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In vitro synergy of azole antifungals and methotrexate against *Candida albicans*

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ABSTRACT

Objective: This study aims to evaluate the effective of azoles and MTX for patients with invasive candidiasis.
Methods: We used the disk diffusion assay and the checkerboard assay to evaluate the *in vitro* interactions between MTX and antifungals. In addition, we used the transmission electron microscopy to observe the ultrastructure of the effect of MTX and fluconazole on *Candida albicans*.
Results: The rates of synergy for the combination of MTX with fluconazole (FLC), itraconazole (ITC), and voriconazole (VRZ) were 91.3%, 65.2%, and 87% in checkerboard testing. No antagonism was found between methotrexate and azole antifungals in any of the strains. Furthermore, MTX treated *C. albicans* showed extensive cell wall vacuolations and the inhibition of blastospores growth, as observed using transmission electron microscopy. There was an apparent destruction of the cell membrane and cell wall resulting in the destruction of cytoplasm, a phenomenon observed when MTX was combined with azoles.
Conclusion: This study provides evidence that the combination of azoles and MTX is effective for patients with invasive candidiasis, which on the other hand, will reduce the side effects of the drugs.

1. Introduction

Invasive candidiasis is a serious and potentially lethal disease with a mortality rate ranging from 36 to 63% [1–4] *C. albicans* is the most prevalent species in mycological studies (45–65%), particularly in patients that are on treatment with immunosuppressive agents such as methotrexate [5–7]. The choice of appropriate antifungals is very important for patients with a *C. albicans* infection treatment.

Our previous study showed different interactions, which include synergy, antagonism, and no interactive effects between antifungals and methotrexate against *Aspergillus* spp. *in vitro* [9]. Methotrexate is an anti-tumor drug of the folate class. Methotrexate mainly inhibits the synthesis of tumor cells by inhibiting dihydrofolate reductase, thus inhibiting the growth and reproduction of tumor cells. Azole antifungal drugs, including imidazoles and triazole derivatives, mainly inhibit the synthesis of ergosterol by inhibiting cytochrome p450dependent enzyme, and finally exert the antifungal effect.

In this study, we tested the interactions between azole antifungals and methotrexate against *C. albicans*. Therefore, the study provides a scientific basis for the clinical choice of an antifungal agent for patients with a *C. albicans* infection during methotrexate treatment.

2. Materials and methods

2.1. Strains and media

A total of 23 clinical isolates of *C. albicans* from vaginal sample among female patients were received by the Research Center for Medical Mycology at Peking University and the clinical laboratory of the Second Hospital of Harbin Medical University. All strains were confirmed according to standard mycological test methods. *C. parapsilosis* (ATCC 22019) was used for quality control. Sabouraud Dextrose agar (SDA) was used for pure cultures. RPMI agar plates were used for the disk diffusion tests. The medium used for the broth microdilution method was RPMI 1640 (GIBCO BRL, Life Technologies, USA) buffered to pH 5.0 with 0.165 M MOPS (Sigma Chemical Co., USA) and 1 M HCl.

