In Vitro Synergism of Fluconazole and Baicalein against Clinical Isolates of Candida albicans Resistant to Fluconazole

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Candida albicans is the most prevalent fungal pathogen and serious concern for patients with compromised immune systems such as cancer patients, transplant recipients and human immunodeficiency virus (HIV)-infected patients. Compared with bacterial infections, few drugs are available to treat fungal infections. This is largely attributable to the eukaryotic nature of fungal cells and the difficulty in identifying unique targets not shared with human hosts. Fluconazole has been shown effective in the treatment of candidemia and is currently used as first-line drug. However, with the increasing clinical use of fluconazole, fluconazole-resistant isolates are occurring frequently.

Attempts have been made to cope with treatment failures by using combination therapy. However, contradictory results of either synergic or antagonistic actions in various antifungal combinations have been reported. As for fluconazole-resistant C. albicans, few data are available about the synergism of fluconazole with other antifungal agents.

Baicalein (BE) is a major component of Scutellaria baicalensis, a Chinese herb described in the Chinese Pharmacopoeia as a medicine (Fig. 1). In addition to its inhibition effect on lipoygenase, BE was found to have antioxidant, neuroprotective, antibacterial, antiviral, and antifungal activities. To seek a novel combination therapy, we investigated the in vitro interaction of fluconazole and baicalein against fluconazole-resistant clinical isolates of C. albicans.

MATERIALS AND METHODS

Strains and Agents Thirty clinical isolates of fluconazole-resistant C. albicans were used in this study, including previously published isolates FH5 and TL3. BE (Sigma-Aldrich, St. Louis, MO, U.S.A.) was dissolved in dimethyl sulfoxide and fluconazole (Pfizer-Roerig Pharmaceuticals, New York, NY, U.S.A.) in distilled water. Dilutions were made in RPMI 1640 medium (Gibco, U.S.A.) supplemented with l-glutamine and buffered with morpholinepropanesulfonic acid (MOPS).

Checkerboard Microdilution Assay Assays were performed on all 30 isolates according to methods of the CLSI (formerly NCCLS) (M27-A2). The initial concentration of fungal suspension in RPMI 1640 medium was 10^6 colony forming unit (CFU)/ml, and the final concentrations ranged from 0.125 to 128 μg/ml for fluconazole and from 0.25 to 128 μg/ml for BE. Plates were incubated at 35 °C for 24 h. Optical density was measured at 630 nm, and background optical densities were subtracted from that of each well. Each isolate was tested in triplicate. The definition of minimum inhibition concentration (MIC) is the lowest concentration of a drug at which growth of tested strain is not observed. MIC80 and MIC50 mean the concentrations at which 80% and 50% of tested strain cannot grow. The fractional inhibitory concentration (FIC) index is defined as the sum of the MIC of each drug when used in combination divided by the MIC of the drug used alone. Synergism and antagonism were defined by FIC indices of ≤0.5 and >4, respectively. An FIC index result of >0.5 but ≤4 was considered indifferent.

Time–Kill Curves C. albicans in RPMI 1640 medium was prepared at the starting inoculum of 10^9 or 10^5 CFU/ml. The concentrations were 16 μg/ml for BE (a concentration of BE without effect on the growth curve) and 10 μg/ml for fluconazole (in vivo achievable concentration of fluconazole). DMSO comprised <1% of the total test volume. At predetermined time points (0, 12, 24, 36, 48 h after incubation with agitation at 35 °C), a 100-μl aliquot was removed from each solution and serially diluted 10-fold in sterile water. A 100-μl aliquot from each dilution was streaked on the Sabouraud dextrose agar plate. Colony counts were determined after incubation at 35 °C for 48 h. The experiment was performed in triplicate. Synergism and antagonism were defined as a re...

Fig. 1. Structure of Baicalein (BE)
Rhodamine 6G (R6G) was added to the final concentration used in combination, the MIC 80 of fluconazole and BE was (100%) in terms of MIC80. The corresponding median FIC individual agent. Synergism was observed in all 30 isolates with glucose supply, glucose was added to the final concentration of 1 mM to initiate R6G efflux. At specified intervals after the addition of glucose, the cells were removed by centrifugation, and 100-μl volume of the cell supernatants was transferred to the well of 96-well flat-bottom microplates (BMG Microplates, Black 96 well). The R6G fluorescence of the samples was measured by FLUOstar/POLARstar Galaxy (BMG labtechnologies, Germany). The excitation wavelength was 515 nm, and the emission wavelength was 555 nm.

RESULTS

Checkerboard Microdilution Assay The results of the checkerboard analysis are summarized in Table 1. Both fluconazole and BE showed weak antifungal activity when tested alone. However, the fluconazole–BE combination markedly reduced MICs, especially the MIC50 of either individual agent. Synergism was observed in all 30 isolates (100%) in terms of MIC50. The corresponding median FIC index was 0.069 (range, 0.037 to 0.098). When analyzed with MIC50, synergism was observed in 27 of 30 isolates (90%) and indifference was observed in 3 of the 30 isolates (10%); at MIC80, synergy was observed in all of the isolates. a) MICs in combination are expressed as [FLC]/[BE]. High off-scale MICs were converted to the next highest concentrations.

