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Evaluation of the Antifungal Activity of Fenticonazole on Strains of Candida albicans on Cellular Lines

Untersuchung der antimyzetischen Aktivität von Fenticonazol bei Candida albicans in Zellkulturen

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Schlüsselwörter: Fenticonazol – Candida albicans – Zellkulturen – Antimykotikum

Summary: Antifungal activity of a new imidazolic drug, fenticonazole, on strains of Candida albicans on HEp2 cellular line was studies.

The MIC (minimum inhibitory concentration) and MCIF (minimum concentration inhibiting filamentation) results are similar to those obtained in our previous studies and confirm the good activity of Fenticonazole.

Inhibition of filamentation by fenticonazole at low concentration confirms validity of the use of fenticonazole in the treatment of surface candidosis and shows the effectiveness of this experimental model, HEp2 cell monostrate, which constitutes an "in vitro" situation of parasitism similar to that observed "in vivo".

Zusammenfassung: Die antimyzetische Aktivität eines neuen Imidazol-Antimykotikums, Fenticonazol, wurde an Candida albicans unter Züchtung auf Zellkulturen der Linie HEp2 untersucht.

Die minimale Hemmkonzentration (MIC) und die minimale Konzentration zur Unterdrükkung der Filamentation (MCIF) unterschieden sich kaum von denen, die in früheren Untersuchungen ermittelt worden waren und bestätigen damit die gute Hemmwirkung des Fenticonazol. Durch die Unterdrückung der Filamentation bei niedrigen Wirkstoffkonzentrationen wird auch die klinische Anwendung bei oberflächlichen Candidosen gerechtfertigt. Außerdem wird hierdurch die Brauchbarkeit des benutzten Zellkultur-Modells bestätigt, das in vitro eine Situation schafft, die den Verhältnissen in vivo gleicht.

The concept that, for Candida albicans, hyphae constitute the expression of its pathogenicity is now universally accepted; this is demonstrated by the hyphae findings in various pathological materials taken from patients presenting evident symptoms clearly attributable to the presence of mycetes; it is confirmed by observation of filamentous forms in experimental disease: in fact the treated rabbit dies from hydronephrosis engendered by giant masses of filamentous forms in the renal pelves.

If blastospores of Candida albicans are inoculated on continuous cellular lines, we can observe the appearance of long hyphae within the space of 24–48 hours, on which small conidia develop; this is a characteristic peculiar to Candida albicans, while it is observed very rarely in Candida tropicalis.

The addition of antimycotic drugs to the medium inhibits the filamentation of Candida albicans, so that by making scalar dilutions of the antimycotic it is possible to determine its highest dilution capable of inhibiting the filamentation on cellular lines.

This valuation model of the activity of an antimycotic, devised by Campisi and colleagues (1), has the undeniable advantage of studying the activity of a substance in conditions close to the parasitism of the spontaneous disease and hence of supplying us with more adequante in formation for purposes of therapy; it is thus a useful complement to the traditional procedures for studying antimycotics.

The aim of our research was, on the basis of this model, to evaluate the antifungal activity of a new imidazolic drug, fenticonazole (Rec 15/1476) previously studied in our Institute by A. L. Costa (2), who used liquid media at varying pH (from 3.20 to 5.32). Fenticonazole (Rec 15/1476), kindly supplied to us by Industria Farmaceutica Recordati, has the following empirical formula $C_{24}H_{20}Cl_2N_2OS \cdot HNO_3$[1-2-(2,4-dichlorophenyl)-2-(4-phenylthiobenzyl-loxy) ethyl imidazole nitrate], molecular weight 518.417. It is a crystalline, odourless white powder soluble in the common organic solvents (3–11).

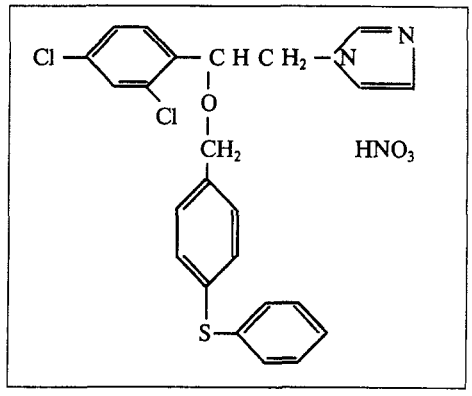

Fig. 1: Structural formula of fenticonazole.

Materials and Methods

Candida albicans strains:

71 strains isolated from various pathological material taken from patients hospitalized at the Messina Polyclinic (Italy) and 6 NCPF strains (National Collection of Pathogenic Fungi) kindly supplied to us by the "Public Health Laboratory Service", London School of Hygiene and Tropical Medicine, Keppel St., London WC1 E7TH.

