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Effect of fluconazole on the growth and adhesion of *Candida albicans* in the presence of antineoplastic agents

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The effect of fluconazole and the antineoplastic agents etoposide and methotrexate on the growth and adhesion of *Candida albicans* were studied. All the tested chemicals inhibited the growth and the adhesion of the yeast to buccal epithelial cells, while fluconazole and etoposide inhibited the adhesion to acrylate surface as well. Our experiments also demonstrated that etoposide and methotrexate interfered with the inhibitory effect of fluconazole on both the growth and cell adhesion. While etoposide strengthened the inhibitory effect of fluconazole, in the presence of methotrexate fluconazole showed lower inhibition on both the growth and adhesion.

Candida albicans is an opportunistic yeast that causes serious diseases in immunocompromised hosts (ODDS 1988). Chronic mucocutaneous and systemic candidiasis are common infections of individuals undergoing immunosuppressive therapy for cancer or organ transplantation (CLIFT 1984, DAROUICHE 1998, BODEY 1988) and in patients with AIDS (REEF and MAYER 1995, KLEIN *et al*. 1984). Immunosuppression caused by cytostatic drugs in cancer chemotherapy may alter the host response, resulting in increased sensitivity to microbial infections. The antineoplastic agents etoposide (ETP) and methotrexate (MTX) are widely used in cancer chemotherapy. ETP, a semi-derivative of podophyllotoxin, is one of the most active drugs available for small cell lung cancer, and it is also clearly active in acute non-lymphoblastic leukemia and lymphoma. Its mode of action appears to be pre-mitosis inhibition, which is possibly secondary to an anti-DNA synthesis effect (ISSEL and CROOKE 1979). MTX is an effective chemotherapeutic agent in the treatment of leukemias, lymphomas and many other solid tumors. It inhibits intracellular dihydrofolate reductase and hence DNA synthesis (MEURMAN *et al.* 1991). Because of the increasing susceptibility of patients with advanced cancer to fungal infection, cancer chemotherapeutic and antifungal agents are often given concomitantly. Fluconazole is a widely used triazole derivative, successfully applied in treating patients undergoing immunosuppressive therapy for cancer with *Candida* infections (AKOVA *et al.* 1994, LAKE *et al*. 1996). There are a limited number of reports on the antimicrobial activity of cytostatic drugs (MEURMAN *et al*. 1991, BODET *et al*. 1985, HAMILTON-MILLER 1984), and surprisingly little information is known about the possible interactions between antineoplastic and antimicrobial agents (MICHEL *et al.* 1979, MOODY *et al*. 1978, GIERINGER *et al.* 1986). The purpose of this study was to examine how MTX and ETP can modify the effect of fluconazole on the growth and the adhesion of the yeast *C. albicans*.

Materials and methods

Chemicals: SABOURAUD dextrose agar (SDA) and mycological peptone were purchased from OXOID (Basingstoke, Hampshire, England). Fluconazole was obtained from PFIZER UK (Sandwich, United Kingdom). Etoposide (200 mg/10 ml) and methotrexate (50 mg/2 ml) were products of TEVA-group (Netanya, Israel). All other chemicals were of analytical grade.

Organisms: Two *Candida albicans* strains were investigated. *C. albicans* FS (fluconazole-sensitive) is a clinical isolate, identified by standard morphological and physiological properties (SANDVEN 1990); *C. albicans* FR (fluconazole-resistant) is a mutant strain, which was developed from the FS strain by permanent shaking at increasing fluconazole concentrations as described earlier (FEKETE-FORGÁCS *et al*. 1999). Both strains were maintained on SDA-slopes at 4 °C.

Susceptibility testing: The MICs of the two antineoplastic agents were determined by agar dilution method using SDA as medium. ETP and MTX were serially diluted into sterile 0.9% NaCl solution and incorporated into SDA plates. Five microlitres of cell suspension (4×10^6 yeast cells per ml in distilled water) were spotted onto the plates, and were incubated for 48 h at 30 °C.

Growth studies in the presence of fluconazole and antineoplastic agents: Ten-millilitre culture of *C. albicans* was grown overnight in SABOURAUD dextrose broth (SDB) containing 4% glucose and 1% mycological peptone. Cells were added to 20 millilitres of SSB medium [1.8% sucrose 1% mycological peptone] containing adequate concentrations of antineoplastic agents (ETP: 1 mg/ml, 2 mg/ml, MTX: 2.5 mg/ml and 5 mg/ml) and/or fluconazole (5 μg/ml for the FS strain, 100 μg/ml for the FR strain) to achieve a final absorbance of 0.1 (λ = 640 nm). The cultures were incubated at 30 °C. After 24 hours one-millilitre aliquots were removed and the optical density at 640 nm was measured.

