Antifungal Action of Imidazole Derivatives from New Pharmaceutical Forms on Various Strains of Candida

MAGDALENA BIRSAN, ILEANA CORNELIA COJOCARU*, MONICA ILIUTA STAMATE, VLAD IOAN TEODOR, CRISTINA TUCHILUS
Faculty of Pharmacy, Gr. T. Popa University of Medicine and Pharmacy, Iasi, 16, University Str., 700115, Iasi, Romania

The antifungal activity of imidazole derivatives was tested on three types of Candida, respectively C. albicans, C. sake, and C. glabrata. The antifungal activity was compared with the activity of miconazole nitrate in 16 new formulations of oral biomucoadhesive tablets, with the purpose of being used in oral candidiasis. All the 16 formulations of biomucoadhesive tablets which contain 25 mg miconazole have a good antifungal action; the diameter of the inhibition area is over 20 mm in all the three strains of Candida. The second goal was to compare the activity of miconazole nitrate with other antifungal substances: clotrimazole (benzyl imidazole derivative), nistatin (polypenic macrolide), econazole (phenyl – ethyl – imidazole derivative), and fluconazole (triazole derivative). Good results, obtained by measuring the diameter of the inhibition area, were shown by econazole, with a diameter of over 22 mm, but this imidazole derivative does not penetrate the stratum corneum well enough, which implies a much longer treatment than miconazole. The third goal was the assessment of the antifungal activity of the 16 formulations of biomucoadhesive tablets by means of establishing the minimum inhibitory concentration (MIC) and of minimum fungicide concentration (MFC).

Keywords: first-generation azoles, biomucoadhesive tablets, miconazole nitrate, and Candida

Candidiasis is a frequent condition of mucosa and tegument, caused by various Candida species, which, in immunodeficiency conditions, may become invasive. Usually, Candida species are pathogenic, isolating themselves from the microflora of mucosa in 10-15% of normal people. To become pathogenic, predisposing factors are necessary, altering the immunity of the host: sugar diabetes, AIDS, people treated with cytostatic and corticosteroids or antibiotics, and with mucosal ulcerations [1]. Candida species are part of man’s normal flora, being the main source of infection. The transmittance occurs via direct contact with buccal, tegument, and vaginal secretions that contain Candida [2].

Broad-spectrum antibiotic therapy significantly increased the life expectancy in patients with severe infections with approximately 10 years, as some authors state. Unfortunately, these medical successes have brought in the last 15 years the spread of fungal infections, followed by the increase of resistance to antifungal drugs, initially in patients with severe immunodepression: after transplant or after chemotherapy in oncology [3]. Candidemia is more and more signalled in long-term patients in Intensive Care units, while the haematogenous fungal infections are the fourth cause of nosocomial infections in critical patients [4]. More and more patients with HIV/AIDS, neoplastic or transplant are colonized with strains of fungi at the gateways, most often with Candida albicans, but also the strains of Candida nonalbicans are significantly spread.

The most common substances that are used for their antifungal actions are imidazole and triazole derivatives. Triazole derivatives are recommended by some studies due to their reduced toxicity in systemic administering and due to their longer duration of action [5].

First-generation azoles are inhibitors of ergosterol biosynthesis with a broad-spectrum activity, such as dermatophytes, unicellular, dimorphic and filamentous fungi. First-generation co-azoles have an exceptional tolerance, with substantial reduction of duration of treatment and minimum side-effects, comparing with other antifungal drugs. They are lipophilic, little soluble in water, reason why we chose the salt form of antifungal drug, with a better solubility in water. Miconazole nitrate is an antimicotic drug, with a similar structure to econazole, differing only by the presence of 2 chlorine atoms (positions 2 and 4) on benzyl radical.

Imidazole and triazole derivatives inhibit the synthesis and incorporation of ergosterol in the membrane of the fungal cell as a result of blocking sterol-14α-demethylase, a cytochrome P450 dependant enzyme, with a key role in biosynthesis of ergosterol [6]. Accumulation of methylsteroids affects the function of membrane phospholipids and inhibits certain enzymatic systems membrane dependant like ATPase and enzymatic transport systems with inhibition of growth and development of fungi [7]. It acts by affecting the permeability of the membrane of the fungi, selective inhibition of RNA and DNA precursors and its mucopolysaccharides.

The minimum fungicide concentration of miconazole triggers an important growth of dispersion of the intracellular potassium, causing a cellular acidification that would favour the activity of autolytic enzymes.

Miconazole also acts by interfering with the fungal enzymes: cytochrome C oxidase, cytochrome C peroxidase and catalase. It does not act directly on the enzymes, but it seems it interferes with the conversion of

* email: mail.icojocaru@yahoo.com

Scheme 1. Miconazole Nitrate. 1-[2(2,4-dichlorophenyl)-methoxy] -2-(2,4-dichlorophenyl)-ethyl-1H-imidazole

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mitochondrial ATP and thus it helps synthesizing these enzymes. NADH oxidase pathway is not affected, therefore peroxides are produced in cytoplasm, at levels that are incompatible with the viability of the cells. The effect on cell morphology varies from the lysis of cell organelles, loss of plasmalemma and thickening of the cellular wall (in fungistatic doses) to progressive cytoplasmic deterioration and loss of cellular functions with cellular death (in fungicidal doses) [5].

