Sodium butyrate inhibits pathogenic yeast growth and enhances the functions of macrophages

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Received 28 June 2011; returned 12 July 2011; revised 3 August 2011; accepted 8 August 2011

Objectives: Butyrate is a short-chain fatty acid that is produced by several human commensal bacteria, such as Clostridium and Lactobacillus species. Butyrate is also known to inhibit histone deacetylase. In this study we assessed the antifungal activity of sodium butyrate (SB) against the human pathogenic yeasts Candida albicans, Candida parapsilosis and Cryptococcus neoformans.

Methods: The growth and virulence traits of the yeasts were assayed in vitro and during interaction with macrophages in the presence of SB.

Results: SB strongly inhibited yeast growth in a concentration-dependent manner, inhibited virulence traits such as filamentation in C. albicans and melanization and capsule formation in C. neoformans and, importantly, significantly decreased yeast biofilm formation. SB also enhanced the antifungal activity of azole drugs. Notably, SB augmented the antifungal activity of macrophages by enhancing the production of reactive oxygen species. The phagocytic rate and killing activity of macrophages significantly increased in the presence of SB, which coincided with an increase in nitric oxide production.

Conclusions: These results demonstrate that SB exerts significant antifungal activity on pathogenic yeasts and enhances the antimicrobial actions of macrophages in response to these microbes.

Keywords: Candida species, Cryptococcus neoformans, fungal virulence, yeast infection

Introduction

Invasive yeast infections are increasingly recognized as a major threat in critically ill adult and paediatric patients. Yeast diseases can range widely in severity from minor cutaneous infections to life-threatening systemic diseases.1 Candida and Cryptococcus species are the most common causes of invasive fungal infections. For example, Candida albicans infection occurs in ~17% of patients treated in the intensive care unit, and invasive candidiasis is associated with significant morbidity and mortality.1 Moreover, other Candida species, such as Candida glabrata and Candida parapsilosis, are increasingly common, particularly in neutropenic patients, neonates and patients on existing azole therapy due to drug resistance in these species.2 Cryptococcus neoformans is estimated to cause disease in 1000000 patients annually with over 600000 deaths.2 Hence, despite appropriate administration of currently available antifungal drugs, invasive yeast infections, especially in compromised hosts, are frequently lethal. In this setting, it is essential to explore new avenues for therapeutic intervention.

Histone deacetylases (HDACs) are enzymes that primarily deacetylate lysines on histones, but also remove acetyl groups of other proteins.3,4 Although certain HDAC inhibitors, such as valproic acid, have a long history of clinical use, increased interest in HDAC inhibitors has arisen because these compounds have applications in cancer treatment. For example, the HDAC inhibitor vorinostat is FDA approved for the treatment of cutaneous T cell lymphoma. Recent studies have shown that inhibition of HDAC can impair fungal growth. Morphogenesis is vital to the virulence of C. albicans and HDACs are known to regulate this process.5 Moreover, pharmacological or genetic blocking of C. albicans HDACs attenuates virulence.6,7 Interestingly, inhibition of HDAC can also reduce adhesion of C. albicans to host cells, which is a required step in disease pathogenesis.8 HDAC inhibitors have also been reported to have synergy with azole drugs.9,10

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In the present study we sought to determine the effects of sodium butyrate (SB), a known histone deacetylase inhibitor, on the growth of *C. albicans*, *C. parapsilosis* and *C. neoformans*. Furthermore, since butyrate is a short-chain fatty acid that is produced by ruminants and commensal bacteria from human intestinal tracts, we also determined whether SB could alter the activity of macrophages. We found that SB exhibited antifungal activity against both *Candida* species and *C. neoformans* in rich media. We also found that SB synergistically enhanced macrophage activity, resulting in the enhanced killing of the yeasts in vitro. Therefore, our data suggest that SB or its derivatives may be useful in combating invasive yeast infections. As it is readily available and inexpensive, SB has an advantage over newer HDAC inhibitors.

### Materials and methods

#### Strains and culture conditions

*C. albicans* SC5314 and *C. neoformans* serotype A strain H99 were obtained from the ATCC. *C. parapsilosis* GA1 is a well-characterized clinical isolate. Yeast strains were cultured at 30°C in 35% glycerol. Yeast strains were cultured at 30°C on 1% yeast extract/2% bactopeptone/2% glucose, pH 6.5 (YPD) agar plates or grown in liquid YPD overnight with rotary shaking at 150 rpm prior to use.