2.2. Azole antifungals and methotrexate

The three azole antifungals that were used include fluconazole

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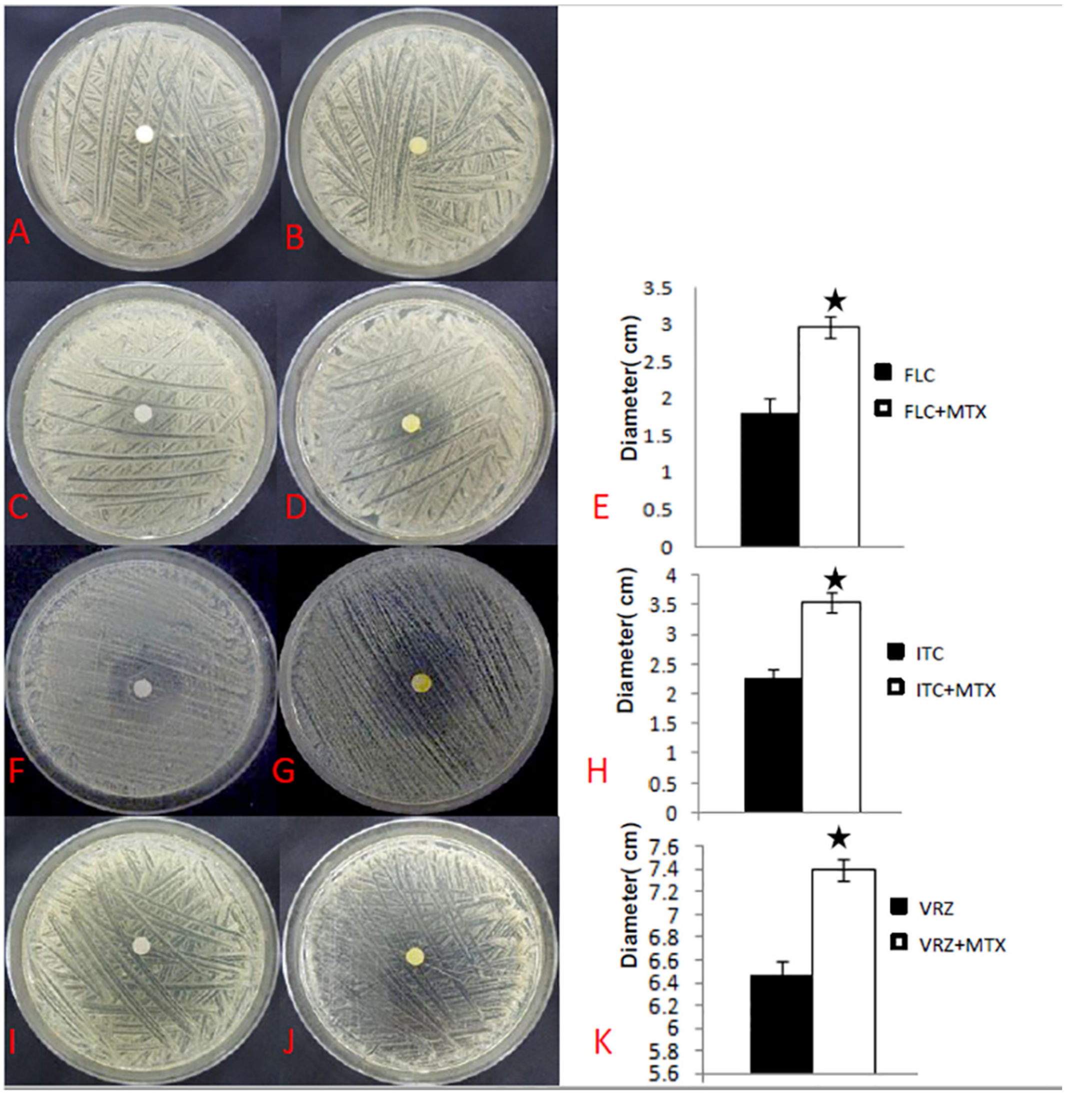

Fig. 1. Disk diffusion assay: The zone diameters of inhibition were recorded as a description of the degree of *C. albicans* growth. Blank paper disks were impregnated with different dosages of MTX, FLC, ITC, VRZ, MTX + FLC, MTX + ITC, MTX + VRZ, and water control. A: water control B: MTX 0.5 mg C: FLC 6.4 μg, D: FLC 6.4 μg + MTX 0.5 mg, E: the inhibition degree of *C. albicans* growth with MTX + FLC is significantly higher than FLC alone. F: ITC 50 μg, G: ITC 50 μg + MTX 0.5 mg, H: the inhibition degree of *C. albicans* growth with MTX + ITC is significantly higher than ITC alone. I: VRZ 8 μg, J: VRZ 8 μg + MTX 0.5 mg, K: the inhibition degree of *C. albicans* growth with MTX + VRZ is significantly higher than VRZ alone. * $P < 0.01$.

Table 1
Checkerboard assay.

