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Synthesis and Bioactivities of Kanamycin B-Derived Cationic Amphiphiles

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Abstract

Cationic amphiphiles derived from aminoglycosides (AGs) have been shown to exhibit enhanced antimicrobial activity. Through the attachment of hydrophobic residues such as linear alkyl chains on the AG backbone, interesting antibacterial and antifungal agents with a novel mechanism of action have been developed. Herein, we report the design and synthesis of seven kanamycin B (KANB) derivatives. Their antibacterial and antifungal activities, along with resistance/enzymatic, hemolytic, and cytotoxicity assays were also studied. Two of these compounds, with a C₁₂ and C₁₄ aliphatic chain attached at the 6''-position of KANB through a thioether linkage, exhibited good antibacterial and antifungal activity, were poorer substrates than KANB for several AG-modifying enzymes, and could delay the development of resistance in bacteria and fungi. Also, they were both relatively less hemolytic than the known membrane targeting antibiotic gramicidin and the known antifungal agent amphotericin B and were not toxic at their antifungal MIC values. Their oxidation to sulfones was also demonstrated to have no effect on their activities. Moreover, they both acted synergistically with posaconazole, an azole currently used in the treatment of human fungal infections.

Graphical abstract

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Supporting Information

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Experimental procedures and characterization data of all new compounds, as well as ¹H and ¹³C NMR spectra of compounds **2a–e**, **3a–e**, and **4c, d** (PDF)

SMILES for compounds **2a–e**, **3a–e**, and **4c, d** (XLSX)

INTRODUCTION

Aminoglycosides (AGs) are a valuable class of antibiotics that exhibit versatility in their biological activities.^{1,2} Widely recognized for their antibacterial properties, naturally occurring AGs have also recently been investigated as antifungal agents.^{3,4} One study evaluated the *in vitro* and *in vivo* activities of several AG antibiotics against six crop pathogenic oomycetes (*Phytophthora* and *Pythium* species) and 10 common fungi,³ while the other examined the *in vitro* activity of AGs against *Pythium insidiosum*, an aquatic oomycete that affects humans, mammals, and birds. These studies showed that, while gentamicin (GEN), neomycin B (NEO), paromomycin (PAR), and streptomycin (STR) might display antifungal activity at a concentration of 64 $\mu\text{g/mL}$, they would be unsuitable for therapeutic use because their antifungal MIC is higher than their safe serum concentration of 30–40 $\mu\text{g/mL}$.⁴

The economic and social burden associated with fungal infections is considerable.^{5–7} Currently, amphotericin B (AmB) and azoles, such as fluconazole (FLC), itraconazole (ITC), and posaconazole (POS), are the drugs of choice in the treatment of fungal infections.⁸ However, the rapid increase in the occurrence of opportunistic fungal infections in immunocompromised or severely ill patients, the difficulty to treat systemic mycoses, and the recrudescence of fungal strains resistant to available drugs,⁹ along with the toxicity associated with azoles,¹⁰ call for the development of new and effective antifungal agents.

Our group and others have recently engaged in the development of semisynthetic AGs with improved antifungal activity. These were notably cationic amphiphilic AGs derived from neamine (NEA) (FG03 and FG08),^{11,12} kanamycin A (KANA) (K20),^{13,14} and tobramycin (TOB) (C₁₄).¹⁵ FG03 and FG08, obtained by glycodiversification, bear a C₈ linear alkyl chain at the 4''-hydroxyl, whereas K20 and C₁₄, obtained by direct modification of KANA and TOB, respectively, have an octanesulfonyl chain and a thiooctadecyl chain, respectively, at the 6''-position. In all cases, it was demonstrated that the hydrophobic moiety, that is, the linear alkyl chain, conferred some amphiphilic properties to the parental AGs and increased

their affinity toward fungal cell membranes.^{14–16} This eventually led to the disruption of the membranes in fungi. With K20 displaying unprecedented inhibitory capabilities toward fungi, and despite the fact that the clinically used¹⁷ kanamycin B (KANB) is a better antimicrobial agent than KANA, there has not been any report to our knowledge of the direct modification of KANB toward the development of antifungal cationic amphiphiles.

