In vitro and *in vivo* study on the effect of antifungal agents on hematopoietic cells in mice

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Abstract

Liposomal amphotericin B, voriconazole, and caspofungin are currently used for systemic and severe fungal infections. Patients with malignant diseases are treated with granulocyte-colony stimulating factor (G-CSF) for the recovery of granulocytes after chemotherapy or hematopoietic cell (HC) transplantation. Since they have a high incidence of fungal infections, they inevitably receive antifungal drugs for treatment and prophylaxis. Despite their proven less toxicity for various cell types comparatively with amphotericin B and the decrease in the number of leukocytes that has been reported as a possible complication in clinical studies, the effect of liposomal amphotericin B, voriconazole, and caspofungin on HCs has not been clarified. The present study aimed to examine the *in vitro* and *in vivo* effect of these three modern antifungals on HCs. Colony-forming unit (CFU) assays of murine bone marrow cells were performed in methylcellulose medium with or without cytokines and in the presence or absence of various concentrations of liposomal amphotericin B, voriconazole, and caspofungin. In the *in vivo* experiments, the absolute number of granulocytes was determined during leukocyte recovery in sublethally irradiated mice receiving each antifungal agent separately, with or without G-CSF. *In vitro*, all three antifungal drugs were nontoxic and, interestingly, they significantly increased the number of CFU-granulocyte-macrophage colonies in the presence of cytokines, at all concentrations tested. This was contrary to the concentration-dependent toxicity and the significant decrease caused by conventional amphotericin B. *In vivo*, the number of granulocytes was significantly higher with caspofungin plus G-CSF treatment, higher and to a lesser extent higher, but not statistically significantly, with voriconazole plus G-CSF and liposomal amphotericin B plus G-CSF treatments, respectively, as compared with G-CSF alone. These data indicate a potential synergistic effect of these antifungals with the cytokines, *in vitro* and *in vivo*, with subsequent positive effect on hematopoiesis.

Keywords: liposomal amphotericin B, voriconazole, caspofungin, hematopoiesis, cell culture, granulocytes

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Introduction

Polyenes, azoles, and echinocandins represent three major classes of antifungal compounds clinically used as therapeutic options for deep fungal infections. Amphotericin B is a polyene agent for which toxicity in clinical use as well as in cell cultures has been observed.^{1,2} Lipid formulations of amphotericin B represent choices developed to reduce toxicity and increase efficacy of amphotericin B. Liposomal amphotericin B is a liposomal formulation of amphotericin B in which the drug is incorporated into

phospholipid-containing small unilamellar liposomes.³ Voriconazole is a second-generation triazole agent, and caspofungin is an echinocandin drug representing two other modern antifungals that have been introduced in an effort to increase efficacy and reduce toxicity.^{4,5} Liposomal amphotericin B, voriconazole, and caspofungin have been shown to be less toxic for various cell types exposed to them, as compared with amphotericin B.^{3,6,7} Moreover, the number of leukocytes in patients receiving the antifungal drugs can be affected. A decrease in leukocyte number

has been reported in clinical studies.^{8–10} However, the effect of modern antifungals on hematopoietic cells (HCs) *in vitro*, *in vivo*, and in clinical practice remains unclear.

During hematopoiesis, HCs proliferate and differentiate within bone marrow (BM) from the immature into the various mature cell types that are released into blood circulation. Cytokines are a group of glycoproteins representing signal molecules provided by the BM microenvironment that are critical for lineage-committed cells and their progenies to complete their differentiation program. Hematopoiesis can occur *in vitro* in the presence of media and cytokines critical for HC proliferation, differentiation, and survival. Semisolid HC cultures represent the appropriate method for hematopoietic progenitor cell (HPC) detection, count, and identification. Under culture conditions and in the presence of proper cytokines, progenitors (colony-forming units, CFUs) reach the final differentiation step forming colonies of easily definable specific lineage mature cells. On the other hand, study and comparison of specific factor activity on a given cell population is enabled by estimation of the number of colonies formed.¹¹

