Synergistic Antifungal Effect of Glabridin and Fluconazole

Wei Liu¹, Li Ping Li¹, Jun Dong Zhang¹, Qun Li¹, Si Min Chen¹, Li Juan He¹, Lan Yan², Guo Tong Xu¹, Mao Mao An¹*, Yuan Ying Jiang¹,²*

¹Tongji University School of Medicine, Shanghai, China, ²New Drug Research and Development Center, School of Pharmacy, Second Military Medical University, Shanghai, China

Abstract

The incidence of invasive fungal infections is increasing in recent years. The present study mainly investigated glabridin (Gla) alone and especially in combination with fluconazole (FLC) against Cryptococcus neoformans and Candida species (Candida albicans, Candida tropicalis, Candida krusei, Candida parapsilosis and Candida Glabratas) by different methods. The minimal inhibitory concentration (MIC) and the minimal fungicidal concentration (MFC) indicated that Gla possessed a broad-spectrum antifungal activity at relatively high concentrations. After combining with FLC, Gla exerted a potent synergistic effect against drug-resistant C. albicans and C. tropicalis at lower concentrations when interpreted by fractional inhibitory concentration index (FICI). Disk diffusion test and time-killing test confirming the synergistic fungicidal effect. Cell growth tests suggested that the synergistic effect of the two drugs depended more on the concentration of Gla. The cell envelope damage including a significant decrease of cell size and membrane permeability increasing were found after Gla treatment. Together, our results suggested that Gla possessed a synergistic effect with FLC and the cell envelope damage maybe contributed to the synergistic effect, which providing new information for developing novel antifungal agents.

Introduction

Despite recent progress in the clinical management, invasive fungal infections are still a tricky problem and have a high mortality. Candida species are the fourth most important cause of hospital-acquired bloodstream infections. Besides, in developing countries systemic cryptococcosis remains large and increasing [1]. The most common isolated Candida species in clinical fungal invasive infection is Candida albicans, followed by Candida tropicalis, Candida parapsilosis and Candida glabratas [2–5]. Cryptococcus neoformans is the first or second most common cause of culture-proven meningitis [1]. One most common agent used in clinic is fluconazole (FLC). However, during long-time or repeated treatment, FLC resistance strains are easily developed [6]. The combination of two or more antifungal agents maybe a feasible policy to solve the problem.

Currently, researches on natural products which have potent synergisms with antifungal drugs have been raised. For example, retigeric acid B, a pentacyclic triterpenoid isolated from a lichen called Lobaria kurokawae Yoshim, can increase the susceptibilities of azole-resistant C. albicans strains in combination with azoles [7,8]. Plagiochin E, a macrocyclic bis (bibenzyl) isolated from the liverwort Marchantia polymorpha, has antifungal activity and resistance reversal effects for C. albicans [9]. Besides, berberine chloride, baicalein, allicin, pure polyphenol curcumin I, pseudolaric acid B, eugenol and methyleugenol were also reported to have synergistic antifungal properties in combination with known antifungals [10–15].

Glabridin (Gla) [4-(8,8-dimethyl-2,3,4,8-tetrahydropyrano[2,3-f]chromen-3-yl)-benzene-1,3-diol] is a major active isoflavan from Glycyrrhiza glabra. It has been reported that Gla had numerous beneficial properties, including antioxidant, anti-cancer, neuroprotective, anti-inflammatory activities, inhibiting fatigue or reversing learning and memory deficits in diabetic rats [16–23]. It possessed weak activity against C. albicans, C. krusei, C. neoformans and other filamentous fungi [24,25]. However, to our knowledge, no study to date has focused on its interaction with FLC.

In this study, synergistic antifungal effect of Gla and FLC against FLC-resistant clinical isolates of C. albicans and other yeast fungi (i.e. C. neoformans, C. tropicalis, C. parapsilosis, C. krusei and C. glabratas) and the possible mechanisms were investigated.

Materials and Methods

Strains and chemicals

25 clinical isolates of FLC-resistant C. albicans, and one C. neoformans 32609, C. tropicalis 2718 and C. parapsilosis ATCC 22019 were kindly provided by the Changhai Hospital, Shanghai, China. C. krusei ATCC2340 and C. glabratas ATCC1182 were kindly provided by professor Changzhong Wang (School of integrated traditional and western medicine, Anhui university of
traditional Chinese medicine, Hefei, China). The susceptibilities of these strains to FLC were measured by broth microdilution method at advance. Frozen stocks of isolates were stored at −80°C in culture medium supplemented with 40% (vol/vol) glycerol and were subcultured twice at 35°C before each experiment. FLC (sigma Aldrich, St. Louis, MO) was obtained commercially. Gla (purity 98%) was obtained from Yuan Cheng Pharmaceutical Co. Ltd, China, and its initial stored concentration was 6.4 mg/ml in dimethyl sulfoxide (DMSO).