Herein, we present the synthesis and antibacterial activity of seven KANB derivatives. Also, the antifungal activities of these compounds alone and in combination with azoles are studied. In addition, we investigated the ability of the most active compounds to delay the development of bacterial and fungal resistance and determined spontaneous resistance frequency against a fungal strain. Finally, we report on their toxicity toward erythrocytes and mammalian cells, along with their time-kill study and mechanism of action against fungi. Two of these compounds, KANB derivatives with a C₁₂ and C₁₄ alkyl chain attached in a thioether bond at the 6''-position, prove to be very promising in all respects.

RESULTS AND DISCUSSION

Design and Chemical Synthesis

In designing the KANB derivatives for this study, our primary focus was to confer cationic amphiphilic properties to them. This could be achieved by attaching hydrophobic residues, such as linear alkyl chains, to the highly polar KANB. Similar techniques have been previously employed in the development of amphiphilic AGs and have led to the discovery of NEO, PAR, KANA, NEA, and amikacin (AMK) derivatives with enhanced antibacterial activity.^{18–28} We envisaged that the length of these alkyl chains could range from C₈ to C₁₆, as these were previously shown to exhibit better potency.^{11–13,26} Finally, the ease of synthesis of the KANB derivatives was another important criterion. Because K20 and C₁₄ were obtained by direct modification at the 6''-position of KANA and TOB, respectively, and hundreds of grams of K20 could be prepared, we reasoned that similar modification of KANB could be accomplished. By incorporating various linear alkyl chains (C₈–C₁₆) at the 6''-position of KANB through a thioether linkage, we could thus generate our target molecules.

In light of this, our KANB derivatives **3a–e** were conveniently synthesized from the known precursor **1**²⁹ via a facile two-step procedure that we recently utilized to generate TOB variants for their evaluation as ribosome-targeting agents³⁰ (Scheme 1). Nucleophilic displacement of the TIPBS group at the 6''-position in **1** by various alkanethiols resulted in the formation of intermediates **2a–e** in good yields (67–78%). Acid treatment of **2a–e** then afforded the expected products **3a–e** as TFA salts in yields ranging from 82% to quantitative.

Antibacterial Activity

The antibacterial activity of KANB and its derivatives **3a–e** was evaluated against an array of Gram-positive and Gram-negative bacterial strains, including strains that are susceptible as well as some that are resistant to the parent AG KANB (Table 1). Among the 11 Gram-positive pathogens were the KANB-resistant and pathogenic methicillin-resistant *Staphylococcus aureus* (MRSA, strain **III**; *S. aureus* USA 100, strain **VI**; *S. aureus* USA

200, strain **VII**; *S. aureus* USA 300, strain **VIII**; and *S. aureus* USA 600, strain **IX**) and vancomycin-resistant *enterococci* (VRE, strain **XI**). The set of six Gram-negative bacteria also included a strain of *Klebsiella pneumoniae* (strain **XV**).

We also assessed the antibacterial activity of KANA. Except against *B. subtilis* 168 (strain **I**), *M. smegmatis* MC2-155 (strain **IV**), and *S. epidermidis* ATCC 12228 (strain **X**), KANA was ineffective at inhibiting the growth of our tested Gram-positive bacteria in vitro, with MIC values >128 $\mu\text{g/mL}$. Moreover, KANB was, in general, more potent than KANA, suggesting that the derivatives of the former AG might be better antibacterial agents.

