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CONCISE ARTICLE

Antifungal activity of 25-azalanosterol against *Candida* species

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Abstract The antifungal properties of 25-azalanosterol was investigated. Compared to normal antifungal reagents, fluoconazole, clotrimazole and voriconazole, it exhibited significant anti-*Candida* activity (the minimum inhibitory concentration [MIC] ranges were 0.125–8, 0.5–8 and 0.5–32 μg/mL against *C. albicans*, *C. krusei* and *C. glabrata*, respectively), but showed little toxicity to mice liver cells at clinical dosage after 24 h of exposure, with the lowest lactate dehydrogenase and the highest ED_{50} compared to four other azoles antifungal agents. 25-Azalanosterol inhibited the incorporation of [*methyl*-$^3H_3$] AdoMet into the C-24 of ergosterol in whole cells of *C. albicans*. Thus, 25-azalanosterol, as an inhibitor of the growth of *C. albicans* in vitro, may have considerable potential as a new class of anti-*Candida* agent that lacks toxic side effects in the mammalian host.

Introduction

The occurrence frequency of human fungal infections have been increasing over the past decade in response to a combination of factors, including advances in invasive surgical techniques which allow for opportunistic pathogen access, immuno-suppression employed in transplantation or resulting from chemotherapy, disease such as acquired immune deficiency syndrome (AIDS), and the frequent use of cytotoxic and/or antibacterial drugs [1]. Candidiasis was caused by *Candida* species; typically, *C. albicans* was the predominant causal organism of most candidiasis [2]. Other species, including *C. krusei*, *C. glabrata*, *C. dubliniensis* and *C. inconspicua*, have been recognised [3].

Until recently, fluoconazole and amphotericin B were the standard therapy for many fungal infections, but a high frequency of renal or hepatic toxicity had limited their use [4]. Although many research groups have carried out intensive research in attempts to develop new antifungal drugs, and some of which have now entered clinical trials, some new candin antifungal agents were also investigated [5]. Moreover, side effects were noted for currently available antimycotics at the therapeutic dosage and reports of fungal pathogens resistant to antifungal agents were increasing in frequency [6]. The success in developing a new drug would depend upon the identification of new antifungal targets that had no mammalian host counterpart, but a broad spectrum of pathogenic fungi [7].

The current treatment of this fungal infection involved chemotherapy with azole antifungal drugs which could inhibit the cytochrome P-450-dependent 14a-demethylation of lanosterol or 24-methylene-24,25-dihydrolanosterol, resulting in a decreased availability of an essential membrane component, such as ergosterol, and a corresponding accumulation of 14a-methylated sterols in cell membranes, which eventually lead to cell growth arrest [8]. We have previously demonstrated a series of sterol substrate analogues which significantly inhibited the growth of *Candida* species [9]. Here, compared to normal antifungal agents, fluoconazole, clotrimazole, ketoconazole and voriconazole, we describe the effects of a lanosterol derivative, 25-azalanosterol (Fig. 1) on: (i) the growth of *Candida* species; (ii) activities of *Candida* sterol C-24

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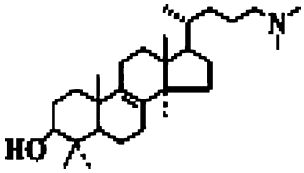
Fig. 1 Structure of 25-azalanosterol



methyltransferase (SMT; E.C. 2.1.1.142); and (iii) mammalian rat liver toxicity. Our results indicated that fungal growth inhibition by low levels of 25-azalanosterol was clearly caused by the inhibition of sterol biosynthesis. Also, 25-azalanosterol had the lowest liver toxicity compared to other antifungal agents. So, 25-azalanosterol may have considerable potential as a novel anti-*Candida* agent that lacks mammalian host toxicity.

