Polymyxin B, in combination with fluconazole, exerts a potent fungicidal effect

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Objectives: The objective of this study was to identify existing clinical compounds that either possess a fungicidal activity alone or can act synergistically with fungistatic antifungals.

Methods: We screened a clinical compound library for drugs that exhibited anti-Aspergillus activity. Among selected compounds, the cationic peptide antibiotic polymyxin B was chosen for further characterization because it can be used parenterally and topically. The fungicidal effect of polymyxin B and its synergistic interactions withazole antifungals were tested against a variety of fungal species. The toxicity of the drug combination of polymyxin B and fluconazole was compared with that of each drug alone in mammalian cell cultures.

Results: We found that polymyxin B possesses a broad-spectrum antifungal activity at relatively high concentrations. However, because of its synergistic interactions withazole antifungals, polymyxin B at much lower concentrations exerts a potent fungicidal effect against Cryptococcus neoformans, Candida albicans and non-albicans Candida species and moulds when combined with azoles. The combination of polymyxin B and fluconazole at concentrations within susceptible breakpoints is particularly potent against C. neoformans isolates, including fluconazole-resistant strains. The drug combination displayed no additional toxicity compared with polymyxin B alone when tested in cell culture.

Conclusions: The combination of polymyxin B and fluconazole has the potential to be used in the clinic to treat systemic cryptococcosis. Our findings suggest that combining cationic peptide antibiotics with azole antifungals could provide a new direction for developing novel antifungal therapies.

Keywords: antifungal, combination therapy, antibiotic, fungal membrane

Introduction

Systemic fungal infections have drastically increased over the past three decades due to the rising immunocompromised population as a result of transplantation, cancer chemotherapy, steroid therapy and, in particular, HIV infection (AIDS). Unfortunately, the outcome of current antifungal therapy is far from satisfactory. For example, the three major global invasive mycoses, aspergillosis, candidiasis and cryptococcosis caused by fungal species of Aspergillus, Candida and Cryptococcus, respectively, typically have mortality rates ranging from 10% to 90%. This poor outcome is in part due to the limited number of clinically available antifungals and the fact that many antifungals either lack potency or are toxic to the host. The emergence of resistant fungal strains to current antifungals, which is exacerbated by the necessity for long-term usage of antifungals in immunocompromised individuals, causes additional difficulty in treatment. For example, a recent global survey of close to 3000 Cryptococcus neoformans isolates indicated that >11% of the isolates are resistant to fluconazole. Therefore, there is an urgent need for new therapies. Through a screen of a clinical compound library, we have found that polymyxin B, an antibiotic used to treat bacterial infections, possesses fungicidal activity. Similar to what has been observed previously, polymyxin B is fungicidal at relatively high concentrations. Interestingly, in combination with fluconazole or itraconazole, polymyxin B at low concentrations demonstrates a killing effect against Aspergillus fumigatus,
Rhizopus oryzae, Candida albicans and non-albicans Candida species. The combination at clinically relevant low concentrations is particularly potent against C. neoformans, including strains of varied resistance to fluconazole.

Materials and methods

Strains and media

For the initial drug screening, the Aspergillus nidulans strain R21 was grown on YAG medium (0.5% yeast extract, 2% agar and 2% glucose). For microscopic analyses of the effects of drugs, R21 and the A. fumigatus strain B5233 were grown on YG liquid medium (0.5% yeast extract and 2% glucose) supplemented with 0.12% uridine and uracil (YN). All yeast strains were maintained on yeast peptone dextrose (YPD) medium. Drug disc diffusion and microdilution assays were performed using RPMI 1640 medium buffered with MOPS.