The aim of this study was to examine whether liposomal amphotericin B, voriconazole, and caspofungin have any effect on HCs *in vitro* using CFU assays, in comparison with conventional amphotericin B. Interestingly, it was found that liposomal amphotericin B, voriconazole, and caspofungin not only were nontoxic, but they also increased the number of murine CFU-granulocyte-macrophage (CFU-GM) colonies in cultures containing them by acting synergistically with the culture's cytokines. This phenomenon prompted further *in vivo* experiments regarding the recovery of granulocytes in sublethally irradiated mice. The modern antifungals increased the number of granulocytes in combination with granulocyte-colony stimulating factor (G-CSF), pointing out a potential synergy with this cytokine *in vivo*.

Materials and methods

Antifungal drugs

Amphotericin B (Fungizone) was obtained from Gibco Life Technologies (Paisley, UK). The clinical formulations of liposomal amphotericin B (AmBisome; Gilead Sciences Limited, Carrigtohill, County Cork, Ireland), voriconazole (VFend; Pfizer PGM, Pocé-sur-Cisse, France), and caspofungin (Cancidas; Merck Sharp & Dohme B.V., Haarlem, The Netherlands) powders for injection were provided by the hospital pharmacy and were reconstituted and further diluted to the appropriate concentration according to the manufacturers' directions, prior to each experiment.

Animals

C57Bl/6 mice were maintained in conventional clean conditions at the hospital's animal breeding and research facility, and were given mouse food and acidified water *ad libitum*. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee and performed in accordance with the European Parliament and Council Directive 2010/63/EU

as well as with the national and institutional guidelines for animal care and use.

Semisolid HC culture (CFU assay)

Fresh whole BM was collected by flushing the tibias from normal 9- to 12-week-old mice in Iscove's modified Dulbecco's medium with 2% fetal bovine serum (Gibco Life Technologies). BM cells were counted on a Neubauer hemacytometer using 3% acetic acid with methylene blue (Stem Cell Technologies, Vancouver, BC, Canada) and cultured at a concentration of 3.5×10^4 cells/mL in semisolid methylcellulose medium (Stem Cell Technologies) according to the manufacturer's specifications. Cultures contained 0.4 ng/mL of stem cell factor (SCF), 2 ng/mL of interleukin-3 (IL-3), 0.1 ng/mL of granulocyte-macrophage-colony stimulating factor (GM-CSF), 40 ng/mL of interleukin-6 (IL-6), and 1.2 U/mL of erythropoietin (all previous cytokines from R&D Systems, Minneapolis, MN, USA). In single cytokine experiments, 50 ng/mL of SCF, 10 ng/mL of IL-3, 10 ng/mL of GM-CSF, 40 ng/mL of IL-6, 10 ng/mL of G-CSF (R&D Systems), or 3 U/mL of erythropoietin was added. HC cultures were also performed in the absence of cytokines. All cultures were plated in duplicate in 35-mm dishes with or without various concentrations of the antifungals amphotericin B (60, 600, 6000 ng/mL), liposomal amphotericin B (6, 60, 600, 6000 ng/mL), voriconazole (4, 20, 100, 500 ng/mL), and caspofungin (0.4, 2, 10, 50 ng/mL). The culture plates were incubated at 37°C for 7 days in humidified atmosphere with 5% CO₂. A colony was defined as a group of > 50 cells. Colonies were counted using an inverted microscope on the basis of standard morphological criteria. Four independent experiments were performed.

Fungal growth test

BM cells were cultured with and without the antifungals as previously mentioned. After seven days of incubation, the cultures were inspected microscopically for the presence of contaminant fungi and inoculated onto Sabouraud dextrose agar plus chloramphenicol (0.5 g/L) (Laboratorios Conda S.A., Madrid, Spain) and malt extract agar (Fluka Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) petri plates. Plates were incubated at 30°C for 10 days and scanned for fungal growth.

