Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 1681–1686

BIOORGANIC & MEDICINAL CHEMISTRY

Anti-*Candida albicans* Properties of Novel Benzoxazine Analogues

Renata Fringuelli,[a,*] Donatella Pietrella,[b] Fausto Schiaffella,[a] Alessia Guarraci,[a] Stefano Perito,[b] Francesco Bistoni[b] and Anna Vecchiarelli[b,*]

[a]*Department of Drug Chemistry and Technology, University of Perugia, Via del Liceo 1, 06123 Perugia, Italy*
[b]*Department of Experimental Medicine and Biochemical Sciences, Microbiology Section, Via del Giochetto, University of Perugia, 06122 Perugia, Italy*

Received 6 September 2001; accepted 21 January 2002

Abstract—We previously reported that azole 1,4-benzothiazine derivatives have appreciable anti-*Candida* activity. In this study, we synthesized 1,4-benzoxazine analogues and examined their possible antifungal activity to further analyze the structure–activity relationships. Results of in vitro and in vivo experiments showed that 1,4-benzoxazine analogues show appreciable antifungal activity. In particular, they have significant capability to cure mice systemically infected with a lethal challenge of *Candida albicans,* as indicated by increased survival time paralleling reduction of colony forming units. Moreover, 1,4-benzoxazine derivatives also showed immunomodulating activity, as indicated by a significant increase of interleukin-12 and interferon-γ production by splenocytes and reinforcement of a T helper type 1 protective immune response to *C. albicans*. In conclusion, the results demonstrate that replacement of sulfur by oxygen may improve immune response against *C. albicans* infection.

Introduction

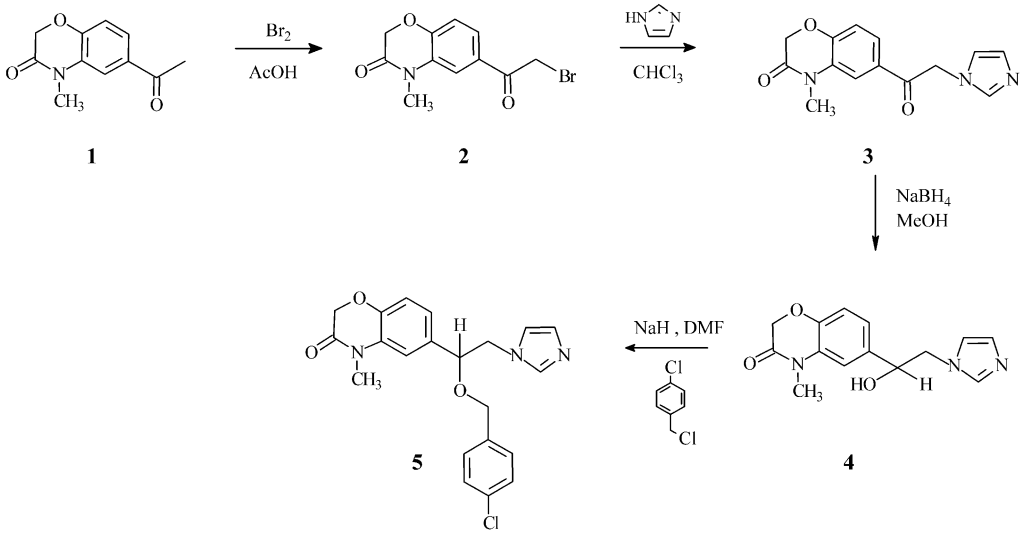
The prevalence of opportunistic fungal infections has increased together with the population of immunocompromised subjects.[1,2] Management strategies are based not only at the site of *Candida* infection but also on other factors including the immunological condition of patients, such as neutropenia, and bone marrow or solid organ transplantation. Fluconazole is the agent of choice for *Candida* infections. However, amphotericin B, with a higher degree of toxicity than fluconazole, is used in fluconazole-resistant infections.[3] This situation is particularly problematic because immunocompromised patients are in need of long-term antifungal therapy to prevent relapses. In addition, neutropenic patients at risk for deep fungal infections benefit from antifungal prophylaxis.[4] Despite the increasing number of fungal infections, progress in the development of antifungal agents is slow with respect to the intensive research devoted to antibacterial therapy.

