1,3-glucan by 1.5-2-fold in biofilm matrix than the complemented and reference strains with no significant difference in biofilm biomass (Nobile et al. 2009). BGL2, XOG1 and PHR1 were assumed in charge of three separate pathways of β-1,3-glucan transportation synthesized by FKS1, a distinct pathway out of the control of ZAP1 (Taff et al. 2012).

In C. albicans 1601 (Figure 2), when fluconazole was used alone, the downregulated IFD6 was a sign of the increase of β-1,3-glucan, consistent with the responses of BGL2, XOG1 and FKS1 (p < 0.05), inferring that the strain would produce and transport more β-1,3-glucan to the fungal cell wall and the outer space to sequester fluconazole. The transportation of β-1,3-glucan could be complemented by the upregulation of BGL2 and XOG1 when PHR1 was inhibited. After SH was employed alone, the selected seven genes obtained notable expressions (p < 0.05). We presumed that SH might be able to suppress the accumulation of β-1,3-glucan outside the fungal cell by the negative regulations of ZAP1 and IFD6. However, the concentration of SH (16 μg/mL) was not sufficient to inhibit the growth of C. albicans 1601, leading to more synthesis and transportation of β-1,3-glucan to the cell wall of growing fungal cells. Exposure to SH and fluconazole simultaneously could further significantly suppress the accumulation of β-1,3-glucan outside the fungal cell as ZAP1 expression still acquired obvious increase (p < 0.05). To our surprise, the expressions of ADH5, XOG1 and FKS1 were significantly upregulated (p < 0.05). We hypothesized that SH could interact with β-1,3-glucan physically or chemically, inducing the enhancement of β-1,3-glucan synthesis and transportation.

In conclusion, we confirmed that SH could be a candidate of synergist with fluconazole against clinical C. albicans isolates. The qRT-PCR analysis of seven genes suggested the antifungal mechanism of SH and/or fluconazole was deeply involved with the synthesis and transportation of β-1,3-glucan.

### Disclosure statement

The authors report no conflicts of interest.

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**Table 2. Interactions of SH and/or fluconazole against clinical Candida albicans strains.**

| C. albicans strains | MIC<sub>90</sub> alone (μg/mL) | MIC<sub>90</sub> in combination (μg/mL) | FICI (Interpretation) |
|---------------------|-------------------------------|---------------------------------------|-----------------------|
| SC5314              | 32                            | 1                                     | 0.75 (IND)            |
| 1601                | 128                           | 16                                    | 0.19 (SYN)            |
| 1604                | 128                           | 32                                    | 0.25 (SYN)            |
| 2009                | 128                           | 32                                    | 0.16 (SYN)            |
| 2209                | 256                           | 32                                    | 0.13 (SYN)            |
| 1110                | 256                           | 64                                    | 0.75 (IND)            |
| 1107                | 128                           | 512                                   | 0.19 (SYN)            |
| 1041                | 128                           | 16                                    | 0.19 (SYN)            |
| 2305                | 64                            | 32                                    | 0.75 (IND)            |
| 2301                | 128                           | 64                                    | 0.53 (IND)            |
| 2103                | 128                           | 1024                                  | 0.19 (SYN)            |
| 2304                | 256                           | 32                                    | 0.19 (SYN)            |
| 2111                | 64                            | 32                                    | 0.25 (SYN)            |
| 2204                | 128                           | 512                                   | 0.38 (SYN)            |
| 1803                | 64                            | 16                                    | 0.5 (SYN)             |
| 2005                | 128                           | 16                                    | 0.38 (SYN)            |
| 2226                | 64                            | 16                                    | <0.31 (SYN)           |
| WY                  | 128                           | 4                                     | 0.75 (IND)            |

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**Figure 2.** qRT-PCR analysis of IFD6, PHR1, ZAP1, ADH5, BGL2, XOG1 and FKS1 expressions under the treatments of no drug (control), 8 μg/mL fluconazole, 16 μg/mL SH, and 8 μg/mL fluconazole +16 μg/mL SH in C. albicans 1601. *p < 0.05, compared with the control.

**Figure 3.** Illustration for the functions of SH and/or fluconazole on β-1,3-glucan transportation and biofilm maturation in fluconazole-resistant C. albicans.
