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Antifungal activity of bis-azasqualenes, inhibitors of oxidosqualene cyclase

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Summary

The antifungal activity and *in vitro* toxicity toward animal cells of two inhibitors of oxidosqualene cyclase, squalene *bis*-diethylamine (SBD) and squalene *bis*-diethylmethylammonium iodide (SBDI) were studied. Minimum inhibitory concentration (MIC) against dermatophytes and other fungi involved in cutaneous and systemic infections (12 isolates from seven species) were determined by the broth microdilution method based on the reference documents M38-A and M27-A2 of Clinical and Laboratory Standards Institute (CLSI). Both compounds exerted fungistatic activities, although with different action. SBDI was the more active compound and displayed low MIC values (in the 3.12–12.5 $\mu\text{g ml}^{-1}$ range) against *Microsporum canis*, *Trichophyton mentagrophytes* and one isolate of *Scopulariopsis brevicaulis*, while SBD showed MIC values against these species in the 3.12–25 $\mu\text{g ml}^{-1}$ range. Toxicity was tested on Madin-Darby canine kidney (MDCK) epithelial cells and human microvascular endothelial cells (HMEC). SBDI proved the less toxic compound: it inhibited *M. canis*, *T. mentagrophytes* and *S. brevicaulis* at concentrations below those found toxic for MDCK cells. HMEC were the more sensitive cells.

Introduction

Since the 1980s, the incidence of fungal infections has increased with a parallel emergence of new fungal pathogens.^{1,2} The past decade has also seen a significantly increased prevalence of resistance to antimicrobial and antifungal agents. Substantial attention has thus been focussed on improving our understanding of the mechanisms of antimicrobial resistance, finding better ways to detect resistance when it occurs, and developing new antimicrobial options to treat infections caused by resistant organisms. The need for effective new antimycotic drugs is also as a result of the spread of

HIV infections, which has contributed to increasing the number of immunocompromised patients, in whom atypical manifestations and more severe and extensive lesions may occur.^{3,4}

2,3-Oxidosqualene cyclase (OSC) (EC 5.4.99.7) is a widely distributed enzyme, which catalyses the cyclisation of (3*S*)-2,3-oxidosqualene (OS) to lanosterol in mammals and fungi and to cycloartenol or to a variety of tetracyclic and pentacyclic triterpenes, such as cucurbitadienol, parkeol and α - or β -amyrin in higher plants.⁵⁻¹² The cyclisation of OS starts with the protonation of the epoxide by a suitable electrophilic residue present in the enzyme, to give a first C-2 carbonium ion intermediate, and it proceeds through the formation of a series of carbonium ion intermediates or high energy intermediates. Various inhibitors of sterol biosynthesis are widely used in therapy as antifungal drugs acting by inhibiting different enzymes of sterol biosynthesis: the azoles are inhibitors of lanosterol C₁₄-demethylase¹³ and the allylamines inhibit squalene epoxidase.¹⁴ For many years, we have studied OSC inhibitors. Initially, they were obtained by mimicking the carbocationic intermediates formed during cyclisation of OS, designing squalene-derived structures in which the positively charged carbocation was replaced by a nitrogen.¹⁵⁻¹⁷

In this study, we determined the minimum inhibitory concentration (MIC) against dermatophytes and other fungi involved in cutaneous and systemic infections of two inhibitors of OSC: squalene *bis*-diethylamine (SBD) and squalene *bis*-diethylmethylammonium iodide (SBDI) (Fig. 1). The *in vitro* toxicity against animal cells was also tested.

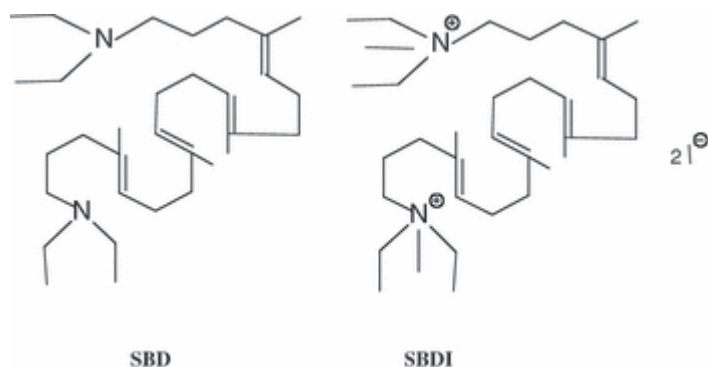


Figure 1. Squalene *bis*-diethylamine (SBD) and Squalene bis-diethylmethylammonium iodide (SBDI) structures.

