Reduced azole susceptibility of oral isolates of *Candida albicans* from HIV-positive patients and a derivative exhibiting colony morphology variation

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Approximately 50% (15/28) of a selection of oral isolates of *Candida albicans* from separate individuals infected with the human immunodeficiency virus (HIV) exhibited low susceptibility to ketoconazole as determined by hyphal elongation assessment. Nine of these isolates exhibited colony morphology variation or switching at 37 °C, of which six expressed low ketoconazole susceptibility. To determine whether colony morphology variation could give rise to derivatives with reduced azole susceptibility, several high-frequency switching variants of three HIV-patient isolates were recovered and assessed. All but one of the variants expressed similar azole susceptibility profiles to their respective parental strains. However, the *C. albicans* derivative 132ACR expressed significantly reduced susceptibility to ketoconazole in comparison to its parental strain 132A. In whole cells, on the basis of total growth the switched derivative 132ACR was markedly less susceptible than its parental isolate 132A to ketoconazole at 10 µM. A much smaller difference was observed with fluconazole at 10 µM, with the switched derivative 132ACR exhibiting a threefold lower susceptibility compared with the parental isolate 132A. The incorporation of [¹⁴C]acetate in control and azole-treated cells of both organisms was higher for the parental strain. When cell lysates of strain 132A and its derivative 132ACR were incubated with [¹⁴C]mevalonic acid and ketoconazole, the IC₅₀ for ¹⁴C-label incorporation into C-4 demethyl sterols was fivefold higher for lysates of the switched derivative 132ACR compared with those of the parental strain 132A. With fluconazole the IC₅₀ value for the derivative 132ACR was 25-fold higher than for strain 132A. The 14-sterol demethylase of the switched derivative 132ACR was possibly less sensitive to azole inhibition than that of the enzyme of strain 132A. These studies indicated that colony morphology variation *in vitro* can generate derivatives with stable, reduced azole susceptibility without prior exposure to azoles.

Introduction

Oropharyngeal candidosis caused by *Candida albicans* is one of the most common manifestations of opportunistic infection among individuals infected with the human immunodeficiency virus (HIV⁺) and patients with acquired immunodeficiency syndrome (AIDS; Gottlieb *et al.*, 1981; Klein *et al.*, 1984; Holmberg & Meyer, 1986; Tavitian *et al.*, 1986a; Phelan *et al.*, 1987; Redfield & Burke, 1988; Syrjanen *et al.*, 1988; Samaranayake &

Holmstrup, 1989; Mandal, 1989; Samaranayake, 1990). Persistence of oral candidosis in these individuals and relapse following treatment with antifungal drugs is very common (Glatt *et al.*, 1988; Samaranayake & Holmstrup, 1989; Larsen, 1990). This is thought to be due to a variety of causes, including the underlying immunodeficiency, poor patient compliance, azole hepatotoxicity, gastrointestinal intolerance and the development of decreased drug susceptibility, and, in the case of the imidazole ketoconazole, poor absorption in the presence of achlorhydria (Tavitian *et al.*, 1986a; Kortring *et al.*, 1988; Epstein, 1990; Larsen, 1990; Fox *et al.*, 1991).

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The *N*-substituted imidazole or triazole antifungal antibiotics, including ketoconazole and fluconazole, are commonly prescribed for the treatment of oral candidosis in HIV⁺ and AIDS patients (Van Den Bossche *et al.*, 1990). Azoles probably act by inhibiting cytochrome *P*-450-dependent 14-sterol demethylase, an important enzyme in ergosterol biosynthesis in fungi (Borgers, 1980). Ergosterol is the main sterol found in *Candida* species and forms a vital constituent of cellular membranes. The depletion of ergosterol and the resulting accumulation of 14-methylated sterols disrupts membrane structure and function, resulting in inhibition of growth (Van Den Bossche *et al.*, 1988).

Many oral isolates ($\geq 30\%$) of *C. albicans* from Irish HIV⁺ and AIDS patients exhibit colony morphology variation at 37 °C on primary isolation, and these occasionally give rise to additional colony variants on subculture (D. C. Coleman & D. E. Bennett, unpublished data). Preliminary studies indicated that some of these variant or switched derivatives exhibited decreased susceptibility to azole antifungals *in vitro*, as determined by agar plate dilution and hyphal elongation assessment tests (Gallagher *et al.*, 1991). Colony morphology variation is a common feature among isolates of both *C. albicans* and other *Candida* species (Soll *et al.*, 1987; Rikkerink *et al.*, 1988; Odds & Merson-Davies, 1989; Soll, 1989; Suzuki *et al.*, 1991; Wickes *et al.*, 1991). Certain switched *C. albicans* derivatives can exhibit a variety of altered morphological, physiological, cell surface and genetic characteristics, including adhesion to epithelial cells, antigenicity, hypha formation, cell size, growth rate, karyotype and genotype variation and decreased susceptibility to the antifungal drugs nystatin, amphotericin B, miconazole nitrate and 5-fluorocytosine (Slutsky *et al.*, 1985; Merz *et al.*, 1988; Soll, 1989; Ghannoum *et al.*, 1990; Iwaguchi *et al.*, 1990; Rustchenko-Bulgac *et al.*, 1990; Rustchenko-Bulgac, 1991; McEachern & Hicks, 1991). In HIV⁺ and AIDS patients, where the levels of oral *C. albicans* can be very high (Tylenda *et al.*, 1989), this variability could allow the generation of altered forms which are better adapted for survival in the oral cavity. Recently Odds & Merson-Davies (1989) have shown that nutrient depletion *in vitro* can induce *Candida* isolates to form colony morphology variants. A parallel situation *in vivo* in the oral cavities of HIV-infected individuals could result in densely packed *Candida* cells depleting available nutrients, resulting in switching. Furthermore, the comprehensive therapeutic regimes often undertaken in HIV⁺ and AIDS patients could possibly also act as a trigger for switching. Thus, phenotypic switching *in vivo* may lead to the generation of substantial numbers of variants possessing altered phenotypes and/or genotypes, some of which may be better suited to survive in the oral cavity.