Materials and methods

Materials and reagents

The fungal strains used in this study were *C. albicans*, *C. krusei* and *C. glabrata*, which were clinically isolated from candidiasis patients by the Department of Clinical Laboratory, Shanxi Modern Woman's Hospital, China. The cells were maintained on slants of Sabouraud's dextrose agar at 4°C. For culture, the cells were inoculated and grown at 35°C for 18 h in YEPD medium (yeast extract, 10 g/L; peptone, 20 g/L; bidest water, 960 mL, after autoclaving, 20mL of sterile glucose solution was added (50% wt/vol), pH 7.0; Meidi Biochemical Product Co., Wuxi, China) [10]. Late exponential phase cells (18 h) were harvested and washed by centrifugation, and used for the preparation of microsomes.

The antifungal agents, fluoconazole, clotrimazole, ketoconazole and voriconazole, were purchased from Kangning Medicine Co. (Shandong, China). 25-Azalanosterol was synthesised and supported by the Shanxi Chemical Industrial Institute. AdoMet iodide salt was purchased from Sigma and [*methyl*-$^3H_3$] AdoMet (10–15 Ci/mmol) was purchased from PerkinElmer Life Sciences. All other reagents and chemicals were purchased from Sigma Co. Ltd.

BALB mice (Experimental Animal Laboratory, Shanxi Medical University), 70 days old, were divided into five different groups (six mice/group) and were housed singly. The liver from six mice in each group at 70 days were dissected separately and cultivated in RPMI1640 medium. The cells were mechanically dispersed by repeated triturating and filtered through a 200-μm metal mesh. Two-millilitre aliquots of the cell suspension were plated at a final density of 10^6 cells/mL for cytotoxicity analysis. Hepatic cytotoxicity was evaluated by lactate dehydrogenase (LDH) activity and MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) in mice liver homogenates.

Inhibition of growth

Minimum inhibitory concentrations (MICs) to *C. albicans* (n=53), *C. krusei* (n=13) and *C. glabrata* (n=21) were estimated after inoculation in RPMI1640 medium containing various doses of antifungal agents, 25-azalanosterol, fluoconazole, clotrimazole, ketoconazole and voriconazole. The preincubation of each *Candida* species were carried out at 35°C, and late exponential phase cells were washed with PBS (pH 7.0) and resuspended in RPMI medium. The antifungal agents were dissolved in dimethyl sulphoxide (DMSO) and then added to inoculation, with a final cell concentration of approximately 1.5×10^6 cells/mL The concentrations of the antifungal agents were varied from 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 to 128 μg/mL. The diluted *Candida* cells with the addition of varied doses of antifungal agents were incubated at 35°C for 24 h. Growth was determined by cell counts and quantisation of the numbers of colony-forming units (CFU) [11].

Microsomal preparation

The preparation of microsomes of *C. albicans* was carried out by a method described previously [12]. The cells were broken with a French Pressure cell at 20,000 psi and the cell debris was removed by centrifugation at 100,000g for 1 h at 4°C. Microsomal pellets were resuspended to a final protein concentration of 10 mg/mL, and the mixture was stored at −80°C until use. Protein concentration was determined at A_{600nm} with bovine serum albumin as a standard. Assays for C24-SMT activity were conducted as described in our earlier studies [13]. The activity of SMT catalysis was characterised with respect to the observed ability of the enzyme to produce hexane-extractable ^3H in the presence of [*methyl*-$^3H_3$] AdoMet. Briefly, the reaction mixture of freshly prepared enzyme in a final volume of 600 μL contained varying concentrations of sterol substrate from 5 to 100 μM, a fixed amount of 100 μM [*methyl*-$^3H_3$] AdoMet (0.6 μCi) and Tween 80 (1%, v/v), and 100 μL of *C. albicans* SMT and was incubated for 60 min. The enzymatic reaction was terminated by the addition of 600 μL of 10% methanolic KOH, followed by boiling for 20 min and extracting with three 2.5-mL portions of hexane [14].