**Antifungal susceptibility testing**

The minimal inhibitory concentrations (MIC) of Gla and FLC against the yeast strains were determined by broth microdilution method as described previously [10]. The yeast at final concentration of 10^6 cells/ml in the RPMI 1640 liquid medium with serial (2×) dilutions of each drug were inoculated in 96-well flat-bottomed microtitration plates. After incubation at 35°C for 24 h or 72 h. Optical densities (OD) were measured at 630 nm with a microtitre plate reader (Thermolabsystems Multiskan MK3), and background optical densities were subtracted from that of each well. MIC was determined as the lowest concentration of the drugs that inhibited growth by 80% compared with that of drug-free wells. DMSO comprised <1% of the total test volume. The quality control strain, C. parapsilosis ATCC 22019 was included in each susceptibility test to ensure quality control. The MIC range of FLC to C. parapsilosis ATCC 22019 was from 0.5 μg/ml to 4 μg/ml, which meant this test was acceptable.

**Checkerboard microdilution assay**

Assays were performed on all isolates according to broth microdilution checkerboard method [10]. The initial concentration of fungal suspension in RPMI 1640 medium was 10^6 cells/ml, and the final combination concentrations ranged from 0.125 to 64 μg/ml for FLC and 1 to 16 μg/ml for Gla. The final concentration for FLC or Gla alone ranged from 0.125 to 64 μg/ml. 96-well flat-bottomed microtitration plates were incubated at 35°C for 24 h or 72 h. OD was measured at 630 nm, MIC was determined as the above.

The data obtained by the checkerboard microdilution assays were analyzed using the model-fractional inhibitory concentration index (FICI) method based on the Loewe additivity theory. FICI was calculated by the following equation: FICI = FIC A+FIC B, where FIC A is the MIC of the combination/the MIC of drug A alone, and FIC B is the MIC of the combination/the MIC of drug B alone. Among all of the FICIs calculated for each data set, the FICImin was reported as the FICI in all cases unless the FICImax was greater than four, in which case the FICImax was reported as the FICI. Synergy was defined as an FICI value of ≤0.5, while antagonism was defined as an FICI value of >4, addition was defined as an FICI value of 0.5< FICI≤1. An FICI result between 1 and 4 (1< FICI≤4) was considered indifferent [7]. The fractional fungicidal concentration index (FICI) was calculated the same.

**Agar disk diffusion test**

*C. albicans* 103 (one FLC-resistant isolate with a MIC of 32 μg/ml for Gla) and other yeast strains were tested by agar diffusion test [10]. 3 ml of aliquot of 10^6 cells/ml suspension was spread uniformly onto the yeast peptone dextrose (YPD) agar plate with or without 64 μg/ml FLC. Then, 6 mm paper disks impregnated with Gla alone or in combination with FLC were placed onto the agar surface. There was 5 μl DMSO in control disks. Photos were taken after incubation at 35°C for 48 h.

**Time-killing test**

*C. albicans* 103 and other yeast strains were prepared at the starting inoculum of 10^6 cells/ml. The concentrations were 4, 8, 16 μg/ml for Gla and 8 μg/ml for FLC, DMSO comprised <1% of the total volume. At predetermined time points (0, 4, 8, 12, 16 and 24 h) after incubation with agitation at 35°C, a 100 μl aliquot was removed from every solution and serially diluted 10-fold in sterile water. A 100 μl aliquot from each dilution was spread on the sabouraud dextrose agar plate. Colony counts were determined after incubation at 35°C for 48 h. Fungicidal activity was defined as a ≥3 log10 reduction from the starting inoculum. Synergism and antagonism were defined as a respective decrease or increase of ≥2 log10 CFU/ml in antifungal activity produced by the combination compared with that by the more active agent alone [26].

