SHORT COMMUNICATION

Inhibition of Sterol Biosynthesis in *Candida albicans* by Imidazole-containing Antifungals

By M. S. MARRIOTT

Pfizer Central Research, Sandwich, Kent CT13 9NJ

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A simple procedure for studying sterol biosynthesis in cell-free homogenates of *Candida albicans* is described. The activities of some imidazole-containing antifungals (tioconazole, clotrimazole, econazole, ketoconazole and miconazole) as inhibitors of sterol C-14 demethylation have been determined using this technique.

INTRODUCTION

Van den Bossche *et al.* (1978) have recently suggested that the activity of imidazole-containing antifungals is due to interference with fungal sterol biosynthesis. More specifically, miconazole was shown to inhibit the removal of the C-14 methyl group from lanosterol.

As in mammalian cells, C-14 demethylation occurs as an early event in fungal sterol biosynthesis (Ohba *et al.*, 1978). Inhibition of C-14 demethylation will also inhibit the removal of the second of the two C-4 methyl groups (Fryberg *et al.*, 1972; Ohba *et al.*, 1978) and result in the accumulation of the more sterically bulky C-4 dimethyl and C-4 methyl sterols, e.g. lanosterol. Such sterols are not integrated as efficiently into biomembranes as are the more planar demethylated sterols, e.g. cholesterol and ergosterol (Demel *et al.*, 1972; Yeagle *et al.*, 1977). The inhibition of C-14 demethylation would thus deprive the fungal cell of its major membrane sterol, ergosterol, and hence limit its growth. In order to determine the activity of a number of imidazole antifungals, we have developed a rapid and simple technique for studying sterol biosynthesis in a cell-free homogenate from *Candida albicans*. This method can be used to measure the comparative intrinsic activity of such antifungals as inhibitors of C-14 demethylation.

METHODS

Organism and growth conditions. *Candida albicans* C₆₆, a clinical isolate, was used as the test organism. It was maintained on Yeast Morphology agar (Difco) and grown in Brain Heart Infusion liquid medium (Oxoid) at 37 °C.

Preparation of cell-free homogenate. Cells from an overnight shake culture (1 litre) were harvested by centrifugation, washed with 0.1 M-KH₂PO₄/K₂HPO₄ buffer, pH 7.4, and resuspended in a minimum volume (3 to 4 ml) of similar ice-cold buffer. Samples (2 ml) were disrupted by vortexing for 2 min in a stoppered test-tube with an equal volume of 60 mesh glass beads (BDH). Unbroken cells and walls were removed by centrifugation (5 min, 1500 g). The resulting supernatant (cell-free homogenate) contained 10 to 15 mg protein ml⁻¹ as assayed by the Lowry method.

Sterol biosynthesis. The assay mixture for measuring sterol biosynthesis contained [2-¹⁴C]mevalonate [10 µl; 0.25 µCi (9.3 kBq)], cofactor solution (50 µl; containing 1 µmol NADP, 1 µmol NAD, 3 µmol glucose 6-phosphate, 5 µmol ATP, 3 µmol reduced glutathione in 0.1 M-KH₂PO₄/K₂HPO₄ buffer, pH 7.4), divalent cation solution (10 µl; containing 2 µmol MnCl₂ and 3 µmol MgCl₂), water or imidazole antifungal solution (10 µl) and cell-free homogenate (0.92 ml). The assay mixture was incubated for 3 h at 37 °C and the reaction was stopped by the addition of freshly prepared 15% (w/v) KOH in 90% (v/v) ethanol. The mixture was then saponified by refluxing for 30 min at 80 °C in a water bath.

Fig. 1. Autoradiogram of non-saponified lipids separated by thin-layer chromatography: (a) control (no miconazole in assay mixture); (b) 5×10^{-8} M-miconazole in assay mixture.

Sterol fractionation. Following saponification, non-saponified lipids (sterols and sterol precursors) were extracted three times with 2 ml petroleum ether (b.p. 40 to 60 °C). The combined extracts were evaporated to dryness under nitrogen and redissolved in 3 ml petroleum ether. Samples (100 μ l) were removed for assay of total incorporation of radioactivity by liquid scintillation counting (Instagel scintillant, Packard Instrument Corporation).

