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Antifungal Activity of Aureobasidin A in Combination with Fluconazole against Fluconazole-Resistant *Candida albicans*

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ABSTRACT

Background: Aureobasidin A is known as a cyclic depsipeptide antibiotic with toxic effects against yeasts such as *Candida* spp at low concentration. Combination therapy is used as a conventional treatment for fungal infections, especially drug-resistant cases. The current study aimed to investigate the combined effects of fluconazole and Aureobasidin A on fluconazole-resistant *C. albicans* isolates using broth microdilution method.

Materials & Methods: Antifungal activity of Aureobasidin A (AbA) compared to fluconazole against *C. albicans* ATCC 76615 strain was determined using the standardized broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI, document M27-Ed4) guidelines. The checkerboard method was used to test the combined effects of Aureobasidin A and fluconazole. The synergy, indifference, and antagonism were defined based on the fractional inhibitory concentration values below 0.5, 0.5-4, and more than 4 µg/mL, respectively.

Findings: MIC_{50} and MIC_{90} evaluations of Aureobasidin A and fluconazole were done at concentrations of 0.25-2 and 32-64 µg/mL against *C. glabrata* isolates, respectively. The synergy between fluconazole and Aureobasidin A was observed against *Candida* isolate. A reduced MIC was demonstrated against *C. albicans* isolate when fluconazole was combined with Aureobasidin A at 4 to 0.12 µg/mL concentrations.

Conclusion: The present study findings revealed that Aureobasidin A combined with fluconazole exhibited potent inhibitory effects against fluconazole-resistant *C. albicans* isolates. Further studies is recommended to investigate the synergistic effects of Aureobasidin A and other antifungal drugs.

Keywords: Minimum inhibitory concentration (MIC), Fluconazole-resistance, Aureobasidin A, *Candida albicans*.

CITATION LINKS

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Introduction

C. albicans is one of the most frequent nosocomial pathogens that cause a variety of disorders, including skin and mucosal infections requiring simple treatment or even systemic life-threatening candidiasis, especially in immunocompromised patients [1-2]. Although the prevalence of candidiasis infection is increasing, there are limited therapeutic options available for life-threatening cases of this infection. Furthermore, the increased resistance of *Candida* species to these available antifungal drugs has brought about major concerns [3]. Azoles, including fluconazole (FLC), are among the most common antifungal drugs frequently used for mucosal and superficial candidiasis. Previous studies have shown that FLC has the same efficacy as Amphotericin B in the treatment of candidemia in patients without neutropenia [4]. Recently, Azoles have been more commonly used as the first-line treatment in clinics since the usual form of Amphotericin B is toxic, and its new lipidic form is expensive. Another antifungal agent that could be considered in this regard is Aureobasidin A, which has been shown to be highly active in vitro against many pathogens [5], including *Toxoplasma gondii* [6], *Candida spp.* [7], *Saccharomyces cerevisiae* [8], *Cryptococcus neoformans* [12], *Leishmania [Leishmania] amazonensis* [22], *Blastomyces dermatitidis, Histoplasma capsulatum* [12], and some *Aspergillus spp.* [9,10]. This compound was found to be fungicidal and exhibit inhibitory activity against inositol phosphorylceramide synthase, a major enzyme required for the synthesis of sphingolipid inositol phosphorylceramide [5, 10].

Finding a safe antifungal compound with high efficacy and low toxicity seems to be essential for the treatment of *C. albicans* infection. Although various studies have been performed to explore and evaluate the safety of new antifungal agents, available data about the antifungal activity of Aureobasidin A is scarce.

Objectives: The current study aimed to investigate the combined effects of fluconazole and Aureobasidin A on fluconazole-resistant *C. albicans* isolates using the checkerboard microdilution method.

Materials and Methods

Fungal strain: In the present study, the standard isolate of fluconazole-resistant *C. albicans* ATCC 76615 was obtained from the Fungi Culture Collection of Pasteur Institute of Iran (http://www.pasteur.ac.ir) and characterized using standard molecular methods.