**Cell growth test**

*C. albicans* 103 was prepared at the starting inoculum of 10^6 cells/ml in glass tubes. Different concentrations of Gla (2, 4, 8, 16 μg/ml) and FLC (2, 4, 8, 16, 32, 64 μg/ml) alone or the combinations of Gla (2, 4, 8, 16 μg/ml) and FLC (2, 4, 8, 16, 32, 64 μg/ml) were added into tubes. After incubation with agitation at 35°C for 24 h, pictures were taken. Aliquot was removed from each tube and serially diluted 10-fold in sterile water. A 100 μl aliquot from each dilution was spread on the sabouraud dextrose agar plate. Colony counts were determined after incubation at 35°C for 48 h.

**Table 1. MICs and MFCs of Gla alone and in combination with FLC against 25 clinical FLC-resistant C. albicans.**

|                | MIC (μg/ml) | MFC (μg/ml) |
|----------------|-------------|-------------|
|                | median      | range       | median      | range       |
| FLC            | 128         | 64–>256     | >256        | >256        |
| Gla            | 32          | 32–64       | 64          | 32–64       |
| FLC/Gla*       | 1/4         | 1–1/4–4     | 8/16        | 4–16/8–16   |
| FIC index      | 0.13        | 0.04–0.14   | 0.27        | 0.26–0.31   |
| Interaction effect (n/%)b | Syn (25/100) | Syn (25/100) |

*aMIC and MFC in combination expressed as [FLC]/[Gla].

bSyn, synergism. The number of strains and percentage for the interaction effect were shown.

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Cell membrane permeability

Membrane permeabilization of *C. albicans* was detected according to a previous study [27]. Briefly, *C. albicans* 103 (1 × 10^10 cells/ml) were incubated with 10 μM calcein acetoxy-methyl ester (Fanbo biochemicals, China) for 2 h. The cells were then washed three times and *C. albicans* (1 × 10^10 cells/ml) was transferred to tubes. After treatment with or without FLC (64 μg/ml), MCZ (64 μg/ml) and Gla (16 μg/ml, 32 μg/ml, 64 μg/ml) for 3 h. The cells were washed three times and about 10,000 cells were acquired for flow cytometry analysis (Maflo Astrios flow cytometer). Experiments were repeated at least two times independently on separate days.

Cell wall inhibitors sensitivity test

Congored and calcofluorwhite (CFW) were incorporated into YPD agar plates at 100 μg/ml and 15 μg/ml, respectively. Yeast cells were grown in YPD medium with or without Gla (8, 16 μg/ml) for 12 h and 3 μl drops of serially diluted suspensions were inoculated into plates. After incubation for 48 h at 30°C, pictures were taken.

Results

The combination of Gla and FLC against clinical FLC-resistant *C. albicans*

The MIC values of Gla tested alone or in combination with FLC in FLC-resistant *C. albicans* were shown in Table 1. According to the interpretive breakpoints for FLC (≤8 μg/ml and ≥64 μg/ml, respectively), 25 clinical FLC-resistant *C. albicans* were selected. The MICs of Gla against all tested strains ranged from 32 μg/ml to 64 μg/ml. When MIC-like assays were performed for FLC in the presence of fixed subinhibitory concentrations of Gla (4 μg/ml), the median MICs of FLC decreased from 128 μg/ml to 1 μg/ml in resistant strains (32-fold to 512-fold reductions). According to the analysis of FICI method, synergism was observed in all 25 tested strains (FICIs < 0.2).

The combination of Gla and FLC against other varied FLC-susceptibility strains

We also tested antifungal effects of Gla alone or in combination with FLC in FLC-sensitive *C. albicans* and the other yeast strains (*C. neoformans, C. tropicalis, C. krusei, C. parapsilosis* and *C. glabratas*) (Table 2). In these strains, the range of MICs of Gla tested alone was from 16 μg/ml to 64 μg/ml, when in combination with FLC the MICs of Gla ranged from 1 μg/ml to 16 μg/ml. In *C. tropicalis* and *C. krusei*, the MIC of FLC were reduced from >64 μg/ml to 4 μg/ml or 8 μg/ml respectively after in combination with Gla. In *C. glabrata*, no synergistic effect was observed.