Candida albicans is a dimorphic fungus that resides on the skin and mucosa of immunocompetent individuals. The balance between the phase of innocuous commensalism and infection appears to be dependent on the immunological status of the host.[4] Studies have clarified the mechanism of protection against *C. albicans* infections and the critical role of efficient functional status of natural effector cells, including polymorphonuclear cells and macrophages,[5,6] and the development of an effective T helper type 1 (Th1) response that plays a pivotal role in protection against *C. albicans*.[7] There is general consensus that an efficient Th1 response is driven and maintained by the proinflammatory cytokine IL-12.[8] Thus, agents displaying direct antifungal properties associated with immunopotentiation of immunological antifungal mechanisms may have an optimal antifungal effect in *C. albicans* infection.

In a program aimed at analyzing the antifungal activity of 1,4-benzothiazine derivatives, in order to evaluate the effect of substituting the aromatic ring present in the most commonly used drugs with 1,4-benzothiazine nucleus that, in itself, shows some antifungal activity,[9] a series of azole 1,4-benzothiazines structurally related to fluconazole and econazole were recently synthesized.[10–12]

*Corresponding author. R. Fringuelli tel.: +39-075-585-5134; fax: +39-075-585-5161; A. Vecchiarelli tel.: +39-075-585-7407; fax: +39-075-585-7407; e-mail: fringuel@unipg.it; vecchiar@unipg.it

0968-0896/02/$ - see front matter
PII: S0968-0896(02)00038-X



Scheme 1.

These derivatives differ in respect to N-4 substitution, the S-1 and C-3 oxidation state, the insertion of the side chain on C-6, C-7 or C-8 positions of benzothiazine nucleus, the nature of azolic substituent (triazole or imidazole).

In particular, structure–activity relationships showed that: (1) imidazole derivatives were more active than triazole ones; (2) various substitutions at N-4 pointed out that methyl and ethyl groups were the best substituents, in fact the non-substituted or the isopropyl derivatives were inactive; (3) by changing the side chain insertion on the benzothiazine nucleus we observed that, while the C-8 position was decidedly unfavourable, the C-7 and C-6 positions were important in maintaining the activity; (4) the oxidation degree of S-1 and C-3 plays a role in influencing the effect against the fungus: while sulfones were practically inactive, sulfoxides showed an appreciable increase in activity. In contrast, the reduction of the amidic carbonyl group in position 3 led to inactive derivatives; (5) the presence of an ethereal function in the side chain improves activity.

In order to complete structure–activity relationships, here we report the synthesis and in vitro and in vivo antifungal activity of 1,4-benzoxazine imidazole analogues **4** and **5** to evaluate the effect of sulfur substitution on activity.

Derivatives **4** and **5** were synthesized starting from 6-acetyl-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one. The bromination and subsequent condensation with 1*H*-imidazole led to ketone **3** that, by reduction with sodium borohydride, gave the secondary alcohol **4**. The alcohol was subsequently converted into **5** by alkylation with 4-chlorobenzyl chloride. The synthetic pathway is shown in Scheme 1.