Compounds and drugs: Aureobasidin A was obtained from Takara Bio Laboratories (CAT No. 630499), USA. The drug powder of fluconazole was purchased from Pfizer Co. and Himedia Laboratories, Mumbai, India. Fluconazole stock solution was prepared in 1 % (v/v) DMSO (Sigma, Germany) and stored at −20 °C. Aureobasidin A stock solution was diluted in 1 % (v/v) pure methanol and stored at 4 °C until used.

Antifungal susceptibility assay and synergy: The minimum inhibitory concentration (MIC) of Aureobasidin A was initially screened against *C. albicans* strains using broth microdilution method according to CLSI (Clinical and Laboratory Standards Institute document M27-Ed4) guidelines [11]. The overnight grown cultures were diluted to 2.5× 10^3 cells/mL in RPMI 1640 medium without bicarbonate and with L-glutamine, buffered to pH 7.0 by morpholinepropanesulfonic acid (MOPS). Then 100 µL of two-fold serial diluted concentrations of Aureobasidin A in RPMI media (ranging from 16−0.031 µg/mL) and 100 µL of cell suspension in RPMI were added into each well of MTP and incubated at 35 °C for 48 hrs. The lowest concentration inhibiting the growth of fungal cells was considered as

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the MIC [11].

Fluconazole at different concentrations ranging from 0.125 to 64 µg/mL was tested as drug control. The RPMI medium with fungal cells was also used as drug-free control. The MIC_{50} and MIC_{90} values were represented as the concentrations of fluconazole and Aureobasidin A that could inhibit 50 and 90 percent of the isolates based on the visible growth compared to control [11].

As previously demonstrated in the Clinical Microbiology Procedures Handbook, a two-dimensional checkerboard procedure was used to test the combined effects of Aureobasidin A and fluconazole [12]. To acquire effective concentrations of antifungal agents in combination, different concentrations of each antifungal agent were used. A total volume of 50 µL of each Aureobasidin A and fluconazole at different concentrations was added to the Columns 1 to 7 and Rows A to G. Following the addition of 100 µL of inoculum, the plates were incubated at 35 °C for 48 hrs, and the MIC endpoints were determined for the combinations of these agents as explained above for each agent tested separately.

The fractional inhibitory concentration (FIC) was calculated to assess the interaction between the drugs used in combination by the following equation: $FICI = FIC_a + FIC_b = C_a^{comb}/MIC_a^{alone} + C_b^{comb}/MIC_b^{alone}$. Where MIC_a^{alone} and MIC_b^{alone} are the MICs of drugs (a: (Aureobasidin A and b: fluconazole) when act alone, and C_a^{comb} and C_b^{comb} are the effective concentrations of these drugs (a and b) when used in combination, respectively. The sum of the FICs was defined as follows: FIC ≤0.5 was defined as synergism, FIC >0.5 <1 was defined as no antagonism, and FIC ≥4 was defined as antagonism [13-14].

Findings

Antifungal susceptibility assay and synergy: The antifungal effects of Aureobasidin A and fluconazole against the standard strain of *C. albicans* ATCC 76615 were determined (Table 1). The antifungal activities of different combinations of fluconazole and Aureobasidin A are shown in Table 2. Aureobasidin A had

Table 1) *In vitro* antifungal susceptibility assay of Aureobasidin A and fluconazole against C. albicans (MIC; µg/mL)

Antifungal Compound	Mean (Range)	MICs		MFC
		MIC_{50}	MIC_{90}	
Aureobasidin A	16 – 0.031	0.25	2	4
Fluconazole	64 – 0.125	32	64	128

Table 2) Combination and synergistic effects of Aureobasidin A and fluconazole against *C. albicans*

Fungi	MIC in combination		FIC in combination		FICI in combination	INT
	AbA	FLC	AbA	FLC		
C. albicans	0.125	4	0.0625	0.0625	0.125	SYN
	0.0625	8	0.031	0.125	0.44	SYN

FLC: Fluconazole; AbA: Aureobasidin A; INT: Interpretation; SYN: Synergism (FIC ≤ 0.5)

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a potent effect on the tested strain, by itself. Synergistic effect of Aureobasidin A and fluconazole on the isolates was determined by calculating fractional inhibitory concentration indices (FICIs) using the standard broth microdilution method (Table 2).