Sterol biosynthesis assay

Cells of *Candida* species were incubated with radiolabelled substrates, [*methyl*-$^3H_3$] AdoMet. Then, washed cells were incubated in buffer A (50 mM Tris-HCl, 2 mM $MgCl_2$, 2 mM β-mercaptoethanol and 20% glycerol (v/v), pH 6.8) and harvested after 18 h at 30°C by centrifugation at 10,000g for 20 min. The washed cells were resuspended

in buffer B (0.1 mM potassium phosphate buffer, including 1 mM EDTA, 0.5 mM dithiothreitol and 20% (vol/vol) glycerol). Test compounds dissolved in DMSO were added to the cell suspensions (980 μL, 10^8 cells/mL). The reaction mixture also consisted of cofactor solution (100 μl; containing 1 μmol of $NADP^+$, 1 μmol of NADPH, 1 μmol of NAD^+, 3 μmol of glucose-6-phosphate, 5 μmol of ATP and 3 μmol of reduced glutathione) and divalent cation solution (10 μL of 0.5 mM $MgCl_2$ and 5 μL of 0.4 mM $MnCl_2$). The reaction was initiated by the addition of 100 μL of [*methyl*-$^3H_3$] AdoMet and the cells were incubated at 30°C for 1.5 h with shaking (110 rpm). The reaction was stopped by the addition of 1 mL 10% (w/v) KOH, 90% ethanol, and the samples were saponified at 80°C for 30 h [15]. The saponified lipids were then extracted with hexane and combined for separation by high-performance liquid chromatography (HPLC). All of the collections of each peak from HPLC were analysed by combined capillary gas chromatography-mass spectrometry (GC-MS), performed on a Hewlett-Packard 6890 GC-quadropole mass selective detector interfaced with a Hewlett-Packard Chemstation. A fused silica capillary column (0.25 μM/mm, i.d. 30 μm) coated with a 0.25-μm film of ZB-5 purchased from Phenomenex, Torrrance, CA, was employed with He as the carrier gas (10 psi) set at 1.2°C (1 min hold) to 280°C at 20°C/min. EI were recorded at 70 eV with the electron multiplier voltage set at 2,200 V. Full mass spectra were obtained and the products identified by a comparison of the retention time and spectrum to those of the authentic standard referenced to cholesterol [12].

LDH assay for hepatic damage

Liver cells collected from 70-day-old mice were resuspended with a final density of 1.5×10^6 cells/mL in RPMI1640 medium and incubated for 60 min with a certain dose (maximum of each MICs determined above) of antifungal agents at 37°C, 5% CO_2. As a quantitative measure of cellular toxicity, the cytotoxic release of LDH in the culture medium was estimated using the CytoTox96 non-radioactive cytoxicity assay kit purchased from Promega Co. Ltd. The activities of LDH released into the culture medium were determined spectrophotometrically by A_{490nm} at 3, 6, 12 and 24 h separately [16].

MTT assay for hepatic proliferation

MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Promega Co. Ltd.) was a yellow water-soluble tetrazolium dye that was reduced by liver cells to a water-insoluble purple formazan. The amount of formazan could be determined spectrophotometrically. The A_{490nm} of liver cells preincubated with varied concentrations of antifungal agents (0, 2.5, 5, 7.5, 12.5, 25, 37.5 and 50 μg/mL) were recorded and plotted in order to determine the ED_{50}, which is the concentration of antifungal agents giving one-half inhibition to cell proliferation [17].