The azasqualenes studied mimic the transient C-2 carbonium ion arising from the opening of the oxirane ring of OS. We have found that quaternarisation or doubling of the tertiary amine function in the azasqualene backbone enhanced the inhibition activity towards fungi; in particular, among these series, SBD and SBDI displayed the highest inhibition activity towards fungal OSC.

Materials and methods

Chemistry

¹H NMR spectra were recorded on a Bruker AC 300 instrument (Bruker, Karlsruhe, Germany) for samples in CDCl₃ solution at room temperature, with Me₄Si (TMS) as internal standard. Coupling constants (*J*) are given in Hz. Mass spectra were recorded on a Finnigan MAT TSQ 700 spectrometer (San Jose, CA, USA). Microanalyses were determined on an elemental analyser 1106 (Carlo Erba Strumentazione, Milano, Italy) and were within $\pm 0.3\%$ of the theoretical values. The reactions were monitored by TLC on F₂₅₄ silica gel precoated sheets (Merck, Damstadt, Germany); after development, the sheets were exposed to iodine vapour. Flash-column chromatography was

performed on 230–400 mesh silica gel. Tetrahydrofuran was dried over sodium benzophenone ketyl. All solvents were distilled prior to flash chromatography. Squalene, lanosterol and polyoxyethylene 9 lauryl ether were from Sigma Chemical Co. (St. Louis, MO, USA).

Synthesis of SBD and SBDI

Initially the synthesis of hexanorsqualene dialdehyde was obtained, according to a procedure developed by us, by reaction of squalene with *N*-bromosuccinimide in aqueous tetrahydrofuran, which allowed the selective formation of the terminal mono- and di-bromohydrins. After a multistep chromatographic purification procedure repeated then for each reaction step, it was converted to the external diepoxide with K_2CO_3 in methanol, followed by a one-step cleavage with HIO_4 in diethyl ether to afford hexanorsqualene dialdehyde.¹⁵

Squalene-bis-diethylamine (Fig. 1).

Hexanorsqualene dialdehyde, obtained as previously reported (1 g, 2.8 mmol) was dissolved in anhydrous tetrahydrofuran (20 ml) and diethylamine (x10, 1.9 g, 26 mmol) was added under stirring at 0 °C. A solution of HCl in anhydrous tetrahydrofuran was added dropwise up to pH 3, followed by sodium cyanoborohydride ($NaBH_3CN$) (163 mg, 2.6 mmol). After stirring at room temperature for 30 min, the reaction mixture was diluted with water and extracted with petroleum ether. The organic layer was washed with saturated brine, dried over anhydrous sodium sulphate and evaporated *in vacuo*. The crude product was purified by flash chromatography with methanol as eluant, to give 608 mg of SBD (46% yield), as a colourless oil. 1H NMR ($CDCl_3$): δ , 0.97 [t, 12 H, $J = 7$ Hz, 2 (CH_3CH_2)₂N], 1.55–1.63 (m, 16 H, allylic CH_3 and 2 CH_2CH_2N), 1.90–2.05 (m, 16 H, allylic CH_2), 2.38 (t, 4 H, $J = 8$ Hz, 2 CH_2CH_2N), 2.51 [q, 8 H, $J = 7$ Hz, 2 (CH_3CH_2)₂N], 5.00–5.19 (m, 4 H, vinylic CH). MS (EI): m/z 473 (M^+ , 21), 458 (15), 445 (15), 444 (33), 387 (37), 374 (10), 305 (19), 236 (21), 168 (100), 112 (45), 99 (95). *Anal.* $C_{32}H_{60}N_2$ (472.84); calcd: C, 81.29; H, 12.79; N, 5.92; found: C, 81.31; H, 12.80; N, 5.89.

Squalene-bis-diethylmethylammonium iodide (Fig. 1).

SBD (100 mg, 0.212 mmol), absolute ethanol (10 ml), K_2CO_3 (586 mg, 4.24 mmol), CH_3I (301 mg, 2.12 mmol) were added sequentially and the reaction mixture was heated under reflux for 15 h. After evaporation of ethanol, water (50 ml) was added, the reaction mixture extracted with dichloromethane (50 ml \times 3), dried over anhydrous sodium sulphate and evaporated *in vacuo*, to give 152 mg of SBDI. 1H NMR ($CDCl_3$): δ , 1.36–1.70 [m, 28 H, allylic CH_3 , 2 $CH_2CH_2N^+$ and 2 (CH_3CH_2)₂ N^+], 1.88–2.20 (m, 16 H, allylic CH_2), 3.30 (s, 6 H, 2 CH_3N^+), 3.58 [broad q, 12 H, 2 $CH_2N^+(CH_2CH_3)_2$], 5.00–5.19 (m, 4 H, vinylic CH). A satisfactory electron impact mass spectrum could not be obtained.

