Activity of fluconazole and its Cu(II) complex towards Candida species

Adam Ząbek · Justyna Nagaj · Agnieszka Grabowiecka · Ewa Dworniczek · Urszula Nawrot · Piotr Młynarz · Małgorzata Jeżowska-Bojczuk

Received: 28 May 2014 / Accepted: 27 September 2014 / Published online: 9 October 2014
© The Author(s) 2014. This article is published with open access at Springerlink.com

Abstract Candida species, although they are present as commensal organisms in the digestive tract of healthy individuals, can produce a broad spectrum of serious illnesses in compromised hosts. Fluconazole, a water-soluble triazole with bioavailability greater than 90 %, has been extensively used to treat a wide range of Candida infections. However, a growing resistance of microorganisms in the treatment leads to the discovery of new drugs or modifications of existing ones. The aim of the present study was to investigate whether coordination of Cu(II) ions to fluconazole affects its antifungal activity. The in vitro susceptibility tests and antifungal studies were performed with two Candida spp.: Candida glabrata and Candida albicans. Overall, 34 strains of the former and 16 strains of the latter were treated with fluconazole, its Cu(II) complex and free Cu(II) ions. The obtained MIC values in 16 cases of the C. glabrata and in 5 cases of the C. albicans were lower for the complex in comparison to the drug. This implies that the complex is more effective against particular strains than the parent drug. The most significant improvement in the complex drug efficacy was observed for fluconazole-resistant species.

Keywords Fluconazole–Cu(II) complex · Candida spp. · Azole resistance · MIC determination

Introduction

In the last decades, a significant increase in incidence of opportunistic systemic fungal infections has been observed (Pfaller and Diekema, 2007). Although many of them could be successfully cured with available antifungal agents, the mortality due to systemic mycoses is still very high (30–90 %). The population of patients at risk is increasing and embraces mostly immunocompromised patients, particularly with HIV/AIDS, after bone marrow or solid organ transplantation, cancer patients undergoing chemotherapies, intensive care unit patients and preterm neonates (Sobel, 1992; Abi-Said et al., 1997; Ables et al., 2000; Alexander et al., 2005; Vazquez and Sobel, 2002; Tscherner et al., 2011; Kaufman, 2008).

Invasive mycoses can be caused by a broad spectrum of opportunistic fungal pathogens, the most important of which being members of the Candida genus. They represent the fourth most frequent pathogen isolated from the blood. The Candida represents a part of commensal flora of the gastrointestinal tract of 60–90 % of healthy human population. On the other hand, they are responsible for many types of superficial as well as deep seated infections, e.g. oral and vulvovaginal candidiosis or candidaemia. Candida albicans is the species most frequently isolated from infection cases; however, the role of the “non-albicans” species, such as Candida glabrata, Candida parapsilosis, Candida tropicalis and Candida krusei, is growing systematically (Biswa...
Materials and methods

Clinical isolates

Fifty clinical isolates of Candida (16 strains of C. albicans and 34 strains of C. glabrata) were isolated from stool, urine, blood, wound, catheters, sputum and throat swabs. They were identified by ID32 (bioMerieux) test in the Department of Microbiology of the Wroclaw Medical University. All strains were preserved in −80 °C in Tryptic Soy broth (Sigma-Aldrich), supplemented with 10 % glycerol (Sigma-Aldrich) and subcultured onto Sabouraud’s dextrose agar (Sigma-Aldrich) for 24 h to ensure viability and purity prior testing.

Assay media and solutions

For antifungal susceptibility testing, the modified RPMI 1640 medium without bicarbonate (Sigma-Aldrich) buffered to pH 7.0 with 0.165 M 3-(N-morpholino)-propane-sulfonic acid (Sigma-Aldrich) and supplemented with glucose to final concentration of 2 % per litre (RPMI 1640 2 % G) was used.

All used solutions were prepared according to Good Manufacturing Practice. Fluconazole (Sigma-Aldrich) was dissolved in double-strength culture medium RPMI 1640 2 % G at concentration of 256 μg/mL. The blank solution of free copper ions was prepared in the same way. Standard solution of copper ions complex with fluconazole was prepared by adding Cu(II) chloride (Sigma-Aldrich) to solution of fluconazole in 1:1 molar ratio.

