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Potency of tamoxifen against clinical isolates of *Candida* resistant to itraconazole

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ABSTRACT: Tamoxifen (TAM) has been shown to have an antifungal action along with its anticancer activity. Various species of fungi have shown varying susceptibility to TAM. The antifungal activity of TAM was studied against clinical pathogenic species of *Candida* that are resistant to itraconazole through using disk diffusion and dilution methods. Of 50 isolates of *Candida* from women with vulvovaginal candidiasis, 15 isolates were resistant to itraconazole. These isolates consisted of four isolates of *C. albicans*, ten isolates of *C. glabrata* and one isolate of *C. utilis*. All isolates were inhibited by a high concentration of TAM (20 mg/ml). *Candida albicans* was affected by TAM at10 mg/ml (MIC, 5.5-7 mg/ml). High susceptibility with a low MIC value of TAM (3 mg/ml) was observed in *Candida glabrata* isolates. *Candida utilis* was also found to be the isolate with the highest resistance to TAM (MIC, 15 mg/ml). In conclusion; TAM is an effective agent against drug-resistant species of *Candida* in high concentrations. It was also demonstrated that resistant isolates of *C. glabrata* are the most susceptible to TAM. High-dose of TAM is recommended for the treatment of fungal infections caused by drug-resistant species of *Candida*. Further studies are needed in this concern.

KEYWORDS: Candida; Itraconazole; Tamoxifen; Resistant

1. INTRODUCTION

The treatment of fungal infections has always been faced with many challenges that may relate to the similarity between fungi and humans, as both are eukaryotic, or relate to the characters of the antifungal agents such as their limited number, cellular toxicity, low spectrum of activity and resistance development [1]. Many alternative compounds have been identified as effective antifungal agents. Tamoxifen (TAM), which is mainly used as an estrogen receptor antagonist for the treatment of breast cancer, is showing promising results as an antifungal agent [1-2]. Its antifungal activities have been demonstrated against a wide range of types of fungi. Yeasts are the fungi most commonly affected by TAM [3-5]. *Saccharomyces cerevisiae* was the first yeast shown to be affected by TAM where it inhibited after 60 minutes of treatment with 15 and 30 µg/ml of TAM [3]. Growth of *Schizosaccharomyces pombe* was also affected by TAM in which its colonies were completely absent under the effect of 40 µg/ml of TAM [6]. Recently, TAM has been shown to have an antifungal effect against many pathogenic types of yeast such as *Candida* spp. and *Cryptococcus neoformans* [5, 7-9]. The comparison among yeasts proved that *Saccharomyces cerevisiae* is more susceptible to TAM (MIC, 12 µg/ml) than *Candida albicans* (MIC, 32 µg/ml) and *Cryptococcus neoformans* (MIC, 64 µg/ml) [7]. It was shown that TAM had high activity against *C. neoformans*, either *in vitro* at MIC 8 µg/ml or inside macrophages [5, 8]. Meanwhile, treatment of the patient with cryptococcal infection may require a higher concentration of TAM. Randomized controlled trials have demonstrated that increasing the TAM dose from an *in vitro* effective concentration (8 µg/ml) to a high dose (300 mg/day) failed to eliminate *C. neoformans* from the cerebrospinal fluid of patients with cryptococcal meningitis [10]. Among *Candida* species, *C. albicans* is the most common species used to evaluate antifungal action of TAM [2, 9]. This yeast has been found to be more susceptible to TAM than other types of yeast such as *Cryptococcus laurentii* [9].

Isolates of *Candida* resistant to itraconazole were mainly selected to be specific fungi used in this study to evaluate their susceptibility to TAM. Itraconazole resistance is the second health problem that women face during treating candidiasis following the failure of fluconazole therapy [11]. This resistance to the most effective antifungals has encouraged the investigation of new alternative drugs. The antifungal effect of TAM

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on drug-resistant yeasts has been illustrated by few studies [12-13]. Some of these studies indirectly mention the inhibiting activity of TAM on a resistant fungal isolate [9, 14]. In addition, TAM showed the same activity pattern against susceptible and resistant isolates of *C. albicans* [12, 14]. Thus, the antifungal action of TAM on clinical isolates of *Candida* resistant to itraconazole was evaluated.

2. RESULTS

In the present study, fifteen isolates related to three species of *Candida* were found to be resistant to itraconazole. These isolates consisted of four isolates of *C. albicans*, ten isolates of *C. glabrata* and one isolate of *C. utilis*. Antifungal action of TAM was tested on these itraconazole-resistant isolates. The measurement of the zone of inhibition showed that all isolates were inhibited by the higher TAM concentration (20 mg/ml). Isolates of *C. albicans* were susceptible to TAM at a concentration of 10 mg/ml with a MIC ranging from 5.5 to 7 mg/ml. *C. glabrata* was the most susceptible species to TAM in which some of its isolates were inhibited at 5 mg/ml of TAM. It required variable MIC value of TAM for inhibition, ranging from a low (3 mg/ml, for *C. glabrata*-9) to a high MIC (8.5 mg/ml for *C. glabrata*-7). *C. utilis* was the most resistant isolate of *Candida* to TAM (MIC, 15 mg/ml)(Table 1).

