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Combination Effect of Miconazole with Polygodial against *Candida albicans*

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Abstract

The combination effect of miconazole with polygodial against *Candida albicans* was investigated by an *in vitro* checkerboard method. Isobolograms, fractional inhibitory concentration (FIC), and fractional fungicidal concentration (FFC) indices were used for evaluating the interaction between compounds combined. The combination of miconazole with polygodial exhibited strong synergism on both fungistatic and fungicidal action against this opportunistic pathogen.

Keywords: Polygodial, Miconazole, *Candida albicans*, Combination, Synergism

1. Introduction

Opportunistic fungal infection in humans has become a serious and increasingly common problem due to the advent of broad spectrum antibiotics. This has been further exacerbated with the use of corticosteroids and immunosuppressive drugs. Secondary fungal infection in HIV infected patients is also a particularly difficult treatment problem [1-3]. Only a relatively a few number of drugs are available for the treatment of systemic fungal diseases. Of these, many have major weaknesses in spectra, potency, safety, and pharmacokinetic properties [4]. Miconazole (1), an imidazole derivative, has broad activity against most strains of yeasts, dermatophytes, and *Aspergillus* spp. By oral or intravenous administration, it is effective against systemic candidiasis, but its antifungal efficacy is relatively weak and toxicity is high, compared to fluconazole, itraconazole, and ketoconazole. Therefore, miconazole is mainly used topically for yeast and other localized fungal infections [5-7]. In general, miconazole is fungistatic rather than fungicidal. Hence, studies are needed to enhance the total biological activity of miconazole by combining it with "other substances", possibly converting it from fungistatic to fungicidal.

In our continuing search for antifungal agents from non-microbial sources, many plant secondary metabolites have been characterized as active principles. Among them, polygodial (2) (see **Figure 1** for structures) isolated from the sprouts of *Polygonum hydropiper* (Polygonaceae) (reclassified as *Persicaria hydropiper*) [8], used as a food spice in Japan, was noted to possess potent fungicidal activity against yeast-like fungi *Candida albicans*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, and also filamentous fungi, including *Trichophyton mentagrophytes*, *T. ruburum*, and *Penicillium marneffei* [9]. Subsequently, polygodial was found to enhance the antifungal activity of antibiotics such as actinomycin D and rifampicin [10]. The combination of antifungal drugs with phytochemicals may be one way to address to the urgent need for effective and selective antifungal therapeutics [11]. In order to gain new insights into combination effects of two or more antifungal compounds on a molecular basis, the study of miconazole in combination with polygodial against *C. albicans* was performed as a model using an *in vitro* checkerboard method [12].

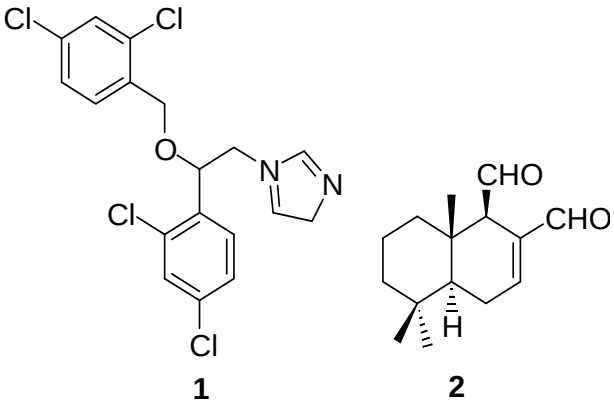

Figure 1. Structure of miconazole (1) and polygodial (2).

2. Methods

The test strain of *Candida albicans* ATCC 18804 used for the experiment was purchased from American Type Culture Collection (Rockville, MD). The procedures used for antifungal assay were the same as previously described [9]. Polygodial was available from our previous work [4,13]. Miconazole was purchased from Sigma Chemical Co. (St. Louis, MO). The FIC index was based on the MICs of the combined compounds. The FIC is calculated as (MIC_a combination/MIC_a alone) + (MIC_b combination/MIC_b alone), where a and b are the two compounds used. The FFC index was based on their MFCs. The calculation method was the same with FIC. The FIC or FFC presented are significant values obtained from the checkerboard matrix. Best values are reported, with the exception of antagonistic activity for which the worst values are reported. The values of FIC and FFC indices were used to define the interaction of combined compounds: synergistic ($X < 0.5$), additive ($1 < X > 0.5$), indifferent ($4 < X > 1$), or antagonistic ($X > 4$) [9,13,14].