Susceptibility testing

Susceptibility testing of each isolate was performed according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing) broth microdilution method (Rodriguez-Tudela et al. 2002), to establish minimum inhibitory concentrations (MICs) of antifungal agents. Sterile 96-well flat-bottom plates containing 100 μL of the twofold serial dilutions of FLZ, FLZ-Cu or Cu(II) ions in double-strength RPMI 1640 medium 2 % G (Sigma-Aldrich) were inoculated with 100 μL of yeast suspensions containing 1–5 × 10⁵ cfu/mL. Drug and complex dilutions were ranged from 0.125 to 128 μg/mL. The plates were incubated at 37 °C for 24 h. The fungal growth was measured at wavelength 530 nm by TECAN Microplate Reader Sunrise™. All assays were performed at least six times, apart from four strains of C. glabrata (1941, 1973, 2098, 2221) and two strains of C. albicans (2210, 2211), which exhibited the best growth reduction in the presence of FLZ-Cu. For these strains, assays were conducted twelve times, and the results were confirmed by
t test for significance (p < 0.05), using STATISTICA 10.0 for Windows (StatSoft, Poland). The data are expressed as mean values, and they are the average of 6 or 12 independent experiments, done in triplicate.

The strains were classified according to the clinical breakpoints (CBPs) developed by EUCAST (Version 6.1, valid from 2013 to 03-11, www.eucast.org/) as susceptible (S) [MIC ≤ 2 mg/L for C. albicans and MIC ≤ 0.002 mg/L for C. glabrata], resistant (R) [MIC ≥ 4 mg/L for C. albicans and MIC ≥ 32 mg/L for C. glabrata] and intermediate susceptible (I) [2 mg/L < MIC < 4 mg/L for C. albicans and 0.002 mg/L < MIC < 32 mg/L for C. glabrata] (Espinel-Ingroff et al., 2013).

Results

Susceptibility of strains and species-specific clinical breakpoints

Antifungal susceptibility tests were performed on fifty yeast strains of Candida spp. For each strain and investigated agent, the percentage distribution of growth reduction and the MICs values were determined. The obtained in vitro results revealed different susceptibility for both C. glabrata and C. albicans strains. According to CBPs for fluconazole, thirteen C. albicans strains were classified as susceptible, three C. albicans and four C. glabrata were resistant, while thirty C. glabrata strains were intermediate susceptible (Supplementary materials, Table S1).

The antifungal effect of the fluconazole–Cu(II) complex

For the purpose of establishing the antifungal effect of the FLZ-Cu complex, all tested strains were classified to an appropriate susceptibility group. Table S1 presents the percentage distribution of growth reduction, and the MICs values obtained for examined strains treated with FLZ and FLZ-Cu. The MIC values indicated that in 16 cases of C. glabrata strains, the studied complex only twice reduced those values. A modest effect was achieved for three out of four resistant (R) C. glabrata strains (1941, 1973, 2098). The MIC values obtained for them were for FLZ above 128 µg/mL (Fig. 2), whereas the susceptibility test result for FLZ-Cu was exactly equal to 188.22 µg/mL. Moreover, the complex revealed a slightly higher influence (p < 0.05) on the percentage growth reduction than FLZ in the range of concentrations 4–128 µg/mL, 16–128 µg/mL and 32–128 µg/mL for C. glabrata 1973, 1941 and 2098, respectively (Table S1, highlighted orange background). This effect was also observed for other strains (e.g. C. glabrata 2221), which exhibited the same MIC values for FLZ and for its cupric complex (Fig. 2; p < 0.05 for range of concentration 32-128 µg/mL). Only for five tested strains of C. albicans, which were susceptible, the complex exhibited a little better antifungal activity than for a free ligand. The percentage growth reduction distribution for two of them is presented in Fig. 3 (Table S1, highlighted orange background). As it can be seen, similarly to the instance of C. glabrata, at a low...
concentration (0.125-0.5 μg/mL FLZ-Cu), the complex was about 10–40 % more effective than FLZ \((p < 0.05)\). This effect was not observed among the drug-resistant strains of \textit{C. albicans} (Table S1). Furthermore, for \textit{C. albicans} 2218, the activity of FLZ-Cu was much lower than that of uncomplexed drug.

