

## Compared effects of azole antifungals on cytokine production of THP-1 cells activated by *Candida albicans*

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### ABSTRACT

In this study we compared the cytotoxic and proinflammatory/antiinflammatory effects of azole antifungals such as clotrimazole, ketoconazole, miconazole and fluconazole on *Candida albicans* co-culture with THP-1 cell line. MICs of several azole antifungal agents were determined with *C. albicans* (NCPF 3179, Bioball™ Multishot 10E8) by the broth microdilution plate method. Cytotoxic effects of antifungal drugs were evaluated on THP-1 cell line by WST-1 test. Cytokine levels of IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12p70 and TNF- $\alpha$  in THP-1

cells stimulated by *C. albicans* were determined by Cytometric Bead Array (CBA). Miconazole, clotrimazole, ketoconazole, and fluconazole exhibited antifungal activity against *C. albicans* Multishot 10E8 with a 8, 16, 64, and 128  $\mu$ g/ml MIC value. The azole antifungals showed low cytotoxicity against THP-1 cell line (IC<sub>50</sub>>512  $\mu$ g/ml). The results demonstrated that IL-8 levels were increased by azole antifungal drugs and particularly, clotrimazole significantly induced not only IL-8 but also TNF- $\alpha$ .

**Keywords:** *Candida albicans*, cytokine, inflammatory, THP-1 cell.

### Introduction

*Candida* is the most important yeast that affects humans and causes opportunistic infections especially in immunocompromised patients, worldwide (1). Azoles are widely used in *Candida* infections treatment for nearly fifty years. In those years, a research program which focused on imidazole chemistry was initiated at Janssen (2). Among many reasons to investigate imidazole chemistry, the moiety of it is present in the neurotransmitter histamine, which in turn is a metabolite of the amino acid histidine, the most important one. Moreover, chloro imidazole, a benzimidazole derivative, displayed antifungal activity. Miconazole seems to suppress adenosine-5'-triphosphate levels (ATP) in *C. albicans* cells (3). Ketoconazole is the first broad-spectrum, orally used, well tolerated antifungal agent which can be used to treat superficial and deep mycoses. Fluconazole was active against *Candida* species including *C. neoformans*, but it was less active against dermatophytes and was not active against *Aspergillus* species (4). Fluconazole was active against *Candida* species and *Cryptococcus neoformans*, less active against dermatophytes and is not active against *Aspergillus* species (4). Fluconazole is only fungistatic but not fungicidal when tested against *Candida* species in both stationary and logarithmic phase (5). Clotrimazole is effective against

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individual *Candida* or fungal cells by altering the permeability of the fungal cell membrane by binding to phospholipids and inhibiting the biosynthesis of ergosterol and other sterols required for cell membrane synthesis. The human monocytic cell line THP-1 is an adequate in vitro model of monocytes/macrophages during interaction with fungal cells. Monocytes are capable of producing chemokines, proinflammatory cytokines, and particularly the immunoregulatory cytokines interleukin-10 (IL-10) and IL-12 (6).

The aim of this study was to determine the antifungal activity of azole antifungals on candida strains, to detect cytotoxic, proinflammatory / antiinflammatory effects of these drugs on THP-1 cell line and , to investigate the changes in the cytokine levels of THP-1 cell line in response to *Candida albicans* (Bioball 10E8) .

## Materials and Methods

### Antifungal agents

Clotrimazole (Sigma, C6019), Ketoconazole (Sigma, K1003), Miconazole (Sigma, M3512) and Fluconazole (TCI-Tokyo Chemical Industry, QAGTF-PG) were obtained in powder form and dissolved in DMSO.

### Anticandidal activity on Bioball:

The antifungal susceptibility profile of *Candida albicans* (NCPF 3179, Bioball™ Multishot 10E8, bioMérieux, sa, Marcy l'Etoile, France) was determined by Broth Microdilution method (BMD). BioBall MultiShot 10E8 is a freeze dried water soluble ball containing a precise number of viable yeast cells. BioBall is produced to the world's highest quality standards, achieving ISO Guide 34, a standard for reference material producers, accreditation (7). Each BioBall was rehydrated in 1.1 mL Re-Hydration Fluid containing 10 doses of 100 uL with  $10^7$  cfu each, resulting in a target concentration of  $5 \times 10^5$  cfu per mL when inoculated in 20 mL of Sabouraud dextrose broth (SDB). Each well of the 96 well plate was filled with 100 uL of each anticandidal drug concentrations (0.5-512 µg/ml) and 100 uL of yeast stock culture was added. After that, plates were incubated at 35°C ( $\pm 1$ ) for 24 h in aerobic conditions; the test was performed in triplicate. After incubation, 20 uL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye was added to each well and viability was evaluated