KANB derivatives **3a–e** exhibited a wide range of antibacterial activity against the tested panel of Gram-positive bacteria. All but **3e** inhibited the growth of MRSA (strain **III**), *S. aureus* USA 100 (strain **VI**), *S. aureus* USA 200 (strain **VII**), and VRE (strain **XI**) at concentrations lower than that required by the parent AG. Furthermore, **3b–d** showed an activity similar to that of KANB against the other two MRSA strains (**VIII** and **IX**). On the other hand, compounds **3a–e** displayed a reduced antibacterial activity against *L. monocytogenes* ATCC 19115 (strain **II**), *M. smegmatis* MC2-155 (strain **IV**), and *S. aureus* ATCC 29213 (strain **V**) as compared to KANB. However, they still exhibited moderate to good antibacterial activity. Finally, while *B. subtilis* 168 (strain **I**) and *S. epidermidis* ATCC 12228 (strain **X**) were both susceptible to KANB (MIC = 4 and 0.5 $\mu\text{g/mL}$, respectively), none of the derivatives showed activity against these pathogens, displaying MICs $\geq$ 128 $\mu\text{g/mL}$. It is worthy to mention that as the length of the thioalkyl group increased from 8 to 12 carbons, the MIC value generally decreased. Once the chain was extended to 14 carbons, an increase in MIC value, sometimes not to a significant extent, was observed. Further elongation of the alkyl chain to 16 carbons resulted in a complete loss of antibacterial activity. Therefore, **3c**, which carries a thiododecyl group (C_{12}) at the 6''-position, exhibited the optimal antibacterial activity, closely followed by **3d** (C_{14}), then **3b** (C_{10}), **3a** (C_8), and **3e** (C_{16}) successively. A similar trend was observed for TOB derivatives that also bear thioalkyl chains at the 6''-position, with their inhibitory efficacy improving from the compound with C_8 chain to C_{14} chain.^{15,26}

Substitution of the 6''-hydroxyl group of KANB by a thioalkyl group appears to significantly decrease its inhibitory activity against Gram-negative bacteria, regardless of the chain length of the substituent. Indeed, while the Gram-negative bacterial strains tested were all susceptible to KANB (MIC $\leq$ 16 $\mu\text{g/mL}$), they regained some resistance in the presence of **3a–e** (MIC >64 $\mu\text{g/mL}$). Only *P. aeruginosa* ATCC 27853 (strain **XVI**) showed intermediate sensitivity to all five KANB derivatives but **3e** (MIC = 32–64 $\mu\text{g/mL}$), as well as *S. enterica* ATCC 14028 (strain **XVII**) to **3d** (MIC = 32 $\mu\text{g/mL}$). Therefore, **3a–e** exhibited higher potency against Gram-positive bacteria than Gram-negative bacteria, as observed with other AG cationic amphiphiles.^{26,31}

To evaluate the effect of metabolic *S*-oxidation on our thioethers, we oxidized our most potent KANB derivatives (**3c** and **3d**) to their corresponding sulfones (**4c** and **4d**; Scheme 1) and assessed their antibacterial activity (Table 1). An increase in MIC value of the sulfone analogues as compared to the parent thioethers was observed across the array of Gram-positive bacteria tested. Only **4d** showed a 64-fold improvement in antibacterial activity

against *M. smegmatis* MC2-155 (strain **IV**) with respect to its corresponding parent thioether **3d**. On the other hand, both sulfones **4c** and **4d** exhibited comparable to slightly better antibacterial activity than their corresponding thioethers **3c** and **3d** against Gram-negative bacterial strains. Therefore, it appears that *S*-oxidation of our KANB derivatives to the corresponding sulfones may decrease their potency against Gram-positive bacteria, while improving their inhibitory efficacy against Gram-negative bacteria.

