A combination approach to treating fungal infections

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Azoles are antifungal drugs used to treat fungal infections such as candidiasis in humans. Their extensive use has led to the emergence of drug resistance, complicating antifungal therapy for yeast infections in critically ill patients. Combination therapy has become popular in clinical practice as a potential strategy to fight resistant fungal isolates. Recently, amphiphilic tobramycin analogues, C₁₂ and C₁₄, were shown to display antifungal activities. Herein, the antifungal synergy of C₁₂ and C₁₄ with four azoles, fluconazole (FLC), itraconazole (ITC), posaconazole (POS), and voriconazole (VOR), was examined against seven Candida albicans strains. All tested strains were synergistically inhibited by C₁₂ when combined with azoles, with the exception of C. albicans 64124 and MYA-2876 by FLC and VOR. Likewise, when combined with POS and ITC, C₁₄ exhibited synergistic growth inhibition of all C. albicans strains, except C. albicans MYA-2876 by ITC. The combinations of FLC-C₁₄ and VOR-C₁₄ showed synergistic antifungal effect against three C. albicans and four C. albicans strains, respectively. Finally, synergism between C₁₂/C₁₄ and POS were confirmed by time-kill and disk diffusion assays. These results suggest the possibility of combining C₁₂ or C₁₄ with azoles to treat invasive fungal infections at lower administration doses or with a higher efficiency.

Invasive fungal infections such as candidiasis have become a major cause of mortality and morbidity, especially among immunocompromised (HIV, cancers) and critically ill patients worldwide. The National Healthcare Safety Network (NHSN) at the Centers of Diseases Control and Prevention (CDC) has reported that Candida spp. ranked the fifth among hospital-acquired pathogens. Candida spp. have also been reported as the fourth most common causative pathogens of nosocomial bloodstream infection claiming more lives in the United States.

Azoles, echinocandins, allylamines, and polyenes are the four major classes of antifungal agents that are used to treat candidiasis as well as other type of fungal infections in humans. Among these four, azoles such as fluconazole (FLC), itraconazole (ITC), posaconazole (POS), and voriconazole (VOR) are considered first line drugs to treat fungal infections, however, has promoted the selection and emergence of drug resistant fungal strains. This necessitates either the development of novel antifungal drugs or improved therapeutic strategy to overcome drug resistance problems by C. albicans. In clinical settings, combination therapy has become a potential alternative to treat invasive fungal infections by improving clinical efficacy of existing drugs such as azoles and reducing their side effects to host by lowering administrative doses. Previously, promising results were observed by combining azoles with different compounds such as tacrolimus (FK506), cyclosporine A, amiodarone, and retigeric acid B against C. albicans strains.

We recently demonstrated that modifying the aminoglycoside tobramycin (TOB) at the 6'-position by incorporating linear alkyl chains (C₆–C₃₂) in a thioether linkage resulted in chain-length-dependent antibacterial and antifungal activities against various bacteria and fungi with resistance to the parent drug, TOB, itself. This was especially true for TOB derivatized with linear alkyl chains of 12 and 14 carbons in length (referred to as C₁₂ and C₁₄ from here on) (Fig. 1). However, synergistic interactions between the amphiphilic aminoglycosides C₁₂ and C₁₄ and azoles against fungal strains have not yet been explored. In this study, in an effort to establish if TOB derivatives could be used in combination with...
currently available antifungal agents, we evaluated the combined effects of \( C_{12} \) and \( C_{14} \) with four azoles against azole-sensitive and azole-resistant \( C. \) albicans by checkerboard, time-kill curve, and disk diffusion assays. Additionally we have determined the in vitro cytotoxicity effect of TOB analogues and azoles in combination against mammalian cells.

**Results**

**In vitro antifungal activities of drugs alone.** Prior to investigating the effect of combining \( C_{12} \) or \( C_{14} \) with four azoles (FLC, ITC, POS, and VOR), the MIC values of these compounds were determined individually against seven strains of \( C. \) albicans (Tables 1 and 2). The clinical sources and susceptibility/resistance profile of these strains, as reported by the American Type Culture Collection (ATCC), are presented in Table S1.

Based on complete inhibition (MIC-0) (Tables 1 and 2), \( C_{12} \) and \( C_{14} \) displayed MIC values of 16–32 \( \mu \)g/mL and 8 \( \mu \)g/mL, respectively, against all \( C. \) albicans strains tested. These MIC values are consistent with those previously reported for these compounds against these specific yeast strains. When compared to \( C_{12} \) and \( C_{14} \), FLC, ITC, POS, and VOR displayed higher MIC values against the majority of the yeast strains tested (MIC values ranged from \( \geq 25 \) \( \mu \)g/mL, 12.5–25 \( \mu \)g/mL, 10–20 \( \mu \)g/mL, and \( \geq 10 \) \( \mu \)g/mL, respectively), with the exception of the \( C. \) albicans ATCC 10231 (A) strain where ITC, POS, and VOR had MIC values of 0.78 \( \mu \)g/mL, 0.62 \( \mu \)g/mL, and 0.31 \( \mu \)g/mL, respectively. The MIC values of azoles against yeast strains were determined based on 50% inhibition or MIC-0 and were consistent with previously reported MICs for these compounds. However, due to the long trailing growth effects by azole susceptible strains \( C. \) albicans MYA-2876 (C) and \( C. \) albicans MYA-2310 (E), we did observe higher MIC-2 values for all azoles against these strains. To validate our MIC data of azoles against these two strains (C and E), we also tested caspofungin as a control in the same set of MIC testing experiments. It is important to note that ATCC has reported these two strains (C and E) as sensitive to caspofungin. Unlike azoles, caspofungin showed complete inhibition at \(< 0.48 \) \( \mu \)g/mL against these strains (Table S2).

