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Novel antifungal agents which inhibit lanosterol 14α-demethylase in *Candida albicans* CCH442

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We have identified four non-azole inhibitors of lanosterol 14α-demethylase in *Candida albicans* CCH442. The most potent compound, A-39806, had IC_{50} values for ergosterol inhibition of 0.9 μM (0.3 mg/L) and 1.9 μM (0.6 mg/L) in whole cell and cell-free extract assays, respectively. A-39806 demonstrated broad in-vitro antifungal activity against several *Candida* species as well as against *Cryptococcus albidus* and *Aspergillus niger*. In-vitro antifungal activity was also demonstrated against a fluconazole-resistant clinical isolate of *C. albicans*.

Introduction

The increase in systemic fungal diseases has resulted in the increased use of broad-spectrum antifungal agents and the initiation of protocols for antifungal prophylaxis in patients at risk.[1,2] The groups at risk include individuals with AIDS, patients with meningitis or neoplastic diseases, burn victims, transplant patients and populations where mycoses are endemic.

The polyene, amphotericin B, has been the most effective antifungal agent against systemic infections. However, the introduction of several new azole compounds with increased potency and safety in the treatment of systemic mycoses has made long-term and prophylactic treatment possible.[3] The long-term use of azoles in some cases has led to drug resistance possibly due to alteration of the drug target, lanosterol 14α-demethylase, or of other sterol biosynthetic enzymes, changes in cellular levels of the demethylase enzyme, or changes in drug permeability.[4–8] A recent 5-year study of several clinically isolated *Candida* species reported a shift in population towards species more resistant to fluconazole during a period of increased azole use.[9]

Our search for non-azole inhibitors of 14α-demethylase that may be effective against azole-resistant organisms has identified several structurally unrelated demethylase inhibitors. One such compound, A39806, was active against fluconazole-sensitive and resistant strains of *Candida albicans* and inhibited the demethylase enzyme in whole cells and cell extracts from *C. albicans* CCH442.

Materials and methods

Chemicals

Miconazole was purchased from Sigma Chemical Co. (St Louis, MO, USA) and fluconazole from Pfizer, Inc. (Groton, CT, USA). Test compounds were synthesized at Abbott Laboratories. Radioisotopes were purchased from either Amersham (Arlington Heights, IL, USA) or DuPont NEN Research (Boston, MA, USA) All other chemicals were obtained from Sigma Chemical Co.

Strains and MICs

Strains used in MIC determinations are listed in Tables III and IV. MICs were determined by the broth micro- and macro-dilution methods.[10,11] *C. albicans* CCH442 was used in the whole cell and cell-free ergosterol assays.

Whole cell and cell-free ergosterol assays

C. albicans CCH442 was grown in yeast nitrogen base (YNB) plus 0.5% (w/v) dextrose or Sabouraud broth. Cells that were concentrated five-fold in 25 mM potassium phosphate buffer, pH 6.5, plus 0.5% dextrose were used in the whole cell assay while cells that were resuspended in 0.1 M potassium phosphate buffer, pH 7.4, were processed in a bead beater for use in the cell-free assay.[12]

The incorporation of [^{14}C]acetate into whole cell and [^{14}C]mevalonic acid into cell-free sterols has been

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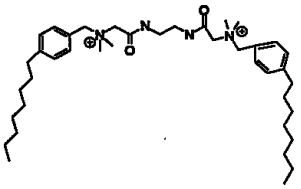
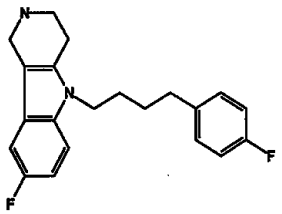
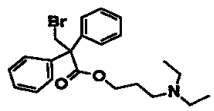
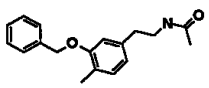
Table I. In-vitro antifungal activity of several demethylase inhibitors against *C. albicans* CCH442

Compound	MIC[a] (mg/L)	Dose[b] (mg/L)	% Inhibition of ergosterol whole cell	cell-free
A4741 	6.3	6.3	42	–
A39806 	3.1	3.1	90	–
A1111 	>100	85	–	50
A16190 	>100	56	–	50

[a] MIC using the micro-dilution method.
[b] Drug concentration used in the ergosterol biosynthesis assays.

described previously.[13,14] The extraction and analysis of radiolabelled sterols was performed as described previously.[12,13] In the whole cell assay, cells (5 mL, OD_{420} = 5.0) were pre-incubated with 50 μL of test compound or DMSO (dimethyl sulphoxide) at 30°C for 10 min, before addition of 7.4×10^4 Bq [U-^{14}C]acetate (0.5 mM, 2.96×10^7 Bq/mmol). Incubation was continued for 2 h at 30°C. Radiolabelled cells were pelleted by centrifugation (5000**g** for 10 min) and cell pellets were saponified at 90°C for 2 h.

