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Full Length Research Paper

Assessment of antifungal effect of omeprazole on *CANDIDA ALBICANS*

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The goal of this study was to investigate the *IN VITRO* effect of omeprazole on *CANDIDA ALBICANS* and analyze the antifungal activity of omeprazole. A total of 150 samples were collected from the patients in Bakirköy Dr. Sadi Konuk Education and Research Hospital and samples were evaluated for *C. ALBICANS*. After the microbiological analyses, fifty one patients (18 men and 33 women) between 0 and 78 of age were found to be *C. ALBICANS* positive and they were included in the study. All consecutive isolates of *C. ALBICANS* were recovered from blood, urine, sputum, oral cavity, vagina, catheter tip and ascitic fluid. Antifungal suspectibility test was carried out by microdilution assay according to the method outlined in the NCCLS document M27-A. It was determined that omeprazole is fairly effective in particular MIC range. Furthermore, it was observed that omeprazole in high concentrations support the growth of fungi.

Key words: *Candida albicans*, omeprazol, antifungal effect, antifungal agents.

INTRODUCTION

The incidence of fungal infections has been increasing dramatically over the last two decades in the world in immunocompetent and immunocompromised (premature newborns, elderly individuals, chemotherapy-treated patients, HIV patients and transplant recipients) patients as well as in patients with leukemia, lymphoma and organ transplantation (Banerjee et al., 2009; Beil and Sewing, 1984; Campbell et al., 1998; Clemons et al., 2006). Also, the incidence of fungal infections is associated with high morbidity and mortality in these patient groups (Beil and Sewing, 1984). The known predisposing factors for fungal infections are immature immune system, breakdown of cellular immunity and colonization seen during the broad-spectrum antibiotic therapy (Campbell et al., 1998; Costa et al., 2010). In particular, yeasts (such as *Candida* spp.) are the most frequently isolated fungi from human infections as well as dermatophytes (Banerjee et al., 2009; Fleischhacker et al., 2008; Garey et al., 2006). *Candida*

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spp. are commonly encountered polymorphic yeasts in the gut lumen and on cutaneous surfaces and they can exist as 2 to 5 µm round to oval cells in shape (blastospores) and can reproduce by budding. Also, they produce mostly pseudohyphae elongating from the cells but few species have ability to produce actual hyphae (Fleischhacker et al., 2008; Gatta et al., 2003). Of the 200 *Candida* spp., *Candida albicans* is one of the most frequently isolated fungal pathogen in humans (Campbell et al., 1998; Fleischhacker et al., 2008). This commensal yeast belongs to the normal flora of skin as well as gastro-intestinal and genital tracts of healthy individuals.

Candida albicans is a Gram-positive and opportunistic yeast that can cause life-threatening systemic infections by entering bloodstream and infecting the organs (Clemons et al., 2006). Their new forms in bloodstream are called chlamydospores. This unique property is a resting stage of yeast and specific to *C. albicans.* Chlamydospores have cylindirical extentions called germ tubes (Fleischhacker et al., 2008; Guery et al., 2009). *C. albicans* produce germ tubes at 33 to 42°C in pH 6 to 8 (Guery et al., 2009). The formation of germ tubes and chlamydo-spores are the distinctive features for *C. albicans* and these characterictics are rarely observed in other *Candida* species (Fleischhacker et al., 2008; Guery et al., 2009; Harrington et al., 2007). The germ tube test and controlling the ability to produce chlamydospores are the most helpful, rapid and reliable methods for the presump-tive clinical identification of *C. albicans* isolates (Heelan et al., 1998).