Results and Discussion

Compound **5** showed appreciable activity in vitro against *C. albicans* (MIC = 15.6–7.8 μg/mL), *Candida krusei* (MIC = 15.6 μg/mL), and *Saccharomyces cerevisiae* (MIC = 7.8–3.9 μg/mL) while no in vitro activity against *C. albicans* (MIC = 250 μg/mL) or *S. cerevisiae* (MIC = 250 μg/mL) was detected with **4**. Appreciable activity of **4** was detected against *C. krusei* (MIC = 31.2 μg/mL). Since the in vitro antifungal effect does not necessarily correlate with in vivo activity,[13–15] to test the antifungal properties of our compounds against disseminated candidiasis, mice were treated ip with 10 mg/kg of **4** or **5** for 6 consecutive days, according to previously defined experimental schedules,[10,11] and challenged with *C. albicans*. Econazole, an analogous agent of **5**, and fluconazole were used as internal controls in our experiments. The results reported in Table 1 show a significant increase of median survival time (MST) of mice treated with compound **5** with respect to diluent-treated mice, while the administration of **4** did not influence MST, although a considerable percentage of mice survived the challenge. It is noteworthy that the mice treated with fluconazole show a significant enhancement of MST with respect to compounds **4** and **5** ($p < 0.01$).

Table 1. Effect of treatment with different compounds on systemic *C. albicans* iv infection (2×10^5 cells) and CFU recovery from kidneys

Treatment[a]	CFU($\times10^3$)[b]	MST[c]	Survival range (days)	D/T[d]
Diluent [e]	97.8 ± 10	12.0	3–34	14/14
4	89.1 ± 9	12.0	3 > 60	9/14
5	70.2 ± 5*	22.5*	3 > 60	8/14
Econazole	73.4 ± 9*	17.5	8 > 60	6/7
Fluconazole	0.1 ± 1*	> 60*	26 > 60	3/14

[a]Diluent and indicated compounds were inoculated ip. The compounds were given at doses of 10 mg/kg 2 h before challenge and once for 6 consecutive days after challenge.
[b]CFU were recovered from the kidneys of mice sacrificed 8 days after infection. Data represent the mean ± SD of triplicate samples from a representative experiment out of three with similar results.
[c]Median survival time.
[d]Dead mice over total animals tested.
[e]DMSO/H_2O, 1:4.
*$p < 0.01$ (compound-treated vs untreated mice).

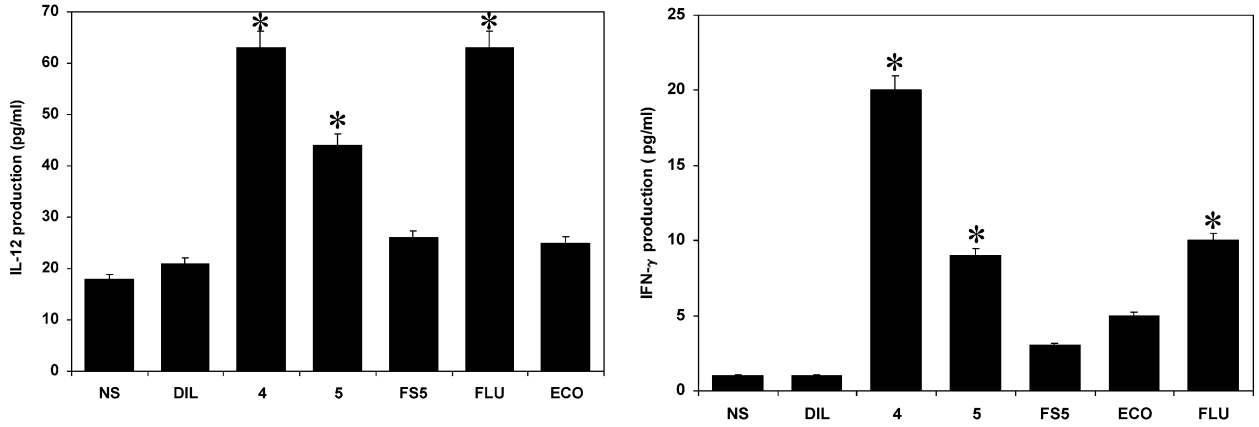


Figure 1. Diluent (DIL) and indicated compounds were inoculated ip. The compounds were given at doses of 10 mg/kg 2 h before challenge and once for 6 consecutive days after challenge. IL-12 and IFN-γ production were determined in supernatant fluids of splenocytes from compound-treated mice. NS, not stimulated cells; DIL, DMSO/H_2O, 1:4; FS5, 7-[1-[(4-chlorobenzyl)oxy]-2-(1*H*-1-imidazolyl)ethyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one; FLU, fluconazole; ECO, econazole. *$p < 0.01$ (treated versus untreated mice).