Clinical compound library screening

The Johns Hopkins Clinical Compound Library (JHCCCL version 1.0), a collection of 1514 FDA-approved (1082) and foreign approved (432) drugs (FAD), was screened for drugs with an inhibitory effect on A. nidulans colony growth. The library was assembled in a 96-well plate format with 25 µL aliquots of 10 mM stocks of each compound in either water or DMSO. An aliquot of 2 µL from each well was spotted onto a YAG plate and incubated at 37°C for 2 days. Drugs that either significantly or completely inhibited A. nidulans colony formation were selected. Selected drugs at 2 µM were tested again for an inhibitory effect on A. nidulans in liquid medium. A. nidulans spores (~10⁷) were inoculated into 500 µL of drug-containing liquid medium and incubated at 37°C overnight in the 8-chambered Lab-Tek Borosilicate Coverglass System. Twenty-three compounds that significantly inhibited colony growth of A. nidulans strain R21 at this concentration were identified. Inhibition of spore germination or germ tube extension of the A. nidulans and A. fumigatus isolates was examined microscopically in the presence of polymyxin B at the indicated concentrations for the time period indicated in the text. Viability of those inhibited cells was examined microscopically after they had been cultured in drug-free medium for 24 h.

Disc diffusion halo assay for antifungal activity

Briefly, yeast cells at a cell density of ~5×10⁶ were spread onto RPMI 1640 agar medium with 1-glutamine and without sodium bicarbonate. The plates were allowed to solidify and dry. Whatman paper discs (7 mm) containing water, fluconazole, polymyxin B and their combination at various concentrations were dried and placed on the solidified agar surface. The cells were incubated for 24–48 h at 37°C.

Microdilution assay for antifungal activity

The microdilution assay was performed according to the CLSI (formerly NCCLS) standard except that the cells were incubated at 37°C. Briefly, yeast cells at a final concentration of ~1×10⁵ cells/mL (or Aspergillus or Rhizopus spores at a final concentration of ~5×10⁴ cells/mL) were inoculated in the RPMI 1640 liquid medium with serial (2×) dilutions of each drug being tested. The concentrations used for polymyxin B and fluconazole are indicated in the text. Wells that contained no drugs or no yeast inoculation were included as positive and negative controls. The MIC₉₀ of polymyxin B or the combination was defined as the lowest drug concentration that resulted in a 100% decrease in absorbance compared with that of the control in drug-free medium. MICs were read after incubation without agitation for 24 h for Candida strains and 48 h for the Aspergillus, Rhizopus or Cryptococcus isolates. Fungicidal effect was examined by counting the cfu of the suspension from each well after plating it onto the YPD medium and incubating for 24 (for Candida strains) to 48 h (for Cryptococcus, Aspergillus and Rhizopus strains). The minimal fungicidal concentration (MFC) was defined as the minimal drug concentration that causes at least 99% of cells to be killed compared with the original inoculum. A synergistic fungicidal effect between fluconazole and polymyxin B for each strain was calculated based on the fractional fungicidal concentration index (FFCI), FFCI = [(F/MFC₉₀) – (P/MFC₉₀)]/(F/MFC₉₀) × 100. Where MFC₉₀ and MFC₉₀ are the MFCs of fluconazole and polymyxin B, respectively, and [F] and [P] are the concentrations at which fluconazole and polymyxin B, in combination, are fungicidal. FFCIs ≤0.5 indicate synergistic interactions, FFCIs >0.5–4.0 indicate no interaction and FFCIs >4.0 indicate antagonistic interactions.

Generation of fluconazole-resistant H99FR

C. neoformans H99 reference strain was cultured in yeast nitrogen base (YNB) liquid medium with fluconazole at an inhibitory but sublethal concentration of 2.4 mg/L. The culture was maintained at 37°C with shaking. An aliquot of the culture was transferred to fresh YNB medium with fluconazole every other day and continued for 16 weeks.