Table 1. Zone of inhibition and minimum inhibitory concentration (MIC) of tamoxifen on *Candida* isolates

Fungal isolate	Zone of inhibition (mm)							MIC (mg/ml)
	Tamoxifen concentration (mg/ml)						Clotrimazole	
	20	10	5	2.5	1.25	0.625	10 µg/ml	
C. albicans-1	17	15	-	-	-	-	15	5.5
C. albicans -2	14	11	-	-	-	-	16	7
C. albicans-3	15	13	-	-	-	-	14	6.5
C. albicans-4	16	12	-	-	-	-	15	7
C. glabrata-1	18	14	10	-	-	-	17	3.5
C. glabrata-2	18	13	11	-	-	-	18	4
C. glabrata-3	13	10	-	-	-	-	16	8
C. glabrata-4	15	10	10	-	-	-	14	3.5
C. glabrata-5	12	12	-	-	-	-	15	7
C. glabrata-6	14	11	-	-	-	-	12	7.5
C. glabrata-7	11	9	-	-	-	-	12	8.5
C. glabrata-8	13	11	-	-	-	-	13	8
C. glabrata-9	15	12	9	-	-	-	15	3
C. glabrata-10	13	10	-	-	-	-	13	8
C. utilis	10	-	-	-	-	-	10	15

-: resistance

3. DISCUSSION

In the present study, *C. albicans* showed higher resistance to TAM, which was also demonstrated by another study [8]. The susceptibility of *Candida* spp. to TAM can be increased by converting TAM to metabolites such as 4-hydroxy-tamoxifen and endoxifen [5,8]. In general, pathogenic *C. albicans* without any azole resistance is usually susceptible to TAM [18]. This fungus usually requires 32 µg/ml of TAM for inhibition [7]. Evaluation of the effect of TAM on the logarithmic growth phase of *C. albicans* indicated that 2×10^{-5} M (7.43 mg/ml) of the TAM had a high fungicidal effect compared to 5×10^{-6} M (1.85 mg/ml) [19]. This is also clarified by the result of another study which showed that the stationary and logarithmic growth phases of *C. albicans* are inhibited by 15×10^{-6} M (5.57 mg/ml) to 20×10^{-6} M (7.43 mg/ml) of TAM [20]. The early and mature biofilm of *C. albicans* was also inhibited by a concentration of 1-4 mg/ml of TAM [21]. In a previous study, *C. albicans* isolated from the oral cavity of breast cancer patients showed greater susceptibility to TAM at concentrations of 5 and 10 µg/ml compared to other yeasts [9]. Such an inhibitory action of TAM has also been found in *C. albicans* isolated from a periodontitis lesion of women with breast cancer in which TAM also reduced the severity of periodontitis after 2 years of treatment with TAM [12]. An *in vivo* study in mice infected with *C. albicans* showed that mice treated with 200 mg/kg of TAM for 7 days reduced the burden of renal candidiasis [7].

Inhibition of itraconazole-resistant isolates of *Candida* in this study required high concentrations of TAM. Other resistant isolates of *C. albicans* are usually susceptible to low concentrations of TAM [12, 14]. Fluconazole-resistant isolate of *C. albicans* from the mouth of breast cancer patients was inhibited by 22.33 µM (8.29 mg/ml) of TAM with 31 times more than by fluconazole [12]. One other fluconazole-resistant isolate of

C. albicans is inhibited by 200 µg/ml of TAM [14]. In addition, TAM was also able to increase the sensitivity of azole-resistant *C. albicans* to fluconazole [13].

Resistant isolates of *C. glabrata* to itraconazole in the current study showed a greater susceptibility to TAM. The normal isolate of *C. glabrata* may, in some cases, require a lower concentration of TAM for inhibition than *C. albicans* [7]. The clotrimazole-resistant isolate of *C. glabrata* was found to be highly inhibited at concentrations of 5 and 10 µg/ml of TAM [9]. Long-term TAM treatment for patients with vulvovaginal candidiasis caused by *C. glabrata* leads to an increased rate of recurrent infection with this fungus [22]. However, *C. glabrata* is usually more resistant to antifungals than *C. albicans* [23]. Thus, the susceptibility of *C. glabrata* to TAM in the present study can make TAM a promising treatment for infections caused by this resistant species.