In parallel experiments, we examined whether this correlated with the clearance of *C. albicans* from the kidneys. Mice were treated with **4**, **5**, econazole, and fluconazole and challenged with *C. albicans* at the same time, doses, and schedules as above. Kidneys were removed 8 days after challenge for CFU evaluation. The results reported in Table 1 showed a significant reduction of CFU in mice treated with **5** in respect to diluent-treated mice. The observed effect was similar to that obtained with econazole. In contrast, CFU recovery from mice-treated with **4** was similar to that observed for diluent-treated mice.

Previous studies showed that important antifungal agents, such as amphotericin B, display a direct anti-*Candida* effect combined with immunostimulating activity,[16,17] thus the possibility arises that **5** could work in a similar manner.

It is well established that the protective response against *C. albicans* is mediated by the Th1 response[18] and the presence of IL-12 is critical for generation of this type of response.[8] To this purpose, IL-12 production by splenocytes from mice treated with our compounds was evaluated. The results reported in Figure 1 show that, differently from benzothiazine derivative 7-[1-[(4-chlorobenzyl)oxy]-2-(1*H*-1-imidazolyl)ethyl]-4-methyl-3,4 - dihydro-2*H*-1,4-benzothiazin-3-one (FS5) previously reported,[11] benzoxazine derivatives **4** and **5** induced a significant increase of IL-12 secretion by splenocytes in respect to untreated or diluent-treated mice. Both agents induced higher levels of IL-12 than econazole. In particular, **4** and **5** induced levels similar to those obtained with fluconazole. To our knowledge, this is the first demonstration that fluconazole induces IL-12 secretion. These results were confirmed in experiments with purified splenic macrophages, suggesting that macrophages are the major biological source of IL-12. As previously observed, IL-12 is a potent inducer of IFN-γ in T cells, as a consequence we examined whether IFN-γ production was altered in splenocytes from mice treated with compounds **4** and **5**. The results reported in Figure 1 show that all agents (**4**, **5**, fluconazole, econazole) were able to increase IFN-γ production of T cells with compound **4** being the best inducer.

In particular IFN-γ release promoted by **4** is significantly higher than that obtained with fluconazole ($p < 0.01$), in contrast, no differences were observed between compound **5** and fluconazole. Given that IFN-γ is a potent macrophage activator, we evaluated whether splenic macrophages from **5**-treated mice had altered antifungal activity. Consistent with this hypothesis, a significant enhancement of anticandidal activity was observed by splenic macrophages. Macrophages from **4**- and **5**-treated mice showed anti-*Candida* activity of 58 ± 5 and 69 ± 7, respectively, which is significantly higher ($p < 0.01$) than that from diluent-treated mice (40 ± 5).

Conclusions

In an attempt to better define structure–activity relationships of a new class of azole derivatives previously reported[10–12], we synthesized 1,4-benzoxazine analogues, and in vitro and in vivo antifungal activity was evaluated.

The alcoholic derivative **4** showed a scarce capability to cure murine candidiasis. In fact, the in vitro activity was not appreciable against *C. albicans* in vitro (MIC results) even when the immunomodulating properties were preserved. Despite enhancement of immune defense against *C. albicans*, compound **4** had a poor effect in vivo.

As in the benzothiazine series, the ether derivative **5** manifested an appreciable capacity to cure murine candidiasis. Indeed, the protection against candidiasis appeared to be the combination of two effects. A direct effect on fungal cells as observed by MIC, as well as an indirect effect on the immune system by promoting or potentiating the protective immune response against *C. albicans*.