Proliferation of HeLa and human monocytic THP-1 cells in the presence of the drugs

HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM l-glutamine and 100 U/mL each of penicillin and streptomycin. THP-1 cells were grown in RPMI 1640 medium with the same supplements. HeLa cells (1×10⁵) were seeded in a well of a 96-well culture plate with 50 µL of DMEM and were grown overnight in a 37°C incubator with 5% CO2. Polymyxin B and/or fluconazole were added to the medium to reach final concentrations of 40 and 100 mg/mL, respectively. At 0, 24, 48 and 72 h after drug addition, cell growth was examined microscopically and quantified. One hundred thousand THP-1 cells were grown in 1 mL of medium and treated similarly. Triplicate samples were taken for each treatment at each timepoint. The experiment was repeated twice.

Results

The antibiotic polymyxin B showed anti-A. nidulans activity

The Johns Hopkins Clinical Compound Library, consisting of 1514 FADs and FDA-approved drugs, was screened for anti-A. nidulans activity. Twenty-three drugs were found to either significantly or completely inhibit the colony growth of the A. nidulans strain R21 when 2 µL aliquots of the drugs from 10 mM stock were directly spotted onto each point inoculum containing ~1×10⁵ spores. Eleven compounds significantly inhibited spore germination in liquid medium at a concentration of 2 µM. The majority of them were known antifungals belonging to the azole family. One antibiotic (polymyxin B sulphate) and three antiseptic compounds were also identified. Because polymyxin B has the potential to treat systemic infections, it was chosen for further studies. The effect of polymyxin B on A. nidulans and its pathogenic relative A. fumigatus is fungicidal. Both spores and pre-germinated germ tubes inhibited by polymyxin B failed to
Polymyxin B with fluconazole is fungicidal

Polymyxin B alone is fungicidal at relatively high concentrations

Because A. nidulans is rarely pathogenic to humans, the susceptibility to polymyxin B of the pathogenic filamentous fungi A. fumigatus and R. oryzae and pathogenic yeasts including C. neoforms, C. albicans, Candida glabrata, Candida krusei and Candida parapsilosis was examined using the standard microdilution assay. Microscopic examination of polymyxin-treated A. fumigatus (B5233) revealed that polymyxin B at 28 or 56 mg/L significantly inhibited germination of A. fumigatus spores compared with the untreated control (Figure 1e). Those spores failed to grow even after removal of the drug, consistent with the fungicidal effect of polymyxin B (Figure 1c and data not shown). Pre-germinated germ tubes also failed to grow in the presence of polymyxin B as evidenced by lack of long hyphae (Figure 1d). However, some spores present in the population were resistant and were able to form hyphae in the presence of the drug after an overnight incubation. Thus, as expected, both B5233 and AF293 isolates showed strong resistance to polymyxin B in the disc diffusion assay (data not shown) and the microdilution assay (MIC<sub>100</sub> > 1000 mg/L) based on visual examination. In contrast, the R. oryzae strain 99-880 is more susceptible, with a MIC<sub>100</sub> of 32 mg/L. The pathogenic yeast strains tested also showed varied susceptibility to polymyxin B, with the MIC<sub>100</sub> ranging from 8 to 256 mg/L (Table 1, columns 1 and 2). This range is consistent with MICs reported in the literature. Given that bacterial strains with MICs > 8 mg/L are considered resistant to polymyxin B, the relatively high MICs observed in these fungal species preclude the clinical use of polymyxin B as a monotherapy in treating fungal infections.

In combination with fluconazole, polymyxin B at lower concentrations is fungicidal for all yeast strains tested

Polymyxins act against Gram-negative bacteria by binding lipopolysaccharide (LPS) and anionic phospholipids in the bacterial membrane, disrupting membrane integrity. It is possible that polymyxin B binds the fungal membrane in an analogous manner, but with reduced efficiency because eukaryotic membranes have low membrane potentials, high levels of sterols and higher contents of neutral lipid. The azole antifungals target the lanosterol 14α-demethylase Erg11 in the fungal ergosterol biosynthesis pathway. This results in a reduced ergosterol level and altered membrane property. We reason that polymyxin B andazole antifungals may have synergistic interactions against fungi. To test this hypothesis, the susceptibility of C. neoforms, C. albicans, C. glabrata, C. krusei and C. parapsilosis strains to fluconazole, polymyxin B and a combination of these two compounds was assessed by disc diffusion halo assay. As shown in Figure 2, the strains demonstrated varied susceptibility to fluconazole, with the C. krusei strain DUMC132.91 and the C. parapsilosis strain MMRL1594 being highly resistant. Polymyxin B alone at 20 μg per disc had no or minimal fungicidal activity against the strains tested (Figure 2b). Interestingly, when fluconazole was combined with polymyxin B, the halo surrounding the disc was significantly more clear (Figure 2a and b), an indication of potential fungicidal activity.