Biological assays

In vitro susceptibility testing.

The fungi tested, with the sole exception of *Aspergillus fumigatus*, were isolated from man and are listed in Table 1. They were preserved, as actively growing cultures, on Sabouraud dextrose agar (Oxoid, Milan, Italy) and stored at 7 °C at the Mycological Collection *Mycotheca Universitatis Taurinensis* (MUT) of the Department of Plant Biology of the University of Turin (Italy). Prior to testing, each isolate was subcultured on potato dextrose agar (PDA; Sigma-Aldrich, Milan, Italy) to check the purity and viability of the inoculum.

Table 1. Tested fungi.

Species	MUT n°	Source
<i>Aspergillus flavus</i> Link var. <i>flavus</i>	3724	man onychomycosis
<i>A. flavus</i> var. <i>flavus</i>	3725	man onychomycosis
<i>Aspergillus fumigatus</i> Fresenius var. <i>fumigatus</i>	2214	air of a compost facility
<i>Cryptococcus neoformans</i> (San Felice) Vuillemin	346	HIV positive man
<i>Microsporium canis</i> E. Boidin	3632	virulent isolates from man
<i>M. canis</i>	3635	virulent isolates from man
<i>Microsporium gypseum</i> (E. Bodin) Guiart & Grigoraki	3622	virulent isolates from man
<i>Scopulariopsis brevicaulis</i> (Saccardo) Bainier	2385	man onychomycosis
<i>S. brevicaulis</i>	2387	man onychomycosis
<i>S. brevicaulis</i>	2389	man onychomycosis
<i>S. brevicaulis</i>	3235	man onychomycosis
<i>Trichophyton mentagrophytes</i> (C.P. Robin) R.Blanchard	3621	virulent isolates from man

Susceptibility testing was carried out following the procedures described by Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards, NCCLS) M27-A2¹⁸ for *Cryptococcus neoformans* and M38-A¹⁹ for the filamentous fungi. The growth medium for broth microdilution susceptibility testing was RPMI 1640 with L-glutamine and without sodium bicarbonate (Sigma-Aldrich) buffered to pH 7.0 with morpholinopropane-sulphonic acid (MOPS; Sigma-Aldrich). Inoculum suspensions of the fungi were prepared by means of a NaCl 0.85% sterile solution, from mature 7- to 14-day-old cultures grown on PDA. Suspensions of filamentous fungi were adjusted spectrophotometrically ($\lambda = 590$ nm) by means of a Turbidimeter (Biolog, USA) to ODs that ranged from 0.09 to 0.11 (78–82% transmittance) and then diluted 1 : 50 in RPMI 1640 to obtain an inoculum size of approximately 0.4×10^4 – 5×10^4 CFU ml⁻¹. Suspension of *C. neoformans* was adjusted to the density of 0.5 McFarland turbidity standard by spectrophotometric method ($\lambda = 600$ nm) (Ultrospec 3300 Pro; Amersham Biosciences, Little Chalfont, UK) and then diluted 1 : 2000 in RPMI 1640 to obtain an inoculum size of approximately 1×10^6 – 5×10^6 CFU ml⁻¹. Inoculum size of all tested fungi was verified by plating in triplicate 0.01 ml of a 1 : 100 diluted inoculum sample on PDA plates. Plates were incubated at 28 °C and colonies were counted as CFU ml⁻¹ when growth became visible. MIC evaluation was carried out in 96-well flat-bottomed plates. Serial twofold drug (SBD, SBDI) dilutions, ranging from 200 to 0.78 $\mu\text{g ml}^{-1}$, were prepared in RPMI 1640 pH 7.0 according to CLSI guidelines. Each well was inoculated with 0.1 ml of the 2x inoculum suspension and with 0.1 ml of the 2x drug dilution. This resulted in the appropriate final concentration in each well of medium (1x RPMI 1640), drugs (100–0.39 $\mu\text{g ml}^{-1}$) and fungal inoculum (approximately 0.2×10^4 – 2.5×10^4 CFU ml⁻¹ for filamentous fungi; 0.5×10^6 – 2.5×10^6 CFU ml⁻¹ for *C. neoformans*). MICs were determined in triplicate for each isolate; growth and sterility controls were included. Microdilution trays were incubated at 28 °C and MIC end points were read visually (by comparing the growth inhibition in each well with that of the control well) at the lowest drug concentration that prevented 100% growth at 24 and 48 h (*A. fumigatus*, *Aspergillus flavus* and *C. neoformans*) or at 72 and 96 h (remaining isolates).

