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The efficiency of the benzothiazole APB, the echinocandin micafungin, and amphotericin B in fluconazole-resistant *Candida albicans* and *Candida dubliniensis*

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This study presents the efficiency of the experimental antifungal agents 6-amino-2-*n*-pentylthiobenzothiazole (APB) and the echinocandin micafungin, and amphotericin B against fluconazole-resistant *Candida albicans* and *Candida dubliniensis* (MIC^{95} for fluconazole ≥ 64 mg/l). The benzothiazole APB was less active against *C. albicans* and *C. dubliniensis* ($MIC^{80} = 8–32$ mg/l, $MIC^{95} = 16–64$ mg/l) than amphotericin B, which was efficient in a concentration range from 0.125 to 2 mg/l. However, the efficiency of micafungin was very high with MIC^{80}, and $MIC^{100} \leq 0.031$ mg/l.

During the last decade fluconazole has become the drug of choice in the treatment of recurrent or more complicated fungal infections caused by *Candida albicans* and *C. dubliniensis* in patients with or without any immune disabilities (Martinez et al. 2002; Perea et al. 2002). Amphotericin B represents a therapeutic alternative mainly in generalized candidiasis. Its use is, however, limited for toxicity. The increase in the number of strains resistant against the antifungal drugs available has initiated the search for new treatment options. The benzothiazole APB is an experimental agent, which showed to be active against *C. albicans in vitro* and *in vivo* in generalized candidiasis and dermatomycoses (Bujdáková and Múčková 1994; Bujdáková et al. 1995). Echinocandins represent a novel class of antifungal compounds with a mechanism of action different from azoles and based on inhibition of beta(1,3)glucan synthase. Micafungin seems to be a very promising echinocandin, which is now under phase III clinical investigation showing very good activity against *Candida* and *Aspergillus* species (Mikamo et al. 2000; Fromtling 2002).

For this study, 16 fluconazole resistant *C. albicans* and *C. dubliniensis* strains were selected out of 60, originally tested for susceptibility to fluconazole. Eight clinical isolates of *C. albicans* (1–8) were isolated from patients with different allergy and cancer diseases (Slovakia), further 6 isolates originating from Brazil (9–11) and Thailand (12–14) were obtained from oropharyngeal candidiasis of HIV infected patients. *C. dubliniensis* NIH 0492 (15) was isolated from a patient from Thailand with stomach cancer and a second *C. dubliniensis* IFM 49832 strain (16) was isolated from a Japanese patient with diabetes. The other 3 strains were obtained from collections: *C. albicans* CY 1123 (Collection of the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University), *C. albicans* CCY 29–3-112 (Slovak Yeast Collection, Bratislava, Slovakia) and *C. dubliniensis* CBS 7987 (Centraal Bureau vor Schimmelcultures, Delft, The Netherlands).

All clinical isolates were cultivated on CHROMagar Candida (Becton Dickinson, UK) overnight at 37 °C. Identification was performed biochemically with the commercial set API 20C AUX (BioMérieux, France) according to manufacturer's manual. The discrimination between *C. albicans* and *C. dubliniensis* was done by PCR assay using set primer pair (Cd-oligo2/F TTTGAAAAGTCGTGCAGCAG, Cd-oligo2/R ACTGACGACTCATGGCA AAT) specific only for *C. dubliniensis*. The standard *C dubliniensis* CBS 7987 was used as positive control. The conditions for PCR were used according to Watanabe et al (2001), with small modifications.

The susceptibility to fluconazole (Pfizer, Switzerland), amphotericin B (Bristol-Myers Squibb, USA), 6-amino-2-*n*-pentylthiobenzothiazole (Sidóová, Chemical Institute, Comenius University, Slovakia) and micafungin (Fujisawa, Japan) were tested by the broth microdilution method according to the NCCLS M27-A reference method in RPMI medium. Amphotericin B and APB were dissolved in dimethylsulfoxide, the final concentration did not exceed 1% (v/v). Fluconazole and micafungin were diluted in sterile water. Concentrations of commercial agents were prepared according to the NCCLS M27-A protocol: for fluconazole 0.12 mg/l–64 mg/l and for amphotericin B 0.03 mg/l–16 mg/l. APB was tested in the concentration range from 2 mg/l to 128 mg/l and micafungin from 0.003 mg/l to 32 mg/l. The inoculum was prepared to 1×10^3 cell/ml. The 96-well plates were incubated at 37 °C in a wet chamber for 24 h. Turbidity was followed visually and measured spectrophotometrically with an automatic reader at 595 nm. The results were given in MIC^{95}or MIC^{100} and MIC^{80}.