### Human cell line

THP-1 (monocytic leukemic-ATCC TIB-202) cell line was obtained from the American Type Culture Collection. The cells were grown in RPMI 1640 (ATCC-formulated RPMI-1640 Medium, Catalog No. 30-2001.) supplemented with

10% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin and 2- mercaptoethanol to a final concentration of 0.05 mM at  $\pm 37^\circ\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$  atmosphere.

### WST-1 assay (Water-soluble Tetrazolium)

$5 \times 10^3$  THP-1 cells were seeded in each well of the 96-well plate and after 24 hours the cells were treated with 0.5-512 µg/ml clotrimazole, ketoconazole, miconazole and fluconazole concentrations. After 24 hour, 0.2 mL WST-1 (Water-soluble Tetrazolium) solution was added to each well. After 3-4 hours of incubation period, the formazan crystals were formed and they were solubilized with 100 µL DMSO. The culture plate was inserted in Cytation 3 (Biotek, VT, USA) microplate reader and the absorbance was measured at 450 nm. This process was repeated for all of the incubation periods. In the experiment, each group was tested in eight wells. The data is mean values from experiment results of four different compounds.

### Coculture conditions

Precise numbers of *C. albicans* 10E8 cultures and THP-1 cells were resuspended in culture medium, and after 4 h. of incubation. Firstly, they were counted with a cell counter (Cedex), plated at  $2 \times 10^6$  cells per well density, and allowed to equilibrate at 37°C for 3 h. After incubation, *Candida* cultures were washed, counted with a the cell counter and plated with THP-1 cells at 4:10 *Candida*-monocyte ratio. Secondly, cocultures were incubated at 37°C in a  $\text{CO}_2$  incubator for 24 h with azole antifungals (8). After incubation collected supernatants from cocultures were tested using an CBA bead array with Accuri C6 flow cytometer. All experiments were performed in duplicate.

### Cytokine detection

Cell culture supernatants were collected, centrifuged, and stored at  $-20^\circ\text{C}$ . Cytokine levels of the supernatants obtained from cultures were quantified for 4 hr by using the BD human inflammatory kit procedure (BD™ Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit Cat. No. 551811). CBA analysis was conducted by using FCAP array in BD Accuri C6 flow cytometer (9).

## Results

### Antimicrobial activity

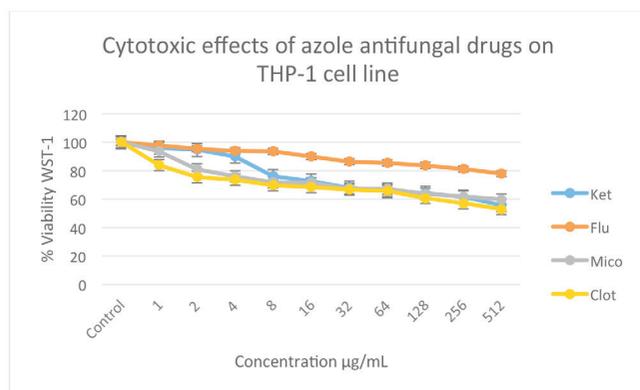
*In vitro* antifungal activities of 4 azoles on *Candida albicans* (NCPF 3179, Bioball™ Multishot 10E8) were investigated by using BMD method. When compared with miconazole (MIC= 8 µg/mL) and were found to be more effective against *C. albicans* than other azole antifungals (fluconazole,

clotrimazole, ketoconazole). Fluconazole (MIC= 128 µg/mL) was less effective than clotrimazole, ketoconazole, miconazole. Clotrimazole inhibited *C. albicans* Bioball at 32 µg/ml, whereas ketoconazole required concentrations of 4 µg/ml. The results could be different because it was used precise number of microorganisms. Therefore Bioball™ Multishot 10E8 was preferred.

WST-1 assay was carried out to evaluate the cytotoxicity of azole antifungals such as clotrimazole, ketoconazole, miconazole and fluconazole on THP-1 cells. Azole antifungals showed low cytotoxic activity on THP-1 cell line ( $IC_{50} > 512$  µg/ml) (Table 1 and Fig. 1).