Toxicity test on Madin-Darby canine kidney epithelial cells and human microvascular endothelial cells

Madin-Darby canine kidney (MDCK) cells were seeded in 24-wells plate (2×10^5 cells/plate) in Dulbecco medium (Sigma–Aldrich) supplemented with 10% foetal bovine serum. Confluent cells were treated with the previously described concentrations of SBD or SBDI. Human microvascular endothelial cells (HMEC) were plated on gelatin-coated plastic in 96-well plates in endothelial basal medium (EMB) containing 10% foetal bovine serum at a density of 1400 cells/well. After 18 h, the medium was replaced with fresh EMB, either alone (control) or supplemented with the previously described concentrations of SBD and SBDI. Cell viability quantisation was assessed 24 and 72 h after treatment, using the colorimetric diphenyltetrazolium bromide cell proliferation kit assay (MTT; Roche Diagnostic, Basel, Switzerland) and following the manufacture’s protocol. Absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 690 nm. The Student’s *t*-test was used to assess the significance ($P \leq 0.05$) of differences between results of treatment and control samples. The morphology of MDCK cells and HMEC after treatment with SBD and SBDI was observed by light microscopy.

Results

MIC values of SBD and SBDI are shown in [Table 2](#). Both compounds exerted activity, although with different action. MIC values ranged from 100 or $>100 \mu\text{g ml}^{-1}$ to $3.12 \mu\text{g ml}^{-1}$, but SBDI displayed MIC values of $50 \mu\text{g ml}^{-1}$ or below in 10 cases out of 12 (all fungi except the two isolate of *A. flavus*), while SBD did so in seven (all dermatophytes and three isolates of *Scopulariopsis brevicaulis*). *Microsporium canis* was the most sensitive species with MIC values ranging from $12.5 \mu\text{g ml}^{-1}$ (SBD toward MUT 3635) to $3.12 \mu\text{g ml}^{-1}$ (SBD toward MUT 3632 and SBDI toward both isolates); it was followed by *S. brevicaulis* MUT 2385 and *Trichophyton mentagrophytes*. *Aspergillus flavus* was the least sensitive species with MIC values of 100 or $>100 \mu\text{g ml}^{-1}$; it was followed by *C. neoformans* and *S. brevicaulis* MUT 2389.

Table 2. MIC of SBD and SBDI on fungus species.

Species	MUT n°	MIC ($\mu\text{g ml}^{-1}$)	
		SBD	SBDI
1. MIC, minimum inhibitory concentration; SBD, squalene <i>bis</i> -diethylamine; SBDI, squalene <i>bis</i> -diethylmethylammonium iodide.			
<i>Aspergillus flavus</i> var. <i>flavus</i>	3724	>100	100
<i>A. flavus</i> var. <i>flavus</i>	3725	>100	100
<i>Aspergillus fumigatus</i> var. <i>fumigatus</i>	2214	100	25
<i>Cryptococcus neoformans</i>	346	100	50
<i>Microsporium canis</i>	3632	3.12	3.12
<i>M. canis</i>	3635	12.5	3.12
<i>Microsporium gypseum</i>	3622	25	50
<i>Scopulariopsis brevicaulis</i>	2385	12.5	6.25
<i>S. brevicaulis</i>	2387	25	25
<i>S. brevicaulis</i>	2389	100	25
<i>S. brevicaulis</i>	3235	25	25
<i>Trichophyton mentagrophytes</i>	3621	25	12.5

Both compounds exerted toxic effects on MDCK and HMEC cells 72 h after treatment, but at different concentrations. On MDCK cells, SBD was toxic at a concentration of $12.5 \mu\text{g ml}^{-1}$ and SBDI at $25 \mu\text{g ml}^{-1}$, while, on HMEC, SBD was toxic at $3.12 \mu\text{g ml}^{-1}$ and SBDI at $6.25 \mu\text{g ml}^{-1}$ (Fig. 2). After 24 h, SBD's greater toxicity and the different sensitivities of both the two cell lines were already noticeable. At $12.5 \mu\text{g ml}^{-1}$ it was seen that neither cell type survived when treated with SBD; on the contrary, when treated with SBDI, a few HMEC remained alive and all MDCK cells survived, although showing signs of some cellular suffering (vacuolisation; Fig. 3). At $25 \mu\text{g ml}^{-1}$, the MDCK cells also died when treated with SBD, but a few cells remained alive when treated with SBDI, despite showing high morphology modifications (pseudopodia formation).