Miconazole nitrate is incompletely absorbed from the gastrointestinal tract. When administered on skin and mucosa, it is absorbed in a limited proportion (~1%). Miconazole easily penetrates the stratum corneum, building effective therapeutic concentrations, that last for more than 4 days from its application on the skin. There is a 1.3% absorption on intravaginal application. It bonds in a 90% proportion to plasmatic proteins. It is metabolized in the liver in inactive compounds, which are eliminated through faeces (50% from the administered dose, as unmodified drug) and through kidneys, as inactive metabolites. The medicine has a good penetrability also in inflamed joints, vitreous body, crystalline lens, and peritoneal cavity. It reaches the saliva and sputum. It also crosses the blood-brain barrier. T½ is 20-30 min.

Miconazole nitrate is indicated for treatment of infections that are provoked by Candida albicans, suprainfected or not by a Gram positive bacterial flora, treatment of dermatophytes, coccidiomycosis, paracoccidiomycosis, kryptocccosis, systemic and mucocutaneous candidiasis (opharyngeal candidiasis), and treatment of acne.

The main goal of this study was to test various substances with antifungal action that are used in buccal candidiasis, therefore we chose three different strains of Candida. Recent studies have shown a very good antifungal activity by means of using polyenic macrolides and semi synthesis antifungal drugs, such as imidazole and triazole derivatives [8-10].

We chose as testing samples 16 new formulations of oral biomucoadhesive tablets, conceived to guide the release of the active substance in the buccal mucosa with the goal to increase their effectiveness [11]. The use of mucosadhension concept with the purpose of controlled release of drug substance is based on the capacity of some natural or synthetic polymers to interact with the layer of mucus that covers the epithelial surface of the mucosa [12]. To improve the bioavailability of drugs that are administered in oral cavity, oral biomucoadhesive tablets have been developed in recent years, that may be applied in various areas of the mouth: palate, mucosa of the cheek, between the gingiva and the upper lip [13]. The tablet adheres to the substrate through various mechanisms and it is kept in the same position until dissolution and/or the completion of release of the active substance. In recent years, the market share of biomucoadhesive systems has significantly increased, according to a recent report that was published by Kalorama, with an estimated value of 6.7 million dollars in 2006 [14] up to 7.9 million dollars in 2010 [15]. This growth may be easily explained by the success of these new pharmaceutical formulations due to the advantages they have.

The second goal was to compare the activity of miconazole nitrate with clotrimazole (benzyl imidazole derivative), nistatin (polyenic macrolide), econazole (phenylethyl imidazole), and with fluconazole (triazole derivative).

The third goal was to assess the antifungal activity of the 16 biomucoadhesive formulations by establishing the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC).

Experimental part

16 new formulations of biomucoadhesive tablets with miconazole nitrate were used as samples that were tested for antifungal activity, in 25 mg concentration and various excipients [11]. The test microorganisms that we used were Candida albicans ATCC 10231, Candida sake, and Candida glabrata ATCC MYA 2950. The strains we used in microbiologic tests are part of collection of microorganisms belonging to department of Microbiology, Faculty of Pharmacy, Gr. T. Popa University of Pharmacy and Medicine, Iasi, and the culture medium was Muller-Hinton Agar for fungi (HiMedia).

Testing the antimicrobial activity

Antimicrobial activity of samples I-XVI was tested for quality, by diffusimetric method [16, 17]. From the culture that was incubated over night at the optimal temperature (30°C), we prepared a suspension, with the density that corresponded the tube of 0.5 in Mc Farland turbidimetric scale.

The suspension was incorporated in 1/10 proportion in Muller-Hinton medium for fungi, melted and cooled down at 50°C. After homogenization, the mixture was distributed in Petri dishes with 12 cm diameter, 25 mL each. After solidification, stainless steel cylinders were disposed on the surface of each Petri dish, in which 0.2 mL of the testing surfaces were distributed. The results were read after keeping the dishes at thermostat, at 24°C, for 48 h.

Each sample was simultaneously tested in three dishes. The diameter of the inhibition areas was measured and recorded. The results are the average of three values that were obtained from measuring the diameter of the inhibition areas of the microbial growth.

We compared the antimicrobial activity of the samples with the activity of the mortar microtabelts: Econazole (50µg), Miconazole (50µg), Clotrimazole (50µg), Fluconazole (25µg), and Nistatin (100 µg/disc).

Quantitative antimicrobial activity was assessed by establishing the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). We used microdilution in liquid Sabouraud medium method [17]. We made double dilutions from samples in liquid Sabouraud medium, obtaining thus concentrations between 0.06-12.5 mg/ml. Each cup with double dilutions of each sample was inoculated with 50 microliters from the solution of each test microorganism (with a density of approximately 10³ UFC/mL). The dishes were incubated at 24°C. After 24 h, MIC value was registered as the smallest sample concentration in which the visible growth of microorganisms is absent.