The strains coming from human pathological material were identified, before the experiment, by means of the following tests:
a) morphologic-macroscopic of colonies on selective media.
b) microscopic fresh and after staining with cotton blue, GRAM and methyl violet.
c) cytochemical (Schiff, Gins, Sheena Story, Hotchkiss, Piekarcki-Robinow).
d) biochemical by means of "Mycotube Roche".
e) serological tests by means of "Candida HA Test Roche" and "Candida IF Test Roche".
f) filamentation test by Taschdjian's method (12).
g) pathogenicity test in rabbits.
h) test of formation of chlamydospores according to Simonetti et al. (13).
i) pathogenicity test by the crystal violet adsorption method according to Miura (14).
l) enzymatic tests according to our previous research (15–21).

Culture media

Sabouraud liquid medium with 2% dextrose at pH 5.80.
Sabouraud liquid medium with 4% dextrose at pH 5.80.
Sabouraud agar medium with 2% dextrose at pH 5.80.
Sabouraud agar medium with 4% dextrose at pH 5.80.
Same Sabouraud liquid and solid media at varying pH (from pH 3.20 to pH 5.32).
Agaracetylglucosamine medium for the production of chlamydospores.
Littman Oxgall broth and agar medium.
Sabouraud agar medium with 4% dextrose with the addition of crystal violet dil. 0.50×10^{-7} for Miura's test.
"Mycoslide Roche" medium for primary cultures.
"Mycoplate MS Roche" medium for isolation and morphological examination.
Ayerst Italiana's Api 20 System.

Drug

Fenticonazole: (REC 15/1476)-crystalline powder, titre 99.90%, Lot N° 4905 (Recordati) solubilized in polyethylene glycole 200 (Carbowax).

Cellular line

HEp2 cellular line was used. The cells were grown in flasks of minimum-essential-medium (MEM) Eagle supplied by Gibco, with the addition of 1% calf serum. From these a cell suspension was prepared after trypsinization and used for production of cultures in tubes. The culture medium was removed after 24 hours and replaced by MEM without serum. The tubes were then put to spin, and after some hours they were used for the tests.

Drug activity

To determine the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of fenticonazole the technique of serial dilution in liquid media at varying pH was used; pH 4.13 was finally adopted since, after repeated tests, it proved to be optimal for the filamentation technique. Series of tubes, each containing 9.80 ml of liquid medium with scalar doses of the test drug were prepared; the concentrations ranged from 160 to 0.0015 mcg/ml. 0.10 ml of a suspension of Candida cells equivalent to 1×10^4 was put into each tube. The controls, with suspension of mycete alone (without antimycotic) were prepared separately.

Before thermostatic incubation the nephelometric reading was taken at wavelength 650 nm.

Incubation was carried out at 37°C, and the final reading made after 48 hours.

Table 1

Turbidimetric tests for determination of MIC, and test on HEp2 monostrate for determination of MCIF (Minimum Concentration Inhibiting Filamentation) of Fenticonazole against Candida albicans strains

Source	Total N° of Candida albicans with differentation in sierological types A and B	MIC, MFC and MCIF values expressed in mcg/ml on liquid media at pH 4.13					
		N° of strain for every MIC value	MIC value	N° of strain for every MFC value	MFC value	N° of strain for every MCIF value	MCIF value
Vaginal secretion	10 (7A, 3B)	1 8 1	2.5 5 10	2 8	10 20	 3 4	1.25 2.5 5
Balanic secretion	8 (6A, 2B)	3 4 1	2 5 10	2 5 1	10 20 40	1 1 2	0.612 1.25 2.5
Conjunctival secretion	6 (5A, 1B)	1 4 1	1.25 5 10	1 1 2 2	5 10 20 40	1 2 3	1.25 2.5 5
Gastric juice	10 (8A, 2B)	2 5 3	2.5 5 10	2 8	10 20	2 3 5	0.612 1.25 5
Buccal cavity	8 (7A, 1B)	5 2 1	5 10 20	1 2 5	10 20 40	1 6 1	1.25 5 10
Expectorate	12 (9A, 3B)	1 1 8 2	1.25 2.50 5 10	2 4 5 1	5 10 20 40	1 2 1 7	0.612 1.25 2.5 5
Faeces	5 (5A)	1 4	2.5 5	2 2 1	5 20 40	1 2 2	0.612 2.5 5
Nail	12 (8A, 4B)	4 6 2	2.5 5 10	2 1 7 2	5 10 20 40	1 3 4 4	0.612 1.25 2.5 5
C. al NCFC London 3153-54-55-56-57-58	6	1 4 1	2.5 5 10	2 1 3	10 20 40	1 2 3	0.612 1.25 5
Total	N° 77	Mean Range Standard deviation	5.52 1.25-20 2.8798		20.58 5-40 10.98		3.55 0.612-10 2.16