In vitro adhesion assays: Adherence of *C. albicans* to plastic surface: *C. albicans* was cultured at 37 °C for 18 h in SDB. Cells were washed three times with PBS (0.02 mol/l Na_2HPO_4/NaH_2PO_4 and 0.15 mol/l NaCl pH 7.2) and were taken up in PBS supplemented with antineoplastic agents (2 mg/ml ETP, or 5 mg/ml MTX) and/or fluconazole (5 μg/ml for FS strain, 100 μg/ml for FR strain) to achieve a 5×10^7 yeast cells ml^{-1} concentration. 2.5 ml of the suspension was rotated end-over-end with an immersed acrylate plate (size $0.5 \times 0.5 \times 0.1$ cm) at 30 °C for 1 h. Then the acrylate plate was removed from the suspension, was washed with sterile PBS to separate non-adherent yeast cells and was incubated in 5 ml SDB at 30 °C for 24 h. The amount of yeast cells was determined spectrophotometrically at 640 nm using standard curves. For comparison of the adherence ability we used optical density corrected for the surface (cm^2) of the acrylate plate (FEKETE-FORGÁCS *et al.* 1999).

Adherence of *C. albicans* to epithelial cells: Human buccal epithelial cells (BEC) were obtained from apparently healthy non-smoking male donors by gentle rubbing of the mucosal surfaces of the cheeks with a sterile plastic spatula (BRAGA *et al*. 1995).

The ability of the two strains to adhere to epithelial cells in the presence of anticancer drugs and fluconazole was investigated by mixing the yeast cells (final concentration: 5×10^6 cells ml^{-1}) with BEC (final concentration: 1.5×10^5 cells ml^{-1}) (BRAGA *et al*. 1996), antineoplastic agents (2 mg/ml ETP, or 5 mg/ml MTX) and/or fluconazole (5 μg/ml for FS strain or 100 μg/ml for FR strain) and rotating at 37 °C for 1 h. After filtration on MILLIPORE membrane (8 μm pore size) the BEC on the filter were washed twice with 5 ml volumes of PBS. A drop was mounted on a glass slide, air dried, heat fixed and stained with crystal violet (GHANNOUM 1992). The mean numbers of yeasts adhering to each 100 epithelial cells were calculated.

Statistical analysis: All the experimental data presented here are means of 3–6 independent measurements. The reproducibility of the analytical procedures were estimated by standard deviations (S.D.). Statistical significance was calculated using STUDENT's t-test.

Results and discussion

In the present study we investigated how the cytotoxic drugs ETP and MTX interfere with the effect of fluconazole on the yeast *C. albicans*. It is known that oral *Candida* infections are common in patients with malignancies (SONIS *et al*. 1978, MEURMAN *et al*. 1997). The adhesion of yeast cells to epithelial cells is widely recognised as the essential step in the process of candidal colonization and subsequent infection (CUTLER 1991, FUKAZAWA and KAGAYA 1997). It is also known that fluconazole has an inhibitory effect on candidal adhesion to epithelial cells (DARWAZEH *et al*. 1991). Therefore we investigated not only the

growth of the yeast, but the adherence ability of *Candida* cells in the presence of antineoplastic agents and fluconazole either to BEC or acrylate surface as well. In these experiments we used two *C. albicans* strains: a fluconazole-sensitive clinical isolate ($MIC_{fluconazole}$ 5 μg/ml) and a fluconazole-resistant laboratory-mutant ($MIC_{fluconazole}$ 80 μg/ml) derived from the former. The FR strain showed not only a higher tolerance to fluconazole, but was superior in all the virulence traits examined including germination, adherence ability, secreted proteinase and phospholipase production. The *in vivo* virulence of this strain was also higher than that of the parent strain (FEKETE-FORGÁCS *et al*. 1999).

Determination of the MIC values

In the case of ETP we got different MIC values for the two strains. With the method described in the Materials and Methods section the MIC_{ETP} of the FS strain was 1.5 mg/ml while this value of the FR strain was found to be slightly, but consistently higher (2 mg/ml). The MIC_{MTX} values for both strains proved to be higher than 10 mg/ml. It is worth noting, that these antineoplastic agents considerably inhibit the growth of bacteria even at low (1–10 μg/ml) concentrations (MEURMAN *et al*. 1991, BODET *et al*. 1985). Thus, our results also suggest that the different antibacterial and antifungal activity of cytostatic drugs can modify the infection patterns with a selective suppression of the normal bacterium flora in cancer patients (BODET *et al*. 1985).

Growth studies

Growth inhibition data for the two strains in the presence of ETP and MTX are given in Table 1.

Both drugs significantly inhibited the growth of both strains at all the concentrations tested. However, the level of growth inhibition in the case of the FR strain was lower with either ETP or MTX.

The combinatory effects of antineoplastic agents and fluconazole are summarized in Table 2.