It has been reported that the antifungal therapies recommended as the first-line treatment have usually been selected based on the clinical status of the patient (Gatta et al., 2003; Johnson et al., 2003; Katiyar et al., 2006). The narrow-spectrum antifungals may not supply sufficient treatment for the patients who are suffering from fungal infections (Gatta et al., 2003). In the recent studies, it has been suggested that early intervention by adequate antifungal agents may considerably reduce mortality in patients (Gatta et al., 2003; Keeling et al., 1985; Kumar et al., 2006; Larner and Lendrum, 1992). The first-line proposed antifungal agents are commonly azoles (especially fluconazole) for patients with fungemia (Johnson et al., 2003; Larsson et al., 1983). Other alter-native antifungal agents such as lipid formulations of amphotericin and echinocandins with considerably broader activities have been preferred at a lower rate due to their high costs than azoles (Larsson et al., 1983). Given the increasing concerns on the resistant yeast species to the antifungal therapies administered in clinical practice (azoles and polyene antibiotics, etc.), newer antifungal agents like omeprazole could be useful in guiding the treatment of fungemia.

Omeprazole, a substituted benzimidazole, is a potent proton pump inhibitor and has been reported in the treatment of acid-peptic diseases of the gastrointestinal tract, duodenal ulcer and Zollinger–Ellison syndrome over the past decade (Martínez et al., 1998; Merlino et al., 1998; Mogensen and Mühlschlegel, 2008; Molero et al., 1998; Monk and Perlin, 1994; Morrell et al., 2005).

The plasma membrane H+-ATPase inhibitor plays a major role in yeast cell physiology. This ion translocation enzyme is responsible for maintaining the electrochemical proton gradient for the adjustment of intracellular pH and nutrition uptake in the fungal cell. The inhibition of H+-ATPase activity by antagonists causes cell death. Therefore, utilization of the plasma membrane H+-ATPase as a molecular target appears to be more attractive approach for the antifungal drug therapies only if the connection is maintained between the inhibition of enzyme activity and suppression of cell growth (Mogensen and Mühlschlegel, 2008).

The aim of this study was to investigate the antifungal activity of omeprazole on *C. albicans* isolated from the clinical samples of blood, urine, sputum, oral cavity, vagina, catheter tip and ascitic fluid of inpatients and outpatients by evaluating its resistance, sensitivity and applicability in candidiasis.

MATERIALS AND METHODS

Sample collection

A total of 150 samples were collected from the inpatients and outpatients in Bakirköy Dr. Sadi Konuk Education and Research Hospital. These samples were collected under the aseptic conditions and placed in sterile sample bags and then, they were immediately transp**o**rted to the laboratory. The samples were evaluated for *C. albicans*. After the microbiological analyses, fifty one patients (18 men and 33 women) between 0 and 78 of age were found to be *C. albicans* positive and they were included in the study. All consecutive isolates of *C. albicans* were recovered from blood, urine, sputum, oral cavity, vagina, catheter tip and ascitic fluid. The reference strains were supplied from The American Type Culture Collection for quality control. These ATCC strains were *C. albicans* ATCC 90028, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019). These strains were supplied and grown as described for *Candida* isolates. The reference strains and *C. albicans* isolates were identified in microbiologically.

Omeprazol activation

Omeprazol was gotten from Eczaciba□i Holding Co. Since antifungal agents like omeprazole become active in acidic environment, the pH of omeprazol was adjusted to pH 2 with HCl after dissolving the agent in dimethyl sulfoxide (DMSO) in accordance with The

National Commmittee for Clinical Laboratory Standards (NCCLS). After an hour, the activation of omeprazol was confirmed by the color change (orange) on the slides. Then, the pH was again increased to pH 7.