Pharmacokinetics and drug toxicity *in vivo* experiment

Groups of female 10- to 12-week-old mice (n=2) were administered intraperitoneally (i.p.) twofold increasing doses of liposomal amphotericin B (2.5–40 mg/kg), voriconazole (40–80 mg/kg), or caspofungin (1–16 mg/kg) once daily for four days. On the fifth day, peripheral blood (PB) from each mouse was collected by retro-orbital puncture with non-heparinized microhaematocrits and centrifuged to isolate serum for the measurement of trough (before the next dosing) levels of the antifungals by drug diffusion bioassays. In parallel, during the experiments as well as after drug discontinuation, mice were observed for potential visible toxicity signs (pain, weight loss, and death).

A toxicity test was performed in the same way in sublethally irradiated mice (5 Gy, drug administration once daily for 26 days).

Drug diffusion bioassay

Microbiological methods were used for the measurements of voriconazole, amphotericin B, and caspofungin levels. A validated and established (in service at the P.H.E. Mycology Reference Laboratory, Bristol, UK) method¹² for the measurement of voriconazole was performed on 24 × 24 cm agar (Scharlau Chemie S.A., Barcelona, Spain) plates by observing the inhibition of fungal (*Candida kefyr*) growth, measuring the inhibition zones around specific concentrations of the drug and forming a calibration curve according to which the unknown samples were quantified. The agar plates were supplemented with yeast nitrogen base (Fluka Sigma-Aldrich Chemie GmbH), glucose (Merck KGaA, Darmstadt, Germany), citrate solution (Mallinckrodt Chemical Works, Los Angeles, CA, USA), and the pH was optimally adjusted to 7.0. Similar methods for amphotericin B and caspofungin were performed on 10 × 10 cm agar (Oxoid Ltd, Basingstoke, England) plates, supplemented with RPMI 1640 and 0.165 mol/L MOPS (AppliChem, Darmstadt, Germany). The pH was adjusted again to 7.0 and the fungal strains used were *Paecilomyces variotii* and *C. kefyr*, respectively.

Absolute granulocyte count *in vivo* experiment

Groups of female 10- to 12-week-old mice (n = 8) were sublethally irradiated (day 0) with 5 Gy delivered by gamma-ray apparatus (IBL 437C), rendering them neutropenic. After irradiation, mice were housed in sterilized cages and had free access to autoclaved food and acidified water containing 0.02 mg/mL of ciprofloxacin (Ciprofloxacin/Generics; Mylan S.A.S., Saint-Priest, France). In the first group, mice received i.p. liposomal amphotericin B (15 mg/kg), voriconazole (60 mg/kg), or caspofungin (8 mg/kg). In the second group, mice were injected subcutaneously (s.c.) with recombinant human G-CSF (100 µg/kg, Filgrastim; Amgen Europe B.V., Breda, The Netherlands). The third group consisted of mice that were coadministered G-CSF (s.c.) and one of the antifungals (i.p.). G-CSF and drugs were given once daily from day 1 until day 26 after irradiation. The antifungals' doses used were chosen based on the pharmacokinetics and drug toxicity *in vivo* experiments mentioned. A fourth group included untreated mice that received sublethal irradiation alone (control group). Four days prior to irradiation as well as every 3–4 days until day 27 after irradiation, PB from each mouse was collected by retro-orbital puncture with heparinized microhaematocrits, and the absolute number of granulocytes was determined by flow cytometry.