	MIC alone	MIC combined	FICI	Note
FLC	4.666	0.814	0.234	Synergy
MTX[a]	> 0.625	0.012		
ITC	1.137	0.257	0.286	Synergy
MTX[b]	> 0.625	0.015		
VRZ	0.226	0.024	0.174	Synergy
MTX[c]	> 0.625	0.013		

Key: a Synergy effect of MTX with azoles in *C. albicans*. FICI ≤0.5 indicates synergistic effect: MTX (methotrexate), mg/ml; ITC (itraconazole), FLC (fluconazole) and VRZ (voriconazole), μg/ml. Data shown are geometric means.
[a] MTX + FLC.
[b] MTX + ITC.
[c] MTX + VRZ.

(FLC), itraconazole (ITC), and voriconazole (VRZ). Stock solutions of powder forms of ITC and VRZ (Melone Pharmaceutical Co., Ltd., China) were dissolved in 100% dimethyl sulfoxide (DMSO). FLC and MTX (Melone Pharmaceutical Co., Ltd., China) were dissolved in distilled water and stored at −20 °C. Antifungals and MTX were diluted by RPMI 1640 and buffered to pH 7.0 by 0.165 M MOPS (Sigma Chemical Co.).

2.3. Disk diffusion assay

Disk diffusion assay was used to evaluate the interactions between methotrexate and azole antifungals [8]. Three strains of *C. albicans* were chosen for the test. After standardized growth and harvest, 100 μl of 10^6 conidia/ml suspension of *C. albicans* was spread uniformly onto RPMI agar plates using a cotton swab. Blank 6-mm-diameter paper disks (Becton Dickinson, Sparks, Md.) were impregnated with different dosages of MTX, FLC, ITC, VRZ, MTX + FLC, MTX + ITC, and MTX + VRZ. After drying, the disks were placed onto inoculated agar plates. All plates were incubated at 35 °C and the inhibition zone diameters were analyzed after 48 h. The zone of inhibition was recorded as a description of the degree of activity against *C. albicans* growth. Each disk diffusion assay was performed twice.

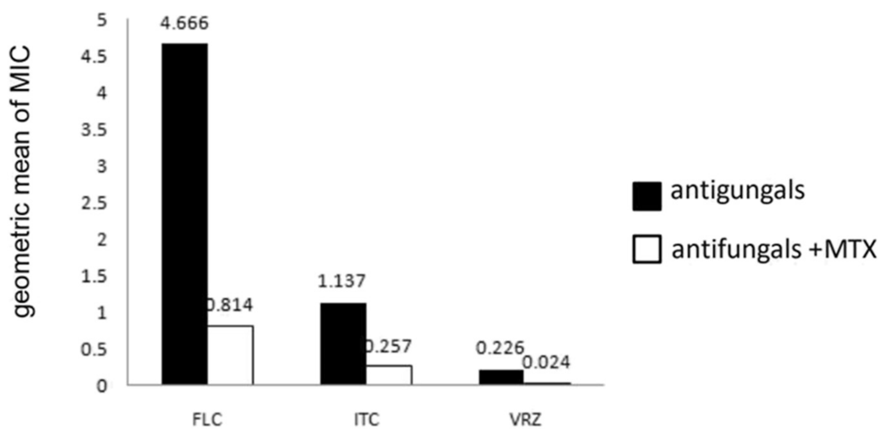

Fig. 2. The MICs of azole antifungals and combinations with MTX for *C. albicans.* The geometric means of MICs of FLC, ITC, and VRZ for *C. albicans* were 4.666, 1.137, and 0.226 respectively. The MICs reduced to 0.814, 0.257, and 0.024 with the combination of MTX (5.7, 4.4 and 9.4 times reduced individually).