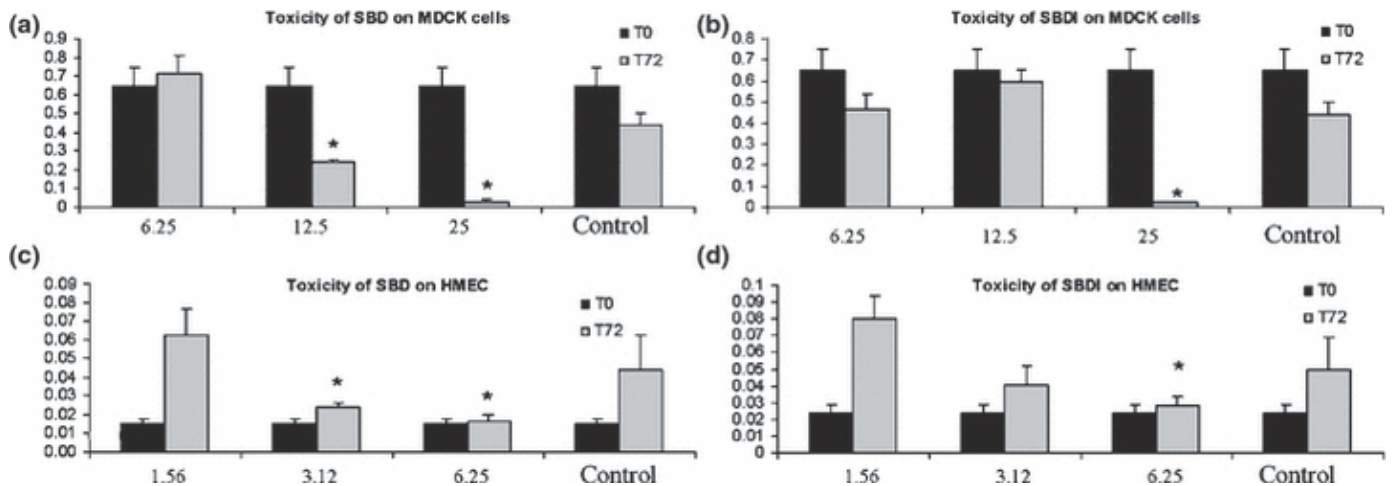


Figure 2. MTT (diphenyltetrazolium bromide test) on cell cultures after 72 h incubation with SBD and SBDI. (a, b): on MDCK (Madin-Darby canine kidney epithelial) Cells. (c, d): on HMEC (human microvascular endothelial cells); T0, beginning of the experiment; T72, MTT after 72 h incubation with SBD and SBDI (*indicates significant differences from control; t -test $P \leq 0.05$).