Flow cytometry

The method was performed following the manufacturers' guidelines. PB cells (50 µL) were stained at room temperature for 15 min with the fluorescein isothiocyanate-conjugated anti-mouse Gr-1 monoclonal antibody

(Ly-6 G/Ly-6 C, clone RB6-8C5; Pharmingen BD Biosciences, San Jose, CA, USA). Then erythrocytes were lysed with FACS lysing solution (dilution 1:10 with distilled water; BD Biosciences, San Jose, CA, USA). A defined number of fluorescent microbeads (Zebra Bioscience BV, Enschede, Overijssel, The Netherlands) was added to permit the acquisition of absolute cell counts even at very low numbers, followed by immediate flow cytometry analysis. Data were acquired using FACSCalibur cytometer and further analyzed with CellQuest Pro software (BD Biosciences) applying a recommended gating strategy.

Statistical analysis

Data were analyzed using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Results are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were performed using paired Student's *t* test (for *in vitro* data) or Student's two-sample independent *t* test (for *in vivo* data). *P* values < 0.05 were considered statistically significant.

Results and discussion

Positive effect of liposomal amphotericin B, voriconazole, and caspofungin on murine HCs *in vitro* in comparison with amphotericin B

The effect of antifungal drugs was tested on the number of colonies in semisolid murine HC cultures containing a cocktail of cytokines (Figure 1). At 60 ng/mL, amphotericin B was not toxic for HCs since the number of CFU-GM colonies was similar to the control culture containing no antifungals. Increasing concentrations, 600 and 6000 ng/mL, caused a concentration-dependent toxicity resulting in significant CFU-GM colony number decrease compared with the control culture (12 ± 1% and 31 ± 2% decrease; *P* = 0.002 and 0.003, respectively). This is consistent with what is already known about the toxic effect of amphotericin B. In contrast, none of the tested concentrations of liposomal amphotericin B, voriconazole, and caspofungin was found to be toxic for HCs in cultures. Interestingly, the number of CFU-GM colonies was significantly increased in all cultures containing them. There was no difference in colony-forming potential among the different antifungals. The addition of 6 ng/mL of liposomal amphotericin B, 4 ng/mL of voriconazole, or 0.4 ng/mL of caspofungin to HC cultures was sufficient to lead to an increase of the CFU-GM colony number compared with the control culture (25 ± 4%, 26 ± 4%, or 25 ± 3% increase; *P* = 0.004, 0.006, or 0.01, respectively). Concentrations of 60 ng/mL of liposomal amphotericin B, 20 ng/mL of voriconazole, or 2 ng/mL of caspofungin appeared to be the optimum promoting the highest increase compared with the control culture (37 ± 5%, 40 ± 7%, or 40 ± 7% increase; *P* = 0.003, 0.007, or 0.007, respectively). A plateau in the increase of the number of CFU-GM colonies compared with the control culture was observed for 600 and 6000 ng/mL of liposomal amphotericin B (37 ± 5% and 36 ± 4% increase; *P* = 0.003 and 0.003, respectively), whereas 100 and 500 ng/mL of voriconazole or 10 and 50 ng/mL of caspofungin led to smaller than their

Figure 1 Effect of liposomal amphotericin B, voriconazole, and caspofungin compared with amphotericin B in CFU assays of murine BM cells in the presence of a cocktail of cytokines. Amphotericin B led to a concentration-dependent toxicity decreasing significantly the number of CFU-GM colonies up to $31 \pm 2\%$ compared with the control culture. On the other hand, modern antifungals were not toxic and significantly increased the number of CFU-GM colonies, with a peak of $37 \pm 5\%$ at 60 ng/mL of liposomal amphotericin B and $40 \pm 7\%$ at 20 ng/mL of voriconazole or at 2 ng/mL of caspofungin, as compared with the control. $n = 4$ independent experiments performed in duplicate (mean $\pm$ SEM). Paired Student's t test (*, $P \leq 0.01$)

optimum concentrations but greater than their lower tested concentrations increase ($36 \pm 5\%$ and $34 \pm 5\%$ or $36 \pm 6\%$ and $31 \pm 6\%$ increase; $P = 0.009$ and 0.007 or 0.004 and 0.01 , respectively). These results indicate not only a non-toxic but also a positive *in vitro* effect of these modern antifungal agents on CFU-GM colony formation, contrary to the toxic effect of amphotericin B.