The aims of the present study were (1) to compare azole susceptibilities among a selection of *C. albicans* oral isolates from Irish HIV⁺ patients and from healthy carriers, and (2) to investigate the basis of significantly decreased azole susceptibility exhibited by one particular switched derivative generated from an azole-susceptible parental isolate.

Methods

Strains, derivatives and culture conditions. *C. albicans* isolates were routinely grown on Potato Dextrose Agar medium (PDA, Oxoid) at pH 5.6, supplemented with 0.4% (w/v) Noble agar (Difco) at 37 °C. For liquid culture, isolates were routinely grown in Yeast Peptone Dextrose Broth [YPD; per litre: 10 g yeast extract (Oxoid), 20 g peptone (Difco), 20 g glucose, pH 5.5] at 37 °C in an orbital incubator (Gallenkamp) set at 180 r.p.m. For ¹⁴C-labelling of whole *C. albicans* cells using [U-¹⁴C]-acetate, YEPD (YPD containing 5 g glucose l⁻¹) was used as the liquid culture medium.

C. albicans isolates and derivatives were identified on the basis of positive germ-tube tests following growth in 2.0 ml fresh human serum at 37 °C for 2 h, by their ability to produce hyphae and chlamydospores on rice-agar-Tween agar (RAT agar; bioMérieux) and by their carbohydrate- and nitrogen-source assimilation patterns using the ID 32C yeast identification system (API Systems).

Forty oral isolates of *C. albicans* were included in this study. These were recovered from saliva or from swab specimens of the mid-dorsum of the tongue by plating on PDA alone and PDA supplemented with the dye phloxine B (5 µg ml⁻¹; PDAB) followed by incubation at 37 °C for 48 h. Differential staining of portions or sectors of oral *C. albicans* colonies with phloxine B following growth on PDAB agar allowed the detection of some clinical isolates which exhibited colony morphology variation (Anderson & Soll, 1987; Odds & Merson-Davies, 1989). For primary isolation purposes, PDA and PDAB plates were supplemented with chloramphenicol (50 µg ml⁻¹). Control isolates were oral-carriage strains recovered from saliva samples of 12 healthy individuals with no known predisposition to immunosuppression. At sampling none of these individuals had any clinical signs of oral candidosis and none were denture wearers. Twenty-eight isolates were recovered from the oral cavities of individual HIV⁺ patients attending the Dublin Dental Hospital. All of these patients presented with overt clinical symptoms of erythematous or pseudomembranous candidosis, the clinical features and incidence of which have been described by Samaranayake & Holmstrup (1989). None of the control subjects or HIV⁺ patients had been treated with antifungal drugs or antibiotics for one month prior to presentation. Nine (~32%) of the 28 isolates from HIV⁺ patients exhibited variation in colony morphology on primary isolation. From three of these, further independent derivatives exhibiting high-frequency switching of colony morphology were recovered *in vitro* following growth on nutrient-depleted medium (see below). Prior to these experiments, the parental isolates were single-colony purified ten times to eliminate the possibility of multiple strains being present. These derivatives were included in the study because this procedure ensured that they originated from a single strain. *C. albicans* cell counts were performed with diluted yeast suspensions freshly prepared in sterile water using an Improved Neubauer haemocytometer (Hudson & Hay, 1989).

Chemicals, enzymes, radioisotopes and antifungal drugs. Chemicals and enzymes were purchased from Sigma, BDH or Boehringer Mannheim. [U-¹⁴C]Acetate (sodium salt; 52 mCi mmol⁻¹; 1.92 GBq mmol⁻¹), RS-[2-¹⁴C]mevalonic acid (dibenzylethylenediamine salt; 52 mCi mmol⁻¹; 1.92 GBq mmol⁻¹) and [α -³²P]dATP (3000 Ci

mmol⁻¹; 110 TBq mmol⁻¹) were purchased from Amersham. Ketoconazole was a gift from Janssen Pharmaceutical (Cork, Ireland) and fluconazole was a gift from Pfizer Central Research (Sandwich, Kent, UK). Stock solutions (1 mM) of ketoconazole and fluconazole were prepared in 0.2 M-HCl and in water, respectively. For azole-susceptibility testing experiments, dilutions of the respective drugs were made in distilled water immediately prior to use and sterilized by filtration through 0.22 µm pore diameter membrane filters (Gelman). Protein concentrations in supernatant fractions following homogenization of *C. albicans* cells were determined using a Bio-Rad Protein Assay kit with bovine albumin as the standard.

Isolation of morphological variants of *C. albicans* by nutrient depletion. Colony morphology variants of the *C. albicans* oral isolates 122A, 132A and 179A (all isolated from separate HIV⁺ patients) were recovered following growth in nutrient-depleted medium. YPD broth cultures (5 ml) of each organism were grown for 24 h at 37 °C and 1×10^3 c.f.u. used to inoculate modified YPD medium (5 ml; YPD with the nutrient components diluted by a factor of 100 or 200), pre-warmed to 37 °C, followed by incubation at 37 °C for 48 h in an orbital incubator at 150 r.p.m. After incubation, cultures were resuspended in sterile water at a cell density of 1×10^3 c.f.u. ml⁻¹ and 200 µl aliquots plated onto six PDA and six PDAB agar plates, followed by incubation at 37 °C for 48 h. PDA and PDAB plates were prepared in 9 cm Petri plates and contained 20 ml of agar per plate. Following incubation, plates were examined for colony morphology variants. Several variant colonies generated from each of the original isolates were chosen for further study.