#### Growth inhibition assays

The growth rates of log-phase yeast cells were analysed in liquid YPD. The growth rates of log-phase yeast cells were analysed in liquid YPD. The growth rates of log-phase yeast cells were analysed in liquid YPD. The growth rates of log-phase yeast cells were analysed in liquid YPD.

#### Biofilm formation and metabolic activity

To evaluate biofilm formation, 96-well polystyrene plates were used as described previously. Briefly, wells were blocked overnight by incubation with 100 μL/well of fetal calf serum at 37°C. Overnight cultures of the yeast strains were washed three times with sterile PBS and suspended at 10⁷ cells/mL in yeast nitrogen base (YNB) containing 50 mM glucose for *Candida* species and in minimal medium (MM; 15 mM dextrose, 10 mM MgSO₄, 29.3 mM KH₂PO₄, 13 mM glycerol and 3 μM thiamine-HCl; pH 5.5) for *C. neoformans*. The cultures were incubated at 37°C without shaking for 48 h. Media were then gently discarded by pipetting. Aliquots of 100 μL of fresh media prepared with different concentrations of SB were added to each well. The plates were incubated for an additional 24 h at 37°C and then gently washed three times with 100 μL of 0.05% Tween 20 prepared in Tris-buffered saline to remove the non-adhered cells. Fungal cells that remained attached to the plastic surface were considered to have formed biofilms. Biofilm formation was expressed as a percentage of metabolic activity.

The metabolic activity was assessed using 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium bromide (XTT). A mixed solution of 50 or 300 μL of XTT (1 mg/mL in PBS) with 4 or 24 μL of menadione solution (1 mM in acetone; Sigma Aldrich) was added to each well of the 96-well or 24-well plates, respectively. The plates were incubated for 5 h at 37°C and colorimetric changes were read with a microtitre reader at 492 nm. Heat-killed cells and YNB medium without cells were used as negative controls. The assays were performed twice using four replicates per sample.

### Susceptibility testing

To examine the synergistic effect of SB with antifungal drugs on yeast susceptibility, yeast cells were grown overnight in YPD, washed, diluted with PBS to OD₆₀₀ 0.1 and serially diluted 1:10 in PBS. Aliquots of 2.5 μL of diluted yeast cells were spotted on YPD plates with or without 20 or 40 mM SB and with or without different concentrations of fluconazole (0.1, 0.5, 1, 2, 4 and 8 mg/L), amphotericin B (0.5, 1 and 2 mg/L) or caspofungin (2, 4, 8 and 16 mg/L). Plates were incubated at 30°C and digitally photographed at a fixed distance after 3 days of incubation.

### Phenotyping assays

To study *C. albicans* filamentation, 2 × 10⁵/mL yeast cells were inoculated in 1 mL of 10% fetal bovine serum (diluted in sterile water) supplemented with or without 20 mM SB. Cultures were incubated without shaking at 37°C for 24 h and filamentation was assessed using microscopy. For inhibition of melanization of *C. neoformans*, 5 × 10⁴ yeast cells were grown in 50 mL of MM with or without supplementation with 1 mM L-3,4-dihydroxyphenylalanine (L-DOPA) and with or without 20 mM SB. Cultures were wrapped with aluminium foil and incubated at 30°C for 7 days in the dark with shaking at 150 rpm. Melanization of cultures was assessed visually and photographed digitally. For capsule size measurements, *C. neoformans* cells were grown overnight in Sabouraud medium. Aliquots of 10⁵ yeast cells/mL were then inoculated in 10% fetal bovine serum in sterile water or in MM with or without 20 and 40 mM SB. Capsule formation was also tested with different concentrations of trichostatin A (TSA; Sigma, USA) in 10% serum medium. Cultures were incubated at 30°C for 2 days with shaking at 150 rpm. Capsules and cell bodies were measured under oil immersion using an Olympus AX70 microscope (Olympus, Melville, NY, USA). The outer margin of the capsule was determined by exclusion of Indian ink. Cell body and capsule size were measured manually using Image J 1.40g software (NIH, Bethesda, MD, USA). Capsule size was the distance from the cell wall to the Indian ink exclusion zone. For each condition at least 500 measurements were calculated. For fluorescence staining of the capsule, TSA-treated and control *C. neoformans* yeast cells were co-stained with Uvitex 2B (cell wall) and monoclonal antibody 1887HU (capsule) conjugated with tetramethylrhodamine isothiocyanate (TRITC). Images were taken with red and blue channels using a fluorescence Olympus microscope (objective ×40).