The positive effect of liposomal amphotericin B, voriconazole, and caspofungin is not due to the fungal inhibition

To confirm that the increase of the number of CFU-GM colonies in cultures containing the modern antifungals was not caused by the elimination of potentially grown fungi in culture medium that might affect the proliferation and differentiation of HPCs, fungal growth tests were performed. No fungi were detected in control culture and in cultures containing the antifungals. Liposomal amphotericin B, voriconazole, and caspofungin seem to induce colony formation in HC cultures that is not mediated by inhibition of potential fungal growth, pointing out another possible mode of action, apart from their antifungal activity.

Synergy between individual cytokines and the antifungal drugs in HC cultures

The fact that the presence of cytokines in HC cultures is essential for proliferation and differentiation of HPCs, together with the above results of additional colony growth for liposomal amphotericin B, voriconazole, and caspofungin in cultures containing a cocktail of cytokines, further prompted the assumption of a similar to cytokines potential growth-promoting activity for these antifungals. Hypothesizing that the antifungal drugs could act as potential cytokines, it is presumed that they might be able to stimulate HPCs alone or in synergy with other factors. To address this point, CFU assays without cytokines but with various concentrations of the three antifungals were employed. No colonies were able to grow at any drug

concentration tested, suggesting that these antifungals cannot exert their positive effect on colony growth in the absence of cytokines. Thus, a synergistic and not an additive effect of them with the culture's cytokines is implied. To better characterize this outcome, CFU assays containing single cytokines and the optimum, on the basis of the earlier findings, concentration of the antifungals were performed (Figure 2). Compared with the respective control cultures (cytokine alone), significant increase of the number of CFU-GM colonies was observed at all different combinations tested, except for the increase caused by SCF plus voriconazole that had a borderline statistical significance ($P = 0.058$). Liposomal amphotericin B, voriconazole, and caspofungin increased the number of CFU-GM colonies in combination with SCF ($20 \pm 8\%$, $30 \pm 11\%$, and $39 \pm 10\%$ increase; $P = 0.03$, 0.058 , and 0.007 , respectively), IL-3 ($15 \pm 3\%$, $16 \pm 1\%$, and $21 \pm 3\%$ increase; $P = 0.006$, 0.005 , and 0.006 , respectively), GM-CSF ($19 \pm 4\%$, $17 \pm 1\%$, and $21 \pm 5\%$ increase; $P = 0.03$, 0.008 , and 0.017 , respectively), G-CSF ($47 \pm 7\%$, $45 \pm 6\%$, and $49 \pm 6\%$ increase; $P = 0.001$, 0.0004 , and 0.002 , respectively), or IL-6 ($45 \pm 14\%$, $35 \pm 7\%$, and $55 \pm 13\%$ increase; $P = 0.006$, 0.0003 , and 0.002 , respectively). These results highlight the necessity of the culture's cytokines for the exertion of antifungal drugs' colony-forming potential, revealing a synergistic contribution to each cytokine's effect for each one of the antifungals.

In vivo experiments: pharmacokinetics and drug toxicity

To test toxicity and tolerance of liposomal amphotericin B, voriconazole, and caspofungin after i.p. injection in mice, pharmacokinetic and toxicity experiments were performed. Treatment with 2.5, 5, 10, 20, and 40 mg/kg of liposomal amphotericin B showed trough levels of 0.44, 0.57, 0.96, 2.41 ± 0.32 , and 5.94 mg/L, respectively. The trough levels for 40 and 80 mg/kg of voriconazole were found to be 0.6 and 4.5 mg/L, respectively. 1, 2, 4, 8, and 16 mg/kg of caspofungin resulted in 0.55 ± 0.05 , 1.1 ± 0.1 , 2 ± 0.82 , 4.83 ± 0.83 , and 9.66 ± 1.66 mg/L, respectively. All antifungals' doses