Results

Effect of 25-azalanosterol on the growth of *Candida* species

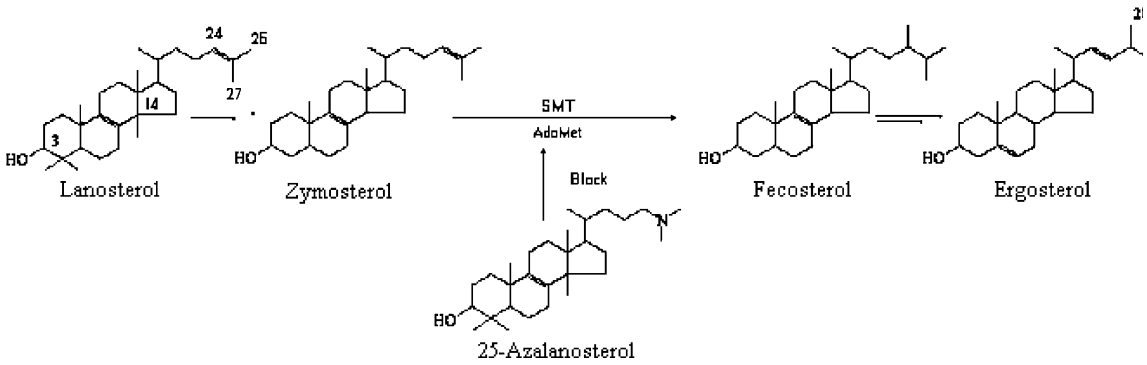
The antifungal activities of 25-azalanosterol, fluoconazole, clotrimazole, ketoconazole and voriconazole against *Candida* species were investigated and the results are summarised in Table 1. 25-Azalanosterol showed a wide range of anti-*Candida* activities, with MIC values ranging from 0.125 to 32 μg/mL. In particular, 25-azalanosterol showed significant potency against *C. albicans*, *C. krusei* and *C. glabrata*, which were close to clotrimazole, ketoconazole and voriconazole, but much greater than fluoconazole.

Effect of 25-azalanosterol on sterol biosynthesis

To determine the mechanism and target of growth inhibition by 25-azalanosterol, the relative rates of total cellular sterol synthesis in whole-cell homogenates of *Candida* species were measured in the presence of 80 μM 25-azalanosterol

Table 1 Minimum inhibitory concentrations (MICs) of 25-azalanosterol and other antifungal agents against *Candida* species

Candida species	MIC range (MIC_{50}, MIC_{90}) (μg/mL)				
	25-Azalanosterol	Fluoconazole	Clotrimazole	Ketoconazole	Voriconazole
C. albicans	0.125–8	0.125–8	0.125–16	0.125–4	0.125–4
(*n*=53)	(0.5, 4)	(0.5, 4)	(0.25, 4)	(0.125, 1)	(0.125, 2)
C. krusei	0.5–8	0.5–32	0.5–8	0.125–8	0.5–16
(*n*=13)	(0.5, 4)	(0.5, 16)	(0.5, 8)	(0.125, 4)	(0.5, 8)
C. glabrata	0.5–32	2–32	0.5–32	0.5–32	0.5–8
(*n*=21)	(1, 16)	(4, 32)	(1, 16)	(2, 16)	(0.5, 8)



Scheme 1 Lanosterol-ergosterol pathway and blockage by 25-azalanosterol

with [*methyl*-$^3H_3$] AdoMet. This approach was based on our earlier observation that 25-azalanosterol is a potent inhibitor of C24-SMT activity (Scheme 1) [14]. In this experiment, [*methyl*-$^3H_3$] AdoMet was added to *Candida* species that had been cultured previously in a medium without amino acids, in phosphate buffer containing glucose (2%, w/v). As shown in Fig. 2, the incorporation of [*methyl*-$^3H_3$] AdoMet into the C-24 of ergosterol was especially inhibited around 50% by the presence of 80μM 25-azalanosterol. The decrease in the incorporation of [*methyl*-$^3H_3$] AdoMet into ergosterol appeared to be a consequence of the inhibition of reactions involving C24-SMT, which was the key enzyme that catalysed the transmethylation of sterols from the precursor. Our previous study also indicated a dose-dependent inhibition of the rate of [*methyl*-$^3H_3$] AdoMet incorporation into ergosterol by 25-azalanosterol (IC_{50} 20μM) [12].

Hepatic toxicity of 25-azalanosterol on mice liver cells

LDH is released when cells are damaged or destroyed. Because of this, the LDH test could be used as a general marker of injury to cells or as a monitor of progressive conditions [17]. So, the LDH of mice liver cells exposed to antifungal agents for certain periods were analysed. From Fig. 3, it is clear that 25-azalanosterol exhibited the lowest inhibition compared to clotrimazole, ketoconazole and voriconazole, and especially much lower than fluoconazole. Meanwhile, since the MTT provided a more efficient format for assessing cellular proliferation, the critical component of the CellTiter 96 AQueous One Solution assay was used in this study to analyse the effect of these five antifungal agents on mice liver cell proliferation. Figure 4 shows the ED_{50} and MICs of mice liver cells