Because the disc diffusion method was not quantitative in determining the susceptibility to polymyxin B due to the relatively large size of this molecule (mol. wt. > 1300 Da), the synergy of the drug combination was further examined using the microdilution assay. Cells from the microdilution assay after incubation with polymyxin B alone, fluconazole alone or the combination at various concentrations were also plated on drug-free medium to count the cfu for determination of the MFC. As shown in Table 1, the strains showed varied susceptibility to polymyxin B and fluconazole, and there is no apparent correlation between the susceptibility towards polymyxin B and the susceptibility towards fluconazole. Consistent with results from the disc diffusion assays, the C. krusei strain DUMC132.91 and the C. parapsilosis strain MMRL1594 are highly resistant to fluconazole (the susceptibility breakpoint for fluconazole is 8 mg/L according to the CLSI standard). The MFC of polymyxin B for each strain tested is similar to the MIC<sub>100</sub> (columns 2 and 3), supporting its fungicidal property. Conversely, the MFC of fluconazole can be much higher than the MIC<sub>90</sub> (columns 4 and 5), and complete cell killing is often not achievable. A synergistic fungicidal interaction between polymyxin B and fluconazole was observed against all fungal strains tested (last two columns). Similar synergistic interactions against the
Table 1. Polymyxin B and fluconazole exhibit a synergistic fungicidal effect (mg/L)

<table>
<thead>
<tr>
<th>Yeast strains</th>
<th>MIC&lt;sub&gt;100&lt;/sub&gt; PMB</th>
<th>MFC PMB</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; FLC</th>
<th>MFC FLC</th>
<th>MFC PMB/FLC</th>
<th>FFCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptococcus neoformans H99a&lt;sup&gt;76&lt;/sup&gt;</td>
<td>8</td>
<td>8</td>
<td>1</td>
<td>8</td>
<td>1–2</td>
<td>0.266</td>
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<tr>
<td>Candida albicans SCS314&lt;sup&gt;a&lt;/sup&gt;</td>
<td>128</td>
<td>&gt;256</td>
<td>0.2</td>
<td>64</td>
<td>6–8</td>
<td>0.125</td>
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<tr>
<td>Candida glabrata PAT21SO&lt;sup&gt;a&lt;/sup&gt;</td>
<td>256</td>
<td>256</td>
<td>8</td>
<td>&gt;64</td>
<td>25–15</td>
<td>0.236</td>
</tr>
<tr>
<td>Candida krusei DUMC132.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32</td>
<td>32</td>
<td>64</td>
<td>64</td>
<td>8–10</td>
<td>0.160</td>
</tr>
<tr>
<td>Candida parapsilosis MMRL1594&lt;sup&gt;a&lt;/sup&gt;</td>
<td>128</td>
<td>256</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>10–10</td>
<td>0.078</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae BY4741&lt;sup&gt;77&lt;/sup&gt;</td>
<td>32</td>
<td>64</td>
<td>4</td>
<td>16</td>
<td>2–4</td>
<td>0.252</td>
</tr>
<tr>
<td>Filamentous fungal strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus fumigatus Af293&lt;sup&gt;78&lt;/sup&gt;</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>0.4</td>
<td>12.8</td>
<td>12–16</td>
<td>0.137</td>
</tr>
<tr>
<td>Rhizopus oryzae 99–880&lt;sup&gt;79&lt;/sup&gt;</td>
<td>32</td>
<td>32</td>
<td>0.4</td>
<td>1.6</td>
<td>4–0.4</td>
<td>0.375</td>
</tr>
</tbody>
</table>

<sup>a</sup>Strains were obtained from Duke University Medical Center.