### Macrophage viability assay

Macrophage viability was assessed as described previously with minor modifications. The macrophage-like cell line J774.16 (derived from a reticulum cell sarcoma) was obtained from the ATCC. The J774.16 cells were grown using Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Carlsbad, CA, USA) containing 10% fetal calf serum, 10% NCTC-109 (Life Technologies), 1% non-essential amino acids (Mediatech, Manassas, VA, USA) and 1% penicillin/streptomycin at 37°C in 5% CO₂. To examine the effect of SB on macrophage viability, the J774.16 cells were plated in 96-well microplates at a density of 3 × 10⁴ cells/well and incubated overnight. The cells were washed and plated in DMEM without or with SB (20 or 40 mM). Viability was assayed by the MTT method. The macrophage-like cell line J774.16 (derived from a reticulum cell sarcoma) was obtained from the ATCC. The J774.16 cells were grown using Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Carlsbad, CA, USA) containing 10% fetal calf serum, 10% NCTC-109 (Life Technologies), 1% non-essential amino acids (Mediatech, Manassas, VA, USA) and 1% penicillin/streptomycin at 37°C in 5% CO₂. To examine the effect of SB on macrophage viability, the J774.16 cells were plated in 96-well microplates at a density of 3 × 10⁴ cells/well and incubated overnight. The cells were washed and plated in DMEM without or with SB (20 or 40 mM). Viability was assayed by the MTT method.
Phagocytosis assays

Phagocytosis assays were performed as described previously. Briefly, macrophage-like J774.16 cells were plated at a concentration of 10^5 cells/500 μL per well in 24-well cell culture polystyrene plates and grown overnight at 37°C in 5% CO₂. Log-phase C. albicans and C. neoformans yeast cells were collected after 24 h of growth and washed three times with PBS. For C. neoformans, an additional step of opsonization with antibody 18B7 at the concentration of 50 mg/L at 37°C for 1 h was performed. C. albicans or C. neoformans cells were added to the macrophages at a ratio of 5:1 (yeasts:macrophage), without or with SB (20 or 40 mM), and the plates were incubated for 1 h at 37°C in 5% CO₂. Wells were then washed with PBS and fixed with a 40% methanol solution. The numbers of macrophages and yeasts were recorded for each field, and at least 200 macrophages were counted. The phagocytosis index was defined as the ratio of the number of intracellular yeasts to the number of macrophages counted.

Macrophage killing assay

Washed, log-phase C. albicans and C. neoformans yeast cells were inoculated at a ratio of 5:1 with J774.16 macrophages with or without addition of SB, as in the phagocytosis assay. The plates were incubated for 2 h at 37°C. The co-cultures were then washed three times with cold PBS and macrophages were lysed by pipetting with sterile water. Equal aliquots from each condition were plated on YPD agar and incubated at 30°C for 2 days. The number of yeast colonies was determined and the colony number recovered was expressed as the percentage of growth relative to yeast incubated in the absence of macrophages.

Measurement of nitric oxide and superoxide release by macrophages

To measure nitric oxide and superoxide production, J774.16 cells were prepared and co-cultured with yeast cells as described above. The macrophages were lysed after 4 h of incubation at 37°C. Aliquots of supernatants from co-cultures or uninfected macrophages were collected. Nitric oxide levels were measured using a commercial Griess reagent kit (Promega, Madison, WI, USA). Similarly, superoxide dismutase activity from the supernatants was determined using a method that involves generation of superoxide and reduction of the tetrazolium dye MTT to its formazan, measured at 570 nm.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Unless otherwise noted, one-way analysis of variance was used to compare the differences between groups, and individual comparisons of groups were done using a Bonferroni post test. The 95% confidence interval was determined in all experiments.