Azole susceptibility tests. *C. albicans* clinical isolates and switched derivatives exhibiting colony morphology variation were assessed for their susceptibility to ketoconazole by a hyphal elongation assessment test using an *N*-acetylglucosamine-yeast nitrogen base-proline (NYP) medium (Marichal *et al.*, 1986). This medium promotes growth in the hyphal form in a humidified atmosphere of 10% (v/v) CO₂ at 37 °C. The procedure exploits the differential effect of azole antifungal agents on the growth and morphology of the dimorphic yeast *C. albicans*. In susceptible strains, inhibition of hyphal outgrowth is observed at lower concentrations (0.0625–0.125 µM) than with less susceptible strains (≥ 10 µM). In susceptible strains only blastoconidia are observed at drug concentrations > 0.125 µM.

Incorporation of [¹⁴C]acetate into sterols and lipids by *C. albicans* whole cells *in vitro*. The abilities of ketoconazole and fluconazole to affect the incorporation of [¹⁴C]acetate into sterols and lipids *in vitro* by whole cells of *C. albicans* strain 132A and its switched derivative 132ACR were determined by the method described by Van Den Bossche *et al.* (1978) with the following modifications. Separate 1 litre conical flasks containing 250 ml YEPD broth supplemented with 2.5 µmol sodium acetate and 6.25 µCi sodium [U-¹⁴C]acetate (52 mCi mmol⁻¹), with or without azole drugs, were inoculated with *C. albicans* strain 132A or its derivative 132ACR to yield a cell density of 4×10^4 c.f.u. ml⁻¹ and placed in an orbital incubator for 20 h at 37 °C, after which time the organisms were in late exponential phase with a cell density of approximately 1×10^8 c.f.u. ml⁻¹. The inocula were prepared by diluting cells from 4 h YEPD broth cultures in sterile saline to yield the required cell density. Cells were homogenized in a Braun cell homogenizer (model MSK; 4000 r.p.m.) using 12 × 20 s cycles with 10 s intermittent cooling using liquid CO₂.

Hydrofluor (National Diagnostics) was used as the scintillation fluid and the radioactivity in each sample was measured using a Packard Tri-Carb Liquid Scintillation 1500 spectrometer.

HPLC analysis of sterols from *C. albicans* whole cells. ¹⁴C-labelled sterols isolated from *C. albicans* whole cells, treated or not treated with azole drugs, were analysed by high-performance liquid chromatography (HPLC) as described by Van Den Bossche *et al.* (1990).

Incorporation of [¹⁴C]mevalonic acid into sterols synthesized *in vitro* using *C. albicans* cell-free extracts. The ability of ketoconazole and fluconazole to affect the incorporation of [¹⁴C]mevalonic acid into sterols synthesized *in vitro* by cytochrome P-450-containing cell-free extracts of *C. albicans* strain 132A and its switched derivative 132ACR, and of a *C. albicans* fluconazole susceptibility testing reference strain Y0109 (obtained from Dr P. Troke, Pfizer Central Research, Sandwich, Kent, UK), was determined using a modification of the method described by Marriott (1980). Yeast cell suspensions prepared in 12 ml (2×10^9 c.f.u. ml⁻¹) 100 mM-potassium phosphate buffer (pH 7.4) containing 30 mM-nicotinamide, 5 mM-*N*-acetylcysteine, 5 mM-MgCl₂ and 0.25 mM-phenylmethylsulphonyl fluoride were lysed using 12 g glass beads (0.45–0.5 mm) in a Braun homogenizer (MSK) using a cycle of 6 × 20 s with 10 s intermittent cooling with liquid CO₂. The homogenate was separated from the glass beads, unbroken cells and cell debris by centrifugation for 5 min at 1500 g. The resulting cell-free homogenate (11 mg protein ml⁻¹) was used as the enzyme-containing preparation for *in vitro* sterol biosynthesis.

The assay mixture for measuring sterol biosynthesis contained 0.5 µCi [2-¹⁴C]mevalonic acid (52 mCi mmol⁻¹) and the cofactor solution in addition contained 1 µmol NADPH.

Samples (100 µl) were removed for assay of total ¹⁴C-incorporation by liquid scintillation counting. The remaining solution was concentrated to dryness and the sterols redissolved in 0.5 ml ethyl acetate. Aliquots (40 µl) were applied 2 cm from the bottom of silica gel F₂₅₄ pre-coated aluminium TLC plates (0.20 mm thick, 20 × 20 cm; Merck no. 5554) and the sterols and sterol precursors separated using a toluene/diethyl ether (9:1, v/v) solvent system (Hitchcock *et al.*, 1989). Following chromatography, sterol bands were located by exposing the TLC plates to UV light (345 nm) both before and after spraying the plates with the autoradiographic image intensifier Autofluor (National Diagnostics). Radioactive bands were located by autoradiography following exposure of TLC plates to Hyperfilm-βmax (Amersham). Sterol-containing bands were then scraped from the TLC plates and levels of ¹⁴C-incorporation measured by scintillation counting as described above. Unlabelled squalene, lanosterol and ergosterol (40 µl of 0.75%, w/v, solutions in ethyl acetate) were applied to all TLC plates as control standards (Barrett-Bee *et al.*, 1986).