Discussion

The present study made use of a standard strain of *C. albicans* ATCC 76615, which was recognized based on biochemical characterization techniques. The antifungal activity of Aureobasidin A was determined in vitro compared to fluconazole as a clinically effective antifungal drug. MICs of fluconazole and Aureobasidin A against *C. albicans* strains were found to be 64 and 2 µg/mL, respectively.

The limited number of antifungal agents available and the increase in resistance to available antifungal drugs such as echinocandins, polyenes, and azoles have posed a remarkable clinical challenge. The physiological and structural similarity between mammalian and fungal cells makes the development of novel antifungals more difficult. *C. albicans* is considered as the most common cause of human infections; it could be found in the normal human microbiota and colonize asymptomatically various parts of the body. Any alteration in the host immune system results in the overgrowth of *C. albicans* cells, leading to a wide range of infections, from superficial mucosal to bloodstream and deep-tissue infections [15-17]. Drug resistance in *C. albicans* strains has less been reported, but in patients with long-term treatment with antifungal agents and also in patients with recurrent ailments, like those with chronic mucocutaneous candidiasis, antifungal resistance has been reported [18]. The incidence of fluconazole resistance in *C. albicans* strains inducing oropharyngeal candidiasis is high, which could be attributed to various factors such as previous OPC infections and fluconazole treatment [19]. The development of drug-resistant *Candida* species causing life-threatening candidiasis has become a major concern [3].

Alternative compounds targeting essential enzymes, including inositol phosphorylceramide (IPC) synthase, have recently become a topic of interest to researchers. These are present in fungal cells but not in mammalian host cells. The phosphoinositol group is transferred from phosphatidylinositol (PI) to the 1-hydroxy group of phytoceramide to form IPC by IPC synthase [20]; previous studies have indicated that this enzyme is necessary for fungi growth and could be targeted by Aureobasidin A [9, 21]. The inhibitory effect of Aureobasidin A has been associated with inhibiting the normal budding process and aberrant actin assembly, probably through membrane integrity break-down, which leads to cell death in Saccharomyces cerevisiae [23]. Takesako et al. (1993) indicated that oral or subcutaneous administration of Aureobasidin A was well-tolerated by mice and was highly efficient in murine systemic candidiasis treatment. They also found that the fungicidal activity of this compound was more effective than Amphotericin B and fluconazole in treating mice with candidiasis [22]. The in vitro susceptibility testing results obtained in this study are consistent with previous studies findings [10] showing that Aureobasidin A MICs against *Candida* isolates are generally low. Similar to the present study, Tan and Tay (2013) examined the antifungal activities of Aureobasidin A against *Candida* planktonic and biofilm cells. In their study, the mean MIC_{50} of Aureobasidin A was reported to be 2 µg/mL, which is similar to the present study finding [24].

A synergistic effect was found using the combination of Aureobasidin A and fluconazole (Table 2). In all the analyzed

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combinations, the MICs of active compounds used in combination decreased compared to that of each compound alone. For example, the combination of Aureobasidin A with fluconazole reduced the MIC of each up to 4 fold compared to the use of each compound separately. This synergistic effect between these agents could be attributed to their different antifungal mechanisms. The fluconazole fungistatic characteristic is mostly due to the decreased ergosterol level in cells, which ultimately leads to cell membrane destruction [25]. The fungal membrane integrity is probably destroyed due to the use of Aureobasidin A in combination with fluconazole via total inhibition of ergosterol biosynthesis and accumulation. Decreased ergosterol biosynthesis and accumulation due to the use of these compounds in combination could lead to membrane destruction, increased cell permeability, and eventually cell death [26],

Conclusion

In conclusion, the peptide Aureobasidin A was shown to be active against fluconazole-resistant *C. albicans* strains. This compound indicated the most favorable antifungal effects in all assays performed and could be considered as a promising candidate in the development of new antifungal medications, especially for combination therapies. The synergistic effect of this peptide in combination with fluconazole is promising due to the increased efficacy of both molecules, decreased active concentrations, and probably decreased toxicity.

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