Considering the different fluconazole sensitivities of the two strains, we used 5 and 100 μg/ml concentrations of the antifungal agent in the case of the FS and FR strains, respectively. These concentrations resulted in approximately 50% inhibition of growth (Table 2). Using the antineoplastic agents together with fluconazole we obtained similar results for both strains. Namely, in the presence of ETP and fluconazole increased inhibition

Table 1
The effect of ETP and MTX on the growth of *C. albicans* FS and FR strains

	FS strain		**FR strain**	
	Absorbance (640 nm)	Inhibition (%)	Absorbance (640 nm)	Inhibition (%)
Control	2.21 ± 0.05	–	2.19 ± 0.06	–
MTX (2.5 mg/ml)	1.62 ± 0.06***	26.7	1.75 ± 0.08***	20
MTX (5 mg/ml)	1.47 ± 0.03***	33.5	1.63 ± 0.04***	25.5
ETP (1 mg/ml)	1.63 ± 0.07***	26.2	1.81 ± 0.08***	17.3
ETP (2 mg/ml)	0.295 ± 0.005***	86.6	0.358 ± 0.006***	83.6

* – $P < 5\%$; ** – $P < 1\%$; *** – $P < 0.1\%$. *P* values were calculated using the STUDENT's t-test

Table 2
The effect of antineoplastic agents and fluconazole on the growth of *C. albicans* FS and FR strains

	FS strain		**FR strain**	
	Absorbance (640 nm)	Inhibition (%)	Absorbance (640 nm)	Inhibition (%)
Control	2.21 ± 0.05	–	2.19 ± 0.06	–
Fluconazole (5 μg/ml)	1.09 ± 0.02***	50.7	–	–
Fluconazole (100 μg/ml)	–	–	1.12 ± 0.02***	48.9
ETP (1 mg/ml)	1.63 ± 0.07***	26.2	1.81 ± 0.08***	17.3
MTX (2.5 mg/ml)	1.62 ± 0.06***	26.7	1.75 ± 0.08***	20
ETP + Fluconazole	0.56 ± 0.02***	74.6	0.76 ± 0.02***	65.2
MTX + Fluconazole	1.60 ± 0.07***	27.6	1.61 ± 0.07***	26.5

* – $P < 5\%$; ** – $P < 1\%$; *** – $P < 0.1\%$. *P* values were calculated using the STUDENT's t-test

was found compared to fluconazole alone (additive effect). Surprisingly, MTX decreased significantly the inhibitory effect of the antifungal agent; the overall inhibition was about the same as we found with MTX alone.

Adherence to acrylate surface and BEC

As we found earlier, the FR strain showed significantly superior adhesion to both BEC and acrylate surface compared to the FS strain (FEKETE-FORGÁCS *et al.* 1999). Fluconazole reduced the adhesion (DARWAZEH *et al*. 1991) of the two strains to plastic surface and to epithelial cells as well (Tables 3 and 4).

In the presence of ETP we detected a significant reduction of yeast adhesion to either acrylate plate or BEC. MTX also inhibited the *in vitro* adhesion of both strains to BEC, however we could not detect any changes in the adhesion abilities to acrylate plates. ETP and fluconazole synergistically affected the adhesion to both acrylate surface and BEC (Tables 3 and 4). MTX decreased the inhibitory effect of fluconazole on the adhesion to

Table 3
The adhesion of *C. albicans* FS and FR strains to acrylate surface in the presence of antineoplastic agents and fluconazole

	FR strain		**FS strain**	
	(A 640nm/cm^2)	Inhibition (%)	(A 640nm/cm^2)	Inhibition (%)
Control	98 ± 4	–	78 ± 4	–
ETP (2 mg/ml)	83 ± 4**	15.3	65 ± 5***	16.7
MTX (5 mg/ml)	98 ± 4	0	77 ± 7	1.3
Fluconazole (5 μg/ml)	–	–	71 ± 1*	8.9
Fluconazole (100 μg/ml)	90 ± 2*	8.1	–	–
ETP + fluconazole	77 ± 4***	21.4	60 ± 2***	23.1
MTX + fluconazole	96 ± 5	2.0	77 ± 5	1.3

* – $P < 5\%$; ** – $P < 1\%$; *** – $P < 0.1\%$. *P* values were calculated using the STUDENT's t-test

Table 4
The adhesion of *C. albicans* FS and FR strains to BEC in the presence of antineoplastic agents and fluconazole

	FR strain		**FS strain**	
	No. of yeast cells adhering to 100 BEC	Inhibition (%)	No. of yeast cells adhering to 100 BEC	Inhibition (%)
Control	899 ± 76	–	284 ± 27	–
ETP (2 mg/ml)	490 ± 21***	45.5	208 ± 27**	26.7
MTX (5 mg/ml)	641 ± 24**	28.7	230 ± 24*	19.0
Fluconazole (5 μg/ml)	–	–	209 ± 27*	26.4
Fluconazole (100 μg/ml)	600 ± 29***	33.2	–	–
ETP + fluconazole	449 ± 8***	50.0	160 ± 20***	43.7
MTX + fluconazole	631 ± 20**	29.8	220 ± 18**	22.5

* – $P < 5\%$; ** – $P < 1\ \%$; *** – $P < 0.1\ \%$. P values were calculated using the STUDENT's t-test

acrylate. A similar tendency was found in the case of BEC, however the changes were less significant.

Since patients undergoing cancer chemotherapy are frequently subject to fungal infection and receive antifungal agents there is the possibility of the interference between the anticancerous and antifungal drugs. Our experiments demonstrate that ETP and MTX can modify the inhibitory effect of fluconazole on the growth and adhesion. Surprisingly while ETP increased the inhibitory effect of fluconazole, in the presence of MTX fluconazole showed lower efficiency.

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