

Effects of in-vitro activity of miconazole and ketoconazole in phospholipid formulations

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Antifungal agents are often used in liposomal formulations in order to improve their pharmacological activity, but how vesicle inclusion can actually affect this is still not fully understood. We report here the results obtained from evaluation of the in-vitro activity against *Candida albicans* ATCC E10231 of miconazole and ketoconazole in various vesicular and non-vesicular preparations, obtained from egg and soya phospholipids, using time–kill curves. In most cases inclusion of miconazole or ketoconazole in liposomes led to a delayed and decreased activity of the drugs, with detectable differences among the various phospholipid concentrations and different liposomal preparations (small unilamellar vesicle, liposomes, multilamellar aggregates and broken liposomal structures). The results obtained may be helpful in the study of new preparations of antifungal agents entrapped in liposomal structures.

Introduction

Miconazole and ketoconazole are imidazole derivatives effective against most pathogenic fungi and some Gram-positive bacteria. Usually they are well tolerated and their low toxicity allows them to be safely used for treating several cutaneous or systemic infections. However, anaphylactic reactions and cardiorespiratory toxicity of miconazole and alterations of hepatic function by ketoconazole, which have been fatal in several cases, have led to premature cessation of therapy in some cases.

Encouraging results have been obtained on the treatment of systemic and topical mycoses with liposomal¹ or lipid² formulations of antifungal drugs: acute and chronic toxicity of amphotericin B was usually reduced and a corresponding remarkable increase of the therapeutic index was observed.³ However, unexpected and conflicting results were sometimes reported. Fluconazole showed different MIC values according to the type of vesicles used, and liposomal fluconazole was more or less active than the non-liposomal drug according to the duration of incubation.⁴

Furthermore, the antifungal activity and the toxicity of amphotericin B were affected by the composition and the size of the liposomes.⁵ At present, it remains unclear as to how vesicle inclusion is actually capable of affecting antifungal activity.

The aim of this work was to investigate the in-vitro behaviour of miconazole and ketoconazole in various vesicular and non-vesicular preparations obtained with egg and soya phospholipids, against *Candida albicans*. Both drugs can be considered as un-ionized molecules in the tested environmental conditions but their octanol-water partition coefficients are quite different ($\log P_{ow} = 5.96$ and 4.74 for miconazole and ketoconazole respectively, as given by the LOGKOW database).

Materials and methods

The free bases of miconazole and ketoconazole were kindly supplied by LPB Istituto Farmaceutico, Milan, Italy. Enriched soya phosphatidylcholine (90% pure) (Phospholipon 90; Natterman Phospholipids GmbH,

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Cologne, Germany) was used for vesicle preparation and high purity (99%) L- α -phosphatidylcholine from egg yolk (Type III-E; Sigma, St Louis, MO, USA) was employed as the reference. Phospholipid B test (Wako Chemicals GmbH, Osaka, Japan) was used for quantitative determinations of these substances. When needed, special HPLC grade reagents were used and distilled water was further purified with a Milli-Q system (Millipore, Bedford, MA, USA). Cholesterol, solvents and all other products used in the present investigation were of analytical grade.

Sonication was performed with a Soniprep 50 apparatus (MSE, Crowley) equipped with an exponential microprobe, operating at 23 KHz and at an amplitude of 6 μ m. Sonication was carried out at 15–20°C because the transition temperature of phosphatidylcholine is lower than this operating temperature.

Liposome dimensions were first determined with a Malvern Autosizer II, Southborough, MA, USA, while reproducibility from preparation to preparation was checked by measuring turbidity with a Perkin Elmer LS5 spectrofluorimeter with excitation and emission wavelengths set at 600 nm.

Miconazole and ketoconazole concentrations were determined by HPLC, as previously described.⁶

Vesicles containing the drug prepared by hydration of a dried lipid film according to an established procedure,⁶ using phospholipon 90 or egg yolk phosphatidylcholine (80 mg), cholesterol (5 mg) and miconazole or ketoconazole (5 mg). Multilamellar vesicles (MLV) were obtained by vortexing the dried lipid film hydrated with 5 mL of distilled water for 5 min. Small unilamellar vesicles (SUV) were prepared by sonication of MLV under a nitrogen stream for 40 min (eight times for 5 min) at 15–20°C. For an appropriate comparison, vesicles without drug were also prepared.