Molecular fingerprinting of *C. albicans* genomic DNA. Total cellular DNA from *C. albicans* isolates and colony morphology variants was isolated using a modification of the methods described by Durkacz *et al.* (1985) and Pearce & Howell (1991) as follows. After RNAase treatment of the extracted DNA, proteinase K was added to a final concentration of 2 mg ml⁻¹ followed by incubation at 37 °C for 2 h. The DNA was then precipitated with 2 vols ethanol at -70 °C for 1 h, and the precipitate was recovered by centrifugation in a microfuge, washed gently with 100 µl 70% (v/v) ethanol, dried and resuspended in TE buffer (10 mM-Tris/HCl, 1 mM-EDTA, pH 7.0). This solution was then extracted once with an equal volume of phenol/chloroform (1:1, v/v) and the resulting DNA-containing extract used directly in restriction endonuclease digestion experiments.

The procedures used for restriction endonuclease digestion of genomic DNA, agarose gel electrophoresis, Southern hybridization analysis, labelling of molecular probes with [α -³²P]dATP and autoradiography have all been described previously (Coleman *et al.*, 1986, 1989).

A *C. albicans*-specific DNA fragment cloned from dispersed, repetitive sequence DNA was used as a molecular probe in hybridization experiments. The probe consisted of a 3.0 kb *Eco*RI fragment cloned in vector plasmid pUC18, and was identical to probe 27A described by Scherer & Stevens (1988). *Escherichia coli* strain 824 (strain JM83 harbouring pUC18 containing the cloned 3.0 kb *Eco*RI insert) was obtained from Professor S. Scherer (Minneapolis, Minnesota, USA).

Results

Susceptibility of oral isolates of C. albicans and colony morphology variants to ketoconazole

In order to determine the susceptibility of oral isolates of *C. albicans* from Irish HIV⁺ patients to azole antifungals, 28 isolates from separate HIV⁺ individuals were tested for their sensitivity to ketoconazole using a hyphal elongation assessment test. The susceptibility of these isolates was compared with that of oral carriage strains from 12 healthy individuals and also with that of several derivatives of isolates from HIV⁺ patients, recovered *in vitro* by culturing the parental organisms in nutrient-depleted medium, which exhibited colony morphology variation or switching. The vast majority (11/12) of the control carriage isolates were susceptible to ketoconazole, whereas about half (15/28) of the isolates from HIV⁺ patients exhibited lower susceptibility (Table 1).

Nine (32%) of these isolates from HIV⁺ patients exhibited variation in colony morphology on primary isolation. In the case of six isolates, the colonies varied between two colony morphology phenotypes, whereas the remaining three isolates switched between three colony morphology phenotypes. To ensure that mixed strains were not responsible for the observed colony morphology variation, total cellular DNA from representative colonies of each phenotype, for all nine isolates, was recovered, digested with the restriction endonuclease *EcoRI* and hybridized with a ³²P-labelled mid-repeat sequence probe identical to 27A (Scherer & Stevens, 1988). The probe was *C. albicans*-specific, with several copies dispersed throughout the genome. When total

Table 1. *Susceptibility of oral C. albicans isolates from healthy carriers and HIV⁺ patients to ketoconazole determined by hyphal elongation assessment*

Isolate source	No. of isolates	No. of isolates with high susceptibility*	No. of isolates with low susceptibility†
Healthy carriers	12	11	1
HIV ⁺ patients	28	13	15

* Hyphal outgrowth inhibited in the presence of ketoconazole concentrations in the range 0.0625–0.125 µM.

† Hyphal outgrowth inhibited only in the presence of ketoconazole concentrations ≥ 10 µM.

cellular DNA from the *C. albicans* isolates was digested and hybridized with the probe, the resulting multiple fragments detected following autoradiography constituted molecular 'fingerprint' patterns which could be used to distinguish between different strains (Scherer & Stevens, 1988; Fox *et al.*, 1989). The molecular fingerprints of all nine isolates were clearly different from each other, whereas the fingerprints of spontaneous variant colonies of the same clinical isolate were in each case indistinguishable (data not shown). Fingerprint patterns generated with cloned, repeat sequence probes can readily distinguish between different strains of *C. albicans* but fingerprints of switched derivatives of a particular strain tend to yield indistinguishable patterns (Soll *et al.*, 1987; Scherer & Stevens, 1988). These findings confirmed that the observed phenomenon of colony morphology variation by the nine oral isolates from HIV⁺ patients was not due to mixed strains. Of

Table 2. *Susceptibility of oral C. albicans isolates from HIV⁺ patients and of their derivatives to ketoconazole as determined by hyphal elongation assessment*

Isolate/derivative	Ketoconazole susceptibility*	Colony morphology	Comments
132A	High	Smooth-white	Oral isolate from HIV ⁺ patient
132A-1	High	Rough-tan	Spontaneous derivative of 132A
132A-2†	High	Rough-tan	Derivative of 132A
132A-3†	High	Wrinkled-tan	Derivative of 132A
132ACR†	Low	Wrinkled-tan	Derivative of 132A
132ACR-1	Low	Wrinkled-white	Spontaneous derivative of 132ACR
132ACR-2	Low	Smooth-white	Spontaneous derivative of 132ACR
122A	High	Smooth-white	Oral isolate from HIV ⁺ patient
122A-1†	High	Wrinkled-tan	Derivative of 122A
179A	Low	Smooth-white	Oral isolate from HIV ⁺ patient
179A-1†	Low	Wrinkled-tan	Derivative of 179A

* High susceptibility, hyphal outgrowth inhibited in the presence of ketoconazole concentrations in the range 0.0625–0.125 µM; low susceptibility, hyphal outgrowth inhibited in the presence of ketoconazole concentrations ≥ 10 µM.