The antifungal effect of Cu(II) ions

In the case of copper ions studies, the obtained results showed the fungal growth reduction on the level of 10–25 % for 30 strains, 26–30 % for 15 strains and >35 % for 5 strains. For those strains of \textit{C. glabrata} which revealed good susceptibility to the FLZ-Cu complex (1941, 1973, 2098, 2221), the values of growth reduction for copper ions were less significant, ranging from 20 to 30 % (Fig. 2). A similar effect was obtained for strains of \textit{C. albicans} (2210, 2211), for which those values reached 28.5 % (Fig. 3, Table S1).

Discussion

Copper complexes have gained a growing interest as pharmaceuticals to be used as diagnostic, antimicrobial, antiviral, anti-inflammatory or antitumor agents (Gielen and Tiekink, 2005; Zhang and Lippard, 2003; Ming, 2003; Iakovidis et al., 2011; Weder et al., 2002; Regtop and Biffin, 1994; Tisato et al., 2010). There are a number of antibiotics named “metalloantibiotics” that require metal ions to act properly, such as bleomycin, streptonigrin, bacitracin and albomycin. The coordinated metal ions play an important role in maintaining a suitable structure and function of these antibiotics (Ming, 2003). It has been shown that copper complexes with non-steroidal anti-inflammatory drugs reveal enhanced anti-inflammatory and antiulcerogenic activity, as well as reduced gastrointestinal toxicity in comparison with uncomplexed drugs (Iakovidis et al., 2011; Weder et al., 2002; Regtop and Biffin, 1994). The best example is the cupric complex of indomethacine, which exhibits a higher anti-inflammatory activity and lower toxicity than the initial agent, i.e. in a metal-free form (Regtop and Biffin, 1994). Other studies presented in literature have focused on chemotherapeutic effects of copper complexes and their use in antitumor therapy, where one of the best examples is bleomycin (Ming, 2003; Tisato et al., 2010).

Taking into account the therapeutic potential of bioinorganic drugs, complexation of the known drugs to metal ions seems to also be an appropriate strategy for the design of antifungal agents. So far, the examples reported in the literature include the enhanced antifungal activity of the fluconazole-Ag(I) complex against \textit{Saccharomyces cerevisiae}, \textit{Mucor mucedo}, \textit{Rhizopus tolonifer}, \textit{Penicillium uncultosum} and \textit{Aspergillus niger} in comparison with the metal-free form (Zhang et al., 2007). Furthermore, there are reports describing higher antifungal activity of other metal ions complexes (Shreaz et al., 2010; Ali et al., 2012). One of them is the paper concerning a good activity of copper and nickel complexes towards different strains of \textit{Candida} (Ali et al., 2012).

The purpose of the presented research was to test the antifungal properties of the FLZ-Cu complex against the fungal strains of \textit{C. glabrata} and \textit{C. albicans}. It should be noted that during conducted study in RMPI medium, the fraction of FLZ-Cu complex has decayed. The NMR studies (data not shown) revealed that copper ions interact with FLZ, and the small remaining fraction of FLZ-Cu is still able to show better activity than FLZ alone. This is clearly seen in Figs. 2 and 3, where the copper ions complexed to the ingredients of the medium exhibit lower activity than those bound to fluconazole. Thus, the effect of FLZ-Cu in the absence of medium components should be much greater.

The obtained results allow to assume that FLZ-Cu complex could be used for exterior purposes as a component of ointment. In this case, the daily doses of the FLZ (the number of repetitions of lubrication), particularly in case of invasive fungal infections, could be significantly reduced by applying its complexed form.
The molecular mechanism of fluconazole action is well known (Charlier et al., 2006). It involves the reduction of ergosterol production of one of the major components of yeast cell membrane, by blocking the activity of the P450 enzyme. A major problem in the treatment of fungal infections is the constantly increasing number of strains resistant to the drugs used. For FLZ, there are three known mechanisms of resistance (Charlier et al., 2006; Löffler et al., 1997; Franz et al., 1998; Parkinson et al., 1995; Orozco et al., 1998). One of them is associated with the overactivity of the efflux pump, which significantly reduces the intracellular concentration of the drug below the effective level. This overactivity is related to the overexpression of the two gene families (CDR and MDR), which can contribute to an increase of the MIC values (Parkinson et al., 1995). As reported by Sanguinetti et al. (2005), among the strains of C. glabrata, the MIC values greater than 32 μg/mL are strongly correlated with the upregulation of efflux transporters. Although with no doubt, more detailed studies are required to explain the action of the FLZ-Cu complex; we can suppose that a complexed form of the drug is able to deliver more molecules of fluconazole into the cell interior, thus overcoming the overactivity of efflux pumps. The process of formation of FLZ-Cu complexes considerably reduces the polarity of the metal ion because of the partial sharing of its positive charge with the ligand donor groups. Such chelation could increase the lipophilic character of the central metal ion. This can be helpful for fluconazole complex in the penetration through the lipid layer of the cell membrane (Gölcü and Dolaz, 2006). On the other hand, the observed effects could not be enough significant to warrant that FLZ-Cu is able to deliver more drug into the cell interior and thus overcome the activity of efflux pumps.