**In vitro synergistic antifungal activities.** Having established the individual MIC values for \( C_{12} \), \( C_{14} \), FLC, ITC, POS, and VOR, the MIC and FICI values of \( C_{12} \) and \( C_{14} \) in combination with the four azoles (FLC, ITC, POS and VOR) were determined in checkerboard assays against the seven strains of \( C. \) albicans (Tables 1 and 2). When combined with FLC or ITC or POS or VOR, \( C_{12} \) and \( C_{14} \) showed strong synergistic inhibitory effects against the majority of the \( C. \) albicans strains tested with FICI values ranging from 0.07–0.5 (FLC or ITC or POS plus \( C_{12} \)) and 0.07–0.27 (VOR plus \( C_{12} \)). The only combinations for which no synergistic effects were observed were FLC plus \( C_{12} \) (FICIs = 0.51 and 1) or VOR plus \( C_{12} \) (FICIs = 0.62 and 0.75) against \( C. \) albicans ATCC 64124 (B) and \( C. \) albicans ATCC MYA-2876 (C), respectively. Likewise, the combination of \( C_{14} \) with FLC or ITC or POS or VOR also exhibited good synergy against the majority of the \( C. \) albicans strains tested, with FICI values ranging from 0.28–0.5 (FLC plus \( C_{14} \)), 0.18-0.5 (ITC plus \( C_{14} \)), 0.18-0.49 (POS plus \( C_{14} \)), and 0.14–0.37 (VOR plus \( C_{14} \)). With \( C_{14} \), the combinations for which no synergistic effects were observed were FLC or VOR plus \( C_{14} \) against \( C. \) albicans ATCC 10231 (A), \( C. \) albicans ATCC 64124 (B), and \( C. \) albicans ATCC MYA-2876 (C), as well as FLC plus \( C_{14} \) (FICI = 1) against \( C. \) albicans ATCC MYA-1003 (G).

**Time-kill studies of drug combinations.** To confirm the synergistic inhibitory effects of \( C_{12} \) or \( C_{14} \) and POS against the azole-resistant \( C. \) albicans ATCC 64124 (B) strain, representative time-kill studies...
### MICs of drugs (μg/mL)

| Drugs and Strains | Alone | In combination | FICIs | Interpretation |
|-------------------|-------|----------------|-------|----------------|
|                   | Azole | C₁₂ | Azole | C₁₂ |        |
| **FLC**           |       |     |       |     |       |
| C. albicans 10231 (A)³ | 25 | 32 | 1.56 | 8 | 0.31 | SYN |
| C. albicans 64124 (B)³ | >25 | 32 | 0.78 | 16 | 0.53 | IND |
| C. albicans MYA-2876 (C)³ | >25 | 16 | 12.5 | 8 | 1 | IND |
| C. albicans 90819 (D)³ | >25 | 32 | 1.56 | 2 | 0.12 | SYN |
| C. albicans MYA-2310 (E)³ | >25 | 16 | 0.39 | 1 | 0.07 | SYN |
| C. albicans MYA-1237 (F)³ | >25 | 32 | 1.56 | 2 | 0.12 | SYN |
| C. albicans MYA-1003 (G)³ | >25 | 32 | 6.25 | 8 | 0.5 | SYN |
| **ITC**           |       |     |       |     |       |
| C. albicans 10231 (A)³ | 0.78 | 32 | 0.049 | 4 | 0.18 | SYN |
| C. albicans 64124 (B)³ | >25 | 32 | 1.56 | 8 | 0.31 | SYN |
| C. albicans MYA-2876 (C)³ | 12.5 | 16 | 3.12 | 4 | 0.5 | SYN |
| C. albicans 90819 (D)³ | >25 | 32 | 0.78 | 4 | 0.15 | SYN |
| C. albicans MYA-2310 (E)³ | 12.5 | 16 | 0.39 | 2 | 0.15 | SYN |
| C. albicans MYA-1237 (F)³ | >25 | 32 | 0.39 | 2 | 0.07 | SYN |
| C. albicans MYA-1003 (G)³ | >25 | 32 | 0.39 | 4 | 0.15 | SYN |
| **POS**           |       |     |       |     |       |
| C. albicans 10231 (A)³ | 0.62 | 32 | 0.15 | 8 | 0.5 | SYN |
| C. albicans 64124 (B)³ | >20 | 32 | 1.25 | 2 | 0.12 | SYN |
| C. albicans MYA-2876 (C)³ | 10 | 16 | 1.25 | 1 | 0.18 | SYN |
| C. albicans 90819 (D)³ | >20 | 32 | 0.31 | 8 | 0.26 | SYN |
| C. albicans MYA-2310 (E)³ | 10 | 16 | 0.62 | 1 | 0.12 | SYN |
| C. albicans MYA-1237 (F)³ | >20 | 32 | 0.31 | 2 | 0.07 | SYN |
| C. albicans MYA-1003 (G)³ | >20 | 32 | 1.25 | 2 | 0.12 | SYN |
| **VOR**           |       |     |       |     |       |
| C. albicans 10231 (A)³ | 0.31 | 32 | 0.07 | 8 | 0.27 | SYN |
| C. albicans 64124 (B)³ | >10 | 32 | 1.25 | 16 | 0.62 | IND |
| C. albicans MYA-2876 (C)³ | >10 | 16 | 2.5 | 8 | 0.75 | IND |
| C. albicans 90819 (D)³ | >10 | 32 | 0.15 | 4 | 0.14 | SYN |
| C. albicans MYA-2310 (E)³ | 10 | 16 | 0.15 | 1 | 0.07 | SYN |
| C. albicans MYA-1237 (F)³ | >10 | 32 | 0.31 | 2 | 0.09 | SYN |
| C. albicans MYA-1003 (G)³ | >10 | 32 | 0.31 | 4 | 0.14 | SYN |