Minimal fungicidal concentrations were established by harvesting micro cultures from double serial dilutions in which the visible microbial growth was absent. MFC was given by the smallest product concentration that kills at least 99.9% of inoculum.

Results and discussions

Diameters of the inhibition areas (in millimetres) that correspond to the tested biomucoadhesive tablets are shown in table 1. Out of all the tested substances, the remarkable antifungal value belongs to miconazole nitrate out of the 16 new formulations, with miconazole as pure substance and econazole as pure substance.
Clotrimazole, a broad-spectrum antifungal agent, indicated in treatment of superficial mucocutaneous infections, provoked by various species of pathogenic dermatophytes and yeast, builds a 21 mm diameter of the inhibition area for Candida albicans strain, 23 mm for Candida sake strain, and 18 mm for Candida glabrata strain. As antifungal activity, this azole has a good activity for Candida albicans and Candida sake, and an average action for Candida glabrata. Unlike miconazole nitrate that is stored in the stratum corneum up to 4 days after application, clotrimazole is active at active therapeutic concentrations up to 3 days. Topically administered, clotrimazole provokes itching, erythema, edema, rash or desquamation, disadvantages that lead to the removal of the substance as the first choice in candidiasis.

Out of the 5 antifungal substances that we used, econazole has the best antimicrobial activity, by building a 24 mm diameter of the inhibition area on Candida albicans, 22 mm on Candida sake, and 27 mm on Candida glabrata. Despite the fact that it makes a very good diameter of the inhibition area, this substance does not last in the tissue as miconazole nitrate or clotrimazole, that is why the treatment with econazole needs a long treatment, up to 2 weeks, to avoid relapse [17].

**Table 1**

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Miconazole nitrate has a similar structure to econazole, differentiating itself from it by the presence of 2 atoms of chlorine in position 2 and 4 on benzyl radical. The presence of chlorine atoms limits the activity of the antifungal with approximately 10%; in exchange, it has the advantage of easily penetrating the stratum corneum, building concentrations that last for more than four days from the topical application. The diameter of the inhibition area that is larger than 20 mm for all the three strains of Candida, together with the advantage of building concentrations that last several days, avoiding this way the risk for relapse, lead to a first choice of miconazole nitrate for the treatment of buccal candidiasis.

Fluconazole, even if it has the advantage of a smaller toxicity in comparison with miconazole nitrate or econazole, had a good activity on Candida albicans and Candida sake strains, but a weak action for Candida glabrata strain (table 1).

Nystatin, polyenic macrolide, made a 19 mm diameter of the inhibition area for the 3 strains of Candida, a small diameter in comparison with miconazole or econazole, but if we consider the low toxicity, it has a good antifungal activity. This polyenic macrolide offers an optimal diameter of the inhibition area for Candida glabrata ATCC MYA 2950 strain.
XI, with over 20 mm diameters of inhibition areas on tested tablets is good, with large inhibition areas, of inhibition areas (table 1). The formulated tablets proved 16 formulations with miconazole nitrate on the strains of Candida albicans ATCC 10231 and Candida glabrata ATCC MYA 2950, and a very good activity on Candida sake strain. One may notice a good activity of the best antifungal action proven by the dimension of the inhibition area of 23 mm is shown by ketoconazole, miconazole, and clotrimazole Ligands, Organometallics, 2015; 34:3809-3815.

For Candida species that we used, the antifungal activity of the tested tablets is good, with large inhibition areas, of over 20 mm diameters of inhibition areas on tested Candida species.

The activity on Candida glabrata ATCC MYA 2950 was lower, proven by smaller values of the diameters of the inhibition areas (table 1). The formulated tablets proved higher activity on the tested C. albicans, C. Glabrata, and C. sake.

Conclusions
We tested the antifungal activity of the 16 new formulations of biomucoadhesive tablets with miconazole nitrate on various Candida strains, respectively Candida albicans ATCC 10231, Candida glabrata ATCC MYA 2950 and Candida sake. One may notice a good activity of the 16 formulations with miconazole nitrate on the strains of Candida albicans ATCC 10231 and Candida glabrata ATCC MYA 2950, and a very good activity on Candida sake strain.

The best antifungal action proven by the dimension of the diameter of the inhibition area of 23 mm is shown by formulations FV and FVII. The antifungal action was compared with the activity of the miconazole nitrate pure substance and with the one of standardized microtablets of econazole, miconazole, clotrimazole, fluconazole, and nistatin.

In our study, first-generation azoles presented a good activity, in comparison with the one of fluconazole, triazole derivative. Out of the results that were obtained on the three strains of Candida spp., it is visible that the most appropriate antifungal drug for buccal candidiasis is miconazole nitrate, formulated in oral biomucoadhesive tablets.

References
15.DUCHENE F, PEPPOS NA, Drug Dev Ind Pharm, 1988; 14: 283.
17.*** Clinical and Laboratory Standard Institute. Reference Method for Broth Dilution Antifungal susceptibility testing of yeasts. 2nd ed. M27-A2