In combination with FLC, Gla at lower concentrations exhibits fungicidal effect for FLC-resistant *C. albicans* by different methods

Cells from the microdilution assays after incubation with Gla, FLC or the combination of Gla and FLC at various concentrations were plated on the sabouraud dextrose agar plate to count the colony forming unit (CFU) for determination of the MFC [100] (the minimal concentration with complete cell killing, i.e. no CFU counted). As shown in Table 1, the MFC of FLC can be much higher than the MIC and complete cell killing was not achievable. The range of the MFC of Gla was from 32 μg/ml to 64 μg/ml. When in combination with FLC (4 μg/ml or 8 μg/ml), Gla at 16 μg/ml showed fungicidal effect against all strains tested.
Further to visualize their synergistic fungicidal effect, different concentrations of Gla and the combination with FLC (8 μg/ml) were analyzed by agar disk diffusion assay. Gla alone at 64, 32, 16, 8 μg per disc had minimal fungicidal activity against the FLC-resistant \textit{C. albicans} 103. While FLC at 8 μg per disc showed weak inhibition effect against \textit{C. albicans}, the halo surrounding the disc was cloudy with colony (Fig. 1A). Interestingly, when FLC was combined with Gla, the halo surrounding the disc was significantly clearer. The diameters of the zones were clearer and larger than those of either drug alone on the plain agar plate, which was an indication of potent synergistic fungicidal activity (Fig. 1D). Similarly, on the agar plate containing 64 μg/ml FLC, Gla also yielded significantly clearer and larger zones at 64, 32, 16, 8 μg per disc (Fig. 1B).

In addition, their synergistic fungicidal effect was confirmed by time-killing test (Fig. 2). Gla alone at 16 μg/ml showed fungicidal effect and led to a decrease of 3.57–log_{10} CFU/ml at 24 h. No appreciable antifungal activity of FLC alone at 8 μg/ml was observed, but the combination of FLC (8 μg/ml) and Gla (4, 8 or 16 μg/ml) yielded 3.14, 3.62 or 4.10–log_{10} CFU/ml reductions compared with Gla alone at 24 h (Table 3). Besides, the combination of Gla at 16 μg/ml and FLC at 8 μg/ml almost resulted in a complete cell-killing at 24 h (Fig. 2C).

In order to determine the relationship between the synergistic effect and the dosage of Gla and FLC, different concentrations of Gla (2 μg/ml–16 μg/ml) and FLC (2 μg/ml–64 μg/ml) were used in the cell growth test. Our results indicated that the synergism of the two drugs depended more on the concentration of Gla than FLC (Fig. 3). 4 μg/ml and 8 μg/ml Gla alone had no antifungal effect, while 64 μg/ml FLC had a weak antifungal activity. The antifungal effect was improved significantly after the two drugs used together at different concentrations except when FLC used at the concentration of 2 μg/ml. More specifically, 16 μg/ml Gla alone had an antifungal effect, while after combining with FLC (4 μg/ml–64 μg/ml), significantly synergistic effects were observed, and even complete cell killing activities were found when the concentration of FLC were above 16 μg/ml. Interestingly, the synergistic effects of the two drugs were unchanged when the dose of FLC declined from 64 μg/ml to 16 μg/ml, but when the combination concentration of FLC was below 16 μg/ml, the synergistic effect was lessened with the dosage of FLC decreasing.

Synergistic effect of FLC and Gla against other yeast strains

The interactions of FLC and Gla against the other yeast strains (i.e. FLC-sensitive \textit{C. albicans}, \textit{C. tropicalis}, \textit{C. neoformans}, \textit{C. parapsilosis}, \textit{C. krusei} and \textit{C. glabrata}) were investigated by
MFCs, agar disk diffusion assay and time-killing test. As shown in Table 2, the strains showed varied susceptibility to Gla and FLC, and there was no apparent correlation between the susceptibility towards Gla and the susceptibility towards FLC. Consistent with results from the disc diffusion assays, the *C. krusei* and *C. glabrata* were highly resistant to FLC. The range of MFC of Gla for each strain tested was from 32 μg/ml to 64 μg/ml, supporting its fungicidal property. Synergistic fungicidal interactions between Gla and FLC were also observed in *C. tropicalis* by counting cells from the microdilution assay. The halo surrounding the discs with FLC and Gla was significantly clearer (Fig. 4) and the diameters of the zones were larger than those of either drug alone on the plain agar plate for FLC-sensitive *C. albicans*, *C. tropicalis* and *C. neoformans*. Besides, the FLC+Gla combination yielded a decreased CFU compared with Gla alone in FLC-sensitive *C. albicans*, *C. tropicalis* and *C. neoformans*, and even greater

**Table 3.** Decrease in log_{10} CFU/ml of yeast strains using different concentrations of Gla combining with FLC at 24 h.