Resistance Studies

The recurring emergence of bacterial resistance to AGs remains one of the major threats associated with their long-term clinical use. To evaluate the ability of bacteria to develop resistance to our newly synthesized compounds, we decided to subject the most susceptible bacterial strain in our study (*L. monocytogenes*; strain **II**) to multiple rounds of MIC testing,³² taking subinhibitory concentrations (1/2 MIC) for the next round of testing for the parent KANB and the two most active derivatives **3c** and **3d** over 15 passages (Figure S25). Although the relative MIC value of **3c** appears to increase by 4-fold as early as at the third passage, it took up to 6 passages to observe a 2-fold change in the MIC of **3d** and a 4-fold change in the MIC of KANB. Surprisingly, compound **3c** developed resistance faster than KANB, which may indicate a different method of resistance development. Furthermore, *L. monocytogenes* seems to be developing resistance to **3d** slower than it does to KANB. This encouraging finding thus suggests that **3d** will be able to delay the emergence of resistance as compared to KANB.

With more than 100 AG-modifying enzymes (AMEs) identified to date,³³ this family of enzymes represents the major causal agent of resistance in bacteria. The development of AG derivatives capable of evading the action of these enzymes thus seems a plausible avenue to overcome resistance issues. We decided to evaluate the ability of AMEs to modify our synthesized KANB derivatives **3a–e** and **4c, d** by measuring the relative activities of six enzymes with these KANB derivatives and comparing them to that of the parent AG, KANB (Figure 1). Among the six AMEs were APH(2'')-Ia,³⁴ AAC(3)-IV,³⁵ AAC(6')-Ie/APH(2'')-Ia (used for its AAC(6')-Ie activity only),³⁵ AAC(6')-IId,³⁶ AAC(2')-Ic,³⁷ and the multiacetylating enzyme Eis,^{37,38} with the latter two both being specific to *Mycobacterium tuberculosis*. Overall, there was a noticeable decrease in activity of these AMEs with all seven derivatives **3a–e** and **4c, d**. This suggests that these enzymes have reduced effect on modifying our newly synthesized KANB derivatives. In addition, out of all six AMEs tested, AAC(6')-IId and APH(2'')-Ia were the least active against **3a–e** and **4c, d**. Meanwhile, the multiacetylating enzyme Eis was the most active of them all. As the length of the alkyl chain attached to KANB increases, the effect of the AMEs seems to decrease from **3a** to **3d**, before increasing back on **3e**. As a result, **3c** (C₁₂) and **3d** (C₁₄), which exhibited the most potent inhibitory activity against bacterial growth, also appeared to be the poorest substrates of AMEs, as was the case with the TOB derivatives.^{15,26,30} Furthermore, their sulfone analogues **4c** and **4d** did not seem to be much more susceptible to the action of the AMEs tested.

AG acetyltransferases (AACs) represent a class of AMEs that regiospecifically acetylate a single amino group of an AG. They include AAC(3), AAC(2'), and AAC(6'), which modify

the amino group at the 3, 2', and 6'-positions of AGs, respectively. As AAC(3) and AAC(6') can sometimes both be found in the same bacterial strain, and because our newly synthesized KANB derivatives could decrease the activity of AAC(2'), we wanted to see the simultaneous effect of multiple AACs on KANB and our best derivative **3d**. By using silica gel thin layer chromatography (TLC),^{37,39,40} we were able to assess the modifications of AAC(3), AAC(2'), and AAC(6'), alone and together, on KANB and **3d** (Figure S26). When individually subjected to AAC(3), AAC(2'), or AAC(6') overnight, KANB was completely converted to its corresponding monoacetylated product (lanes 2, 3, and 4). Meanwhile, in the presence of two enzymes, some of the KANB remained unmodified (lanes 5, 6, and 7). This suggests that the use of two AACs might cause one to inhibit the other. However, this was not the case for **3d**. In both compounds, we observed that whenever AAC(3) was used in combination with AAC(2') (lanes 5 and 13) or AAC(6') (lanes 7 and 15), only the 3-acetylated product was formed. Similarly, only the 6'-monoacetylated adduct was obtained when AAC(2') and AAC(6') were used together (lanes 6 and 14). This is in agreement with the fact that AAC(3) is more catalytically efficient than AAC(6'), which in turn is more catalytically efficient than AAC(2').³⁵ Furthermore, modification at the 3-position appears to prevent further modification of the 6'-position, which is consistent with what we previously observed in the study of sequential modifications of other AGs.^{34,35} When all three enzymes were used simultaneously, KANB was converted to a diacetylated adduct (lane 8), while only the 3-monoacetylated product was obtained for **3d** (lane 16). All together, these results showed that, although our KANB derivative **3d** could not completely stop the action of AMEs, especially when several of them were present, it would still be able to slow their action and, as such, the emergence of bacterial resistance.