In our studies SUV liposomes were 25–45 nm in diameter, while MLV were at least ten times bigger, often with an irregular size distribution.

When necessary, liposomes were broken by six cycles of freezing and thawing (between –20°C and +40°C).

SUV formulations were sterilized by filtration. Drug and phospholipid concentrations, determined before and after filtration, indicated that >95% of each substance was always recovered. In the case of multilamellar aggregates no final filtration was possible because of the larger dimensions of these structures, thus sterile water and sterilized vessels were used.

In some experiments miconazole and ketoconazole (10 g/L) were homogeneously dispersed in sterile distilled water by sonication; the suspension was then diluted in the test medium to a predicted final concentration of 25 mg/L and 3 mg/L.

Strains

Miconazole and ketoconazole activities were tested against *C. albicans* ATCC E10231. Experiments were carried

out in casitone broth (yeast extract 5 g/L, casitone 5 g/L, glucose 5 g/L) and inocula of 1×10^3 , 5×10^3 and 8×10^3 were used.

Samples were incubated at 37°C for different times, usually between 1 and 96 h. Suspensions were then appropriately diluted in saline, spread by inclusion in Sabouraud dextrose agar and incubated at 37°C for 24 h; colony-forming units were then quantified.

Results and discussion

Preliminary experiments indicated that empty phospholipid liposomes (SUV or MLV), and DMSO at the concentration used in the microbial growth experiments (i.e., <1%) showed no detectable activity and neither did miconazole, which is almost water-insoluble, when suspended in sterile water and tested against *C. albicans*.

As seen in Figure 1, liposomal miconazole was always less active against *C. albicans* than the free drug and differences between MLV and SUV inclusion were detectable. A further decrease in miconazole antifungal activity was observed when miconazole-loaded SUV were broken by freezing–thawing.

Of the data obtained with an inoculum of 8×10^3 cells/mL, it is interesting to point out that 50 mg/L free miconazole and MLV miconazole were fungicidal after 4 h and 96 h respectively, while for SUV and broken SUV miconazole, no fungicidal activity was detected even after 96 h, although a fungistatic effect as observed. When 25 mg/L of free miconazole were used, a fungicidal effect was observed only after 96 h while for all other formulations (SUV, MLV and broken SUV), no fungicidal activity was detectable. In addition, the fungistatic activity was notably reduced, in particular for SUV and broken SUV miconazole preparations. Furthermore, free miconazole, at a concentration of 12.5 mg/L, was not fungicidal and the fungistatic effect was again notably reduced for the various phospholipid formulations.

When the *C. albicans* inoculum was lowered to 1×10^3 cells/mL, free miconazole was fungicidal after 24 h for drug concentrations of 25 mg/L and 12.5 mg/L, while a fungistatic effect as observed up to 96 h for a free drug concentration as low as 3.12 mg/L. At the latter concentration, the effect of the presence of phospholipids became particularly evident because no drug activity was detected in the different formulations and *C. albicans* developed just as in untreated samples.

Finally, no appreciable differences between the behaviour of liposomes prepared with pure egg yolk phosphatidylcholine or with phospholipon 90 were observed.

These results suggest that the reduction of antifungal activity may be due to the decreased availability of the drug when included within the vesicular structures, and thus as phospholipid concentration increases a consequent decrease of miconazole activity takes place. Figure 2 shows the effect of increasing egg phosphatidylcholine