The 60 clinical isolates of *C. albicans* and *C. dubliniensis* were collected in the year 2002. All these strains were tested for susceptibility to fluconazole and 16 of them were proved to be fluconazole resistant with $MIC^{95} \geq 64$ mg/l. This resistance was confirmed by the ability of every clinical isolate to grow on a Sabouraud plate in the presence of 64 mg/l of fluconazole. These clinical isolates were tested for susceptibility to amphotericin B and the experimental agents APB and micafungin. The results are summarized in the Table. The antifungal activity was determined at both concentrations, MIC^{80} and MIC^{95} (or MIC^{100}). For fluconazole, MIC^{80} was observed in range from 0.25 mg/l to 8 mg/l, but MIC^{95} was proved higher than 64 mg/l. In this case, it is optimal for clinicians to know both MICs, as fluconazole has a fungistatic effect on the growth of *Candida* cells and a MIC^{95} over 64 mg/l suggests the presence of surviving *Candida* cells resistant to fluconazole. Amphotericin B as well as micafungin are fungicidic, so the determination of MIC^{95} or MIC^{100} is preferred. APB is fungistatically active like fluconazole with MICs from 16 to 64 mg/l, while the majority of strains had a MIC^{95} of 32 mg/l (Table). This com-

Table: Efficiency of fluconazole, amphotericin B, 6-amino-2-*n*-pentylthiobenzothiazole and micafungin on clinical isolates of *C. albicans* and *C. dubliniensis*

Clinical isolates	Fluconazole		Amphotericin B		APB		Micafungin	
	MIC^{80}	MIC^{95}	MIC^{80}	MIC^{95}	MIC^{80}	MIC^{95}	MIC^{80}	MIC^{95}
1. *Candida albicans* 1314	0.5	≥ 64	2	2	$\leq 32 \geq 16$	32	≤ 0.031	≤ 0.031
2. *Candida albicans* 1173	0.5	≥ 64	0.5	0.5	$\leq 32 \geq 16$	32	≤ 0.031	≤ 0.031
3. *Candida albicans* 1167	0.5	≥ 64	0.5	1	$\leq 32 \geq 16$	32	≤ 0.031	≤ 0.031
4. *Candida albicans* 1033	0.5	≥ 64	0.5	0.5	16	32	≤ 0.031	≤ 0.031
5. *Candida albicans* 1212	0.25	≥ 64	0.5	0.5	$\leq 32 \geq 16$	32	≤ 0.031	≤ 0.031
6. *Candida albicans* 1331	1	≥ 64	0.25	0.5	16	16	≤ 0.031	≤ 0.031
7. *Candida albicans* 1395	8	≥ 64	2	2	$\leq 32 \geq 16$	32	≤ 0.031	≤ 0.031
8. *Candida albicans* 1444	8	≥ 64	0.5	0.5	$\leq 32 \geq 16$	32	≤ 0.031	≤ 0.031
9. *Candida albicans* 47604	0.25	≥ 64	0.5	0.5	$\leq 32 \geq 16$	32	≤ 0.031	≤ 0.031
10. *Candida albicans* 47605	0.25	≥ 64	1	1	32	64	≤ 0.031	≤ 0.031
11. *Candida albicans* 47606	0.25	≥ 64	0.5	0.5	32	64	≤ 0.031	≤ 0.031
12. *Candida albicans* 46521	0.5	≥ 64	0.5	0.5	16	32	≤ 0.031	≤ 0.031
13. *Candida albicans* 47500	0.25	≥ 64	1	1	32	64	≤ 0.031	≤ 0.031
14. *Candida albicans* 47504	2	≥ 64	1	1	$\leq 32 \geq 16$	32	≤ 0.031	≤ 0.031
15. *Candida dubliniensis* NIH 0492	0.25	≥ 64	1	2	8	16	≤ 0.031	≤ 0.031
16. *Candida dubliniensis* IFM 49832	0.25	≥ 64	0.5	1	$\leq 16 \geq 8$	16	≤ 0.031	≤ 0.031
Candida albicans CCY 29–3-112	≥ 64	≥ 64	0.125	0.125	32	32	≤ 0.031	≤ 0.031
Candida albicans CY 1123	8	≥ 64	1	2	$\leq 32 \geq 16$	32	≤ 0.031	≤ 0.031

APB – 6-amino-2-*n*-pentylthiobenzothiazole
MIC – minimal inhibitory concentration

pound was less active against *C. albicans* and *C. dubliniensis* than amphotericin B, which was efficient in concentration range from 0.125 to 2 mg/l. The MIC^{80} of APB could not be accurately determined in standard two-fold dilution. Therefore, we used a concentration range from 10 mg/l to 30 mg/l. MIC^{80} for APB was then determined from 18 to 25 mg/l. This agent is interesting for its mechanism of action interfering with ergosterole biosynthesis like azoles, but at the 4-demethylation level (Kuchta et al. 1994). This mechanism of activity is an explanation for the activity of APB against fluconazole-resistant *Candida* strains, as well as for the synergy effect with azoles like clotrimazole, econazole and itraconazole (Múčková et al. 2000). The most active agent was micafungin presenting a new generation agent for the treatment of candidiasis and aspergillosis (Mikamo et al. 2000; Fromtling 2002). Its efficiency was very high, with MIC^{80}, and MIC^{95} values below 0.031 mg/l. In agreement with other reports on the efficiency of the echinocandin caspofungin against fluconazole-resistant strains of *Candida* (Kartsonis et al. 2002; Bachmann et al. 2002), micafungin was proved to be active against fluconazole resistant strains. Thus this agent can represent a very potent alternative antifungal drug in the treatment of candidiasis caused by azole resistant yeasts.

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