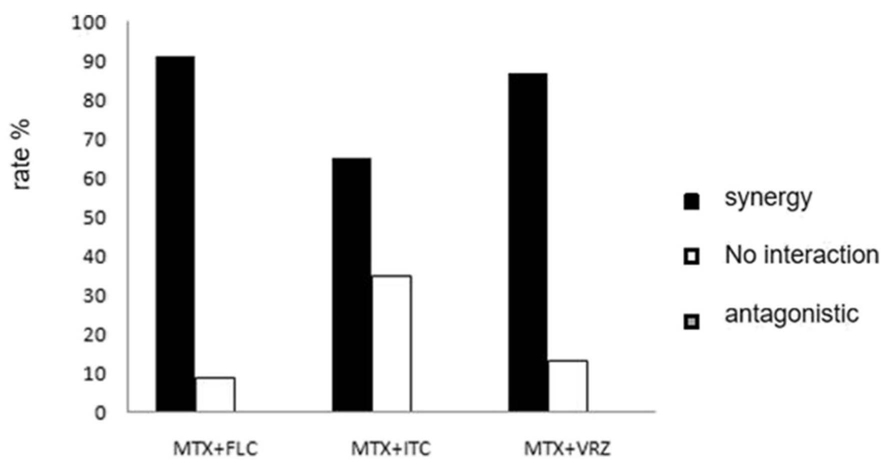

Fig. 3. The rates of the combination of MTX with FLC, ITC, and VRZ. The rates of synergy for the combination of MTX with FLC, ITC, and VRZ were 91.3% (21/23), 65.2% (15/23), and 87% (20/23), respectively. No antagonism was found against any of the strains between methotrexate and the azole antifungals.

2.4. Azole antifungals and methotrexate checkerboard testing

The *in vitro* interactions between MTX and antifungals were evaluated in a checkerboard assay as previously described [9]. Briefly, each isolate was prepared by picking colonies from an overnight culture on Sabouraud Dextrose Agar (Merck, Germany) at 35 °C and suspended in sterile normal saline. The fungal inocula were adjusted to yield a 2× inoculum (1–5 × 10^6 CFU/ml). Then, a working suspension was prepared with a 1:100 dilution, followed by a 1:20 dilution of stock suspension with RPMI1640 broth medium, which resulted in concentrations of (0.5–2.5) × 10^3 CFU/ml. Checkerboard testing was performed in duplicate in microdilution assays for each fungal strain. Combinations included MTX + FLC, MTX + ITC, and MTX + VRZ. The combinations were formed with each drug at the following concentrations: MTX, 10–0.04 mg/ml; FLC, 16–0.125 μg/ml; ITC, 8–0.0625 μg/ml. and VRZ, 2–0.015 μg/ml. Drug interactions were classified as synergistic, no interaction or antagonistic according to the fractional inhibitory concentration index (FICI). The interaction was defined as synergistic if the FICI was < 0.5, no interaction if it was 0.5–4.0, and antagonistic if it was over 4.0 [10].

2.5. Transmission electron microscopy (TEM)

To examine the mechanism of synergy interactions between methotrexate and the azole antifungals on *C. albicans*, a control and FLC treated (48 h) cells were processed for visualization under an electron microscope [11]. One strain of *C. albicans* (No. 26136) was treated with FLC only, MTX only, and the combination of MTX and FLC at the concentration of MIC of the drugs combination at 35 °C for 48 h. Yeast cells were then pelleted by centrifugation and resuspended and fixed with 4% glutaraldehyde. The samples were dehydrated in a graded acetone series (50, 70, 90 and 100%) and embedded in Epon 812 (Electron Microscopy Sciences, Fort, WA, USA). The sections were stained with uranyl acetate and lead citrate and examined using a Hitachi H-7500 (Hitachi High Technologies Corp., Tokyo, Japan) transmission electron microscope operating at 80 kV.

3. Results

3.1. Methotrexate and azole antifungals positively interact based on the disk diffusion assay

A total of 23 clinical isolates of *C. albicans* from vaginal sample among female patients were received. We evaluated the interaction of methotrexate and azole antifungals by a disk diffusion assay. Three strains were chosen for the test. As shown in Fig. 1, positive interactions were found between MTX and FLC (Fig. 1.C, D, E), MTX and ITC (Fig. 1.F, G, H), and MTX and VRZ (Fig. 1.I, J, K) compared to MTX and azole antifungals alone.