The capacity of **5** to disaggregate the *Candida* cell wall may be considered a potential mechanism to cure murine

candidiasis. This effect, may render fungal cells more susceptible to immune effectors, contributing to elimination of the fungus from infected tissues. Moreover, the reinforcement of a **5**-induced protective response against *Candida* is an adjunctive mechanism against *C. albicans* infections. This may be important considering that serious *C. albicans* infections occur in immunocompromised individuals and that the success of antifungal therapy is often related to the neutropenic state of patients,[19,20] showing that the collaboration between the innate immune system and the efficacy of antifungal therapy is essential. Compound **5,** through induction of IL-12 by macrophages, promotes IFN-γ release by T cells that in turn activates natural effectors such as macrophages, excellent immunoeffectors against *C. albicans*.[16,21] Th1 generation necessary to mount a protective immune response to *C. albicans* should be included in the ability of **5** to cure systemic candidiasis.

Experimental

Melting points determined in capillary tubes (Electrothermal, Model 9100, melting point apparatus) were uncorrected. Element analysis was performed on a Carlo Erba element analyzer 1106, and the data for C, H, and N are within ±0.4% of the theoretical values. ^{1}H NMR spectra were recorded at 200 MHz (Bruker AC-200 spectrometer) with Me_4Si as internal standard. Chemical shifts are given in ppm (δ) and the spectral data are consistent with the assigned structures. Reagents and solvents were purchased from common commercial suppliers and used as received. Column chromatography separations were carried out on Merck silica gel 40 (mesh 70–230) and Merck aluminum oxide 90, neutral, activity III (mesh 70–230). Yields of purified product were not optimized. Fluconazole and econazole were purchased from Pfizer (New York). All starting materials were commercially available unless otherwise indicated.

6-Acetyl-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (1). Potassium *tert*-butoxide (1.75 g, 15.70 mmol) was added in one portion to a solution of 6-acetyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (2.00 g, 10.50 mmol) in dry *N*,*N*-dimethylformamide (DMF) (15 mL) under a nitrogen atmosphere at room temperature. The resulting homogeneous mixture was stirred for 15 min followed by the dropwise addition of methyl iodide (MeI) (2.23 g, 15.70 mmol) in dry DMF (5 mL). The solution was stirred for additional 4 h and then evaporated to dryness and chromatographed on silica gel eluting with cyclohexane/ethyl acetate (EtOAc) 90:10 to give **1** in quantitative yield (2.10 g), mp 154–157 °C, ^{1}H NMR ($CDCl_3$) δ 2.57 (3H, s, $COCH_3$), 3.42 (3H, s, NCH_3), 4.68 (2H, s, OCH_2), 7.00 (1H, d, J = 7.9 Hz, H-8), 7.55–7.65 (2H, m, H-5 and H-7).

6-(2-Bromoacetyl)-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (2). Bromine (1.28 g, 8.00 mmol) in acetic acid (AcOH) (10 mL) was added slowly to a solution of acetyl derivative **1** (1.50 g, 7.31 mmol) in AcOH (25 mL) and stirred at room temperature for 30 min. The mixture was evaporated to dryness and chromatographed on silica gel eluting with cyclohexane/EtOAc 90:10 to give **2** (1.40 g, 68%), mp 132–134 °C, ^{1}H NMR ($CDCl_3$) δ 3.45 (3H, s, NCH_3), 4.40 (2H, s, CH_2Br), 4.70 (2H, s, OCH_2), 7.05 (1H, d, J = 7.9 Hz, H-8), 7.60–7.70 (2H, m, H-5 and H-7).