**Table 1.** Antifungal susceptibilities of *Candida albicans* determined by broth microdilution MIC and cytotoxic activity on THP-1 cell line by WST-1 methods.

	<i>C. albicans</i> MIC (µg/ml)	THP-1 cytotoxicity WST-1 (µg/ml)
Clotrimazole	16	>512
Ketoconazole	64	>512
Miconazole	8	>512
Fluconazole	128	>512



**Figure 1.** Cytotoxic effects of azole antifungal drugs on THP-1 cell line.

#### Cytokine levels in *C. albicans*- THP-1 cells.

Six cytokine levels of proinflammatory/antiinflammatory *C. albicans*-stimulated and unstimulated THP-1 cells were compared (Fig. 2). All azole antifungals IL-8 level induced coculture THP-1 cell line after stimulation for 4 h *C. albicans* (pg/mL).

#### Discussion

*Candida* is a fungal opportunistic pathogen commonly located on the mucosal surfaces of the gastrointestinal tract, skin and/or female genital tract in the majority of humans (10). *Candida* infections in immune system response via form monocytes secrete cytokines. Cytokines form a complicated network that modulates numerous cellular events (11). Human monocytes and macrophages are important components of host defense against pathogens. Mannan, an important component of the *C. albicans* cell wall, as well as following exposure to some cytokines. There are several methods available to measure cytokine levels, mostly used are conventional ELISA technics. ELISA methods are generally cost-effective, however they are restricted to measuring one cytokine at a time, and also require multiple sample aliquots and repetitive execution of the procedures for each cytokine (12). Therefore, we used a microparticle-based flow cytometric immunoassay which is an efficient method for simultaneously measuring six cytokines of interest from a various test samples (13-15). This method is more sensitive and analytically comparable with conventional ELISA (16). In this study, the anticandidal, cytotoxic and proinflammatory/antiinflammatory effects of four azole antifungal drugs were compared for the first time. Commonly, MIC values are determined by BMD method. In this method, microbial cell number /mL is adjusted according to McFarland standard. *C. albicans* NCPF 3179, Bioball™ Multishot 10E8 contains precise number of the microorganism. It should not be expected same results according to turbidimetric method. Bioball™ Multishot 10E8 is a newer, more reliable and more validated than McFarland. For this reason it was preferred. The cytotoxic effects of azole drugs were demonstrated by WST-1 method. According to our results, these azole antifungal drugs showed lower cytotoxic effects on THP-1 human monocyte cell line (Fig.2). This data shows that these drugs have selective toxicity on *C. albicans*. Cytokine levels of IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12p70 and TNF- $\alpha$  in THP-1 cells stimulated by *C. albicans* were determined by Cytometric Bead Array (CBA). In this study, we demonstrated that high levels of proinflammatory cytokines IL-8 and TNF were produced by *C. albicans*-stimulated THP-1 cells (Table 1). The chemokine IL-8 plays an important role in the pathogenesis of an inflammation. Because of the powerful properties of IL-8, its extracellular release must be tightly regulated. Monocytes are important components of cellular defense mechanisms in humans (17). When stimulated by microorganisms, arachidonic acid is produced and released, and production of cytokines including

tumor necrosis factor alpha (TNF- $\alpha$ , interleukin-1b (IL-1b), IL-6, IL-8 is realized (18). Fluconazole is a drug that inhibits the formation of *C. albicans* hyphae and is concentrated in human phagocytes (19-20). Clearly, these differences could be actively reinforced by the immunomodulating properties of mannan and other mannoprotein constituents of the yeast. These results support the use of selected cytokines as adjunctive therapy in disseminated candidiasis. TNF could be induced, in a dose-dependent manner, by stimulation of human monocytes with a phospholipomannan antigen of the yeast. These responses by the monocytes may be an important mechanism both for candida infections and for the initiation of a local inflammatory reaction (21). The results described in our study clearly indicate that the microbicidal activity of human monocytes is closely associated with the activities of proinflammatory cytokines.