3.2. In vitro FIC index between methotrexate and azole antifungals

To further confirm the results, we did the checkerboard testing for 23 strains of *C. albicans*, as shown in Table 1, Figs. 2 and 3. The MICs of

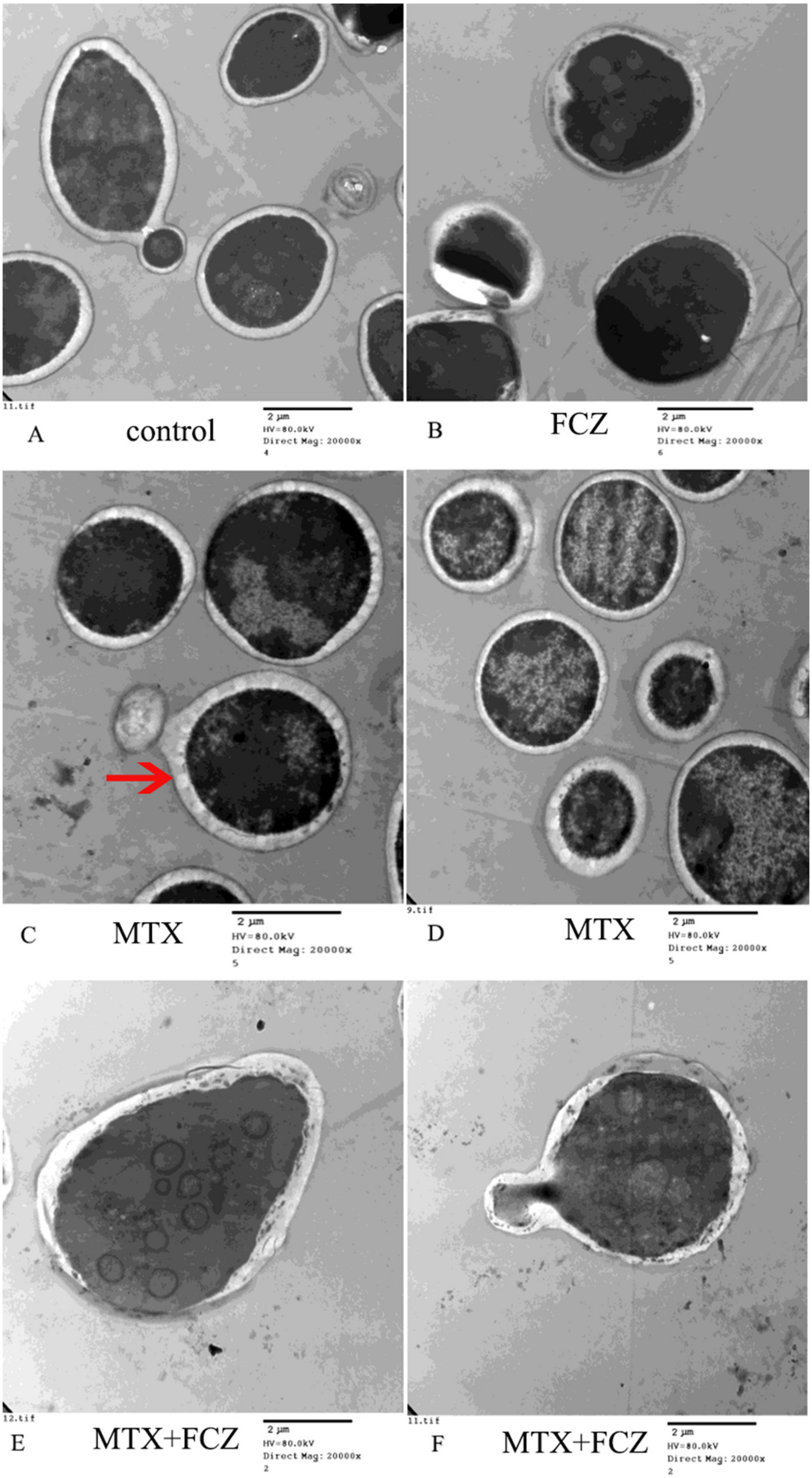

Fig. 4. Transmission electron microscopic pictures of *C. albicans* with or without exposure to MTX and/or FCZ. (A) Untreated *C. albicans* showed an intact cell structure with normal morphologies of nucleus and cytoplasm. (B) FCZ-treated *C. albicans* showed destroyed membranes resulting in the destruction of the cytoplasm.FCZ:1 μg/ml (C,D) MTX-treated *C. albicans* showed extensive cell wall vacuolations (arrow), cytoplasm electron density decrease, and the inhibition of the growth of blastospores. MTX: 0.0049 mg/ml (E,F) combination of MTX and FLC. Apparent defects on cell morphologies included a deformed and augmented body, an extensive cell wall, membrane disruption, and an apparent destruction of the cytoplasm. FCZ: 1 μg/ml, MTX: 0.0049 mg/ml.