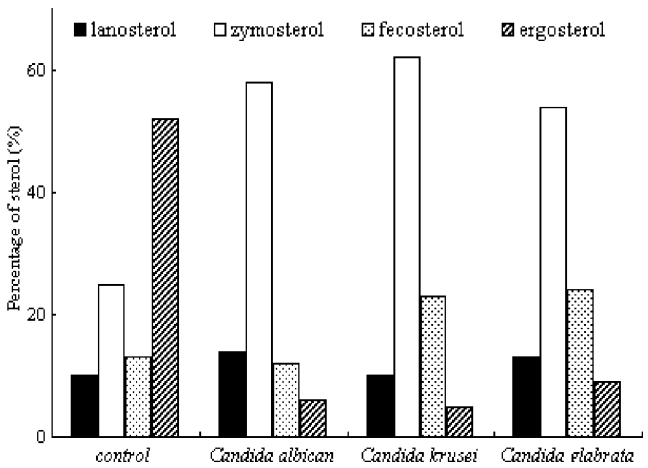


Fig. 2 Sterols biosynthesised in *Candida* species after incubation with the addition of 80 μM 25-azalanosterol. The sterol concentrations in the supernatant of cell homogenates were analysed by high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). The experimental results are shown as the average of three repetitions

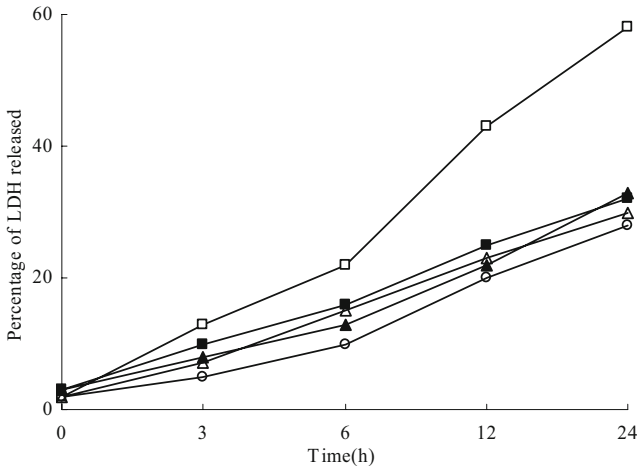


Fig. 3 Percentage of cytotoxic release of lactate dehydrogenase (LDH) in the culture medium of mice liver cells exposed to certain doses of antifungal agents: 32 μg/mL fluoconazole (*open squares*); 32 μg/mL ketoconazole (*closed squares*); 32 μg/mL clotrimazole (*open triangles*); 16 μg/mL voriconazole (*closed triangles*); 32 μg/mL 25-azalanosterol (*open circles*). The results are shown as the average of three repetitions

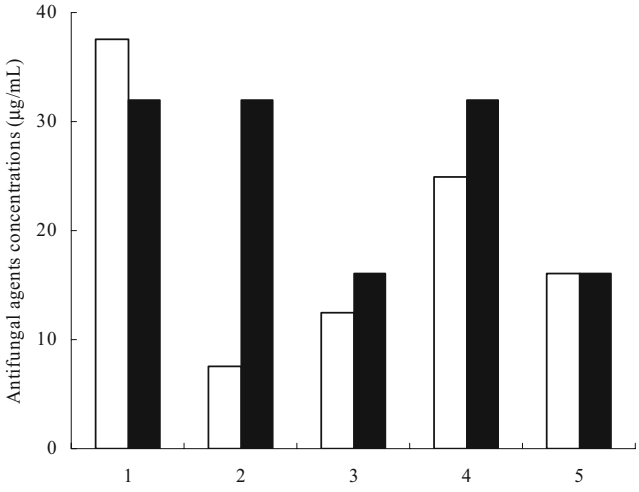


Fig. 4 Comparison of minimum inhibitory concentrations (MICs) (*solid bars*) to the ED_{50} (*open bars*) of mice liver cells exposed to certain concentrations of antifungal agents. 1: 25-azalanosterol; 2: fluoconazole; 3: clotrimazole; 4: ketoconazole; 5: voriconazole. The results are shown as the average of three repetitions