Microbiological identification of *CANDIDA* species

All the collected strains were analysed microscopically for the identification of *Candida* by 10% KOH or sterile physiological saline solution. The clinical isolates were streaked onto the Saboraud-Dextrose agar (SDA) plates which is a selective media for the growth and identification of fungi (Shindo et al., 1998). They were incubated at 35°C for 24 h. The growing yeast colonies on SDA were examined for *C. albicans* by germ tube test in serum, colony colour on *CANDIDA* ID2 and identification by API Candida (BioMerieux, France**)**. Firstly, yeast cultures were incubated with human serum placed into blood culture bottles for 2-2.5 h at 37°C and then the presence or absence of germ tubes recorded. All germ tube positive yeast isolates were accepted as identified as *Candida albicans*. The same samples were cultured on the chromogenic agar plates of *CANDIDA* ID2 which is a commercially a ready-to-use medium allowing the specific identification of *Candida* spp. The plates were read and results interpreted after the incubation for 24 h at 37°C according to the manufacturer's instructions. *C. albicans*, *Candida dubliniensis* and *C. krusei* were characterized with blue, turquoise and pink colonies in the *CANDIDA* ID2 agar plates, respectively. The blue colored colonies, identified as *C. albicans* on the basis of their typical appearances, were enrolled in the study. Then, definitive identification of *C. albicans* was made with the API *Candida* test kit (BioMerieux, France) on the basis of biochemical reactions from the microbiological aspect. Each pure isolate of *C. albicans,* identified microbiologically before, were stored in the medium at -20°C until use (Stevens et al., 2006). The inoculums of yeast strains were prepared based on the NCCLS document. The stored isolates of *C. albicans* and reference strains were re-streaked onto SDA and incubated for 24 h at 35°C. Then, three or five colonies, which were in similar morphology and ≥1 mm in diameter, were picked up and suspended in 5 ml of 0.85% sterile physiological saline solution by vortexing. The density and turbidity of the suspension were adjusted to 1.5 x 10^6 CFU/ml and a Mc Farland standard of 0.5. Then, the suspensions were diluted (1:100 and 1:20) in RPMI 1640 medium. In this way, the final concentrations of the inoculums were adjusted to 0.5 to 2.5 x 10^3 CFU/ml.

Antifungal suspectibility testing

Antifungal suspectibility testing was carried out by microdilution assay according to the method outlined in the NCCLS document M27-A (1997) (Stevens et al., 2006). In the antifungal suspectibility testing, RPMI 1640 medium, which was supplemented with both glutamine and pH indicator, without sodium bicarbonate, was used. The pH of this medium was adjusted to pH 7 at 25°C with morpholinepropanesulfonic acid (MOPS) until the final concentration was 0.165 mol/L and sterilized by filtering. *C. albicans* ATCC 90028, *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as reference strains. During the experiments, these reference strains were periodically streaked onto SDA plates and checked for their purity to prevent contamination.

The dilution intervals of omeprazole

The powdered omeprazole was diluted in DMSO in quantities specified as in Tables 1 to 3 and three omperazole suspensions in different concentrations were prepared.

The sterile microdilution plates (96-well and U-bottom) were used for the microdilution technique M27-A recommended by the NCCLS. During all the experiments, freshly prepared samples, which were incubated for 48 h at 35°C, were used. The omperazole suspensions ranging from 32 to 6 µg/L in concentration were distributed in 96-well microdilution plates in amounts of 100 µl per well. Then, the *Candida* suspensions, which were adjusted to a Mc Farland standard of 0.5, were distributed to the wells in amounts of 100 µl loaded with omeprazole. One well was used as control for the yeast growth and only yeast cells were placed and omeprazole was not added to the wells. The last wells were loaded only with medium to control the contamination of the test medium. The plates were incubated for 48 h at 35°C. Same procedure was used for the second and third concentrations of omperazole given in Tables 2 and 3.

RESULTS AND DISCUSSION

Since *Candida* spp. are commonly encountered opportunistic yeasts in normal flora of healthy individiuals, they have critically important role in terms of high morbidity and mortality. There are several studies aiming to reduce the rates of fungal morbidity and mortality but antifungal drug discovery are still needed to be developed (Sümer et al., 2005). The (H^++K^+)-ATPase inhibitor omeprazole is an effective treatment with a favourable safety profile for acid-peptic disease of the gastrointestinal tract, duodenal ulser and Zollinger–Ellison syndrome over the past decade (Martínez et al., 1998; Merlino et al., 1998; Mogensen and Mühlschlegel, 2008; Molero et al., 1998; Monk and Perlin, 1994; Morrell et al., 2005).

Johnson et al. (2003) showed that omeprazole increased the serum concentration of itrakonazole and positively affected its antifungal effect. In the other preliminary studies, it was determined that omeprazole interacts with fungal ATPase and inhibits this enzyme as well as gastric (H^++K^+)ATPase in a similar pattern (Sümer et al., 2005; Thomas et al., 2001).