Table 1. *In vitro* susceptibility of yeast strains to C₁₂ and azoles alone and in combination. ³All of the strains are from ATCC. ²Indicates strains that are resistant to FLC, ITC, and VOR according to ATCC. ³Indicates strains that are susceptible to FLC, ITC, and VOR according to ATCC. Note: SYN indicates synergy (FICI ≤ 0.5) whereas IND indicates indifferent (FICI >0.5–4).

were performed (Fig. 2). At 8 or 4 μg/mL, C₁₂ or C₁₄ alone did not show inhibition to the growth of *C. albicans* ATCC 64124 (B). In contrast, POS, at 10 μg/mL, showed inhibition for the first 3 h of growth of the yeast strain, and after that the growth was similar to that of the growth control (no drug). However, the combined administration of C₁₂ (2 μg/mL) with POS (1.25 μg/mL) and C₁₄ (2 μg/mL) with POS (1.25 or 2.5 μg/mL) against *C. albicans* ATCC 64124 (B) yielded a ≥2 log₁₀ decrease in CFU/mL after 9 h and 12 h of treatment compared with each compound alone, respectively (Fig. 2). The results obtained by time-kill studies are consistent with those from the checkerboard assays.

**Disk diffusion assays.** To examine the nature of the drug interactions between C₁₄ with POS or ITC against the azole-resistant *C. albicans* ATCC 64124 (B) strain, disk diffusion assays were performed in duplicate. C₁₄ (500 or 700 μg/mL), POS (100 μg/mL), and ITC (150 μg/mL) alone, when applied on disk, did not show a zone of inhibition against *C. albicans* ATCC 64124 (B). However, when co-spotted, C₁₄
(500 μg/mL) and POS (100 μg/mL) or C14 (700 μg/mL) and ITC (150 μg/mL) resulted in a visible zone of inhibition against this strain, which confirmed the synergistic antifungal interactions of these compounds (Fig. 3).

**Cytotoxic effect of drug combinations.** To investigate the cytotoxic effects of C12 or C14 and POS alone and in combinations, assays were performed against A549 and BEAS-2B cells (Fig. 4 and Tables S3-5). As we previously reported11, C12 or C14 at their respective highest antifungal MIC values of 32 μg/mL and 8 μg/mL, basically did not show toxicity against the A549 and BEAS-2B cell lines. On the other hand, the newly tested POS at 20 μg/mL, which is a concentration below its antifungal MIC value against C. albicans ATCC 64124 (B), exhibited severe toxicity against the A549 and BEAS-2B cell lines, resulting in ≤37% cell survival in both cases. On a very positive note, when tested at 8-fold higher concentrations of POS (10 μg/mL) plus C12 or C14 (16 or 8 μg/mL) in combinations than their synergistic antifungal MIC values (Note: the synergistic MIC values for POS and C12 or C14 in combinations are 1.25 and 2 or 1), only minimal or no toxicity were observed against the A549 and BEAS-2B cell lines, resulting in ≥47% cell survival in both cases.

**Discussion**

Opportunistic fungal infections have become a serious threat to human health due to the rising population of immunocompromised patients as result of HIV infections, chemotherapy, and organ transplant12. Azoles are drugs of choice for antifungal therapy for various fungal infections in humans, including candidiasis. However drug-drug interactions, severe side effects, and development of resistance have limited their therapeutic efficacies against fungi13. Thus, new strategies are warranted to overcome antifungal drug resistance and side effects due to use of high doses of these drugs.