In the cell-free assay, cell extract (2 mg protein) was pre-incubated with aerated phosphate buffer and 1% (v/v) DMSO or test compound in DMSO for 10 min at 30°C. Co-factors[14] and 3.7×10^4 Bq [2-^{14}C]mevalonic acid lactone (0.5 mM, 1.48×10^8 Bq/mmol) were added for a final volume of 0.5 ml and the mixture was incubated at 30°C for 2 h. Reaction was terminated by saponification at 90°C for 30 min under nitrogen. Extraction and analysis of radiolabelled sterols proceeded as described below.

Table II. Inhibition of ergosterol biosynthesis in *C. albicans* CCH442

Assay	IC_{50} (μM) A39806	miconazole	fluconazole
Whole cell	0.9	1.7	–
Cell-free	1.9	17.8	85

Table III. In-vitro antifungal activity of A39806, miconazole and fluconazole

Organism	MIC (mg/L) (n = 2)[a] A39806	miconazole	fluconazole
C. albicans ATCC62376	0.78–3.12	–	3.12–12.5
C. albicans ATCC10231	3.12–6.25	6.25–25	6.25
C. albicans 579A	1.25	12.5	3.12–6.25
C. albicans CCH442	3.12–6.25	12.5–25	3.12–6.25
C. tropicalis NRRL-Y-112	6.25–12.5	6.25–12.5	12.5–25
Candida (*Torulopsis*) *glabrata* ATCC15545	3.12	6.25–12.5	12.5–50
C. albidus ATCC34140	12.5	–	50–>100
A. niger ATCC16404	6.25	–	>100

[a] MICs were determined by the micro-dilution method.

Extraction and analysis of radiolabelled sterols

Non-saponifiable lipids (NSL) were extracted in petroleum ether. 5α-Dihydro[4-^{14}C]testosterone (37–74 Bq) was added to each tube as an internal standard before extraction. The petroleum ether was evaporated to dryness under nitrogen and the residue was taken up in 50 μL of cyclohexane, then spotted (10 μL) in triplicate on Merck no. 11845, 20 cm × 20 cm silica gel 60 plates with pre-concentration zone. Plates were developed in chloroform and NSL spots ergosterol, lanosterol, and squalene were quantified on a radioactive thin-layer chromatography scanner (Packard, Downers Grove, IL, USA). Radio active counts of each NSL were adjusted to the control values using the internal standard as a reference.

Results and discussion

We have identified four inhibitors of ergosterol biosynthesis in *C. albicans* CCH442 (Table I). These non-azole compounds increased 4,4-dimethyl sterols with a concurrent decrease in desmethyl sterols indicative of lanosterol 14α-demethylase inhibition (data not shown). Two of these compounds, A4741 and A39806, demonstrated in-vitro antifungal activity as well as inhibition of whole cell ergosterol biosynthesis.

The most potent compound in our screen of ergosterol inhibitors, A39806, produced IC_{50} values two- and nine-fold lower than the values obtained with miconazole in the whole cell and cell-free assays, respectively (Table II). Fluconazole in the cell-free assay had IC_{50} values 45-fold higher than values obtained with A39806. We could not obtain a reproducible linear dose-response with fluconazole in the whole cell assay.

A39806 demonstrated in-vitro activity against several *Candida* species with MICs ranging from 0.78 to 12.5 mg/L (Table III). This compares well with miconazole (MIC range = 6.25–25 mg/L) and fluconazole (MIC range = 3.12–50 mg/L). *Cryptococcus albidus* and *Aspergillus niger* were also more sensitive to A39806 than to fluconazole. Organisms such as *Candida (Torulopsis) glabrata* are intrinsically more resistant to some azoles.[3]

The susceptibility of several fluconazole-sensitive and -resistant clinical isolates of *C. albicans* to A39806 was investigated (Table IV). Fluconazole-sensitive (MIC ⩽ 0.5 mg/L) strains had A39806 MICs ranging from 0.5 to 1.0 mg/L, while strains moderately sensitive (MIC = 4–8 mg/L) to fluconazole had A39806 MICs ranging from 0.06 to 1.0 mg/L. Two highly resistant strains (fluconazole MIC = 32 and 64 mg/L) remained sensitive to A39806 (MIC = 1.0 mg/L).

Long-term or prophylactic azole treatment may result in the selection for azole-resistant organisms such as *C. (Torulopsis) glabrata* or in the development of azole-resistant candida strains which has occurred in some

Table IV. In-vitro antifungal activity of A39806 and fluconazole against azole sensitive and resistant strains of *C. albicans*[a]

Organism	MIC (mg/L)[b] A39806	fluconazole
C. albicans ATCC90028	0.5	0.5
C. albicans OY2-76	1.0	0.25
C. albicans OY2-79	1.0	0.5
C. albicans OY3-55	0.5	4.0
C. albicans 1378-17	0.06	4.0
C. albicans 1378-48	1.0	8.0
C. albicans OY8-47	1.0	32
C. albicans 04-81	1.0	64

[a] Strains were obtained from Dr M. A. Pfaller, Department of Pathology, University of Iowa College of Medicine, Iowa City, IA 52242, USA.
[b] MICs were determined by the macrodilution method.

AIDS patients suffering from oropharyngeal and oesophageal candidiasis.[9,15,6] The discovery of A39806 demonstrated the feasibility of identifying novel demethylase inhibitors with broad-spectrum antifungal activity.

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