3. Results and Discussion

Antifungal activity of miconazole and polygodial was tested against *C. albicans* and the results are listed in **Table 1** [13,14]. Polygodial exhibited potent fungicidal activity nearly comparable to amphotericin B. Based on a time-kill curve experiment, polygodial showed strong and fast fungicidal activity against *C. albicans* under growing conditions and this activity was strongly increased at non-growing conditions. This result is agreeable with previous reports [13,14]. The difference in the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of polygodial is no more than 2-fold, indicating no residual fungistatic activity. In a time-kill curve experiment, polygodial showed strong and fast fungicidal activity against *C. albicans* under growing conditions [15]. In contrast to polygodial, the difference in MIC and MFC of miconazole is 8-fold. Combination effects of miconazole with polygodial were investigated by comparing their MICs and MFCs against *C. albicans* using a checkerboard method [12]. The MIC and MFC of both compounds were used as standards for evaluating their combination effects.

Table 1. Antifungal activity (μg/mL) of miconazole, polygodial and amphotericin B against *C. albicans*.

Compounds Tested	MIC (*MFC*)
Miconazole	6.25 (50)
Polygodial	3.13 (6.25)
Amphotericin B	1.56 (3.13)

In combination with miconazole, polygodial exhibited a potent synergistic effect on antifungal action as shown in **Figure 2**. Namely, when 0.2 μg/mL of miconazole was combined with 0.313 μg/mL of polygodial, the growth of *C. albicans* was completely inhibited. The FIC was calculated as 0.16 [18]. The combination of polygodial and miconazole demonstrated strong synergism based on their FFC of 0.19. More importantly, the MFC of miconazole was decreased from 50 μg/mL to 3.13 μg/mL when it was combined with 0.78 μg/mL of polygodial. It appears that polygodial exhibits strong synergistic effect to both

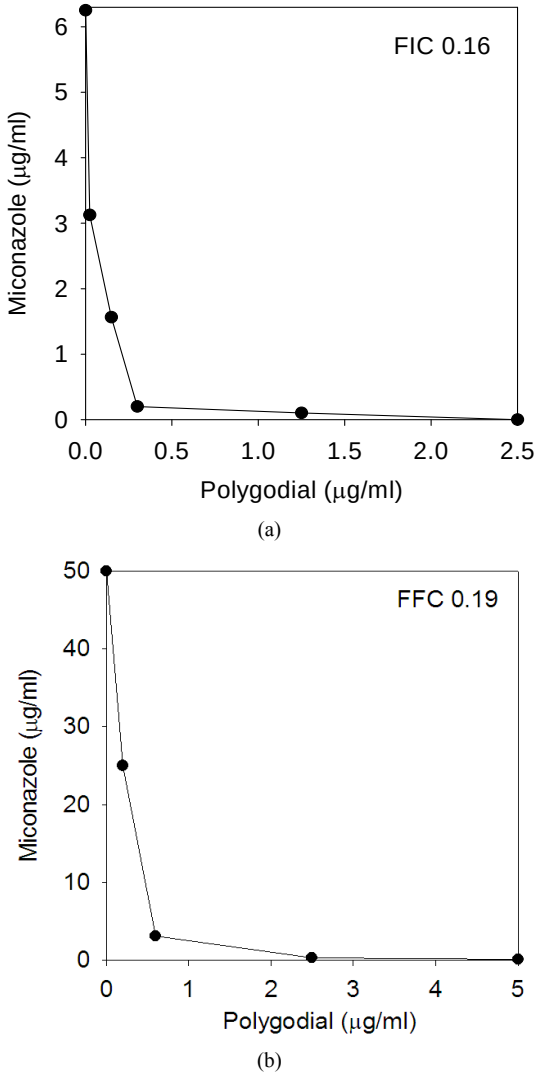

Figure 2. Resulting isobologram of the MICs (a) and MFCs (b) obtained with combinations of miconazole and polygodial against *C. albicans*. The fractional inhibitory concentration (FIC) or fractional fungicidal concentration (FFC) index was calculated with the MICs or MFCs of the combined compounds that exhibited the best antifungal combination effect. Data were obtained by the checkerboard broth dilution technique at 30˚C [13,18].