Keeling and coworkers (1985) reported that the inhibition by omeprazole, degraded by acid, was more pronounced in Na^+, K^+-ATPase rather than H^+, K^+-ATPase. Furthermore, they emphasized that H^+, K^+-ATPase could be inhibited by considerably high omeprazole concentrations (Mogensen and Mühlschlegel, 2008).

Beil and Sewing have reported that omeprazole inhi-bited the (H^++K^+)-ATPase activity of preparations isolated from parietal cells of guinea pig (National Committee for Clinical Laboratory Standards, 1997; Reboli et al., 2007). In the other study, it was showed that omperazole was unstable in the acidic solutions and the inhibition of

Table 1. The first concentration range of omeprazole (32 to 0.06 µg/ml).

Potency	100%
Weight (mg)	100
Desired stock concentration (µg/ml)	12800
DMSO quantity	7.8125
Used microplaque number	10
Initial concentration (mcg/ml)	32

Step	Requiredconcentration	Total volume	Sampleconcentration	Sample quantity	DMSO quantity	Proportion	Final volume	Diluted (1:50) RPMI
1	3200	0.4	12800	0.1	0.5	0.25	0.2	9.8
2	1600	0.2	3200	0.1	0.1	0.5	0.2	9.8
3	800	0.2	3200	0.05	0.15	0.25	0.2	9.8
4	400	0.4	3200	0.05	0.35	0.125	0.2	9.8
5	200	0.2	400	0.1	0.1	0.5	0.2	9.8
6	100	0.2	400	0.05	0.15	0.25	0.2	9.8
7	50	0.4	400	0.05	0.35	0.125	0.2	9.8
8	25	0.2	50	0.1	0.1	0.5	0.2	9.8
9	12.5	0.2	50	0.05	0.15	0.25	0.2	9.8
10	6.25	0.4	50	0.05	0.35	0.125	0.2	9.8
11	ATIK		6.25	0.2				

$(H^+ + K^+)$-ATPase activity by omeprazole was highly dependent upon pH (Reboli et al., 2007; Rex and Sobel, 2001; Richardson and Elewski, 2000).

Resistance to antifungal drugs has become a major problem worldwide and has become a significant problem increasingly in pathogenic mycology. In general, the antifungal-resistant strains arised from haphazardly use of antifungal drugs in repeated dosages. It is known that some strains of *Candida* are resistant to the antifungal agents such as azoles and flukanazole. In recent studies, it was reported that this resistance problem can occur due to the developing mutations depending on their origin, living conditions and its host. Nowadays, our knowledge on the mechanism of the resistant strains of *C. albicans* to antifungal drugs has increased. On the other hand, some antifungal drugs are mostly effective on both pathogenic fungi and host due to the high similarities of eucaryotic cells. Since many antifungal drugs have side-effects on humans, it is important to discoverfairly new drugs targeting the non-shared features between host and fungi or application of antifungal drugs with less side-effects. Thus, it has been considered that omperazole, a proton pomp inhibitor in humans, can be effective on fungal plasma membrane ATPases and might be used in fungal diseases.

In our study, the patient age range of study group (19 males and 32 females) was from 0 to 78 years of age. There was no significant difference in terms of age distribution within the patient groups ($p>0.05$).

Theminimuminhibitoryconcentration(MIC)ofomeprazole was evaluated by the antifungal suspectibility test in a total of 51 isolates of *C. albicans* which were isolated from blood, urine, dental plaque, oral cavity, sputum, vagina, catheter tip and ascitic fluid and reference strains of *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 in accordance with the NCCLS M27-A microdilution method. MICs were defined as the highest concentration that showed a sharp decline in the density of growth. 30 of 51 *C. albicans* strains (59%) were susceptible to 0.06 µg/ml of omeprazole in the first

Table 2. The second concentration range of omeprazole (512-1 µg/ml).