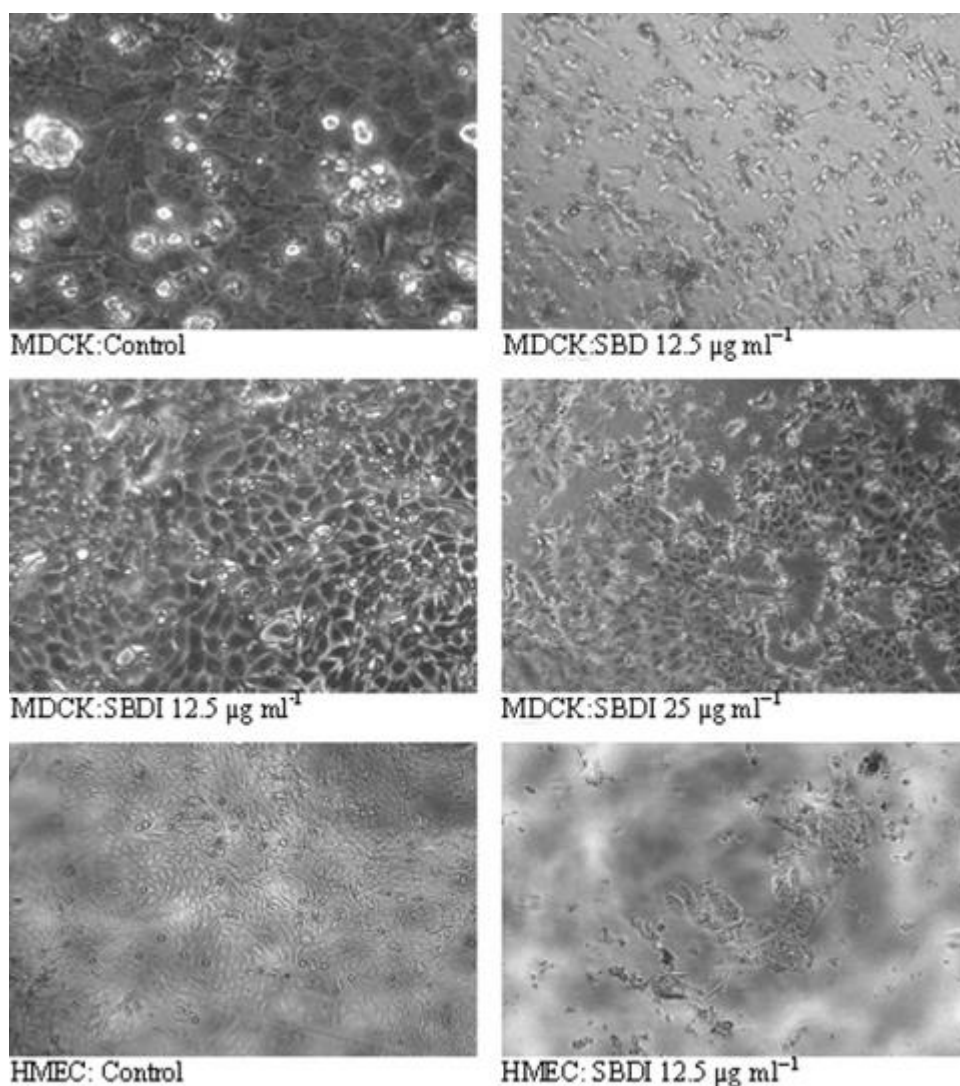


Figure 3. SBD and SBDI toxicity on MDCK (Madin-Darby canine kidney epithelial) cells and HMEC (human microvascular endothelial cells), after 24 h incubation.

Discussion

SBD and SBDI, as others oxidosqualene cyclase inhibitors developed by us,^{20,21} displayed *in vitro* fungistatic activity towards dermatophytes commonly isolated from superficial fungal infections (*M. canis* and *T. mentagrophytes*) and opportunistic fungi, such as *A. fumigatus*, *C. neoformans* and *S. brevicaulis*, more and more involved in various types of mycoses. However, SBD and SBDI showed a different inhibition ability: SBDI possessed better fungistatic activity than SBD, showing generally lower MIC values (particularly towards *M. canis* MUT 3635 and *T. mentagrophytes*) and being able to inhibit to a certain extent also highly refractory opportunist fungi such as *A. fumigatus*, *S. brevicaulis* and *C. neoformans*. SBDI proved to be the less toxic compound as well. It inhibited *M. canis* (both isolates) and *S. brevicaulis* (MUT 2385) at concentrations below those found toxic for MDCK cells. Toxicity of both compounds to HMEC was higher: SBD was already toxic at $3.12 \mu\text{g ml}^{-1}$, while SBDI at 6.25 . The toxicity *in vitro* tests, however, has only a rough value, particularly when a topical use of the molecules is envisaged. An increase in the use of topical agents in the present-day treatment of dermatophytosis accounts for the many adverse effects of the systemic therapy.³ The real toxicity will have therefore to be verified by *in vivo* tests.

The comparison of the activity of SBD and SBDI with that of other antifungal drugs in clinical use, revealed that the inhibition of *M. canis* by SBDI was similar to that of fluconazole, terbinafine, amphotericin B, griseofulvine, ketoconazole, itraconazole, clotrimazole, miconazole, eberconazole and amorolfine,^{3,22–30} while inhibition of *T. mentagrophytes* was similar to that of griseofulvine²² and fluconazole.^{27,28} Moreover, as compared with amorolfine,³⁰ SBDI displayed higher inhibition towards *T. mentagrophytes*. Inhibition of *A. fumigatus* by SBD was comparable to those of some derivatives of 2,4-dihydroxythiobenzanilides,⁴ while that of SBDI to itraconazole, amphotericin B fluconazole and flucytosine.^{31–35} Inhibition of *C. neoformans* by SBD was similar to that of flucytosine,³⁶ while that of SBDI of that of fluconazole.³⁶ The fungistatic activity of both compounds against the four tested isolates of *S. brevicaulis* was similar or higher than that of derivatives of 2,4-dihydroxythiobenzanilides,⁴ that of SBDI was higher than that of flucytosine and voriconazole and fluconazole.^{28,35} *Scopulariopsis brevicaulis* is among the most frequent causes of non-dermatophytic nail infections, though over the last 20 years, severe illnesses have been described in hosts displaying factors that predispose them to infection. *In vitro* resistance to many antifungals, such as amphotericin B, flucytosine, azoles, terbinafine, and capsosungin³⁵ has led to *in vitro* evaluation of the activity of antifungal combinations.³⁷ Synergy however has only been noted in a few cases and with some isolates. The activity displayed by SBD on *S. brevicaulis* (MIC range 12.5–100 µg ml⁻¹) and especially by SBDI (MIC range 6.25–25 µg ml⁻¹) can thus be regarded as an important and promising result with regard to this emerging and refractory pathogen.