the fungistatic and the fungicidal actions of miconazole. It appears that the antifungal action of miconazole can be converted to fungicidal when it is combined with small amounts of polygodial. In general, antimicrobial therapy is dependent on both drugs' growth inhibition and the host's immune system. However, systemic fungal disease occurs commonly in patients with seriously impaired immune systems, so fungicidal properties of antifungal agents are considered to be very important [16]. Since azole antifungal agents, including miconazole are only fungistatic [7], the combination of miconazole and polygodial could be useful. Miconazole and polygodial were found to have a particularly strong synergistic effect on fungicidal action against *C. albicans*. Hence, this combination was further investigated by their time-kill curve against this yeast-like pathogen [17]. For the inoculum of 5×10^6 colony forming units (CFU)/mL, 6.25 μg/mL of polygodial completely killed the initial inoculum within 12 h, whereas a concentration of 3.13 μg/mL did not show fungicidal activity as shown in **Figure 3**. Miconazole alone did not exhibit any lethal activity at the concentration of 25 μg/mL within 48 h. However, when it was combined with 3.13 μg/mL (equivalent to 1/2 MFC) of polygodial, the complete lethal action of miconazole was observed within 24 h. Subsequently, the concentration of miconazole could be further reduced to 0.78 μg/mL when the assay was extended to 48 h.

The fungicidal mechanism of polygodial is associated with its specific dialdehyde structure. It does not act by a single defined process but rather, has multiple functions by which it exerts the potent fungicidal action [13]. However, its fungicidal activity primarily comes from its ability to act as a nonionic surface-active agent (surfactant), disrupting the lipid-protein interface. For example, polygodial is known to induce leakage by disrupting the membrane surface [10,13]. The binding of polygodial to cell surface alone is unlikely to explain its entire antifungal mechanism, but may be large part of it. The surfactant concept may also explain in part the permeability of foreign molecules. In combination with polygodial, more miconazole may enter the cells through pores derived from membrane damage by polygodial [10]. Once inside the cells, miconazole is known to inhibit Δ14-lanosterol demethylase, a microsomal cytochrome P450-dependent enzyme, causing ergosterol depletion [18]. The subsequent replacement of ergosterol with lanosterol results in alterations to the plasma membrane, particularly its permeability. The similar potentiation mechanism of actinomycin D by this sesquiterpene was previously described [10,19]. It seems that polygodial facilitates the transmembrane transport of miconazole (foreign compound) into the cells [14,20].

Polygodial is known to inhibit the plasma membrane

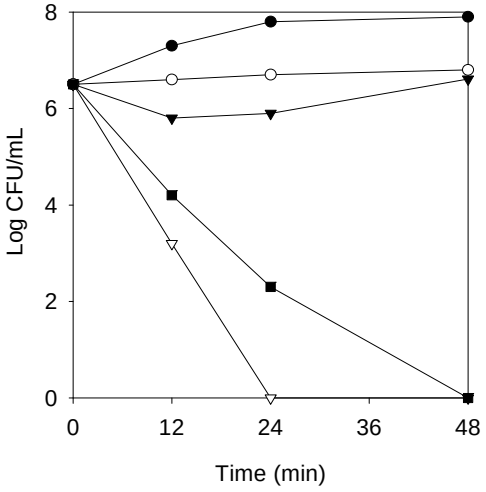

Figure 3. Effect of miconazole and polygodial on the growth of *C. albicans*. Symbols indicate the concentration of polygodial and miconazole: Drug free (●); poylgodial 3.13 μg/mL (○); miconazole 25 μg/mL (▼); 3.13 μg/mL (▽), 0.78 μg/mL (■) of miconazole in combination with polygodial 3.13 μg/mL.