The remaining solution was concentrated and applied to silica gel F₂₅₄ pre-coated aluminium thin-layer chromatography plates (Merck). The sterol components were separated using the solvent system heptane/di-isopropyl ether/acetic acid (60:40:4, by vol.). Radioactive components were located either by autoradiography or by the use of a Berthold thin-layer plate scanner. Radioactive zones were cut from the plate and assayed for radioactivity by liquid scintillation counting. The positions of the various compounds were based on relative mobilities of authentic standards of ergosterol, lanosterol and squalene (all from Sigma).

Materials. [2-¹⁴C]Mevalonate, dibenzylethylenediamine salt, specific activity 0.17 mCi mmol⁻¹ (6.3 MBq mmol⁻¹), was supplied by The Radiochemical Centre, Amersham. Miconazole and ketoconazole were gifts from Janssen Pharmaceuticals, clotrimazole was a gift from Bayer and econazole and tioconazole were synthesized by Pfizer Central Research.

RESULTS

The solvent system used separated C-4 dimethyl and C-4 methyl sterols from C-4,14 demethyl sterols. In the presence of miconazole (Fig. 1) no C-4,14 demethyl sterols were synthesized and only C-4 dimethyl and C-4 methyl sterols were found. In all experiments the presence of imidazole-containing antifungal did not inhibit the total incorporation of radioactivity into non-saponified lipids. The proportion of [2-¹⁴C]mevalonate incorporated was 10 to 15% of the amount added. Recoveries of radioactivity following thin-layer chromatography were in the range 60 to 90%.

Table 1 shows the distribution of radioactivity between the various components in the presence and absence of miconazole. It is apparent that 5×10^{-7} M-miconazole caused a total inhibition of C-14 demethylation whilst 5×10^{-8} M-miconazole had no effect. In subsequent experiments the IC₅₀ (concentration causing a 50% reduction in the proportion of radioactivity incorporated into C-14 demethyl sterols) was estimated to be $3.5 \pm 1.5 \times 10^{-7}$ M-miconazole.

Table 1. *Distribution of radioactivity in non-saponified lipid components separated by thin-layer chromatography*

Component	Radioactivity (% of total)				
	Miconazole concn in assay mixture				
	0 (control)	5×10^{-5} M	5×10^{-6} M	5×10^{-7} M	5×10^{-8} M
Squalene	36	46	47	47	31
Unknown	5	4	4	5	5
C-4 Dimethyl sterol	23	36	35	37	22
C-4 Methyl sterol	19	12	12	10	17
C-4,14 Demethyl sterol	17	0	0	0	20
Origin	1	2	2	2	4

The activities of some clinically important imidazole antifungals were compared using this technique. Tioconazole (Jevons *et al.*, 1979) and ketoconazole (Thienpont *et al.*, 1979) were the most active compounds tested with IC_{50} values in the range 5×10^{-8} to 8×10^{-8} M. Econazole and clotrimazole had IC_{50} values of 9×10^{-8} M and 1.2×10^{-7} M, respectively. Imidazole itself was also a weak inhibitor of C-14 demethylation with an IC_{50} of approximately 7×10^{-4} M.

DISCUSSION

The assay system described provides a simple and relatively rapid method for determining the activity of imidazole-containing antifungals as inhibitors of C-14 demethylation. Whilst the present work has concentrated on *C. albicans*, the method can, in principle, be extended to other pathogenic or opportunistic fungi. Although the technique has been designed to measure inhibition of C-14 demethylation it would, of course, identify compounds which inhibited other steps in sterol biosynthesis, e.g. the biosynthesis of squalene or the conversion of squalene to lanosterol.

The results obtained do not prove that inhibition of sterol biosynthesis is the antifungal mode of action of these imidazole derivatives. In fact their activities against *C. albicans* C_{66} , in terms of minimum growth inhibitory concentration, do not correlate with the IC_{50} values. However, a number of other factors can influence the sensitivity of individual fungal species, e.g. penetration of the antifungal to the active site and the relative sensitivity of the C-14 demethylase enzyme. These are currently being investigated.

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