The antifungal activity against dermatophytes (*M. canis* and *T. mentagrophytes*), *S. brevicaulis* and *A. fumigatus* and moderate *in vitro* toxicity of these inhibitors of the OSC fungal enzyme are thus worthy of more extensive investigation in a broader spectrum of isolates and species. It would be interesting to evaluate their toxicity on other cell lines, and *in vivo*. Further studies are also required to clarify whether the antifungal activity is a consequence of depletion of ergosterol or of accumulation of intermediates of the sterol biosynthetic pathway at concentrations that are toxic for the fungi.

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References

- 1 De Hoog GS, Guarro J, Gené J et al. Atlas of Clinical fungi, 2nd edn. Utrecht, The Netherlands: Centraalbureau voor Schimmelcultures, 2000.
- 2 John W, Baddley MD, Stephen A, Moser PhD. Emerging fungal resistance. *Clin Lab Med* 2004; 24: 721–35.
- 3 Fernandez-Torres B, Carrillo AJ, Martin E et al. In vitro activities of 10 antifungal drugs against 508 dermatophyte strains. *Antimicrob Agents Chemother* 2001; 45: 2524–8.
- 4 Niewiadomy A, Matysiak J, Maćik-Niewiadomy G. In vitro evaluation of 2,4-dihydroxythiobenzanilides against various moulds. *Eur J Pharm Sci* 2001; 13: 243–8.
- 5 Benveniste P. Biosynthesis and accumulation of sterols. *Annu Rev Plant Biol* 2004; 55: 429–57.
- 6 Abe I, Rohmer M, Prestwich GD. Enzymatic cyclization of squalene and oxidosqualene to sterols and triterpenes. *Chem Rev* 1993; 93: 2189–06.
- 7 Corey EJ, Virgil SC, Cheng H et al. New insights regarding the cyclization pathway for sterol biosynthesis from (S)-2,3-oxidosqualene. *J Am Chem Soc* 1995; 117: 11819–20.
- 8 Wendt KU, Schulz GE, Corey EJ, Liu DR. Enzyme mechanisms for polycyclic triterpene formation. *Angew Chem Int Ed Engl* 2000; 39: 2812–33.
- 9 Xiong Q, Rocco F, Wilson WK, Xu R, Ceruti M, Matsuda SPT. Structure and reactivity of the dammarenyl cation: configurational transmission in triterpene synthesis. *J Org Chem* 2005; 70: 5362–75.
- 10 Milla P, Athenstaed K, Viola F et al. Yeast Oxidosqualene cyclase (Erg7p) is a major component of lipid particles. *J Biol Chem* 2002; 277: 2406–12.
- 11 Nes WD, Koike K, Jia Z et al. Cycloartenol analysis by ¹H and ¹³C NMR, crystallographic observations, and molecular mechanics calculations. *J Am Chem Soc* 1998; 120: 5970–80.
- 12 Moreau RA, Nes WD, Bach TJ, Parish EJ, Zawistowski J. Recent advances in sterol research presented at the 99th AOCS annual meeting & expo in Seattle Washington, May 2008. *Lipids* 2008; 43: 1091–3.
- 13 Vanden Bossche H, Marichal P, Coene M-C et al. Target for antifungal agents and herbicides. In: Nes W.D., Parish E.J., Trzaskos J.M. (eds), *Regulation of Isopentenoid Metabolism*. Washington D.C: American Chemical Society, 1992: 219–30.
- 14 Ryder NS, Stuetz A, Nussbaumer P. Squalene epoxidase inhibitors: structural determinants for activity and selectivity of allylamines and related compounds. In: Nes W.D., Parish E.J., Trzaskos J.M. (eds), *Regulation of Isopentenoid Metabolism*. Washington D.C: American Chemical Society, 1992: 192–204.
- 15 Ceruti M, Balliano G, Viola F, Cattel L, Gerst N, Schuber F. Synthesis and biological activity of azasqualenes, bis-azasqualenes and derivatives. *Eur J Med Chem* 1987; 22: 199–08.
- 16 Ceruti M, Balliano G, Viola F, Grosa G, Rocco F, Cattel L. 2,3-Epoxy-10-aza-10,11-dihydrosqualene, a high-energy intermediate analogue inhibitor of 2,3-oxidosqualene cyclase. *J Med Chem* 1992; 35: 3050–8.
- 17 Cattel L, Ceruti M. Inhibitors of 2,3-oxidosqualene cyclase as tools for studying the mechanism and function of the enzyme. *Crit Rev Biochem Mol Biol* 1998; 33: 353–73.
- 18 National Committee for Clinical Laboratory Standards. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standards. Wayne, PA: NCCLS, 2002 (NCCLS document M27-A2).
- 19 National Committee for Clinical Laboratory Standards. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi: Approved Standard. Wayne, PA: NCCLS, 2002 (NCCLS Document M-38A).