† Derivatives recovered following growth in nutrient-depleted medium. Three colonies of each organism were tested in separate experiments.

Fig. 1. Colony morphologies of *C. albicans* isolate 132A and its derivative 132ACR and of colony morphology variants of derivative 132ACR on PDA medium. *C. albicans* strain 132A (a) was an oral isolate recovered from a HIV⁺ individual. Derivative 132ACR (b) was a tan-coloured wrinkled colony morphology variant derived from strain 132A following growth in nutrient-depleted medium. Derivative 132ACR switches at a frequency of approximately 1% between three colony morphology phenotypes including tan wrinkled, white wrinkled and smooth white colony forms. Panel (c) shows examples of the white wrinkled and smooth white colony forms.

these nine isolates, six exhibited lower susceptibility to ketoconazole. Two colony morphology phenotypes of each of these six isolates were tested and all exhibited reduced susceptibility to ketoconazole.

Derivatives exhibiting high-frequency switching of colony morphology (colony morphology alternated between two or three colony variant forms at frequencies of approximately 1%, i.e. about 1 in 100 c.f.u. from a single colony would give rise to a variant colony form) were recovered from three of the nine isolates by culturing them in nutrient-depleted medium. All the derivatives tested exhibited similar ketoconazole susceptibility profiles to their respective parental isolates, except for one morphological variant (designated 132ACR) of the ketoconazole-susceptible parental *C. albicans* isolate 132A, which exhibited decreased ketoconazole susceptibility (Table 2).

Colonies of derivative 132ACR were predominantly tan-coloured, possessing wrinkled surfaces on PDA agar, and readily gave rise to a further two types of colony morphology variants on subculture on PDA agar at 37 °C, at frequencies of approximately 1% (i.e. about 1 in 100 c.f.u. from a single colony of derivative 132ACR would give rise to either colony variant form; Fig. 1). One of these additional variant forms consisted of white wrinkled colonies and the other, of normal smooth white colonies. Several representative single-colony isolates of both of these types of colony morphology variant also exhibited similar decreased susceptibility to ketoconazole (Table 2). Derivative 132ACR continued to shed these two types of variant colony forms following 100 consecutive single-colony purifications on PDA medium at 37 °C.

These findings indicated that colony morphology variation *in vitro* can, in some cases, lead to generation of derivatives exhibiting decreased ketoconazole susceptibility, as determined by hyphal elongation assessment

experiments. Because the reduced ketoconazole susceptibility expressed by derivative 132ACR and its colony morphology variants was not acquired as a result of prior exposure to the drug, it was decided to investigate this phenomenon further. The effects of the azole antifungals ketoconazole and fluconazole were investigated with respect to (i) growth of 132ACR and its parental strain 132A, (ii) lipid and sterol patterns of whole cells of both organisms, and (iii) the biosynthesis of sterols *in vitro* using cytochrome *P*-450-containing cell-free extracts of strain 132A and its derivative 132ACR.

Effects of ketoconazole and fluconazole on growth of C. albicans 132A and its derivative 132ACR

Separate YEPD broth cultures of *C. albicans* strain 132A and its switched derivative 132ACR containing standardized inocula were incubated for 20 h at 37 °C in the presence or absence of varying concentrations of azole. In control experiments without azole the growth of the parental strain 132A was greater than that of its derivative 132ACR in more than ten separate experiments; following 20 h growth the cell density of broth cultures of strain 132A was 1.4 times greater than cultures of its derivative 132ACR. Ketoconazole had a greater effect in reducing the cell densities of broth cultures of *C. albicans* strain 132A than of broth cultures of its derivative 132ACR over a range of drug concentrations (Fig. 2a). Thus the cell densities (expressed as a percentage of those of drug-free control cultures) of broth cultures of derivative 132ACR were 5% greater than those of broth cultures of strain 132A at a ketoconazole concentration of 0.01 µM and 13% greater at 0.1 µM (Fig. 2a). The cell density of derivative 132ACR was reduced to 2% of the control value following growth in the presence of 10 µM-ketoconazole, whereas that of the parental strain 132A was reduced to 2% at a ketoconazole concentration of only 0.1 µM. Both organisms were less susceptible to growth inhibition by fluconazole than by ketoconazole over the same concentration range. Derivative 132ACR exhibited a greater susceptibility to fluconazole at the lower azole concentrations (0.1 and 1.0 µM) than its parental strain 132A, whereas it exhibited a decreased susceptibility in comparison to strain 132A at 10 µM-fluconazole (Fig. 2b).

Biosynthesis of lipids and sterols in whole cells of C. albicans strain 132A and its derivative 132ACR

In order to determine the effects of azoles on the biosynthesis of lipids and sterols in whole cells of *C. albicans* strain 132A and its derivative 132ACR, these

Fig. 2. Cell densities of *C. albicans* strain 132A (▨) and its derivative 132ACR (■) following growth in YEPD medium at 37 °C in the presence and absence of azoles. Cell densities are expressed as a percentage of azole-untreated cultures for each respective organism. For strain 132A, the mean value for 100% ($n = 3$) was 13.4×10^7 c.f.u. ml⁻¹ and for derivative 132ACR, 9.75×10^7 c.f.u. ml⁻¹. (a) Ketoconazole-treated cultures; (b) fluconazole-treated cultures. Similar results were obtained in three separate sets of experiments.

organisms were grown in the presence of [¹⁴C]acetate and of various concentrations of ketoconazole and fluconazole.