Figure 1. Effects of SB on fungal growth. (a) SB inhibited the growth of C. albicans, C. parapsilosis and C. neoformans in YPD. Error bars represent standard deviations from two independent experiments with five replicates. Significant differences of at least P<0.01 relative to controls were achieved for each yeast species at ≥10 mM. *P<0.01, **P<0.001, ***P<0.0001. (b) SB reduced fungal biofilm formation on a polystyrene surface. Error bars represent standard deviations from two independent experiments with four replicates. Significant differences of P<0.0001 relative to controls were achieved for each yeast species at ≥10 mM. (c) SB synergistically increased susceptibility of yeasts to fluconazole. Spot assays of yeast cells without (Mock) or with the indicated drug concentrations without (top panels) or with (lower panels) 40 mM SB are shown. Experiments were performed twice with similar results. AMB, amphotericin B; FLC, fluconazole; CAS, caspofungin.
Results

**SB inhibits pathogenic fungal growth**

To test the ability of SB to inhibit growth, yeast cells were inoculated in liquid YPD with or without various concentrations of SB. We found that SB significantly inhibited yeast growth in a concentration-dependent manner (Figure 1a). Concentrations of 10 mM SB inhibited the growth of *C. albicans* and *C. parapsilosis* by ~25% at 24 h of incubation (*P*<0.01). SB at 20–60 nM reduced the growth of Candida yeast by 33%–57% compared with controls (*P*<0.001). Interestingly, we found that 10 mM inhibited the growth of *C. neoformans* by 40% (*P*<0.001). SB at 20–60 mM SB resulted in a 60%–82% growth inhibition in *C. neoformans* (*P*<0.001).

Biofilm formation is associated with fungal virulence. To test whether SB impedes biofilm formation *in vitro*, we induced yeast cells to form biofilm on polystyrene surfaces for 2 days. SB was then added to the biofilm cultures. We found that addition of 10 mM SB impeded biofilm formation by ~65% in *C. neoformans* and *C. parapsilosis*, whereas it inhibited 80% of *C. albicans* biofilm growth (Figure 1b, *P*<0.0001 for all yeast strains).

To examine whether SB potentiated the effects of commonly used antifungals, we tested the susceptibility of the yeast cells to antifungal drugs in the presence of different concentrations of SB. Concentrations of 20 or 40 mM SB did not increase the antifungal activity of amphotericin B or caspofungin, whereas 40 mM SB significantly enhanced the susceptibility of all of the yeasts to fluconazole (Figure 1c). Concentrations of less than 40 mM SB did not increase the antifungal activity of amphotericin B or caspofungin, whereas 40 mM SB significantly enhanced the susceptibility of all of the yeasts to fluconazole (Figure 1c). Concentrations of less than 40 mM SB did not increase the antifungal activity of amphotericin B or caspofungin, whereas 40 mM SB significantly enhanced the susceptibility of all of the yeasts to fluconazole (Figure 1c).

**Figure 2.** SB inhibits *C. albicans* filamentation and *C. neoformans* capsule formation and melanization. (a) SB impeded the filamentation of *C. albicans* in serum medium. Images were taken with an Olympus microscope (objective ×20). Scale bar = 20 μm. (b) SB inhibited *C. neoformans* capsule formation in MM and in 10% serum. Images were taken using bright-field microscopy (objective ×40). Scale bar = 5 μm. (c) Capsule and cell body sizes of *C. neoformans* cells were measured in cultures shown in (b). A minimum of 500 cells were used for measurements of capsule and cell body sizes for each indicated condition. SB significantly reduced capsule size (left panels; **P**<0.001; ***P**<0.0001), but did not affect the cell body size (right panels). Error bars represent standard deviations. (d) TSA inhibited *C. neoformans* capsule formation in 10% serum. Yeast cells were co-stained with Uvitex 2B (blue) for the cell wall and monoclonal 18B7-conjugated TRITC (red) for the capsule. Images were taken with an Olympus fluorescence microscope (objective ×40). Scale bar = 5 μm. (e) SB inhibited melanization of *C. neoformans* cells in MM. Images were taken with a digital camera after 7 days of incubation. All experiments were performed twice with similar results. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
not significantly enhance yeast susceptibility to fluconazole, although yeast colonies were smaller (data not shown).