In this study, we investigated the in vitro antifungal synergy of two amphiphilic TOB derivatives, C12 and C14, with four azoles (FLC, ITC, POS and VOR) against seven azole-resistant and azole-sensitive strains of C. albicans. Our results demonstrated that C12 and C14 exhibit potent antifungal synergy in vitro with all four azoles against the majority of the C. albicans strains tested. Despite displaying less antifungal activity when used alone, C14 alone (16–32 μg/mL) compared to C14 alone (8 μg/mL), C12 demonstrated better synergistic inhibitory effects when combined with azoles against all strains of C. albicans tested with FICI values ranging from 0.07–0.5. The only combinations for which no synergy was detected were those of C12 and FLC or VOR against C. albicans ATCC 64124 (B) and C. albicans ATCC MYA-2876 (C) (Table 1). Similarly, C14 also did not display synergy when used in combination with FLC and VOR against these strains. Although C14 also displayed good antifungal synergy in combinations with all azoles against the majority of the fungal strains tested (FICI values ranging from 0.14–0.5), more combinations yielded no synergy. In addition to the C14 with FLC or VOR against strains B and C, indifference was observed with the combinations of FLC or VOR with C14 against C. albicans ATCC 10231 (A), FLC with C14 against C. albicans ATCC MYA-1003 (G), as well as ITC with C14 against C. albicans ATCC MYA-2876 (C) (Table 2). It is also noteworthy to mention that the MIC values of all azoles were greatly reduced in presence of C12 or C14 against various fungal strains. For example, the MIC values of POS were reduced by 64-fold against C. albicans ATCC 90819 (D) in the presence of C12 or C14. Also, POS lowered the MIC values of C12 or C14 by 4-fold against same strain in both case. Alternative methods, such as time-kill studies and disk diffusion assays, were also performed to evaluate the drug interactions of C12 and C14 with POS or ITC (used for disk diffusion assays only) against C. albicans ATCC 64124 (B). The results obtained further confirmed the synergistic interactions of C12 and C14 with POS and were in agreement with the results obtained by checkerboard analysis against specific yeast strains. Interestingly, although we did observe zones of inhibition for C14 and C14 with POS or ITC against C. albicans ATCC 64124 (B) in our disk diffusion assay, these were small. Probably, the higher molecular weight of TOB analogues may have contributed the poor diffusion of these compounds through agar14 or interaction of these polycationic compounds with sulfates and acids of agar polymer may have resulted reduced inhibition with minor zone of inhibition. Interestingly, when tested with antifungals other than azoles such as caspofungin (an echinocandin) and naftifine (an allylamine), C12 and C14 did not show synergy at least against one strain of C. albicans, C. albicans ATCC 64124 (B) (data not shown).

In this study, we included clinical isolates of C. albicans strains that are reported as azole (FLC, ITC and VOR) resistant strains, except for two strains, C. albicans ATCC MYA-2876 (C) and C. albicans ATCC MYA-2310 (E), which are reported as azole-sensitive. In the majority of cases, C12 and C14 exhibited synergistic inhibitory effects with azoles against these strains. These observations indicates that combination therapy using C12 or C14 with an azole may provide a new strategy to fight fungal infections caused by resistant strains like C. albicans ATCC 64124 (B) that has mutations in its ERG11 sequences16,17.

Having established the synergistic antifungal interactions of C12 and C14 with azoles, and knowing their non-cytotoxicity effects against A549 and BEAS-2B mammalian cell lines11, we further evaluated the cytotoxicity effects of C12 and C14 in combination with POS against the A549 and BEAS-2B cell lines. At above 8-fold higher than or equal to their synergistic antifungal MIC values, C12 and C14 with POS exhibited minimal to no toxicity against these cell lines resulting in ≤47% cell survival (Fig. 4 and Tables S3-S6). These results may suggest that the clinical efficacies of azoles can be resumed by achieving low
### Table 2. In vitro susceptibility of yeast strains to C14 and azoles alone and in combination.