Rhodamine 6G Efflux Assay C. albicans cells from YPD cultures grown in the absence or presence of drugs were collected by centrifugation (3000×g, 20 °C, 5 min) and washed three times with phosphate buffered saline (PBS). The cells were subsequently resuspended in PBS (about 5×10^7 cells/ml) and incubated 2 h to exhaust the energy. Rhodamine 6G (R6G) was added to the final concentration of 10 μM. Cell suspensions were incubated at 30 °C with shaking (200 rpm) for 90 min to allow R6G accumulation. The cells were washed three times and the final concentration of the cells was kept at 5×10^7 cells/ml exactly. For the groups with glucose supply, glucose was added to the final concentration of 1 μM to initiate R6G efflux. At specified intervals after the addition of glucose, the cells were removed by centrifugation, and 100-μl volume of the cell supernatants was transferred to the well of 96-well flat-bottom microplates (BMG Microplates, Black 96 well). The R6G fluorescence of the samples was measured by FLUOstar/POLARstar Galaxy (BMG labtechnologies, Germany). The excitation wavelength was 515 nm, and the emission wavelength was 555 nm.

Table 1. Interaction of Fluconazole (FLC) and Baicalein (BE) against 30 Clinical Isolates of *C. albicans* Resistant to Fluconazole by Checkerboard Microdilution Assay

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MIC50 (μg/ml)</th>
<th>MIC80 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLC</td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>BE</td>
<td>64</td>
<td>≥32</td>
</tr>
<tr>
<td>FLC+BE</td>
<td>0.25/1</td>
<td>≤0.125—1/0.5—4</td>
</tr>
<tr>
<td>FIC index</td>
<td>0.179</td>
<td>0.058—1.377</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td></td>
<td>128</td>
<td>≥64</td>
</tr>
<tr>
<td></td>
<td>128</td>
<td>&gt;64</td>
</tr>
<tr>
<td></td>
<td>0.5/8</td>
<td>0.125—2/1.6—16</td>
</tr>
<tr>
<td></td>
<td>0.069</td>
<td>0.037—0.098</td>
</tr>
</tbody>
</table>

a) When analyzed with MIC50, synergism was observed in 27 of 30 isolates (90%) and indifference was observed in 3 of the 30 isolates (10%); at MIC50, synergy was observed in all of the isolates. b) MICs in combination are expressed as [FLC]/[BE]. High off-scale MICs were converted to the next highest concentrations.

The isolate was exposed to 10 μg/ml of fluconazole (FLC), 16 μg/ml of baicalein (BE), or a combination (10 μg/ml FLC plus 16 μg/ml BE). CFU were determined after 0, 12, 24, 36, and 48 h of incubation.

Fig. 2. Time–Kill Curves of *C. albicans* Isolate Tian25 (a Clinical Fluconazole-Resistant Isolate) Obtained by Using an Initial Inoculum of 10^5 CFU/ml (A) and 10^7 CFU/ml (B) The isolate was exposed to 10 μg/ml of fluconazole (FLC), 16 μg/ml of baicalein (BE), or a combination (10 μg/ml FLC plus 16 μg/ml BE). CFU were determined after 0, 12, 24, 36, and 48 h of incubation.

The fluorescent dye Rhodamine 6G is known to be the substrate of multidrug resistance pumps and its increased efflux has been demonstrated in a number of organisms that maintain multidrug resistance. Therefore we tested the Rhodamine 6G efflux in *C. albicans*. As shown in Fig. 3, without glucose supply, the function of efflux transporters was weak, and neither fluconazole nor BE had marked effect on the efflux ability. When glucose was added, the function of efflux transporters was enhanced significantly in all groups, among which the level of Rhodamine
6G in the supernatant of the cells grown in the presence of BE was clearly lower than that in untreated cells while a little higher than that in cells grown in the presence of fluconazole. Combination of fluconazole and BE resulted in an even lower level of Rhodamine 6G in the supernatant of the cells.

**DISCUSSION**

In this work, we demonstrate that BE is able to inhibit strongly the proliferation of clinical isolates of *C. albicans* in combination with subinhibitory concentrations of fluconazole. As one of the main ingredients of Chinese herb, *Scutellaria baicalensis*, BE has been studied extensively and reported to show a wide range of biological activities. The inhibitory activity of BE alone on microorganism was reported several years ago. Recently, Fujita et al. showed the synergism between BE, and tetracycline, and BE and β-lactams against methicillin-resistant *Staphylococcus aureus*. Chang et al. found synergism of BE and gentamicin against vancomycin-resistant *Enterococcus*. Here our findings of chemosensitising activity of BE suggest promising clinical application of this compound against drug-resistant fungi.

It is well known that enhanced extrusion of drugs through efflux pumps, which is observed in all cells from microorganisms to mammalians, constitutes a major cause of multidrug resistance (MDR). BE has been reported as an inhibitor of multidrug efflux pumps. To explore the mode of the antifungal action of BE, we measured Rhodamine 6G in the supernatant of *C. albicans*, which represents the function of efflux pumps. The reduced efflux of Rhodamine 6G of cells grown in the presence of BE indicates that BE might reduce the extrusion of drug out of the yeast cells by inhibiting efflux pumps, thus increasing the susceptibility of the cells to antifungals. Of note, combination of fluconazole and BE resulted in an additive effect on Rhodamine 6G efflux, although the result of MIC<sub>90</sub> was clearly synergistic. This suggests that inhibition of efflux pumps was not the only mechanism of BE against fluconazole-resistant *C. albicans*. Indeed, besides active efflux of drugs, several other mechanisms for resistance to azoles have been reported, such as target enzyme alteration and metabolism shift. We postulated that BE may exert the synergistic effect with fluconazole also by affecting some other factors on resistance.

Further studies are needed to uncover the exact mechanism of BE action. Nevertheless, its chemosensitising activity in combination with fluconazole may be of interest in the development of new strategies to combat resistant fungal infections.

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**REFERENCES**