MFC is defined as the lowest drug concentration at which at least 99% of cells were killed compared with the original inocula. Suspensions from the microdilution assay after 24 or 48 h (for Cryptococcus) of incubation were plated on drug-free medium to obtain the numbers of cfu to determine the MFC. Successive 2<sup>x</sup> serial dilutions of the drugs were used. When fluconazole was used alone, sometimes the MFC could not be achieved in the concentration range tested and is indicated by ‘>’.

PMB stands for polymyxin B (susceptible breakpoint for bacteria is 2 mg/L according to the BSAC or 4 mg/L according to the CLSI); FLC, fluconazole (susceptible breakpoint for yeasts is 8 mg/L); ITC, itraconazole (susceptible breakpoint for moulds is 1.0 mg/L). The highest concentrations tested for PMB, FLC and ITC were 256 (except for A. fumigatus, where 1000 mg/L was the highest concentration tested), 64 and 6.4 mg/L, respectively. The fractional fungicidal concentration index (FFCI) = [FLC]/MFC<sub>FLC</sub> + [PMB]/MFC<sub>PMB</sub>, where MFC<sub>FLC</sub> and MFC<sub>PMB</sub> are the concentrations at which fluconazole and polymyxin B, respectively, and FLC and ITC are the concentrations at which fluconazol<sup>e</sup>e and polymyxin B, respectively, were examined (Table 1). These strains belong to three major C. neoformans molecular types of serotype A (VNI, VNII or VNB) and are of either α or α mating type (Table 2).<sup>51–53</sup>

Despite the variations in susceptibility towards polymyxin or fluconazole, the combination of polymyxin B at 2 mg/L and fluconazole at 8 mg/L was able to achieve >99% cell killing for all strains tested except for two strains, Bt81 and A2–102–5, where 93–96% killing was achieved. We also obtained a relatively fluconazole-resistant strain H99<sup>F8</sup> by culturing susceptible H99 in the presence of sublethal fluconazole for 16 weeks. The strain H99<sup>F8</sup> showed a 16-fold increase in MIC<sub>90</sub> of fluconazole (Table 2) and significantly increased resistance to itraconazole (data not shown). Again, a synergistic interaction against H99<sup>F8</sup> was observed between polymyxin B and fluconazole, and the combination of polymyxin B at 2 mg/L and fluconazole at 8 mg/L was again effective (Table 2).

The combination of polymyxin B and fluconazole at clinically relevant concentrations is effective against Cryptococcus strains with varied fluconazole resistance

Because the C. neoformans strain H99 is more susceptible to polymyxin B than the Candida strains tested (Table 1), we decided to further examine whether the susceptibility to polymyxin B and to the drug combination is specific to the H99 strain or is a general trait of Cryptococcus. C. neoformans has three serotypes (A, D and the less common AD hybrids). Serotype A causes >95% of cryptococcosis cases, and cryptococcosis caused by serotype A is also more severe.<sup>45–49</sup> Serotype A isolates are in general more resistant to antifungals compared with serotype D.<sup>30</sup> Thus, additional clinical and environmental C. neoformans serotype A isolates that are genetically distinct were examined (Table 1). These strains belong to three major C. neoformans molecular types of serotype A (VNI, VNII or VNB) and are of either α or α mating type (Table 2).<sup>51–53</sup>

The combination of polymyxin B and fluconazole displays no adverse effect on the growth and proliferation of HeLa and THP-1 cells when used at a concentration >10 times higher than that required for anti-Cryptococcus activity