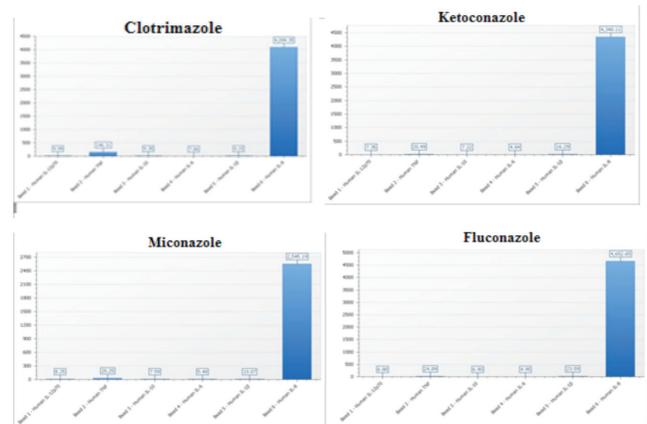
**Table 2.** Cytokine levels of *Candida albicans* stimulated and unstimulated THP-1 cell line after 4 hour (pg/mL).

Cytokines	THP-1 unstimulated	<i>Candida albicans</i>				
		4 h stimulated	Clotrimazole	Ketconazole	Miconazole	Fluconazole
IL-12p70	7.60	8.19	9.99	7.35	8.25	6.68
TNF	7.19	22.58	146.31	20.49	28.25	24.89
IL-10	7.42	7.46	9.35	7.22	7.59	6.90
IL-6	4.52	5.39	7.06	4.84	5.48	4.95
IL-1 $\beta$	-	-	9.15	16.29	10.07	23.59
IL-8	23.92	130.27	4094.35	4340.11	2545.19	4652.65

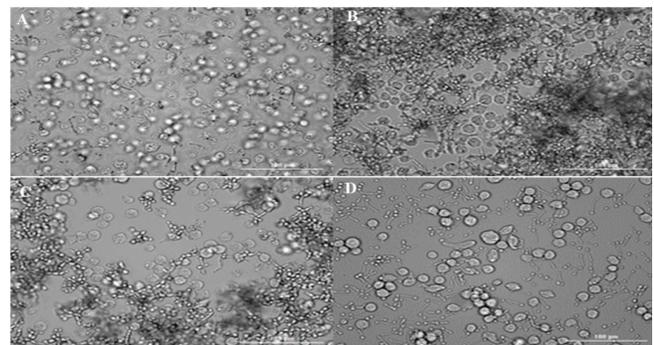
### **Candida albicans ile aktive edilmiş THP-1 hücrelerinin sitokin üretim seviyelerine azol grubu antifungallerin karşılaştırmalı etkileri**

#### **ÖZ**

Bu çalışmada klotrimazol, ketokonazol, mikonazol ve flukonazol gibi azol grubu antifungallerin sitotoksik ve proinflatuar/antiinflatuar etkilerini, *Candida albicans* ile kokültüre edilmiş THP-1 hücreleri üzerinde karşılaştırdık. *C. albicans* (NCPF 3179, Bioball™ Multishot 10E8) kullanılarak bazı azol grubu antifungal ilaçların MIC değerleri sıvı mikrodilüsyon yöntemi ile belirlendi. WST-1 yöntemi ile antifungal ilaçların sitotoksik



**Figure 2.** Determined of Clotrimazole, ketoconazole, miconazole and fluconazole effects of IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12p70 and TNF- $\alpha$  in THP-1 cells stimulated by *Candida albicans* (pg/mL).



**Figure 3.** 16 µg/ml concentration light microscope view of *Candida albicans* infected THP-1 cells (x40) A: Clotrimazole, B: Miconazole, C: Fluconazole, D: Ketoconazole

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#### **Conflicts of Interest**

All the authors declared no competing interests.

etkileri THP-1 hücreleri üzerinde gösterildi. *C. albicans* ile stimüle edilmiş THP-1 hücrelerinin sitokin seviyeleri (IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12p70 ve TNF- $\alpha$ ) antikor kaplı boncuk sistemi ile flow sitometride belirlendi. Mikonazol, klotrimazol, ketokonazol, ve flukonazolün MIC değerleri sırasıyla *C. albicans* Multishot 10E8 8, 16, 64 ve 128 µg/ml olarak bulundu. Azol antifungaller THP-1 hücreleri üzerinde düşük sitotoksik gösterdi (IC<sub>50</sub>>512 µg/ml). Azol antifungal ilaçlar IL-8 seviyesinde artışa neden olurken, özellikle klotrimazol sadece IL-8 değil TNF- $\alpha$  seviyesinde de önemli bir artışa neden olmuştur.

**Anahtar Kelimeler:** *Candida albicans*, sitokin, inflammatuar, THP-1 hücreleri.

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