**SB inhibits filamentation in C. albicans and capsule and melanin formation in C. neoformans**

We evaluated the impact of SB on well-known virulence factors of *C. albicans* and *C. neoformans*. Filamentation is associated with virulence in *C. albicans*, and we found that 20 mM SB significantly suppressed *C. albicans* filamentation (Figure 2a). Capsule formation is the major virulence factor of *C. neoformans*, and SB significantly inhibited capsule formation by the yeast in inducing conditions (Figure 2b and c). This inhibitory effect of SB was concentration-dependent in the capsule-inducing condition with 10% serum. Notably, there was no effect on cell body size. We also tested whether the HDAC inhibitor TSA had a similar effect on capsule inhibition. Indeed, we found that TSA (9 mg/L) inhibited capsule formation in 10% serum medium (Figure 2d). Melanin formation is another important virulence factor of *C. neoformans*, and we observed that 20 mM SB significantly inhibited melanization of *C. neoformans* (Figure 2e). Thus, in addition to inhibiting yeast cell growth, SB affects the expression of key fungal virulence factors.

**SB is protective to macrophages infected with pathogenic yeasts**

To assess the effect of SB on macrophages, we examined the effect of SB on macrophage viability. The metabolic activity of J774.16 macrophages was examined in the MTT reduction assay at 2 and 4 h with or without various concentrations of SB. We observed that there were no significant changes in macrophage viability when 20 or 40 mM SB was added to the medium at 2–4 h of incubation (Figure 3). We then examined the viability of macrophages in the presence of *C. albicans* and *C. neoformans* yeast cells and different SB concentrations (Figure 3). In the presence of 20 mM SB, the viability of *C. albicans*-infected macrophages was significantly greater than that of non-treated samples after 2 or 4 h (Figure 3a and b, *P* < 0.05). With 40 mM SB, the protective effect in the presence of *C. albicans* was evident only at 4 h. For *C. neoformans*, SB significantly enhanced the viability of infected macrophages at 20 mM at 4 h and at 40 mM at 2 and 4 h (*P* < 0.05). Therefore, SB enhances macrophage viability during infection with pathogenic yeasts.

**SB enhances effector functions of macrophages**

Since SB enhances macrophage survival upon challenge with yeast cells, we investigated the phagocytic rate and killing activity of J774.16 cells with or without SB. We found that J774.16 cells phagocytosed *C. albicans* and *C. neoformans* yeasts more effectively in the presence of SB (Figure 4a).
macrophages were exposed to C. albicans, the addition of 40 mM SB significantly increased phagocytosis by ~50% in comparison with the control (Figure 4a, P<0.05). For C. neoformans, the addition of 20 or 40 mM SB significantly increased phagocytosis by ~25% and 80%, respectively (Figure 4a, P<0.05 for 20 mM SB and P<0.0001 for 40 mM SB). In addition to enhanced phagocytosis, we found that intracellular killing of the yeast cells by macrophages was significantly increased in the presence of SB. The addition of 20 or 40 mM SB increased the killing of both C. albicans and C. neoformans by 25% and 50%, respectively (Figure 4b, P<0.05).

Butyrate increases production of nitric oxide, but not superoxide, by macrophages infected with pathogenic yeasts

To further elucidate the possible killing mechanisms of the macrophages against yeast cells in the presence of SB, we measured the release of nitric oxide and superoxide by J774.16 macrophages with and without SB. We found that the nitric oxide level in the supernatants of cultures with J774.16 macrophages was enhanced in presence of C. albicans (P=0.1002) or C. neoformans (P<0.001) yeast cells. Furthermore, the addition of 20 mM or 40 mM SB to the macrophages challenged with either of these yeasts resulted in further significant increases in nitric oxide levels compared with macrophages and yeast cells without SB (Figure 5a). Addition of SB did not alter nitric oxide levels of J774.16 cells in the absence of yeast cells (data not shown). In contrast to the enhanced nitric oxide production, we observed a decrease in superoxide levels after addition of 20 mM SB to co-cultures with C. albicans and 20 and 40 mM to co-cultures with C. neoformans (Figure 5b). Nevertheless, the levels of superoxide in macrophage cultures with yeast cells were significantly increased relative to uninfected macrophages (P<0.001). Addition of SB did not alter superoxide levels of J774.16 cells in the absence of yeast cells (data not shown). Thus, we concluded that SB leads to increased macrophage nitric oxide production in response to these pathogens.