The antifungal activity of TAM depends principally on the targeting of fungal calmodulin in the same way as for mammalian cells [7]. The presence of TAM prevents the binding of the EF hand motifs of the protein calmodulin with its calcineurin substrate which ultimately inhibits the calmodulin-calcineurin pathway [2, 4-8, 18]. Blockage of this pathway can affect many fungal cell activities, such as increased cell lysis, disturbed polarization of the membrane actin, and decreased formation of reproductive bud and germ tube [7]. The treatment of *C. albicans* with 16 µg/ml of TAM showed a reduction in filament formation and a modification in cell wall architecture by decreasing the amounts of mannoproteins covering 1,3-β-glucan [18]. In addition, the antifungal effect of TAM is affected by the pH in which its fungicide action against *C. albicans* decreases with acidity and increases with pH from 7 to 7.5 in a concentration-dependent manner [24].

TAM has a number of features that make it appropriate for use as an antimicrobial or antifungal agent. The most important pharmaceutical characteristics of TAM are its low adverse effects, its oral availability and its easy distribution to many tissues for rapid arrival at the site of infection [4]. Other features of TAM that make it a good antimicrobial agent are its activation of macrophage immune activity by interacting with the macrophage lipid mediators or signaling pathway and its easy penetration of macrophages to reach an internal pathogen [4-5]. TAM is also able to exert a synergistic effect on many antifungal agents against various fungal pathogens [2, 13]. The combination of different azoles with TAM increases their antifungal action against *C. neoformans* more than when they are used alone because TAM inhibits protein synthesis and azole inhibit ergosterol synthesis [6]. The antifungal activity of fluconazole against *C. neoformans* is also increased by combination with TAM at *in vitro* and *in vivo* levels [5]. However, combining may not always be effective or useful for many antifungals in some cases of fungal infections. No synergic effects were observed between fluconazole or amphotericin B and TAM against *C. neoformans* [10]. Fungicidal and fungistatic effects of miconazole against *C. albicans* are also unaffected by the combination with TAM [25].

4. CONCLUSION

Tamoxifen has variable effects on drug-resistant pathogenic species of *Candida*. Its antifungal action on *C. albicans* and *C. utilis* is not sufficiently strong to recommend its usage during the treatment their infections. TAM may be useful for treating infections caused by itraconazole-resistant isolates of *C. glabrata*. Considering TAM as a new antifungal agent for the treatment of an untreatable fungal infection is primarily dependent on increasing its concentration.

5. MATERIALS AND METHODS

5.1 Fungal isolation

In total, 50 *Candida* isolates were collected by vaginal swab from women volunteers with clinical confirmation of vulvovaginal candidiasis (23-57 years) from June to July 2021. The swabs were immediately microscopically examined and cultured on a Mueller-Hinton agar (MHA)(HiMedia, India) then incubated at 35º C for 24-48 hours. The morphological features of the isolates were identified as primary diagnosis by staining with crystal violet of Gram stain. Confirmation diagnosis was made using VITEK2 compact system with VITEK® 2 YST ID cards for yeast (BioMérieux, France).

5.2. Antimicrobial susceptibility test

Susceptibility of isolates was determined using a disk diffusion method referred by the CLSI-M44 (2018) [15]. A suspension of fungal growth was prepared by cultivating about 5 colonies of isolate from refresh subculture in 2 ml of sterilized Mueller-Hinton broth (MHB)(Himedia, India) and adjusted to the standard 0.5 McFarland to reach 1.5×10^8 cfu/ml. A swab from inoculate was stripped over the MHA. A two-fold serial dilution was used to prepare different concentrations of TAM, ranging from 0.625 mg/ml to 20 mg/ml by

dissolving it in ethanol. A number of 6 mm diameter disks were made from filter paper and sterilized. The disks with different concentrations of TAM were prepared at the time and loaded onto inoculated plates. Plates were incubated at 35° C for 24 hours. Disks with sterilized distilled water (D.W.) or ethanol were used as negative controls. Clotrimazole (10 µg/ml), a standard antifungal agent, was used as a positive control. Diameter of the zone of inhibition was measured in mm around the effective disk. Resistance to itraconazole was confirmed when the zone of inhibition around the itraconazole disk (10 µg) (Torrejón de Ardoz, Madrid, Spain) was less than 24-25 mm [16].

5.3. Determination of minimum inhibition concentration (MIC)

The MIC value of the TAM was determined using the dilution method referenced by CLSI-M60 (2017) [17]. Isolated yeasts were subcultured in the MHB and incubated at 35°C for 24 hours. A plastic microdilution plate (96 wells) was used to determine the MIC value. Each well of plate was equipped with 100 µl of free of MHB, 50 µl of adjusted fungal counts, and 50 µl of each TAM concentration. Controls used in the disk diffusion method were also used in a microdilution plate. Inoculated plates were incubated at 35°C for 24 hours. Results were recorded depending on the presence or absence of growing was visually observed.

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