| Drugs and Strains<sup>a</sup> | MICs of drugs (μg/mL) | Alone | In combination | FICIs | Interpretation |
|-------------------------------|----------------------|-------|----------------|-------|----------------|
| **FLC**                      |                      |       |                |       |                |
| C. albicans 10231 (A)<sup>b</sup> | 25 8 6.25 4 0.75 | IND   |                |       |                |
| C. albicans 64124 (B)<sup>b</sup> | >25 8 1.56 4 0.56 | IND   |                |       |                |
| C. albicans MYA-2876 (C)<sup>c</sup> | >25 8 12.5 4 1 | IND   |                |       |                |
| C. albicans 90819 (D)<sup>b</sup> | >25 8 6.25 2 0.5 | SYN   |                |       |                |
| C. albicans MYA-2310 (E)<sup>c</sup> | >25 8 25 8 0.78 2 0.56 | IND   |                |       |                |
| C. albicans MYA-1237 (F)<sup>b</sup> | >25 8 0.78 2 0.28 | SYN   |                |       |                |
| C. albicans MYA-1003 (G)<sup>b</sup> | >25 8 12.5 4 1 | IND   |                |       |                |
| **ITC**                      |                      |       |                |       |                |
| C. albicans 10231 (A)<sup>b</sup> | 0.78 8 0.19 2 0.5 | SYN   |                |       |                |
| C. albicans 64124 (B)<sup>b</sup> | >25 8 0.78 2 0.28 | SYN   |                |       |                |
| C. albicans MYA-2876 (C)<sup>c</sup> | 12.5 8 1.56 4 0.62 | IND   |                |       |                |
| C. albicans 90819 (D)<sup>b</sup> | 25 8 0.39 2 0.26 | SYN   |                |       |                |
| C. albicans MYA-2310 (E)<sup>c</sup> | 12.5 8 0.78 1 0.18 | SYN   |                |       |                |
| C. albicans MYA-1237 (F)<sup>c</sup> | >25 8 3.12 1 0.25 | SYN   |                |       |                |
| C. albicans MYA-1003 (G)<sup>c</sup> | >25 8 0.78 2 0.28 | SYN   |                |       |                |
| **POS**                      |                      |       |                |       |                |
| C. albicans 10231 (A)<sup>b</sup> | 0.62 8 0.15 2 0.49 | SYN   |                |       |                |
| C. albicans 64124 (B)<sup>b</sup> | >20 8 1.25 1 0.18 | SYN   |                |       |                |
| C. albicans MYA-2876 (C)<sup>c</sup> | 10 8 1.25 2 0.37 | SYN   |                |       |                |
| C. albicans 90819 (D)<sup>b</sup> | >20 8 0.31 2 0.26 | SYN   |                |       |                |
| C. albicans MYA-2310 (E)<sup>c</sup> | 10 8 0.31 2 0.28 | SYN   |                |       |                |
| C. albicans MYA-1237 (F)<sup>c</sup> | >20 8 1.25 1 0.18 | SYN   |                |       |                |
| C. albicans MYA-1003 (G)<sup>c</sup> | >20 8 1.25 2 0.31 | SYN   |                |       |                |
| **VOR**                      |                      |       |                |       |                |
| C. albicans 10231 (A)<sup>b</sup> | 0.31 8 0.03 4 0.59 | IND   |                |       |                |
| C. albicans 64124 (B)<sup>b</sup> | >10 8 5 4 1 | IND   |                |       |                |
| C. albicans MYA-2876 (C)<sup>c</sup> | 10 8 5 2 0.75 | IND   |                |       |                |
| C. albicans 90819 (D)<sup>b</sup> | >10 8 0.31 1 0.15 | SYN   |                |       |                |
| C. albicans MYA-2310 (E)<sup>c</sup> | 10 8 0.15 1 0.14 | SYN   |                |       |                |
| C. albicans MYA-1237 (F)<sup>c</sup> | >10 8 0.31 1 0.15 | SYN   |                |       |                |
| C. albicans MYA-1003 (G)<sup>c</sup> | >10 8 2.5 1 0.37 | SYN   |                |       |                |

<sup>a</sup>Allo of the strains are from ATCC. <sup>b</sup>Indicates strains that are resistant to FLC, ITC, and VOR according to ATCC. <sup>c</sup>Indicates strains that are susceptible to FLC, ITC, and VOR according to ATCC. Note: SYN indicates synergy (FICI ≤ 0.5) whereas IND indicates indifferent (FICI > 0.5–4).

Doses with less toxicity when combined with C12 or C14 to treat stubborn mycoses. Besides, the results may provide flexibility to extrapolate the range of concentrations that can be used in combination to perform in vivo experiments.

Certain amphiphilic aminoglycosides such as FG08 and K20 were reported to inhibit fungi by disrupting fungal membrane<sup>18</sup>–<sup>20</sup>. Recently, we reported that C12 and C14 inhibit fungi by inducing apoptosis leading to fungal membrane disruption<sup>11</sup>. On the other hand, azoles kill fungi by inhibiting the cytochrome P450-dependent enzyme sterol 14-α-demethylase involved in ergosterol biosynthesis. The mechanism by which C12 and C14 synergize with azoles remains to be established in studies that are out of scope for this manuscript. One of the major mechanisms of resistance to azoles by fungi is due to up-regulation of efflux pumps (CDR1 and CDR2) that lower the intracellular drug concentrations<sup>21</sup>. When azoles are combined with C12 and C14, it could be expected that C12 and C14 could enhance azoles permeability to fungi by altering fungal membrane integrity that may intensify the fungal killing. However, the cascades of multiple secondary effects such as reactive oxygen species (ROS) accumulation,
mitochondrial membrane potential dissipation, and DNA condensation and fragmentation as a result of membrane disruption action cannot be overlooked as a cause of death 22.

Conclusions

In conclusion, our study demonstrated the synergistic combination effects between C12 or C14 and four azoles against the majority of the C. albicans strains tested. These synergistic interactions were further confirmed by time-kill curves and disk diffusion assays. The combination effects of C12 or C14 and azoles appears non toxic to mammalian cells at higher or equal to synergistic antifungal MIC values of these drugs against fungi. C12 or C14-azoles combination therapy might be mainly beneficial to treat invasive fungal infections like candidiasis. Future studies in our laboratory will be focused on establishing the mechanism of action of these drugs in combination.
Materials and Methods

Materials. Tobramycin (TOB) was purchased from AK scientific (Union City, CA). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO) and used without further purification. TOB analogues with linear alkyl chains $C_{12}$ and $C_{14}$ were synthesized as described previously $^{10}$ and were dissolved in double distilled water (ddH$_2$O) at a final concentration of 10 g/L for storage at $-20^\circ$C.