Although both polymyxin B and fluconazole have been used in human populations to treat infectious diseases for years and their toxicity profiles are known, the potential side effects caused by the combination of these two drugs in animals or humans remain unknown. As polymyxin B is accumulated to a much higher level in the kidney (nephrotoxicity), we decided to test the toxicity of the drug combination at higher doses than those required for the anti-Cryptococcus activity in tissue.
Polymyxin B with fluconazole is fungicidal

**Figure 2.** Synergistic interaction between fluconazole and polymyxin B against *Candida* and *Cryptococcus*. Discs containing water, polymyxin B, fluconazole and the drug combination were dried and overlaid on a lawn of yeast cells derived from the strains indicated. Cells were incubated for 24 h (*Candida* species) or 48 h (*Cryptococcus*). Inhibition of fungal growth in regions surrounding the disc produces a halo. A completely clear halo indicates fungicidal activity. (a) Disc diffusion halo assays of the *C. albicans* strain SC5314 produced a clear halo when fluconazole and polymyxin B were used in combination. (b) Microscopic observations reveal significant clearing of the zone of inhibition (halo) when fluconazole and polymyxin B were used in combination. The left-hand side of each image shows the edge of the disc. PMB, polymyxin B; FLC, fluconazole.

**Table 2.** In combination with fluconazole, polymyxin B at low concentrations is effective against different *Cryptococcus neoformans* isolates

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Source</th>
<th>Virulence</th>
<th>MIC&lt;sub&gt;100&lt;/sub&gt; PMB</th>
<th>MFC PMB</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; FLC</th>
<th>MFC FLC</th>
<th>MFC PMB/FLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>H99 (&lt;a&gt;)&lt;sup&gt;76&lt;/sup&gt;</td>
<td>VNI (A1)</td>
<td>clinical</td>
<td>high</td>
<td>8</td>
<td>8</td>
<td>1</td>
<td>4</td>
<td>1–2</td>
</tr>
<tr>
<td>C45 (&lt;a&gt;)&lt;sup&gt;51&lt;/sup&gt;</td>
<td>VNI</td>
<td>clinical</td>
<td>high</td>
<td>20</td>
<td>24</td>
<td>2</td>
<td>6</td>
<td>2–8</td>
</tr>
<tr>
<td>C23 (&lt;a&gt;)&lt;sup&gt;51&lt;/sup&gt;</td>
<td>VNII</td>
<td>environmental</td>
<td>low</td>
<td>16</td>
<td>20</td>
<td>4</td>
<td>&gt;64</td>
<td>2–8</td>
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<tr>
<td>Bt31 (&lt;a&gt;)&lt;sup&gt;52,53&lt;/sup&gt;</td>
<td>VNB (A4)</td>
<td>clinical</td>
<td>—</td>
<td>8</td>
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<td>12</td>
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<tr>
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<td>VNB (A21)</td>
<td>clinical</td>
<td>—</td>
<td>8</td>
<td>8</td>
<td>12</td>
<td>&gt;64</td>
<td>2–8&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>A2-102-5 (&lt;a&gt;)&lt;sup&gt;51&lt;/sup&gt;</td>
<td>VNI (A2)</td>
<td>environmental</td>
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<td>10</td>
<td>10</td>
<td>16</td>
<td>32</td>
<td>2–8&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>VNB (A19)</td>
<td>clinical</td>
<td>—</td>
<td>8</td>
<td>8</td>
<td>16</td>
<td>&gt;64</td>
<td>2–8</td>
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<tr>
<td>H99&lt;sup&gt;RI&lt;/sup&gt;</td>
<td>VNI (A1)</td>
<td>laboratory</td>
<td>—</td>
<td>5</td>
<td>6</td>
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<td>48</td>
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<td>VNB (A4)</td>
<td>clinical</td>
<td>—</td>
<td>6</td>
<td>8</td>
<td>32</td>
<td>&gt;64</td>
<td>2–8</td>
</tr>
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PMB, polymyxin B; FLC, fluconazole.
The highest concentration of fluconazole tested was 64 mg/L.
MICS/MFCs are given in terms of mg/L.