Total lipid extract. An increase in the ¹⁴C-incorporation into the lipid extracts of strain 132A and its derivative 132ACR grown for 20 h in YEPD with [¹⁴C]acetate was observed with increasing concentrations of both ketoconazole and fluconazole compared with azole-untreated control cultures (Fig. 3). For both drugs, the incorporation of the ¹⁴C-label was higher by the parental strain 132A than by its switched derivative 132ACR, with the greatest difference (approximately 10-fold) observed at 0.1 μM -ketoconazole (Fig. 3). However, gross lipid analysis of strain 132A and its derivative 132ACR by TLC showed no obvious differences (data not shown). The highest ¹⁴C-incorporation (38.9%) was recorded in lipid extracts prepared from 250 ml YEPD broth cultures of strain 132A grown in the presence of 10 μM -fluconazole and [¹⁴C]acetate (9×10^6 d.p.m. per 250 ml medium).

Sterols. Incubation of strain 132A and its derivative 132ACR for 20 h in the presence or absence of ketoconazole or fluconazole or solvent alone and [¹⁴C]-acetate followed by TLC of the lipid fractions demonstrated that almost 75% and 72%, respectively, of the total ¹⁴C-label present in the cell extracts was incorporated into complex lipids (Table 3). ¹⁴C-incorporation into free sterols recovered from cultures of strain 132A and its derivative 132ACR represented about 17% and 19%, respectively, of the total ¹⁴C-incorporation with organisms grown in the absence of azoles (Table 3). There was no apparent difference in the amount of incorporated ¹⁴C-label in the ergosterol-containing C-4

Fig. 3. [¹⁴C]Acetate incorporation into lipids and sterols in whole cells of *C. albicans* strain 132A and its derivative 132ACR following 20 h growth in the presence of [¹⁴C]acetate and with or without various concentrations of ketoconazole or fluconazole. Insufficient growth of strain 132A was obtained in the presence of 10 μM -ketoconazole for analysis to be performed. ■, Strain 132A grown with ketoconazole; ▲, strain 132A grown with fluconazole; □, derivative 132ACR grown with ketoconazole; △, derivative 132ACR grown with fluconazole. Similar data were obtained with three separate sets of experiments.

demethyl sterol fractions of azole-untreated control cultures of strain 132A and its derivative 132ACR (Table 3). However, clear-cut differences were observed in the ¹⁴C-levels incorporated into C-4 demethyl-, 4,14-dimethyl-, and 4,4',14-trimethyl sterols between control and azole-treated cells of either organism (Table 3). A 50% decrease in [¹⁴C]acetate incorporation into the C-4 demethyl sterols fraction was obtained at a ketoconazole concentration of $\sim 0.01 \mu\text{M}$ and a fluconazole concentra-

Table 3. Effects of ketoconazole and fluconazole on the incorporation of [^{14}C]acetate into sterols and complex lipids *in vitro* by whole cells of *C. albicans* strain 132A and its derivative 132ACR

Concn of azole (μM)	^{14}C -label incorporated as % of total ^{14}C -incorporation into indicated classes of lipids*							
	Complex lipids		C-4 demethyl sterols		4,14-dimethyl sterols		4,4',14-trimethyl sterols	
	132A	132ACR	132A	132ACR	132A	132ACR	132A	132ACR
Ketoconazole								
Control	75	72	15	15	1	2	1	1
0.01	71	66	9	8	2	3	4	3
0.1	65	68	5	4	5	3	6	5
10.0	ND	46	ND	2	ND	3	ND	4
Fluconazole								
Control	74	71	12	12	1	1	1	2
0.01	75	78	13	11	1	1	1	1
0.1	67	70	6	5	2	2	1	4
10.0	64	76	4	2	4	2	3	5

ND, Not determined; insufficient growth of strain 132A for analysis. Complex lipids remained at the origin following chromatography.

* Total radioactivity found in the lipids and sterols extracted from whole cells was taken as 100% for each respective strain. Data are presented for a single set of experiments; similar data were obtained in three independent sets of experiments. R_F values of the sterols were determined by reference to the relative mobility of authentic standards (R_F values in parenthesis) as follows: C-4 demethyl sterols, (0.14); 4,14-dimethyl sterols, (0.20); 4,4',14-trimethyl sterols (0.22).

Table 4. Incorporation of [^{14}C]acetate into sterols in whole cells of *C. albicans* strain 132A and its derivative 132ACR in the presence or absence of ketoconazole as detected by HPLC analysis

<i>C. albicans</i> strain	Ketoconazole concn (μM)	^{14}C -label incorporated as % of total ^{14}C -label incorporation into indicated sterols and squalene								
		Sterols & squalene*: Retention time:	E 6.6	3,6 diol 8.2	14ME 17.9	4DE 18.9	OF 21.0	L 22.2	24MET 24.7	S 40.9
132A†	Control		100	0	0	0	0	0	0	0
	0.01		38	26	0	0	3	17	13	3
	0.1		0	36	1	4	12	16	21	9
132ACR	Control		100	0	0	0	0	0	0	0
	0.01		66	3	0	0	0	19	12	0
	0.1		0	28	0	0	23	9	39	0
	10.1		0	81	0	0	8	3	8	0

* Total radioactivity detected in sterol and squalene preparations was taken as 100%. Sterols were characterized on the basis of their retention times (min) as shown in the table. Abbreviations: E, ergosta-5,7,22-trien-3-ol (ergosterol); 3,6 diol, 14-methylen-ergosta-8,24 (28)-dien-3,6-diol; 14ME, 14-methyl-ergosta-8-24 (28)-dien-3-ol; 4DE, 4,14-dimethyl-ergosta-8,24-dien-3-ol; OF, 4,14-dimethyl-ergosta-8,24 (28)-dien-3-ol; L, 4,4',14-trimethyl-cholesta-8,24-3-ol; 24MET, lanost-8,24 (28)-dien-3-ol; S, squalene.