Discussion

Several recent studies have shown that inhibition of HDAC can suppress yeast growth and virulence.25–27 SB is a well-established HDAC inhibitor.23,24 In the present work we tested the activity of SB against several medically important fungi. Our results show that SB is a potent inhibitor of C. albicans, C. parapsilosis and C. neoformans growth and the inhibition is dose-dependent. Furthermore, we show that SB also inhibits biofilm formation by these pathogenic yeasts. Since fungal biofilm formation is associated with virulence and drug resistance,25 our data suggest that SB might be advantageous for combating biofilm infections. Furthermore, SB enhanced yeast susceptibility to fluconazole, suggesting that SB could augment the effects of fluconazole and potentially increase therapeutic efficacy.

Filamentation of C. albicans26 and capsule formation by C. neoformans26 are well-established virulence traits that are strongly suppressed by SB. The effects of SB on C. albicans morphological switching were anticipated based on prior studies showing that filamentation and genes associated with this switch are affected by HDAC inhibitors.25–27 The most distinctive feature of C. neoformans is a polysaccharide capsule that enlarges depending on environmental stimuli and growth conditions.27 This capsule has been associated with diverse deleterious effects on the immune response.27–28 We observed that SB specifically inhibited the yeast capsule, but not the cell body. A reduction in capsule size diminishes the capacity of the yeast to evade phagocytosis and toxic host effector molecules. Hence, in addition to inhibiting the growth of C. albicans and C. neoformans, SB disturbs the development of virulence determinants, which further diminishes the pathogenic capacity of these fungi.

Macrophages are major effector cells in the host response to pathogenic fungi. SB did not impact the viability of macrophages. Moreover, SB provided a survival advantage for macrophages infected with either C. albicans or C. neoformans. Additionally, SB increased the phagocytic efficiency of macrophages for C. albicans and C. neoformans and macrophages exposed to SB more effectively killed the ingested yeasts. Furthermore, SB enhanced the production of nitric oxide by macrophages, which correlated with the reduced survival of yeasts in SB-treated macrophages. SB has previously been found to enhance nitric oxide production in bovine mammary epithelial cells during infection with Staphylococcus aureus, which correlated with increased nitric oxide synthase (iNOS) mRNA expression.29 SB has also been shown to enhance iNOS expression and nitric oxide production in intestinal epithelial cells.30

Figure 5. Butyrate enhances macrophage nitric oxide production. (a) The presence of 20 or 40 mM SB increased the release of nitric oxide by J774.16 cells. (b) SB reduced superoxide production by J774.16 cells. Control, uninfected macrophages. *P<0.05, **P<0.01, ***P<0.001. Error bars represent standard deviations. All experiments were performed three times.
cells in response to treatment with lipopolysaccharide and interferon γ. These findings suggest that SB might indirectly trigger the expression of iNOS. In concert with previously published data, our findings suggest that SB augments the capacity of macrophages to combat yeast infection. However, it is not clear how SB modified superoxide production in response to the yeast infections.

In addition to increasing nitric oxide synthesis by macrophages, SB can impact diverse cellular activities, including cell differentiation, proliferation, motility, the induction of cell cycle arrest and apoptosis. SB can also induce antimicrobial peptide production in mammalian colonic epithelium. Because of its capacity to up-regulate protective host responses in the intestine, SB has recently been promoted as a therapeutic agent for Shigella infection and it has been shown to protect against Salmonella infection in birds. It is notable that, in our work, SB exerted its activity against yeast at concentrations as high as ~80 mM in the colon and gastrointestinal tracts. Thus, since infusions of SB have been reported to result in serum levels of ~60 μM, current formulations of SB might be most relevant for oral or topical applications. However, derivatives of SB can be formulated to have enhanced serum stability to achieve higher, sustained drug levels. Since SB inhibits fungal replication, restricts biofilm formation, interferes with the production of virulence determinants, and enhances the microbicidal activity of host effector cells, we propose that SB or derivatives should be exploited in antifungal drug development.

Funding
This study was supported in part by a Hirschl/Weill-Caulier Career Scientist Award (to J. D. N.). Livia C. L. Lopes was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Transparency declarations
None to declare.

References