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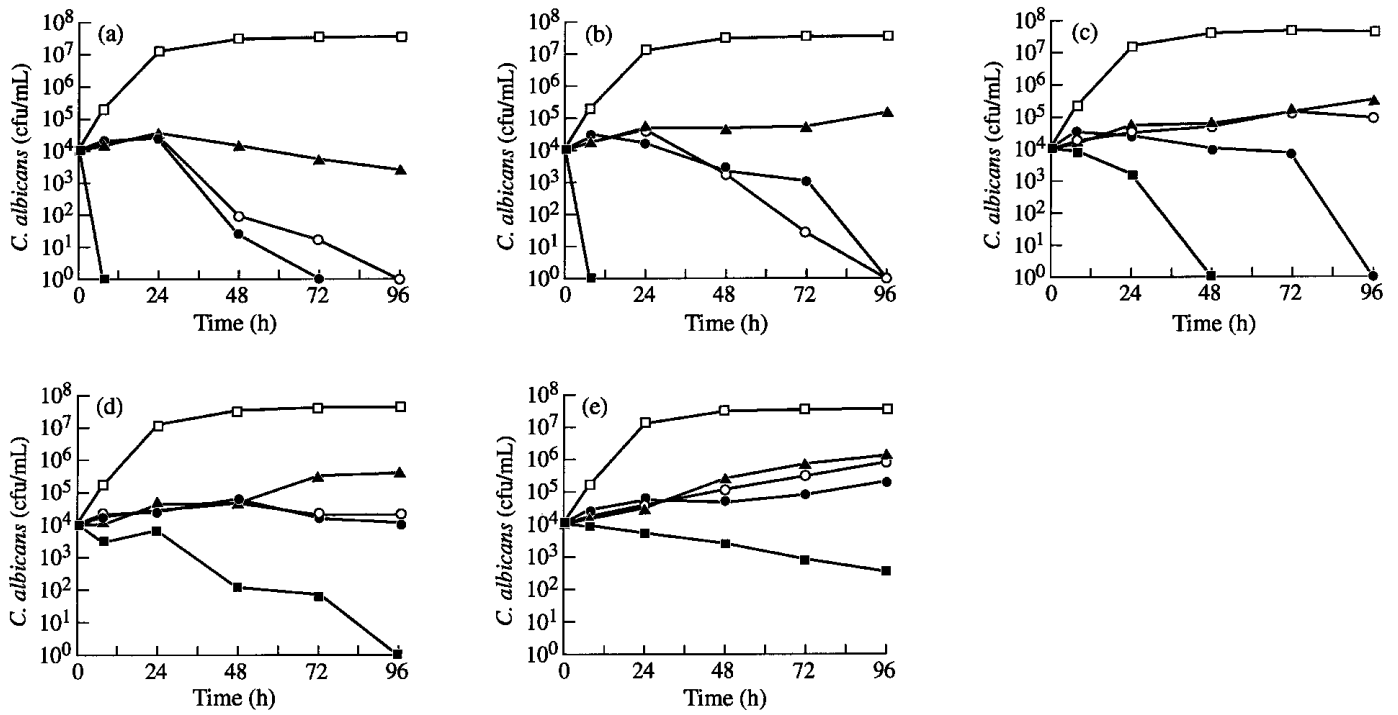


Figure 1. In-vitro activity against *C. albicans* ATCC E10231 of MLV (●), SUV (○), broken SUV (▲) and free miconazole (■) compared with the growth of the control (□). Reported results refer to an inoculum of 8×10^3 cfu/mL. The drug concentrations tested were 150 mg/L (a), 100 mg/L (b), 50 mg/L (c), 25 mg/L (d) and 12.5 mg/L (e).