| FLC+Gla (μg/ml) | Mean (±SD) decrease in log_{10} CFU/ml compared with Gla alone |
|-----------------|---------------------------------------------------------------|
|                 | *C. albicans 103* | *C. albicans SC5314* | *C. tropicalis 2718* | *C. neoformans 32609* |
| 8+4             | 3.14 (0.08)       |                     |                     |                     |
| 8+8             | 3.62 (0.11)       | 1.51 (0.08)         | 1.64 (0.09)         | 4.42 (0.12)         |
| 8+16            | 4.10 (0.30)       | 1.82 (0.09)         | 3.16 (0.11)         | 4.50 (0.11)         |

Figure 2. Time killing curves of *C. albicans* 103 treated with different concentrations of Gla and FLC. FLC-resistant *C. albicans* 103 were treated with FLC (8 μg/ml), Gla (4 μg/ml) and FLC+Gla (4+8) μg/ml (A), Gla (8 μg/ml) and FLC+Gla (8+8) μg/ml (B) or Gla (16 μg/ml) and FLC+Gla (8+16) μg/ml (C) for 24 h. Aliquots were obtained at the indicated time points and serially dilutions were spreaded on agar plates. Colony counts were determined after 48 h incubation.

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reductions were observed in *C. neoformans* and *C. tropicalis* (>3 Log_{10} CFU/ml decrease, fungicidal effect can be defined) (Table 3). However, the synergistic fungicidal effect of FLC and Gla was not observed in *C. krusei* and *C. glabrata* by agar disk diffusion assay and time-killing test (results not shown), which was consistent with FFCI values of the previous tests.

### Effect of Gla on the cell envelope

Flow cytometry analysis (side scatter [SSC]-forward light scatter [FSC]) showed that *C. albicans* 103 treated with FLC underwent weak cell shrinkage, while the cells treated with MCZ exhibited a significant decrease in cell size, as evidenced by the decrease in forward light scattering. Interestingly, changes in cell size were also observed after exposure to Gla, especially to Gla at the concentration of 32 µg/ml and 64 µg/ml (Fig. 5).

Calcein AM is a non-fluorescent derivative of calcein that can readily diffuse across membranes. Once within the cytoplasms of target cells, calcein AM is hydrolyzed by cytoplasmic esterases, yielding membrane-impermeable calcein which could be loaded into intact cells. After incubation with calcein AM, the cellular fluorescence of calcein was detected and quantified by flow cytometry to evaluate the effect of Gla on the cell membrane permeabilization. Results showed that cellular calcein was markedly decreased by treatment of *Candida* cells with different concentrations of Gla (16 µg/ml, 32 µg/ml, 64 µg/ml), while by FLC a slight reduction of cellular calcein was observed (Fig. 6).

We also investigated the effect of Gla on the cell wall carbohydrates. Spot assays indicated that 16 µg/ml Gla treatment made *C. albicans* become more sensitive to both cell wall inhibitors (CFW and congored) compared with the control cells (Fig. 7). We used concanavalin A, calcofluorwhite (CFW) and specific anti-β-glucan primary antibody to stain the carbohydrates (mannan, chitin and glucan). However, fluorescence micrographs did not show obvious change in the three cell wall layers (Fig. S1).
Figure 4. Agar disk diffusion assay of Gla alone or in combination with FLC against *C. albicans* SC5314, *C. tropicalis* and *C. neoformans*.

Upper agar plates of disks were impregnated with 64, 32, 16 and 8 µg of Gla or 5 µl of DMSO as control disk. In lower agar plates, disks were impregnated with FLC+Gla (64+8) µg, FLC+Gla (32+8) µg, FLC+Gla (16+8) µg, FLC+Gla (8+8) µg, FLC (8 µg) or 5 µl of DMSO as control disk. Left sketch panels describe the images for the right agar plates.

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Figure 5. Changes in cell size (forward scatter-side scatter) in the presence of Gla. FLC-resistant *C. albicans* 103 were treated with or without Gla (16, 32, 64 µg/ml), FLC (64 µg/ml), MCZ (64 µg/ml) for 3 h. Then the cells were analyzed by flow cytometry.

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Discussions

A large number of natural products from plants are reported to possess potent antifungal properties in recent years, such as terpene derivatives, flavans, nucleosides, peptides, alkaloids, saponins and sterols [28]. Gla is an isoflavan from *G. glabra* root. Previous studies have been reported that the alcohol or ethanol extract of the roots of *G. glabra* possessed weak antifungal activity against *C. albicans*, *Arthrinium sacchari*, *Chaetonotum funicola* and various filamentous fungi [24,29,30]. Synergistic activity of Gla and nystatin against oral *C. albicans* has been demonstrated previously [25]. But to our knowledge, there was no investigation on the combination of Gla and FLC against fungi.