6-[2-(1*H*-1-Imidazolyl)acetyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (3). 1*H*-Imidazole (0.72 g, 10.59 mmol) was added to a solution of bromoderivative **2** (1.00 g, 3.52 mmol) in chloroform ($CHCl_3$) (25 mL) and the mixture was stirred at room temperature for 2 h. The mixture was evaporated to dryness and chromatographed on silica gel eluting with $CHCl_3$/methanol (MeOH) 98:2 to give **3** (0.70 g, 75%), mp 216–219 °C, ^{1}H NMR ($CDCl_3$) δ 3.42 (3H, s, NCH_3), 4.75 (2H, s, OCH_2), 5.40 (2H, s, CH_2N), 6.95, 7.15 and 7.55 (each 1H, s, H imidazole), 7.10 (1H, d, J = 7.9 Hz, H-8), 7.60–7.65 (2H, m, H-5 and H-7).

6-[1-Hydroxy-2-(1*H*-1-imidazolyl)ethyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (4). $NaBH_4$ (0. 07 g, 1.84 mmol) was added in small fractions over 1 h to a solution of **3** (0.50 g, 1.84 mmol) in MeOH (20 mL). The mixture was then evaporated to dryness and the residue chromatographed eluting with $CHCl_3$/MeOH 90:10 to give **4** (0.46 g, 92%), as an amorphous solid, ^{1}H NMR (DMSO-d_6) δ 3.25 (3H, s, NCH_3), 3.95–4.20 (2H, m, $CHCH_2$), 4.60 (2H, s, OCH_2), 4.75–4.85 (1H, m, $CHOHCH_2$), 5.75 (1H, bs, $CHOHCH_2$), 6.85–7.65 (6H, m, aromatic and imidazolic H).

6-[1-[(4-Chlorobenzyl)oxy]-2-(1*H*-1-imidazolyl)ethyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (5). To a solution of **4** (0.50 g, 1.83 mmol) in DMF (6 mL), sodium hydride (NaH) (60% mineral oil dispersion, 0.22 g, 5.50 mmol) was added in small fractions to prevent any heating. 4-Chlorobenzyl chloride (1.20 g, 7.45 mmol) in DMF (4 mL) was then added dropwise. The mixture was stirred at room temperature for 2 h and the excess of hydride was decomposed with small amounts of EtOAc. After evaporation to dryness, the crude residue was suspended in water and extracted with $CHCl_3$. The combined organic layers were evaporated to dryness to afford a crude residue that was purified by chromatography on aluminum oxide eluting with $CHCl_3$ to give **5** (0.42 g, 60%) as an oil, ^{1}H NMR ($CDCl_3$) δ 3.30 (3H, s, NCH_3), 4.15 (2H, d, J = 5.0 Hz, $CHCH_2$), 4.20 and 4.45 (each 1H, d, J = 12.0 Hz, CH_2Ph), 4.45–4.55 (1H, m, $CHCH_2$), 4.65 (2H, s, OCH_2), 6.70–7.45 (10H, m, aromatic and imidazolic H).

Mice

Female CD1 mice (8–10 weeks old, weighing 25–30 g) were obtained from Charles River Breeding Laboratories (Calco, Lecco, Italy).

C. albicans, *C. krusei* and *S. cerevisiae*

C. albicans CA-6, used in this study, was isolated from a vaginal swab and identified according to the taxonomic

criteria of van Uden and Buckley.[22] *C. krusei* strain 6258 (American Type Culture Collection, Rockeville, MD, USA) and *S. cerevisiae* strain 9763 (ATCC) were used in this study. The yeasts were grown at 28 °C in Sabouraud dextrose agar. Under these conditions the organisms grew as a pure yeast-phase population. Before use, yeast cells were harvested from a 24 h culture, suspended in pyrogen-free saline, washed twice, quantified by hemocytometry, and adjusted to the desired concentration.

Systemic candidiasis model

Mice were infected intravenously (iv) with 2×10^5 *C. albicans* blastoconidia via the lateral tail vein. Diluent ($DMSO/H_2O$, 1:4) or chemicals (**4**, **5**, fluconazole, and econazole) were administrated intraperitoneally (ip) at a dose of 10 mg/kg of body weight 2 h before infection and then daily for 6 consecutive days. The dose and administration schedules used in this study were selected from previously reported data[10] and based on dose and treatment schedules reported in the literature for a novel azole with a broad antifungal spectrum, such as ER-30346.[23] For survival studies, mice were observed for 60 days. Three mice per group were killed by CO_2 asphyxiation 8 days after infection for quantitative culture of both kidneys.