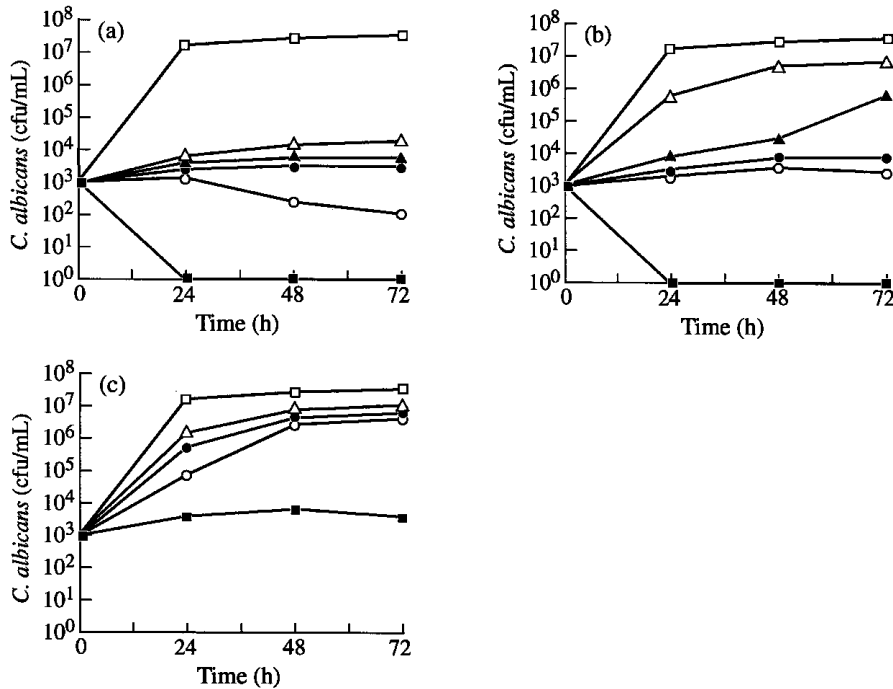


Figure 2. Effect of phospholipid concentration (0.125 mg/L (○), 0.25 mg/L (●), 0.5 mg/L (▲) and 1 mg/L (△)) on the in-vitro activity against *C. albicans* ATCC E10231 of MLV miconazole formulations. Free drug, ■; control, □. Reported results refer to an inoculum of 1×10^3 cfu/mL and to drug concentrations of 25 mg/L (a), 12.5 mg/L (b) and 3.125 mg/L (c).

concentrations on the activity of MLV miconazole against *C. albicans*.

The patterns of variations between liposomal and free ketoconazole differed from those seen with miconazole. SUV and MLV ketoconazole formulations were less active than the free drug against *C. albicans* only at 25 mg/L, while no differences were detectable at higher or lower concentrations.

Results obtained from the various experiments showed that liposomal inclusion of miconazole led to an increased survival of *C. albicans*, with respect to the free drug, especially in experiments carried out with long incubation times. These results are consistent with findings of other investigators regarding the in-vitro activity of antifungal agents in similar vehicles, such as amphotericin B in lipid complex, MLV⁷ or SUV.⁸ It must be emphasized, however, that often there is no correlation between the in-vitro and in-vivo activities of antifungal drugs in liposomal formulations. This is particularly evident for amphotericin B, which when included in liposomes is less toxic both *in vivo* and *in vitro*⁹ and has a higher therapeutic index than the free drug. Furthermore, it has been demonstrated that high density, but not low-density, lipoproteins play a major role in the lower toxicity *in vitro*, of liposomal amphotericin B to LLC PK1 renal cells with respect to the free drug, while the antifungal activity *in vitro* of liposomal and free amphotericin B against *C. albicans* is not altered in the presence of high- or low-density lipoproteins. Therefore, the lowered toxicity *in vivo* of the liposomal form compared with the free drug appears to be due to the greater association to high-density lipoproteins in human serum.¹⁰

With regard to ketoconazole, the time-killing curves show that the liposomal encapsulation of the drug allowed it to maintain its in-vitro antifungal activity and, in most cases, it was similar to that observed with the free drug. Such an effect can be related to the different lipophilicity of the two drugs as indicated by the higher log P_{ow} value of miconazole with respect to that of ketoconazole, as indicated. Consequently, vesicular inclusion of miconazole is more effective both on entrapment efficiency and on in-vitro antifungal activity of this drug.

At the same time it is interesting to point out that in most cases the fungicidal activity of the tested drugs was slightly higher for the vortexed phospholipid dispersion (MLV) than for the SUV preparation; such differences can be interpreted in terms of a faster release from the large unilamellar aggregates. On the other hand, the further decrease of antifungal activity, observed with broken SUV, can be related to a partial precipitation, during the freezing-thawing treatment, of the poorly soluble drug that led to a reduction of its actual concentration. This proposed explanation for the different response to the tested antifungal drugs in the presence of SUV, MLV and broken vesicles is supported by the reversibility of the observed effects: when multilamellar and broken liposome formulations containing miconazole were sonicated, such treat-

ment led to preparations that showed the same activity previously obtained with intact SUV.

Furthermore, the comparison of the behaviour of miconazole and ketoconazole, particularly at the lower tested concentration, indicated that the effect of a liposomal formulation is not always predictable and that different drugs, in spite of their similar activity, may be differently affected by vesicle inclusion.

Finally, the comparison between pure egg yolk phosphatidylcholine and phospholipon 90 vesicles proved that the origin (egg or soya), and consequently the actual phospholipid composition, never significantly affected drug activity.

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