exposed to five antifungal agents for 24 h. Clearly, the ED_{50} of 25-azalanosterol was the highest, which meant that its toxicity to liver cells was the lowest. Also, their ED_{50} and MICs were significantly different. Except for 25-azalanosterol, the ED_{50} of the other three antifungal agents, fluoconazole, clotrimazole and ketoconazole, were much lower than their maximal MICs, while the two values of voriconazole were close. This meant that 25-azalanosterol was the only agent which exhibited, at the clinical dosage level, little toxicity to liver cell, because its ED_{50} was higher than the maximum MIC, which suggests that 25-azalanosterol may have some form of indirect effect on C24-SMT in vivo.

It is well known that mammals and fungi share many reactions in their pathways of sterol biosynthesis [18], so we investigated whether lanosterol analogue, 25-azalanosterol, also inhibited mammalian cholesterol biosynthesis. As shown in Fig. 2, 25-azalanosterol (80 μM) almost completely blocked ergosterol biosynthesis by causing the accumulation of lanosterol and zymosterol, which, otherwise, would have been demethylated by 14-demethylase in *Candida* species, but exhibited virtually much less inhibition to mice hepatic cholesterol biosynthesis, and little toxicity to mammalian cells at the clinical dosage level.

Discussion

The azoles are fungistatic drugs by the inhibition of the cytochrome P-450-mediated removal of the C-14 methyl group from the ergosterol precursor, lanosterol, which are then subjected to the accumulation of resistant phenotypes due, in part, to the need of continuous administration to patients who are immuno-compromised [19]. Resistance has been reported in *C. albicans* as well as in other species of *Candida* [20, 21]. In addition, other fungal pathogens, including species of *Histoplasma*, *Cryptococcus* and *Aspergillus* [2], have been the subjects of recent reports on azole resistance. The increase in infections, coupled with the reduced efficacy of the currently available drugs, makes the discovery and development of new antifungal agents an urgent matter. The pathway for fungal sterol biosynthesis had provided an excellent target for antifungal development, but there remain additional sites in the pathway that have not been thoroughly investigated.

The identification of a novel antifungal drug with unique modes of action is desirable, since fungi resistant to currently available antifungal agents would unlikely be cross-resistant to these newer drugs. Based on our previous results that described the anti-*Candida* activities of 25-azalanosterol [12, 18], here, we set out a new class of drug derived from lanosterol that may be targetted against major enzymes involved in fungal growth. Our results indicated that the chemical modification of the natural lanosterol, such as the addition of a methyl, fluoro or bromol group, could lead to significant improvements in inhibitory activity against the growth of *C. albicans* [13, 22]. This modification was likely to have made the compound more lipophilic in nature, which, in turn, may have made it a more effective inhibitor of enzymes involved in lipids. However, this modification appeared to have changed its inhibitory behaviour against C24-SMT in vitro. In contrast, the inhibition abilities of fluoconazole or clotrimazole were not related to the inhibition ability of sterol C-14 demethylation, which were consistent with the results obtained in other studies [23]. Borgers et al. [24] reported that, in addition to their effects on sterol synthesis, the triazole antifungal agents affected ATPase as well as other membrane enzymes, membrane transport and fatty acid metabolism, which caused physical membrane damage. Thus, although 4-methyl-lanosterol effectively blocked the C24-SMT reaction in vivo, it failed to directly inhibit this enzyme activity in vitro. One explanation for this result may be that 25-azalanosterol was metabolised by the fungus to an inhibitor of C24-SMT activity. Alternatively, 25-azalanosterol may have had an, as yet unidentified, indirect effect on the C24-SMT reaction in vivo, which may mean that derivatives of lanosterol will represent a new class of antifungal compound with low host toxicity. These compounds may provide a safer alternative to fluoconazole or clotrimazole.

In conclusion, our results suggested that 25-azalanosterol has promising selective anti-*Candida* activity and deserves further in vitro and in vivo investigation.

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