At the same time, the mechanism associated with ROS generation can be disregarded as it has been reported earlier (Nagaj et al., 2012). An additional proof of absence of ROS activity in all isolates is small influence of free copper ions on their growth reduction. Copper concentrations used in those studies were not toxic for growth of the investigated yeast of genus Candida. Copper concentrations used are over 1.5 g/L (Adamo et al., 2012; Avery et al., 1996). Therefore, the probable mechanism of impact of Cu(II) ions on the investigated strains should have another reason.

**Conclusion**

An increase a number drug-resistant strains of the Candida species caused systemic invasive fungal infections has become the inspiration to perform studies of antifungal activity of fluconazole modified by copper ions binding. The antifungal activity of FLZ-Cu was elaborated and explored. However, only slightly improved effect on the drug-resistant strains of C. glabrata and a moderate on susceptible C. albicans was observed. Despite the same value of MICs, the percentage growth reduction of individual strains of C. glabrata and C. albicans (free living planktonic forms of Candida spp.) was greater by approximately 10–40 % for the complex in comparison with the copper-free drug. The obtained results indicate modest activity of the FLZ-Cu complex for chosen strains but may help to define a new direction for studies of antifungal drugs.

**Acknowledgments** The research was supported by Wroclaw Research Center EIT+ under the Project “Biotechnologies and advanced medical technologies - BioMed” (POIG 01.01.02-003/08-00) financed from the European Regional Development Fund (Operational Programme Innovative Economy. 1.1.2).

**Open Access** This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited.

**References**

Abi-Said D, Anaissie E, Uzón O, Raad I, Pinzcowski H, Vartivarian S (1997) The epidemiology of hematogenous candidiasis caused by different Candida species. Clin Infect Dis 24:1122–1128

Ables AZ, Blumer NA, Nancy A, Valainis GT, Godenick MT, Kajdasz DK, Palesch YY, Yuku Y (2000) Fluconazole prophylaxis of severe Candida infections in trauma and postsurgical patients: a prospective, double-blind, randomized, placebo-controlled trial. Infect Dis Clin Pract 9:169–175

Adamo GM, Brocca S, Passolunghi S, Salvato B, Lotti M (2012) Laboratory evolution of copper tolerant yeast strains. Microb Cell Fact 11:1–11

Alexander BD, Schell WA, Miller JL, Long GD, Perfect JR (2005) Candida glabrata fungemia in transplant patients receiving voriconazole after fluconazole. Transplant 80:868–871

Ali I, Wani WA, Khanb A, Haquea A, Ahmadb A, Saleema K (2012) Synthesis and synergistic antifungal activities of a pyrazoline based ligand and its copper(II) and nickel(II) complexes with conventional antifungals. Microb Path 53:66–73

Avery SV, Howlett NG, Radice S (1996) Copper toxicity towards Saccharomyces cerevisiae: dependence on plasma membrane fatty acid composition. Appl Enviro Microbiol 62:3960–3966

Biswas S, Van Dijck P, Datta A (2007) Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of Candida albicans. Microbiol Mol Biol Rev 71:348–376

Charlier C, Hart E, Lefort A, Ribaub P, Dromer F, Denning DW, Lortholary O (2006) Fluconazole for the management of invasive candidiasis: where do we stand after 15 years? J Anti-microb Chemother 57:384–410

Dery MA, Hasbun R (2011) Fluconazole-resistant Candida: mechanisms and risk factor identification. Curr Fungal Infect Rep 5:23–28

Eggimann P, Garbino J, Pittet D (2003) Epidemiology of Candida species infections in critically ill non-immunosuppressed patients. Lancet Infec Dis 311:685–702