*Information was obtained from previous reports.<sup>51–53</sup> VNI, VNII and VNB indicate the three molecular types of *C. neoformans*. An upper case ‘A’ followed by a number indicates further genotypic classification based on the amplified fragment length polymorphism (‘AFLP’) genetic typing pattern. The symbol ‘—’ indicates that no information is available.

<sup>b</sup>The combination at the indicated concentrations achieved 93%–96% rather than >99% killing.
adverse effect on the growth or the morphology of HeLa cells or THP-1 cells (Figure 3 and data not shown).

Discussion

Here we showed that the combination of polymyxin B and fluconazole (or itraconazole) at low concentrations is fungicidal against a variety of pathogenic fungal species and also relatively fluconazole-resistant strains, indicating the potential of this combination in treating systemic and localized fungal infections. Because polymyxin B (≤1000 mg/L) is already used in eye drop/ointment and in creams to treat bacterial infections, it could be combined with azole antifungals to treat non-systemic fungal infections, such as fungal keratitis and mucocutaneous candidiasis (oral thrush, vaginal candidiasis) that affect the general as well as the immunocompromised populations.

This drug combination could also potentially be an effective treatment for cryptococcosis that involves the brain. Polymyxin B can penetrate the brain tissue with a low but appreciable efficiency, and has been used to treat bacterial meningitis when administered intravenously, intrathecally or intraventricularly. Thus, it is conceivable that the combination of polymyxin B and fluconazole could be efficacious against cryptococcal meningitis. Even if polymyxin B could not reach a level at which it can act synergistically with fluconazole in the brain tissue when it is administered intravenously, more effective clearance/reduction of the fungal burden in other tissues/organs may exert a sink effect that could help further reduce brain fungal burden. Assessing the efficacy of the drug combination against systemic cryptococcosis with different routes of administration in animal models in the future will provide valuable information regarding the potential efficacy of this drug combination in the clinic.

Based on the well-known mechanism by which polymyxin B kills Gram-negative bacteria, we hypothesize that polymyxin B kills fungi through binding anionic lipids on fungal membrane and disruption of membrane integrity. This cationic heptapeptide with a hydrophobic tail derives its bactericidal activity from its electrostatic interaction with negatively charged lipids (LPS or anionic phospholipids) and its hydrophobic interaction with membrane lipids. These interactions allow polymyxin B to form channels to destroy the integrity of the cytoplasmic membrane. The observation that magainin 2, another antibiotic that is structurally different from polymyxin B but shares a similar mode of action against bacteria, is also fungicidal and acts synergistically with fluconazole against H99 (data not shown) indicates a conserved mechanism of cationic peptide antibiotics against fungi.

The lower efficiency of polymyxin B alone (or other cationic peptide antibiotics such as magainin 2) against eukaryotes compared with bacteria could be partly due to the presence of sterols in eukaryotic membrane, as sterols have been shown to reduce the insertion of cationic peptides into anionic mixed membranes to form pores. The fact that azole treatment lowers fungal ergosterol levels and renders fungi more susceptible to polymyxin B supports this hypothesis.

Although our toxicity study in cell culture suggests that the specificity of azoles against fungal ergosterol biosynthesis (without affecting mammalian cholesterol biosynthesis) may prevent increased toxicity of the drug combination towards hosts compared with polymyxin B alone, the safety concern of the current formulation of polymyxin B might limit its use in the clinic to treat systemic fungal infections. However, the resurging interest in using polymyxin B to treat bacterial infections caused by multidrug-resistant isolates may drive the development of safer and more effective new formulations. Regardless of the clinical potential of polymyxin B per se in treating fungal infections, given the recent discoveries of cationic peptides as potential antifungals, investigation into the fungicidal activity of polymyxin B and the synergy between polymyxin B and azoles will probably have a far-reaching impact on the development of novel, effective and safer antifungal therapies.

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Transparency declarations

None to declare.
Polymyxin B with fluconazole is fungicidal

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