Here, we demonstrated that Gla had weak antifungal activity against different fungi such as *Candida* species and *C. neoformans*, consisting with the previous reports [24,25,29,30]. When combining with FLC, Gla exerted a strong synergistic antifungal effects at lower concentrations. Different assays indicated a potent synergistic effect of Gla and FLC against FLC-resistant *C. albicans*, *C. neoformans* and *C. tropicalis*. The synergistic effects depended more on the concentration of Gla. According to Celine Messier’s study on the toxic effect of Gla for oral epithelial cells (67% cell viability at 10 μg/ml), the concentration of Gla required for reducing the MIC of FLC ranged from 1 μg/ml to 4 μg/ml below the concentration which significantly reduced cell viability [25]. In order to determine whether this property of glabridin is specific to other isoflavans, we selected a second isoflavan equol and tested the interaction of equol and FLC against FLC-resistant *C. albicans* by checkerboard microdilution assay. The results showed a weak synergistic effect between equol and FLC. The MIC of equol alone was >1280 μg/ml, only when the concentration of equol was at 32 μg/ml a synergism was observed between equol and FLC, the concentration of FLC was reduced from >64 μg/ml to 4 μg/ml. This may suggested that there were synergistic effects between isoflavans and FLC, but the antifungal activities and synergisms of isoflavans and FLC were different for their distinct chemical structures.

The synergistic antifungal effect of Gla has not been characterized. Similar studies on the mode of synergism as follows: increasing reactive oxygen species (ROS) to accelerate apoptosis [31], inhibiting drug efflux pumps to increase intracellular drug concentration [11,32], targeting the ergosterol biosynthesis pathway to increase the fluidity for the resulted ergosterol depletion [32]. We tested the membrane sterols of *C. albicans* after treated with Gla alone or in combination with FLC by GC/MS, but none obvious sterols change was observed in Gla treatment cells (data...
This suggested that the synergism of Gla and FLC may not be related to the inhibition of membrane sterols synthesis. Previous study have displayed that isoflavan equol was capable of changing Candida cell membrane integrity by formation of membrane lesions and cell surface abnormalities against C. albicans [33]. Besides, flavans catechin hydrate and epigallocatechin gallate were also identified to have synergistic effect with FLC against C. tropicalis, cell shrinkage and plasma membrane damage were observed in the combination [34]. In our study, similar cell envelope changes were found in the Candida cells treated with Gla. A significant decrease in cell size and an increase of cell membrane permeability were observed in C. albicans after the treatment with Gla. However, Candida cells treated with Gla became more sensitive to cell wall inhibitors. We further stained the carbohydrate of the cell wall (mannan, chitin and glucan), while no obvious change of cell wall after Gla treatment was observed (Fig. S1).

In conclusion, the present study first demonstrated that Gla could enhance the antifungal effect of FLC, especially showed strong synergistic effect against C. albicans, C. neoformans and C. tropicalis. Their synergism maybe related to the effect of Gla on the cell envelope. Gla may serve as a pro-natural product for fungal infection treatment. Further studies should be carried out to identify its relationship of activity and structures.

Supporting Information

Figure S1 Fluorescence micrographs of the cell wall structures of Candida albicans by the treatment of Gla. Exponentially growing cells treated with or without 32 µg/ml Gla were stained by 50 µg/ml Concanavalin A Alexa fluor 488 conjugate for mannan, 30 µg/ml Calcofluorwhite for chitin, or specific anti-β-glucan primary antibody and Cy3-labeled goat-anti mouse secondary antibody for glucan. Then cells were scanned under a Leica confocal laser scanning microscope and micrographs were acquired.

(TIF)

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Author Contributions

Conceived and designed the experiments: WL MMA YYJ. Performed the experiments: WL LPL JDZ QL HS SMC LJH LY. Analyzed the data: WL JDZ LY GTX MMA YYJ. Contributed reagents/materials/analysis tools: WL LPL JDZ QL HS SMC LJH LY. Wrote the paper: WL MMA YYJ. Final approval of manuscript: WL LPL JDZ QL HS SMC LJH LY GTX MMA YYJ.

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