H^+-ATPase by disrupting and disorganizing the hydrogen bonds at the lipid bilayer-protein interface [13]. The previous report suggests that the plasma membrane H^+-ATPase is also a site of antifungal action for miconazole [21]. This ATPase is mainly involved intercellular pH regulation and hence, evolves into potential target for rational drug design [22]. However, the inhibition of the glucose-induced medium acidification around MFC of polygodial was 25%, which was rather weak. The high cell density such as 10^8 cells/mL was needed for the acidification assay as compared with MIC and MFC assay performed by 10^6 cells/mL. Therefore, the fungicidal potency of polygodial was thought to be weakened by the high cell density. The inhibition of plasma membrane H^+-ATPase alone could not explain the fungicidal action of polygodial. The data obtained are consistent with an effect on the lipid bilayer-protein interface rather than a direct interaction of H^+-ATPase. All of these are agreeable with the previous report that the primary active site of polygodial is at the membrane [19]. In addition to H^+-ATPase, other plasma membrane proteins may also be disrupted by polygodial. For example, the resistance mechanism of *C. albicans* is known to involve changes in cellular efflux mechanisms [23], the fungal equivalent to the miconazole resistant efflux pumps. Polygodial may disrupt the efflux pump (membrane protein), similar mechanisms described for the H^+-ATPase. The drug efflux pumps are based on energy-dependent efflux and both miconazole and polygodial are common inhibitors of the plasma membrane H^+-ATPase (P-type) and the mitochondrial ATPase (F-type) [24].

In addition to polygodial, anethole also acts synergistically with several antifungal agents. For example, anethole was reported to enhance the fungicidal activity of polygodial 128-fold against *C. albicans* in combination with a sublethal concentration of anethole [17]. On the other hand, anethole exhibited a strong synergistic effect on fungistatic action of an azole antifungal agent, miconazole, against this opportunistic fungus but a marginal synergistic effect on fungicidal action. Thus, *C. albicans* cells appeared to adapt to this combination stress, eventually recovering and growing normally. Interestingly, a structurally similar phenyl propanoid, eugenol characterized from the same source, did not show this synergistic activity at all, although it was found to exhibit fungicidal activity against *S. cerevisiae* and *C. albicans* with each MFC of 800 μg/mL.

Anethole was previously described to exhibit *in vivo* synergistic effect on the fungicidal activity of the anethole/-polygodial-containing compound against *C. albicans*, supporting its present clinical application [25]. On the basis of the result obtained with polygodial, the study was further extended to see if the enhancing activity to miconazole is specific to only polygodial's dialdehyde structural feature. In order to facilitate it, a structurally simple primary aliphatic alcohol, decanol was selected since amphipathic primary aliphatic alcohols exhibit antifungal activity against *C. albicans*. Among these alcohols, decanol was noted to exhibit the most potent fungicidal activity with an MFC of 50 μg/mL against *C. albicans*. Hence, miconazole was combined with decanol to see if the same combination effect can also be observed against *C. albicans*. The combination of miconazole and decanol synergistically retarded the growth of *C. albicans*, but had only marginal synergism on their fungicidal action. Thus, *C. albicans* cells appeared to adapt to this combination stress, eventually recovering and growing normally. To reveal the different combination effect between polygodial and decanol, the leakage of 260 nm absorbing materials and K^+ ion from *C. albicans* cells was checked. Polygodial induced leakage of 260 nm absorbing materials, similar to those described for combination against *S. cerevisiae* [10]. In contrast to polygodial, decanol did not induce a leak of 260 nm absorbing materials although it did induce a leak of K^+ ion. The leakage of K^+ ion by decanol was observed within 30 min treatment accompanied by significant loss of cell viability, suggesting that decanol quickly affects the plasma membrane of *C. albicans* cells forming rather smaller size pores than polygodial. The size formed may not be large enough to facilitate the transmembrane transport of miconazole into the cells.

In conclusion, the combination effect of miconazole with polygodial is truly synergistic, as shown by the various concentrations less than half-MIC or MFC of the combined counterpart. It seems that the combination of miconazole and polygodial targets the extracytoplasmic region, and thus does not need to enter the cell, thereby avoiding most cellular pump-based resistance mechanisms. The combination strategy of antifungal drugs and phytochemicals for the purpose of effectively controlling systemic fungal pathogens is promising.

4. Acknowledgements

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