Quantification of *C. albicans* in the kidneys

The kidneys of mice were aseptically removed and homogenized with 3 mL of sterile distilled water. The number of colony forming units (CFU) was determined by a plate dilution method. Colonies of *C. albicans* cells were counted after 48 h of incubation at room temperature and results were expressed as the number of CFU per organ.

Susceptibility testing

Susceptibility testing was performed by the M27-A micro-dilution method of the National Committee for Clinical Laboratory Standards[24] in 0.165 M MOPS (morpholinepropanesulfonic acid)-buffered (pH 7) RPMI 1640 medium (Gibco BRL, Paisley, UK). The activity of compounds against *C. albicans* was tested using serial dilutions ranging from 0.9 to 500 mg/mL. The MIC was the lowest concentration of chemical that produced an 80% reduction in the turbidity compared to chemical-free normal subjects.

Preparation of splenocytes

After in vivo treatment with chemicals, spleens were removed aseptically and placed in 5 mL of RPMI 1640 and single cell suspensions were prepared. One portion was cultured to examine cytokine production from total spleen cells; the second portion was used to test the killing activity against *C. albicans*. To evaluate cytokine production, splenocytes (20×10^6 cells/mL) were cultured for 18 h in RPMI plus 10% fetal calf serum. After incubation, supernatants were recovered and stored at −80 °C. Cytokine levels in culture supernatant fluids were measured with an ELISA kit for mouse IL-12 (Endogen Inc., Woburn, MA, USA) and mouse interferon-γ (IFN-γ, Endogen Inc).

Inhibition of *C. albicans* growth by splenic macrophages. Anti-*Candida* activity was evaluated by comparing the percentage of viable yeasts incubated in the presence of effector cells with the growth of normal subjects in the absence of effector cells. Viable yeasts were determined by a quantitative plate assay. Briefly, effector cells (5×10^5/well) were plated in 96-well plates and infected with viable *C. albicans* (5×10^4/mL). After 3 h of incubation at 37 °C under 5% CO_2, Triton X-100 (final concentration, 0.1%) was added to each well and the plates were shaken vigorously. Serial dilutions from each well were made in distilled water and plated on Sabouraud dextrose agar. The colonies of *C. albicans* were counted after 24 h at 37 °C. The results were expressed as the percent reduction of CFU according to the following formula: 100−(CFU experimental group/CFU normal cultures)×100.

Statistical analysis

Differences in median survival time were determined by Mann–Whitney U test. Student's *t*-test was used to evaluate the significance of all other data. Each experiment was repeated three to five times.

Acknowledgements

We thank Eileen Mahoney Zannetti and Jo-Anne Rowe for excellent editorial assistance. This study was supported by the National Research Project on AIDS (Opportunistic Infections and Tuberculosis) contract 50B.39, Italy.