methotrexate for *C. albicans* were higher than 0.625 mg/ml, which indicates that MTX has no activity against *C. albicans* in the blood concentration range in the human body (the blood concentration range of MTX is as high as 1×10^{-3} M). The combination MICs of FLC, ITC, and VRZ with MTX were 5.7, 4.4 and 9.4 times reduced individually compared with the MICs of the azoles treated *C. albicans* alone (Fig. 2). Regarding interactions, the rates of synergy for the combination of MTX with FLC, ITC, and VRZ were 91.3% (21/23), 65.2% (15/23), and 87%

(20/23), respectively. No antagonism was found against any of the strains between methotrexate and the azole antifungals (Fig. 3).

3.3. Ultrastructural observation of the effect of MTX and FLC on Candida albicans

Untreated *C. albicans* showed an intact cell structure with a normal nucleus and cytoplasm morphology (Fig. 4.A). In contrast, FLC-treated *C. albicans* showed destroyed membranes resulting in the destruction of cytoplasm (Fig. 4.B). MTX treated alone showed extensive cell wall vacuolations, cytoplasm electron density decrease, and the inhibition of the growth of blastospores, (Fig. 4.C, D). The combination of MTX and FLC showed apparent defects on cell morphologies including a deformed and augmented body, an extensive cell wall, membrane disruption, and an apparent destruction of the cytoplasm (Fig. 4.E, F).

4. Discussion

Several factors have significantly increased the incidence of human fungal infections but the primary influences are related to medical practices such as immunosuppressive therapy, including methotrexate. Methotrexate has been widely used in the treatment of osteosarcomas, lymphomas, and graft-*versus*-host disease (GVHD) prevention, *etc.* However, patients receiving this drug are at a high risk of infection by opportunistic pathogens during treatment. The most prevalent fungus infecting immunosuppressed patients is *C. albicans* [7]. Moreover, serious adverse reactions, including myelosuppression, orointestinal mucositis, and nephrotoxicity limit its clinical application [12–14]. Azole antifungals are widely used to treat Candida spp. infections [15]. These compounds inhibit the 14a-demethylation of lanosterol by interacting with cytochrome P450, which is a crucial enzyme in the ergosterol biosynthetic pathway [16]. The resulting decrease in ergosterol levels and the accumulation of toxic sterol intermediates in the cytoplasmic membrane lead to growth inhibition [17]. Nevertheless, the side effects of azoles such as hepatic dysfunction are also reported [18].

In our previous study, we found a high rate of synergy for the combination of terbinafine and MTX against *Aspergillus* spp. *In vitro*. However, no interaction was detected for the combination of MTX and itraconazole against *Aspergillus* spp. [9]. It was reported that MTX significantly decreased the inhibitory effect of fluconazole [19], However, it was also reported that the combination of methotrexate and itraconazole caused greater inhibition of germ tube elongation than MTX alone [20]. In the present study, we concluded that the combination of azole antifungals and MTX was synergistic *in vitro* against *C. albicans*. From the transmission electron microscopy, we observed that MTX treated *C. albicans* showed extensive cell wall vacuolations and the inhibition of the growth of blastospores. Combinations of MTX and azoles showed apparent defection of cell morphologies and an apparent destruction of the cytoplasm. The results presented that the dose of combination of MTX and azoles will be lower than the individual compounds. The mechanism of the interactions between antifungals and MTX still needs further investigation.

5. Conclusion

Our study reveals the synergy of azole antifungals and methotrexate against *Candida albicans*. We suggest that the combination of azoles and MTX will be effective for patients with an invasive candidiasis infection.

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Declaration of competing interest

The authors declare that they do not have any conflict of interest.

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