Potency	100%
Weight (mg)	100
Desired stock concentration (µg/ml)	51200
DMSO quantity	1.95
Used microplaque number	10
Initial concentration (mcg/ml)	512

Step	Requiredconcentration	Total volume	Sampleconcentration	Sample quantity	DMSO quantity	Proportion	Final volume	Diluted (1:50) RPMI
1	51200	0,4	51200	0,4	0	1	0,2	9,8
2	25600	0,2	51200	0,1	0,1	0,5	0,2	9,8
3	12800	0,2	51200	0,05	0,15	0,25	0,2	9,8
4	6400	0,4	51200	0,05	0,35	0,125	0,2	9,8
5	3200	0,2	6400	0,1	0,1	0,5	0,2	9,8
6	1600	0,2	6400	0,05	0,15	0,25	0,2	9,8
7	800	0,4	6400	0,05	0,35	0,125	0,2	9,8
8	400	0,2	800	0,1	0,1	0,5	0,2	9,8
9	200	0,2	800	0,05	0,15	0,25	0,2	9,8
10	100	0,4	800	0,05	0,35	0,125	0,2	9,8
11	ATIK		100	0,2				

concentration range of omeprazole (32 to 0.06 µg/ml), while 16 (31.7%) were susceptible to 1 µg/ml of omeprazole in the second concentration range of omeprazole (512 to 1 µg/ml). On the other hand, almost all these strains (except 3 strains) were fairly resistant to omeprazole in the third concentration range of omeprazole (1 to 0.001 µg/ml). As a result, the effective MIC range of omperazole was found to be 1 to 0.06 µg/ml and this efficacy disappeared in higher or lower concentrations. This phe-nomenon is called “the eagle effect” or “paradoxical effect” in the literature. There are several reports analy-zing the effects of high concentrations of three antifungal substances, including caspofungin, on the growth of *Candida* spp. and it was demonstrated that *in vitro* effi-ciency on *Candida* spp. was reduced by increasing anti-fungal dosesm (Wallmark et al., 1983; Whitley-Williams, 2006; Zomorodi and Houston, 1996).

Sümer and her colleagues (2005) examined the effi-ciency of omeprazole on *C. albicans* and they observed that most of the strains of *Candida* spp. showed increasing suspectibility to the high concentration of omeprazole (320 µg/ml). In the other parallel studies examining the different antifungal drugs, it was observed that this concentration was considerably high. Therefore, it was concluded that utilization of different antifungal drug combinations or experiencing different preparations was more efficacious to reduce the concentration of antifungal drugs, and also local application instead of systemic utilization was necessary.

In this study, we determined that omeprazole is fairly effective on *C. albicans* in 1 to 0.06 µg/ml and surely appliciable in candidiasis. On the other hand, we observed that omeprazole application in high concentrations sup-ported the growth of fungi. Especially, to chose and apply the most effective antifungal therapy is essential for controlling the nosocomial infections and epidemiological studies and also for maintaining public health. In conclu-sion, we believe that omeprazole therapy in *Candida*

Table 3. The third concentration range of omeprazole (1 to 0.001 µg/ml).

Potency	100%
Weight (mg)	100
Desired stock concentration (µg/ml)	12800
DMSO quantity	7.8125
Used microplaque number	10
Initial concentration (mcg/ml)	32

Step	Requiredconcentration	Total volume	Sampleconcentration	Sample quantity	DMSO quantity	Proportion	Final volume	Diluted (1:50) RPMI
1	51200	0,4	51200	0,4	0	1	0,2	9,8
2	25600	0,2	51200	0,1	0,1	0,5	0,2	9,8
3	12800	0,2	51200	0,05	0,15	0,25	0,2	9,8
4	6400	0,4	51200	0,05	0,35	0,125	0,2	9,8
5	3200	0,2	6400	0,1	0,1	0,5	0,2	9,8
6	1600	0,2	6400	0,05	0,15	0,25	0,2	9,8
7	800	0,4	6400	0,05	0,35	0,125	0,2	9,8
8	400	0,2	800	0,1	0,1	0,5	0,2	9,8
9	200	0,2	800	0,05	0,15	0,25	0,2	9,8
10	100	0,4	800	0,05	0,35	0,125	0,2	9,8
11	ATIK		100	0,2				

infections is clearly effective and promising application, and also can solve the major antifungal drug-resistance problem.

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