References and Notes

1. Imwidthaya, P.; Poungvarin, N. *Postgrad. Med. J.* **2000**, *76*, 85.
2. Steenbergen, J. N.; Casadevall, A. *J. Clin. Microbiol.* **2000**, *38*, 1974.
3. Sheehan, J. D.; Hitchcock, C. A.; Sibley, C. M. *Clin. Microbiol. Rev.* **1999**, *12*, 40.
4. Walsh, T. J.; Hiemenz, J.; Pizzo, P. A. *Clin. Infect. Dis.* **1994**, *18*, 793.
5. Monari, C.; Casadevall, A.; Pietrella, D.; Bistoni, F.; Vecchiarelli, A. *J. Infect. Dis.* **1999**, *180*, 1542.
6. Vecchiarelli, A.; Dottorini, M.; Puliti, M.; Todisco, T.; Cenci, E.; Bistoni, F. *Am. Respir. Dis* **1991**, *143*, 1049.
7. Cenci, E.; Mencacci, A.; Fè d'Ostiani, C.; Montagnoli, C.; Bacci, A.; Del Sero, G.; Perito, S.; Bistoni, F.; Romani, L. *Res. Immunol.* **1998**, *149*, 445.
8. Trinchieri, G. *Adv. Immunol.* **1998**, *70*, 83.
9. Baruffini, A.; Pagani, G.; Amoretti, L. *Il Farmaco* **1967**, *22*, 528.
10. Fringuelli, R.; Schiaffella, F.; Bistoni, F.; Pitzurra, L.; Vecchiarelli, A. *Bioorg. Med. Chem.* **1998**, *6*, 103.
11. Pitzurra, L.; Fringuelli, R.; Perito, S.; Schiaffella, F.; Barluzzi, R.; Bistoni, F.; Vecchiarelli, A. *Antimicrob. Agents Chemother.* **1999**, *43*, 2170.
12. Schiaffella, F.; Guarraci, A.; Fringuelli, R.; Pitzurra, L.; Bistoni, F.; Vecchiarelli, A. *Med. Chem. Res.* **1999**, *9*, 291.

13. Anaissie, E. J.; Karyotakis, N. C.; Hachem, R.; Dignani, M. C.; Rex, J. H.; Paetznick, V. *J. Infect. Dis.* **1994**, *170*, 384.
14. Rex, J. H.; Galgiani, J. N.; Bartlett, M. S.; Espaniel-Ingroff, A.; Ghannoum, M. A.; Lancaster, M.; Odds, F. C.; Rinaldi, M. G.; Barry, A. L. *Clin. Infect. Dis.* **1997**, *24*, 235.
15. Rex, J. H.; Nelson, P. W.; Paetznick, V. L.; Lozano-Chiu, M.; Espinel-Ingroff, A.; Anaissie, E. J. *Antimicrob. Agents Chemother.* **1998**, *42*, 129.
16. Bistoni, F.; Vecchiarelli, A.; Mazzolla, R.; Puccetti, P.; Marconi, P.; Garaci, E. *Antimicrob. Agents Chemother.* **1985**, *27*, 625.
17. Vecchiarelli, A.; Verducci, G.; Perito, S.; Puccetti, P.; Marconi, P.; Bistoni, F. *J. Antibiot. (Tokyo)* **1986**, *39*, 846.
18. Stevens, D. A.; Walsh, T. J.; Bistoni, F.; Cenci, E.; Clemons, K. V.; Del Sero, G.; Fè d'Ostiani, C.; Kullberg, B. J.; Mencacci, A.; Roilides, E.; Romani, L. *Med. Micol.* **1998**, *36*, 174.
19. Peltroche-Llacsahuanga, H.; Schnitzler, N.; Schmidt, S.; Tintelnot, K.; Lutticken, R.; Hasse, G. *FEMS Microbiol. Lett.* **2000**, *191*, 151.
20. Richardson, M. D.; Rennie, S.; Marshall, I.; Morgan, M. G. .; Murphy, J. A.; Shankland, G. S.; Watson, W. H.; Soutar, R. L. *J. Hosp. Infect.* **2000**, *45*, 288.
21. Vecchiarelli, A.; Dottorini, M.; Beccari, T.; Cociani, C.; Todisco, T.; Bistoni, F. *Am. Rev. Respir. Dis.* **1993**, *147*, 414.
22. Vecchiarelli, A.; Mazzolla, R.; Farinelli, S.; Cassone, A.; Bistoni, F. *J. Gen. Microbiol.* **1988**, *134*, 2583.
23. Hata, K.; Kimura, J.; Miki, H.; Toyosawa, T.; Nakamura, T.; Katsu, K. *Antimicrob. Agents Chemother.* **1996**, *40*, 2237.
24. National Committee for Clinical Laboratory Standards. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast*. Approved standard NCCLS document M27-A., 1997.