Antifungal agents. The antifungal agents fluconazole (FLC), itraconazole (ITC), posaconazole (POS), and voriconazole (VOR) were obtained from AK Scientific, Inc. (Mountain View, CA). FLC, ITC, POS, and VOR were dissolved in DMSO at a final concentration of 5 g/L. All of these solutions were stored at $-20^\circ$C.

Fungal strains and culture conditions. The yeast strains $C. albicans$ ATCC 10231 (A), $C. albicans$ ATCC 64124 (B), and $C. albicans$ ATCC MYA-2876 (C) were kindly provided by Dr. Jon Y. Takemoto (Utah State University, Logan, UT, USA). The yeast strains $C. albicans$ ATCC 90819 (D), $C. albicans$ ATCC MYA-2310 (E), $C. albicans$ ATCC MYA-1237 (F), and $C. albicans$ ATCC MYA-1003 (G) were

Figure 3. Disk diffusion assay (done in duplicate; series 1 and 2) showing that $C_{14}$, when used in combination with the azoles POS or ITC, kills $C. albicans$ ATCC 64124 (strain B). Note: at the concentrations tested, $C_{14}$, POS, or ITC do not kill the fungal strain. $a1$ and $a2 = POS (100\mu g) + C_{14} (500\mu g)$; $b1$ and $b2 = POS (100\mu g)$; $c1$ and $c2 = C_{14} (500\mu g)$; $d1$ and $d2 = H_2O$; $e1$ and $e2 = ITC (150\mu g) + C_{14} (700\mu g)$; $f1$ and $f2 = ITC (150\mu g)$; $g1$ and $g2 = C_{14} (700\mu g)$; $h = H_2O$. 

\[ a1 = a2 = POS (100\mu g) + C_{14} (500\mu g) \]
\[ b1 = b2 = POS (100\mu g) \]
\[ c1 = c2 = C_{14} (500\mu g) \]
\[ d1 = d2 = H_2O \]
\[ e1 = e2 = ITC (150\mu g) + C_{14} (700\mu g) \]
\[ f1 = f2 = ITC (150\mu g) \]
\[ g1 = g2 = C_{14} (700\mu g) \]
\[ h = H_2O \]
purchased from the ATCC (Manassas, VA, USA). All yeast strains were cultivated at 35°C in RPMI 1640 medium.
**In vitro antifungal activities.** Based on the previously reported MIC values for C12, C14, FLC, ITC, POS, it is important to note that the MIC values for C12, C14, FLC, ITC, POS, VOR, and FOR against different fungal strains were determined as described in the CLSI document M27-A2. Some of our fungal strains, such as C. albicans ATCC 64124 (strain B), tend to produce pseudohyphae (filaments) in RPMI 1640 medium, which has been found to compromise cell counting when using a hemocytometer. Therefore, we used potato dextrose broth (PDB) to prepare yeast inocula and later diluted in RPMI 1640 medium to perform MIC value determination, as well as checkerboard and time-kill assays. Modifications included growing yeast cells in potato dextrose broth (PDB) for 24–48 h at 35 °C, diluting the yeast culture in RPMI 1640 medium to a concentration of 1 × 10^6 cells/mL (as determined by using an hemocytometer) and using a final inoculum size of 5 × 10^4 cfu/mL for all the assays (Note: identical results were obtained when using 5 × 10^6 cfu/mL and 5 × 10^4 cfu/mL as a final inoculum size when tested against strain B. As it is known that a higher inoculum size of cells can raise the MIC values determined, we selected 5 × 10^4 cfu/mL to provide conditions that would lead to the highest MIC values possible for our compounds so that we could really determined their potential). Two-fold serial dilution of C12, C14, FLC, ITC, POS, and VOR was prepared using RPMI 1640 medium (100 μL) and cell suspension (100 μL) was added to 96-well microtiter plates to achieve final drug and inoculum concentration of 0.15–10 mg/L and 5 × 10^4 cfu/mL, respectively. Plates were incubated for 48 h at 35 °C. The MIC values for all azoles studied were defined as the lowest drug concentration that inhibits 50% of fungal cell growth or MIC-2. The MIC values for C12 and C14 were defined as the lowest drug concentration that yielded complete growth inhibition or MIC-0.

**Determination of percentage of yeast cell growth inhibition used to determine MIC-2 values for FLC, ITC, POS, VOR, and caspofungin.** To confirm the susceptibility profile of yeast strains C and E, we determined the percentage of C. albicans ATCC MYA-2876 (C) and C. albicans ATCC MYA-2310 (E) growth inhibition by FLC, ITC, POS, VOR, and caspofungin. The experiments were performed as described above for the in vitro antifungal activities and percentages of growth at concentrations varying from 0.48-31.25 μg/mL of azoles or caspofungin were measured by reading absorbance at 600 nm (A600) using a SpectraMax M5 plate reader.