Figure 2 Effect of liposomal amphotericin B, voriconazole, and caspofungin in CFU assays of murine BM cells in the presence of the single cytokines SCF (a), IL-3 (b), GM-CSF (c), G-CSF (d), or IL-6 (e). Liposomal amphotericin B in combination with each cytokine significantly increased the number of CFU-GM colonies to $20 \pm 8\%$, $15 \pm 3\%$, $19 \pm 4\%$, $47 \pm 7\%$, or $45 \pm 14\%$, respectively, as compared with the respective controls. Voriconazole plus SCF induced a $30 \pm 11\%$ increase with borderline statistical significance, whereas its combination with IL-3, GM-CSF, G-CSF, or IL-6 significantly increased the number of CFU-GM colonies to $16 \pm 1\%$, $17 \pm 1\%$, $45 \pm 6\%$, or $35 \pm 7\%$, respectively, as compared with the respective controls. Caspofungin in combination with SCF, IL-3, GM-CSF, G-CSF, or IL-6 significantly increased the number of CFU-GM colonies to $39 \pm 10\%$, $21 \pm 3\%$, $21 \pm 5\%$, $49 \pm 6\%$, or $55 \pm 13\%$, respectively, as compared with these cytokines alone. $n = 4$ independent experiments performed in duplicate (mean $\pm$ SEM). Paired Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P \leq 0.001$)

Figure 3 Effect of caspofungin (a), voriconazole (b), and liposomal amphotericin B (c), alone and in combination with G-CSF, on the recovery of granulocytes in sublethally irradiated mice as determined by flow cytometry at specific time points. From day 3 until day 13 after irradiation, a very low number of granulocytes was observed in all groups. Daily s.c. injections of G-CSF alone significantly increased the number of granulocytes at all time points after day 13, as was expected. Daily i.p. injections of the antifungals alone induced no significant change in granulocyte counts as compared with the untreated mice. G-CSF plus caspofungin resulted in significant increase in the number of granulocytes on days 13, 20, and 23, as compared with the G-CSF-treated group. Days 16 and 27 showed also higher counts but not statistically significant. G-CSF plus voriconazole resulted in non-statistically significant increase in the number of granulocytes at all time points after day 16, as compared with the G-CSF-treated group. G-CSF plus liposomal amphotericin B resulted in non-statistically significant increase in the number of granulocytes on days 23 and 27, as compared with the G-CSF-treated group. $n = 8$ mice per group (mean $\pm$ SEM). Student's *t* test (*, $P < 0.05$; **, $P \leq 0.01$)

were generally well tolerated in nonirradiated mice, whereas, in sublethally irradiated mice, the maximum well-tolerated doses for liposomal amphotericin B, voriconazole, and caspofungin were 15, 60, and 8 mg/kg, respectively.

Effect of liposomal amphotericin B, voriconazole, and caspofungin, alone or with G-CSF, on the recovery of granulocytes in mice after sublethal irradiation

To further investigate whether liposomal amphotericin B, voriconazole, and caspofungin have a similar effect *in vivo*