- 20 Airaudi D, Ceruti M, Giannetta A, Filipello Marchisio V. Preliminary screening of some squalenoid derivates for toxicity test towards dermatophytes. *Mycoses* 1995; 38: 311–5.
- 21 Airaudi D, Ceruti M, Bianco C, Filipello Marchisio V. In vitro susceptibility of fungi to acyclic inhibitors of 2,3-oxidosqualene cyclases. *Mycoses* 1996; 39: 51–6.
- 22 Alio AB, Mendoza M, Zambrano EA, Diaz E, Cavallera E. Dermatophytes growth curve and in vitro susceptibility test: a broth micro-titration method. *Med Mycol* 2005; 43: 319–25.
- 23 Cetinkaya Z, Kiraz N, Karaca S et al. antifungal susceptibilities of dermatophytic agents isolated from clinical specimens. *Eur J Dermatol* 2005; 15: 258–61.
- 24 Brilhante RSN, Cordeiro RA, Medrano DJA, Monteiro AJ, Sidrim JJC, Rocha MFG. antifungal susceptibility and genotypical pattern of *Microsporum canis* strains. *Can J Microbiol* 2005; 51: 507–10.
- 25 Esteban A, Abarca ML, Cabañes FJ. Comparison of disk diffusion method and broth microdilution method for antifungal susceptibility testing of dermatophytes. *Med Mycol* 2005; 43: 61–6.
- 26 Fernandez-Torres B, Izna I, Guarro J. Comparison of in vitro antifungal susceptibilities of conidia and hyphae of dermatophytes with thick-wall macroconidia. *Antimicrob Agents Chemother* 2003; 47: 3371–2.
- 27 Santos DA, Hamdan JS. Evaluation of broth microdilution antifungal susceptibility testing conditions for *Trichophyton rubrum*. *J Clin Microbiol* 2005; 43: 1917–20.
- 28 Carrillo-Muñoz AJ, Giusiano G, Guarro J et al. In vitro activity of voriconazole against dermatophytes, *Scopulariopsis brevicaulis* and other opportunistic fungi as agents of onychomycosis. *Int J Antimicrob Agents* 2007; 30: 157–61.
- 29 Fernandez-Torres B, Izna I, Guarro J. In vitro activities of the new antifungal drug Eberconazole and three other topical agents against 200 strains of dermatophytes. *J Clin Microbiol* 2003; 41: 5209–11.
- 30 Li RY, Wan Z, Wang AP et al. In vitro susceptibility testing of amorolfine in pathogenic fungi isolated from dermatomycosis patients in China. *Mycoses* 2003; 47: 402–6.
- 31 Mallie M, Bastide JM, Blancard A et al. In vitro susceptibility testing of *Candida* and *Aspergillus* spp. to voriconazole and other antifungal agents using Etest (R): results of a french multicentre study. *Int J Antimicrob Agents* 2005; 25: 321–8.
- 32 Meletiadis J, Mouton JW, Meis JFGM et al. Comparison of the Etest and the sensititre colorimetric methods with the NCCLS proposed standard for antifungal susceptibility testing of *Aspergillus* species. *J Clin Microbiol* 2002; 40: 2876–85.
- 33 Shimokawa O, Niimi M, Kikuchi K, Saito M, Kajiwara H, Yoshida S. Relationship between MIC and minimum sterol 14 α -demethylation-inhibitory concentration as a factor in evaluating activities of azoles against various fungal species. *J Clin Microbiol* 2005; 43: 5547–9.
- 34 Espinel-Ingroff A, Johnson E, Hockey H, Toke P. Activities of voriconazole, itraconazole and amphotericin B in vitro against 590 moulds from 323 patients in the voriconazole Phase III clinical studies. *J Antimicrob Chemother* 2008; 61: 616–20.
- 35 Cuenca-Estrella M, Gomez-Lopez A, Mellado E, Buitrago MJ, Monzon A, Rodriguez-Tudela JL. *Scopulariopsis brevicaulis*, a fungal pathogen resistant to broad-spectrum antifungal agents. *Antimicrob Agents Chemother* 2003; 47: 2339–41.
- 36 Perkins A, Gomez-Lopez A, Mellado E, Rodriguez-Tudela JL, Cuenca Estrella M. Rates of antifungal resistance among spanish clinical isolates of *Cryptococcus neoformans* var. *neoformans*. *J Antimicrob Chemother* 2005; 56: 1144–7.
- 37 Cuenca-Estrella M, Gomez-Lopez A, Buitrago MJ, Mellado E, Garcia-Effron G, Rodriguez-Tudela JL. In vitro activities of 10 combinations of antifungal agents against the multiresistant pathogen *Scopulariopsis brevicaulis*. *Antimicrob Agents Chemother* 2006; 50: 2248–50.