**Antifungal checkerboard analysis.** The synergistic interaction between C12, C14, with four azoles (FLC, ITC, POS, and VOR) was evaluated against various strains of C. albicans using a microdilution checkerboard assay according to CLSI M27-A3. The test was performed in 96-well plates using RPMI 1640 medium. It is important to note that the MIC values were also determined for all azoles and TOB analogues alone in the same set of experiments in checkerboard assays for comparison. These MIC values are not from previous reports. The final concentration of yeast cells used was 1 × 10^5 cfu/mL as verified by colony counting. The final concentration of drugs ranged from 0.25–32 μg/mL for C12, 0.06–8 μg/mL for C14, 0.39–25 μg/mL for FLC, 0.39–25 μg/mL for ITC, 0.31–20 μg/mL for POS, and 0.31–10 μg/mL for VOR. Plates were incubated for 48 h at 35 °C. Each test was performed in duplicate. A non-parametric model based on Loew Additivity (LA) theory was used to analyze the nature of in vitro interaction of C12 and C14, and all four azoles using fractional inhibitory concentration index (FICI). According to LA theory, FICI can be defined as the sum of the ratios of the MIC values of each drug when used in combination to their respective MIC values when used alone. Drug interactions were classified as synergistic (SYN), indifferent (IND), or antagonistic (ANT) according to the fractional inhibitory concentration index (FICI). The interaction was defined as synergistic if the FICI was ≤ 0.5, indifferent if > 0.5–4, and antagonistic if > 4.

**Time-kill studies of drug combinations.** Representative time-kill studies were performed to investigate the activity of C12 and C14 in the presence or absence of POS against oneazole-resistant strain, C. albicans ATCC 64124 (B). These assays were performed in 15 mL culture tubes using RPMI 1640 medium as previously described. Different sets of cell suspensions were prepared with C12 (8 μg/mL), C14 (4 μg/mL), and POS alone (10 μg/mL), or combinations of C12 (2 μg/mL) plus POS (2.5 μg/mL) or C14 (2 μg/mL) plus POS (1.25 and 2.5 μg/mL), or growth control (no drug) and sterility control (no cells and no drug). The final inoculum size of yeast cells used was 10^6 cfu/mL as confirmed by colony count. The cell suspensions were then incubated at 35 °C with constant shaking (200 rpm). Aliquot of 100 μL from each tube were removed at 0, 3, 6, 9, 12, and 24 h, and serially diluted in sterile ddH2O. 50 μL of each dilution was plated onto potato dextrose agar (PDA) and then incubated at 35 °C. Colony counts were determined after 48 h of incubation. The experiments were performed in duplicate.

**Disk diffusion assays.** The disk diffusion assays were performed in duplicate according to the CLSI document M44-A2. C. albicans ATCC 64124 (B), at a density of ~ 5 × 10^6 cfu/mL, were spread onto PDA plates. Sterile filter disks (~0.6 cm) were placed on the agar surface. 10 μL aliquot of POS (100 μg/mL), ITC (150 μg/mL), and C14 alone (either 500 or 700 μg/mL), or combinations of C14 (500 μg/mL) plus POS
survival, each well was treated with 10 μl of fluorescent dye resorufin, which can be detected at A560 excitation and A590 emission wavelengths by resazurin – drug, and test value represents cells

\[ \text{Test value} = \frac{\text{cells} + \times 100}{\text{control value}} \]

described27 with minor modifications. The human lung carcinoma epithelial cells A549 and the normal human bronchial epithelial cells BEAS-2B were grown in DMEM containing 10% fetal bovine serum (FBS) and 1% antibiotics. The confluent cells were then trypsinized with 0.05%-trypsin-0.53 mM EDTA and resuspended in fresh medium (DMEM). The cells were transferred into 96-well microtiter plates at a density of 3000 cells/well and were grown overnight. The following day, checkerboard plates were prepared to evaluate the cytotoxic effects of POS, and C12 or C14 alone and in combination against A549 and BEAS-2B cells. The checkerboard plates were prepared in a new 96-well microtiter plates as described above in antifungal checkerboard analysis except that drugs were diluted in DMEM medium in a final volume of 200 μL. The final concentration of drugs ranged from 0.25–32 μg/mL for C12, 0.06–8 μg/mL for C14, and 0.31–20 μg/mL for POS. The media containing cells were then replaced by 200 μL of fresh culture media containing drugs either alone or in combinations from the checkerboard plates. The cells were incubated for additional 24 h at 37°C with 5% CO2 in a humidified incubator. To evaluate cell survival, each well was treated with 10 μL (25 mg/L) of resazurin sodium salt (Sigma-Aldrich) for 3–6 h. Metabolically active cells can convert the blue non-fluorescent dye resazurin to the pink and highly fluorescent dye resorufin, which can be detected at A560 excitation and A590 emission wavelengths by using a SpectraMax M5 plate reader. Triton X-100® (1%, v/v) gave complete loss of cell viability and was used as the positive control. Percent cell survival was calculated as: (control value – test value) × 100/ control value, where control value represents cells + resazurin – drug, and test value represents cells + resazurin + drug.