† Insufficient growth of strain 132A was obtained in the presence of 10 μM -ketoconazole for analysis to be performed.

tion of $\sim 0.1 \mu\text{M}$ for both strain 132A and its derivative 132ACR (Table 3). An increased incorporation of ^{14}C -label was also observed in the sterol fractions containing 4,14-dimethyl sterols and 4,4',14-trimethyl sterols for both organisms (Table 3).

Greater resolution of the three free sterol bands detected by TLC in extracts from whole cells of strain 132A and its derivative 132ACR following 20 h growth in the presence of ketoconazole was achieved by HPLC (Table 4). In cells of *C. albicans* strain 132A after 20 h growth in the presence of 0.01 μM -ketoconazole, incor-

poration of ^{14}C -label into ergosterol was reduced to 38% of the control value, whereas in *C. albicans* derivative 132ACR incorporation of ^{14}C -label into ergosterol was only reduced to 66% of the control value (Table 4).

Biosynthesis of sterols *in vitro* using cell-free extracts of *C. albicans* strain 132A and its derivative 132ACR

In order to investigate whether the decreased azole susceptibility exhibited by *C. albicans* derivative 132ACR could be correlated with decreased sensitivity

of its 14-sterol demethylase, the effects of ketoconazole and fluconazole on the biosynthesis of sterols *in vitro* were investigated using cell-free extracts of strain 132A and its derivative 132ACR. Cell-free extracts of both organisms were prepared by homogenization and the biosynthesis of ^{14}C -labelled sterols from $[2\text{-}^{14}\text{C}]$ mevalonic acid as substrate was monitored in the presence and absence of azoles. The proportion of $[2\text{-}^{14}\text{C}]$ mevalonate incorporated into sterols by lysates of *C. albicans* strain 132A and its derivative 132ACR was 31% and 22%, respectively. Sterols were separated by TLC following isolation of non-saponifiable lipids. The identity of the separated sterols was determined by reference to the relative mobilities of authentic standards. Non-saponified lipids synthesized from extracts of both strain 132A and its derivative 132ACR were separated into the following compounds: C-4 demethylated sterols, 4,14-dimethylated sterols, 4,4',14-trimethylated sterols, an unknown compound and squalene.

In experiments with cell-free extracts from strain 132A, 0.01 μM -ketoconazole and 0.04 μM -fluconazole, respectively, inhibited sterol demethylation by 50% (IC_{50} values). The corresponding IC_{50} values for cell-free extracts of derivative 132ACR were 0.05 μM -ketoconazole and 1.0 μM -fluconazole, respectively. Not only were there differences in the IC_{50} values between strain 132A and its derivative 132ACR but there were also differences in the IC_{50} values obtained with ketoconazole and fluconazole. Liquid scintillation counting analysis of ^{14}C -label incorporation revealed a concomitant increased incorporation of ^{14}C -label into the 4,14-demethylated sterols and 4,4',14-trimethylated sterols with lysates from both organisms at azole concentrations above the IC_{50} values.

For comparison with the results obtained with *C. albicans* strain 132A, additional IC_{50} determinations were made with a fluconazole reference test *C. albicans* strain, strain Y0109, whose characteristics have been investigated (P. Troke, personal communication). The results for strain Y0109 were similar to strain 132A: IC_{50} value of 0.01 μM -ketoconazole and 0.05 μM -fluconazole.

Discussion

Oral and oesophageal candidosis in HIV⁺ and AIDS patients is a difficult condition to treat effectively. The introduction of the orally active azole antifungals ketoconazole and fluconazole has produced dramatic improvements in the management of the condition in immunocompromised patients (Fazio *et al.*, 1983; Dupont & Drouhet, 1988; Epstein, 1990; Larsen, 1990; Hay, 1991). However, even with these drugs, protracted treatment is necessary if remission of infection is to be

achieved and sustained (Esposito *et al.*, 1990; Leen *et al.*, 1990; Larsen, 1990). The situation has been compounded by an increasing number of reports of azole resistance in oral isolates of *Candida* species from HIV⁺ and AIDS patients (Tavitian *et al.*, 1986b; Kortling *et al.*, 1988; Warnock *et al.*, 1988; Fox *et al.*, 1991; Kitchen *et al.*, 1991; Fan-Havard *et al.*, 1991).

The majority of *C. albicans* strains and many isolates of other *Candida* species have the ability to exhibit colony morphology variation or switching, which recent studies have shown is often associated with genetic variation and which may provide, non-sexual *Candida* strains with the ability to adapt rapidly in their natural environment (Rustchenko-Bulgac *et al.*, 1990; Rustchenko-Bulgac, 1991; Suzuki *et al.*, 1991; Wickes *et al.*, 1991). This ability may contribute to pathogenicity of the switched derivatives and to the development of derivatives with decreased susceptibility to antifungal drugs (Slutsky *et al.*, 1985; Soll, 1989; Ghannoum *et al.*, 1990). Approximately 30% of oral isolates of *C. albicans* recovered from more than 200 individual Irish HIV⁺ and AIDS patients exhibited colony morphology variation at 37 °C on primary isolation (D. C. Coleman and others, unpublished). In the present study variants were readily generated from several oral *C. albicans* isolates from HIV⁺ patients and one of these was found to exhibit substantially reduced susceptibility to ketoconazole *in vitro*, in comparison to its parental isolate.