during the intensive activation of hematopoiesis, absolute granulocyte count experiments were performed in sublethally irradiated mice (Figure 3). The fact that G-CSF is the most commonly used cytokine in clinical practice to increase the number of circulating granulocytes in neutropenic patients¹³ prompted its selection for the *in vivo* experiments. From day 3 to day 13 postirradiation, the number of granulocytes remained at very low levels in all groups of mice. In G-CSF-treated group, as expected, the number of granulocytes was significantly higher at all time points after day 13, compared with the control group. The granulocyte count showed no significant difference between the antifungal drug-treated mice and the control group, indicating that these three antifungals had no effect when used alone. These data coincide with the observed lack of effect of these antifungal drugs alone on proliferation and differentiation of HPCs in CFU assays. Coadministration of G-CSF and caspofungin significantly increased granulocyte numbers on day 13 (239 ± 53 vs. 119 ± 19 ; $P=0.037$), day 20 (2145 ± 395 vs. 1029 ± 131 ; $P=0.01$), and day 23 (3213 ± 370 vs. 1736 ± 270 ; $P=0.003$), compared with the G-CSF-treated group. Counts on days 16 and 27 were also higher but not statistically significant. G-CSF plus voriconazole treatment induced an increase in the number of granulocytes at all time points after day 16, compared with the G-CSF-treated group, which was not statistically significant. These results underline a possible positive *in vivo* activity of these antifungals on the recovery of granulocytes, observed only in the presence of G-CSF thus revealing a potential synergy of each of them with this cytokine. Liposomal amphotericin B plus G-CSF showed no significant difference compared with G-CSF alone, resulting in a lower than caspofungin plus G-CSF and voriconazole plus G-CSF increase only on days 23 and 27 that was not statistically significant (1743 ± 574 vs. 1514 ± 188 ; $P=0.7$ and 2614 ± 692 vs. 2419 ± 353 ; $P=0.8$, respectively).

All the presented *in vitro* and *in vivo* data of this study reveal a possible positive effect of caspofungin, voriconazole, and liposomal amphotericin B on HCs, in the presence of cytokines. Toll-like receptors (TLRs) might play a role in the understanding of the above described findings. In addition to their direct antifungal effects, through unique mechanism of action,^{1,3-5} the three antifungals have immunomodulatory properties too. More specifically, these compounds act on mononuclear cells and granulocytes inducing expression of TLRs and TLR-dependent signaling (TLR4 for liposomal amphotericin B,¹⁴ TLR2 for voriconazole,¹⁵ and TLR9 for caspofungin¹⁶). TLRs are able to recognize antigens of pathogens thus activating immune responses.¹⁷ Apart from mature immune cells, hematopoietic stem and progenitor cells also express functional TLRs that influence hematopoiesis in response to pathogens during infection, and especially there is a tendency towards myeloid rather than lymphoid differentiation. This stimulation of myeloid differentiation pathways enables rapid replacement and/or increase of mature myeloid cells. It was shown that TLR2, TLR4, TLR9, and their coreceptors are expressed by the progenitors of myeloid lineage, but not the progenitors dedicated to megakaryocyte and erythroid differentiation. Upon TLR ligation, myeloid progenitors

give rise to monocytes and/or macrophages and/or granulocytes, *in vitro* and *in vivo*.¹⁸⁻²¹ Thus, the possible stimulatory effect of the three modern antifungal agents on HCs might be explained within the context of the myeloid progenitor cell TLR signaling.

In conclusion, the reported data suggest that caspofungin, voriconazole, and liposomal amphotericin B not only are nontoxic in HC cultures, but they also stimulate the proliferation and differentiation of HPCs increasing the number of CFU-GM colonies in cultures. This positive effect is not due to the inhibition of potential grown fungi in culture medium that might affect the growth of colonies, but through a possible synergy of the antifungals with the culture's cytokines. This synergy is also observed *in vivo*, as an increase in the number of granulocytes during the recovery from the neutropenia. The present study demonstrates a possible different mode of action for caspofungin, voriconazole, and liposomal amphotericin B, apart from their antifungal effect, necessitating further investigation of the underlying mechanisms.

Author contributions: MS performed the research, analyzed and interpreted the data, and wrote the manuscript. DB performed the flow cytometry analysis. TAV conducted the fungal growth tests and the drug diffusion bioassays. JM performed the drug diffusion bioassays. NM performed the statistical comparisons. ES contributed to the animal handling. AS and AA provided critical comments and expert consultation during the execution of the study. DS conceived and designed the research and supervised the project, analyzed and interpreted the data, and contributed to the manuscript writing. All authors reviewed the manuscript and approved its final version.

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