References
1. Horn, D. L. et al. Epidemiology and outcomes of candidemia in 2019 patients: data from the prospective antifungal therapy alliance registry. Clin. Infect. Dis. 48, 1695–1703 (2009).
2. Pfaffer, M. A. & Diekema, D. J. Epidemiology of invasive mycoses in North America. Crit. Rev. Microbiol. 36, 1–53 (2010).
3. Sievert, D. M. et al. National Healthcare Safety Network, T.; Participating, N. F. Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010. Infect. Control Hosp. Epidemiol. 34, 1–14 (2013).
4. Wisplinghoff, H. et al. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin. Infect. Dis. 39, 309–317 (2004).
5. Falci, D. R. & Pasqualotto, A. C. Profile of isavuconazole and its potential in the treatment of severe invasive fungal infections. Infect. Drug Resist. 6, 163–174 (2013).
6. Sun, S. et al. In vitro interactions between tacrolimus and azoles against Candida albicans determined by different methods. Antimicrob. Agents Chemother. 52, 409–417 (2008).
7. Marchetti, O., Moreillon, P., Glauser, M. P., Bille, J. & Sargand, D. Potent synergism of the combination of fluconazole and cyclopenthine in Candida albicans. Antimicrob. Agents Chemother. 44, 2373–2381 (2000).
8. Guo, Q., Sun, S., Yu, J., Li, Y. & Cao, L. Synergistic activity of azoles with amiodarone against clinically resistant Candida albicans tested by checkerboard and time-kill methods. J. Med. Microbiol. 57, 457–462 (2008).
9. Sun, L. et al. In vitro activities of retigeric acid B alone and in combination with azole antifungal agents against Candida albicans. Antimicrob. Agents Chemother. 53, 1586–1591 (2009).
10. Herzog, I. M. et al. 6’-Thioether tobramycin analogues: towards selective targeting of bacterial membranes. Angew. Chem. Int. Ed. Engl. 51, 5652–5656 (2012).
11. Shrestha, S. K., Fosso, M. Y., Green, K. D. & Garneau-Tsodikova, S. Amphiphilic tobramycin analogues as antibacterial and antifungal agents. Antimicrob. Agents Chemother. 59, 4861–4869 (2015).
12. Fisher, M. C. et al. Emerging fungal threats to animal, plant and ecosystem health. Nature 484, 186–194 (2012).
13. Doltom, M. J. & et al. Multicenter study of posaconazole therapeutic drug monitoring: exposure-response relationship and factors affecting concentration. Antimicrob. Agents Chemother. 56, 5503–5510 (2012).
14. Cavenaghi, L. A., Biganzoli, E., Danese, A. & Parenti, F. Diffusion of teicoplanin and vancomycin in agar. J. Antimicrob. Chemother. 44, 2373–2381 (1999).
15. Kakeya, H. et al. Genetic analysis of azole resistance in the Darlington strain of Candida albicans. Antimicrob. Agents Chemother. 44, 2985–2990 (2000).
16. Chang, C. W. et al. Antifungal activity of eravopine, a novel fungicide, against Candida albicans. J. Antimicrob. Chemother. 63, 667–672 (2010).
17. Shrestha, S., Grilley, M., Fosso, M. Y., Chang, C. W. & Takemoto, J. Y. Membrane lipid-modulated mechanism of action and non-cytotoxicity of novel fungicide aminoglycoside FG28. PLoS One 8, e73843 (2013).
18. Shrestha, S. K. et al. Antifungal amphiphilic aminoglycoside K26: bioactivities and mechanism of action. Front. Microbiol. 5, 671 (2014).
19. Ghanounou, M. A. & Rice, L. B. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin. Microbiol. Rev. 12, 501–517 (1999).
20. Hao, B., Cheng, S., Clancy, C. J. & Nguyen, M. H. Caspofungin kills Candida albicans by causing both cellular apoptosis and necrosis. Antimicrob. Agents Chemother. 57, 326–332 (2013).
21. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts - Approved standard. CLSI document M27-A3. Wayne, PA. 2008.
22. Meletiadis, J., Mouton, J. W., Meis, J. F. & Verweij, P. E. In vitro drug interaction modeling of combinations of azoles with terbinafine against clinical Scedosporium prolificans isolates. Antimicrob. Agents Chemother. 47, 106–117 (2003).
25. Klepser, M. E., Malone, D., Lewis, R. E., Ernst, E. J. & Pfaller, M. A. Evaluation of voriconazole pharmacodynamics using time-kill methodology. *Antimicrob. Agents Chemother.* **44**, 1917–1920 (2000).

26. Clinical and Laboratory Standards Institute. *Method for antifungal disk diffusion susceptibility testing of yeasts - Approved guidelines, 2nd edition.* CLSI document M44-A2. Wayne, PA. 2009.

27. Lafleur, M. D. *et al.* Potentiation of azole antifungals by 2-adamantanamine. *Antimicrob. Agents Chemother.* **57**, 3585–3592 (2013).

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**Author Contributions**

M.Y.F. synthesized the TOB derivatives used in this study. S.K.S. performed all of the experiments. S.K.S. and S.G.T. analyzed data, wrote the manuscript, and prepared all figures. All authors reviewed the manuscript.

**Additional Information**

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