The discussion of the results of these experiments has to account for two interacting sets of variables: those arising from the differences between the parental strain 132A and its derivative 132ACR, and those that follow from the differences between the drugs ketoconazole and fluconazole. The results of the whole-cell studies showed that the derivative 132ACR grew less efficiently than its parental strain 132A. The pattern of growth inhibition for the two drugs differed: ketoconazole produced a dose-related effect, with 132A being more susceptible than 132ACR, while the pattern obtained with fluconazole was quite different. Derivative 132ACR was more susceptible to the effect of fluconazole at 0.1 and 1.0 μM than 132A and although this pattern was reversed at 10 μM , it is notable that 132A was still able to achieve substantial growth in the presence of fluconazole.

The chromatographic analyses showed that 132A and 132ACR produced a similar pattern of lipids and sterols, including ergosterol. Incorporation of $[^{14}\text{C}]$ acetate in control and azole-treated cultures of both organisms was higher for the parental strain 132A than for the derivative 132ACR, reflecting the more efficient growth of the strain. This parameter was highest in the fluconazole-treated cultures of strain 132A and the greatest difference between incorporation into 132A and 132ACR was observed with 0.1 μM -ketoconazole, thus

paralleling data obtained from the cell growth experiments. The concentration required to reduce ^{14}C -label incorporation into C-4 demethyl sterols for ketoconazole ($0.01\ \mu\text{M}$) and fluconazole ($0.1\ \mu\text{M}$) was the same for both 132A and 132ACR. The data from these experiments are of the same magnitude as the data from cell growth studies, both in the comparative activities of the two drugs and in the approximate drug concentrations required to inhibit cell growth by 50%. The pattern of incorporation into the different lipid fractions was consistent with inhibition of ergosterol biosynthesis and the accumulation of precursors.

The results using the cell-free system also showed that in both 132A and 132ACR the synthesis of ergosterol could be inhibited by the two drugs and the increased incorporation into precursors occurred at concentrations above the IC_{50} values. However, it was notable that the IC_{50} values for the two organisms differed, with 132A being more sensitive to inhibition than 132ACR, and that ketoconazole was more potent than fluconazole. The parental strain 132A would not seem to be atypical since the results obtained with the fluconazole reference test strain (Y0109) were in line with those for 132A and are similar to data published by Marriott & Richardson (1987). These results suggest, therefore, that the process of 14-demethylation in 132ACR is less readily inhibited by these two drugs than the corresponding process in 132A.

The lack of correlation between the IC_{50} values obtained in the cell-free system (ketoconazole-132ACR: $0.05\ \mu\text{M}$; fluconazole-132ACR: $1.0\ \mu\text{M}$) with the approximate values for 50% reduction of ^{14}C acetate incorporation into whole cells (ketoconazole-132ACR: $0.01\ \mu\text{M}$; fluconazole-132ACR: $0.1\ \mu\text{M}$) and, if the comparison is made, though not strictly valid, with the cell growth experiments then the divergence appears to be enhanced. These results are difficult to explain. A number of factors, whether acting separately or in combination, may be important. The concentrations of azoles in the cell growth and whole-cell experiments are those of drug present in the media and we have no way of knowing how much drug entered the cells and remained there as the active moiety, unlike in the cell-free system. This makes a comparison of the concentrations difficult. However, in the cell-free system the sterol biosynthetic pathway is isolated from the micro-environment from which the entire process from acetate uptake to sterol incorporation in the cell membrane occurs in a highly regulated manner. Given that azoles may act at more than one point in this process it is conceivable that the difference between the cell-free and whole-cell experiments reflects this. Other workers have also reported that there was no correlation between the results obtained with a triazole when using a cell-free system and broth

dilution (Hitchcock *et al.*, 1987). The results of the present study do not point to the cell wall of 132ACR being less permeable to azoles than the wall of 132A since they are the opposite of what would be expected in that instance as others have shown (Barrett-Bee *et al.*, 1988). Since 132ACR can switch between three colony morphology phenotypes, it is not possible to know which one or which combination of variants is predominant under specific experimental conditions. But if this type of derivative were to arise *in vivo* the same situation could obtain.

Derivative 132ACR appears to be resistant to the effects of the imidazole ketoconazole and the triazole fluconazole compared to its parental strain 132A. It does not seem to differ in its gross pattern of lipid and sterol production and the resistance may result from an alteration(s) in the sterol biosynthesis pathway. It is not possible to determine from these experiments whether the resistance is a result of the increased production of enzyme or a change in the target site. The emergence of azole resistance *in vivo* following protracted treatment has been reported previously and is a major cause for concern (Smith *et al.*, 1986). The development of decreased azole susceptibility in switched variants of *C. albicans* in the absence of prior exposure to azoles, as demonstrated in this study, could be equally important. Nutrient depletion has been shown to allow the isolation of colony morphology variants from *Candida* strains both at $25\ ^\circ\text{C}$ (Odds & Merson-Davis, 1989) and at the more physiological temperature of $37\ ^\circ\text{C}$ in this study. Levels of oral *Candida* in HIV⁺ and AIDS patients can be very high (Tylanda *et al.*, 1989; Samaranyake & Holmstrup, 1989) and it is possible that the prevalence of *Candida* cells in the oral cavities of these individuals could result in localized environments becoming nutrient-depleted. Such conditions might then provide the necessary stimulus for switching to occur and hence increase the possibility that some derivatives with reduced azole susceptibility be formed. This sequence of events could be one of the main reasons why treatment failure following oral and systemic azole therapy of oral candidosis in HIV⁺ and AIDS patients is being reported more frequently. The consequences, if this is the case, for the treatment of these patients will be of great importance in determining which antifungal drugs should be used and how they should be used.

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