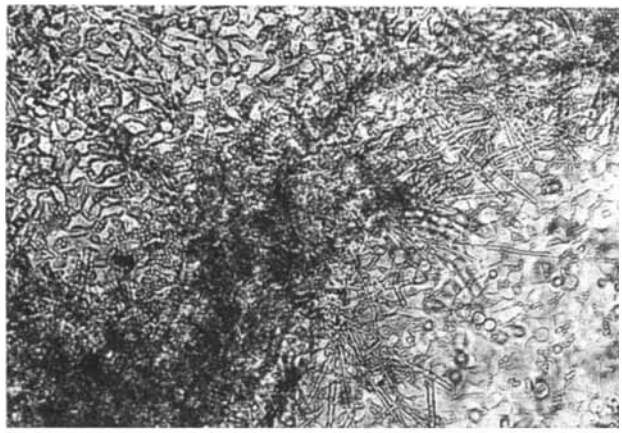

Fig. 2: HEp2 cells and interlacement of Candida albicans hyphae and blastospores in presence of fenticonazole in concentrations lower than 2.80 mcg/ml.

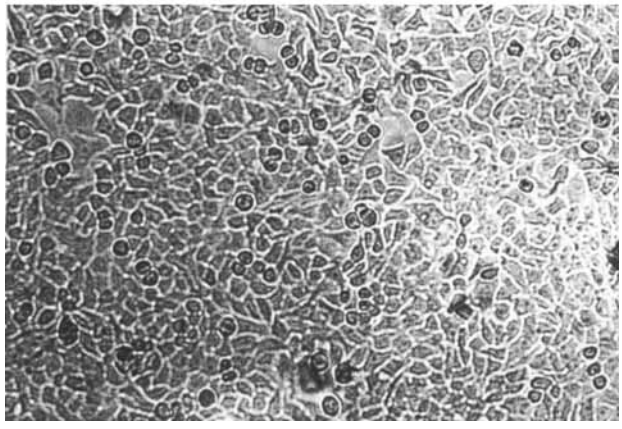

Fig. 3: HEp2 cells and rare Candida albicans blastospores. Inhibition of filamentation in presence of fenticonazole in concentrations equal to or greater than those of the MIC.

After determination of MIC, the MFC was evaluated by forming subcultures from the test tubes that did not exhibit growth development and ascertaining the minimum concentration of drug capable, after an adequate incubation period, of preventing the growth of mycetes in such subcultures.

Cellular line tests

Scalar doses of Fenticonazole and the Candida albicans suspensions were added to the cell maintenance medium for each series of tubes on the basis of the same procedure as for determination of MIC and MFC. Controls were made separately with tubes containing MEM without antimycotic, but with Candida suspension in order to ascertain filamentation, and tubes containing MEM without Candida suspension, but containing the antimycotic at the highest cell alteration concentration.

Results and Conclusions

The results of MIC (minimum inhibitory concentration), MFC (minimum fungicidal concentration) and MCIF (minimum concentration inhibiting filamentation), collected in Table 1, show the full agreement of the values obtained from the three tests performed simul-

taneously in the same physicochemical conditions (pH, incubation, temperature, culture media), with the support of controls in all tests.

The total mean of MIC for fenticonazole on 77 strains of Candida albicans gave a value of 5.52 mcg/ml, while for MFC the value was 20.58 and for MCIF 3.55.

The MCIF values were slightly higher than half of the MIC values and slightly higher than a sixth of the MFC value.

In conclusion, the MIC and MFC results are similar to those obtained in our previous studies, and this demonstrates and confirms the antimycotic activity of fenticonazole. Inhibition of filamentation by fenticonazole at low concentration (total mean 3.55) on the one hand justifies the use of fenticonazole in the treatment of surface candidosis and in particular in systemic candidosis, and on the other shows the effectiveness of this experimental model using an HEp2 cell monostrate, with constitutes an in vitro situation of parasitism similar to that observed in vivo.

To illustrate the activity of fenticonazole on Candida albicans strains grown on cellular lines the following photographs (light microscopy) are demonstrated (Figs. 2 and 3). The first figure shows HEp2 cells and interlacements of Candida albicans hyphae and blastospores in presence of fenticonazole at concentrations lower than 2.80 mcg/ml, while the second figure evidences the total inhibition of filamentation in presence of fenticonazole at concentrations equal to or higher than those of MIC.

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