Antifungal Activity

Certain amphiphilic KANA and TOB derivatives were shown to have potent antifungal and/or antibacterial activities.^{11,14,15} These reports have prompted us to explore the in vitro antifungal activities of our newly synthesized amphiphilic KANB derivatives **3a–e**. The MIC values of KANB, its derivatives **3a–e**, and three azoles (POS, FLC, and ITC) against various fungi are summarized in Table 2. The fungal strains tested included *Candida* and *Aspergillus* species, which are the most common causes of fungal infections.⁴¹ With *C. albicans* species being among the most prevalent fungal pathogens⁴² and accounting for a large number of invasive fungal infections observed in intensive care units,⁴³ our collection of fungal strains included *C. albicans* clinical isolates from immunocompromised patients that are either susceptible (*C. albicans* ATCC MYA-2876 (strain **C**) and *C. albicans* ATCC MYA-2310 (strain **E**)) or resistant (*C. albicans* ATCC 10231 (strain **A**), *C. albicans* ATCC 64124 (strain **B**), *C. albicans* ATCC 90819 (strain **D**), *C. albicans* ATCC MYA-1237 (strain **F**), and *C. albicans* ATCC MYA-1003 (strain **G**)) to azoles. Like amphiphilic TOB analogues, our KANB derivatives also exhibited chain length-dependent antifungal activities against fungi in the order **3a** (C₈) < **3b** (C₁₀) < **3c** (C₁₂) < **3d** (C₁₄) < **3e** (C₁₆). Compounds **3c**, **3d**, and **3e** showed moderate to excellent activity against the fungal strains tested, with MIC values ranging from 1.95 to 31.2 µg/mL, 1.95 to 15.6 µg/mL, and 1.95 to 3.9 µg/mL, respectively. However, the parent AG KANB and its derivatives **3a** and **3b** were inactive against all yeast strains tested, with MIC values 125 µg/mL. Compound **3a** only displayed moderate activity against the filamentous fungi *A. nidulans* ATCC 38163 (strain **H**) (MIC =

15.6 $\mu\text{g/mL}$), while **3b** was very active (MIC = 1.95 $\mu\text{g/mL}$). It is noteworthy mentioning that **3c**, **3d**, and **3e** showed either comparable or, in most cases, enhanced antifungal activity against all yeast strains when compared to MIC-2 (50% inhibition) values of all three commercial antifungal azoles. More intriguingly, **3b–e** also exhibited higher inhibitory effect to *A. nidulans* ATCC 38163 (strain **H**), with MIC values = 1.95 $\mu\text{g/mL}$, which were superior to FLC, but comparable to POS and ITC. These results demonstrate that our KANB derivatives **3a–d** are more active against filamentous fungi than their TOB counterparts previously reported.¹⁵ Indeed, while TOB analogues with C₈, C₁₀, C₁₂, and C₁₄ alkyl chains displayed MIC values of 125, 62.5, 7.8, and 7.8 $\mu\text{g/mL}$ against *A. nidulans* ATCC 38163, we observed an 8-, 32-, 4-, and 4-fold decrease in MIC values of their corresponding KANB derivatives **3a**, **3b**, **3c**, and **3d**, respectively. *Aspergillus* species are one of the most common and emerging causes of invasive aspergillosis in immunocompromised individuals, causing a high rate of morbidity and mortality worldwide.⁴⁴ Moreover, compounds **3c**, **3d**, and **3e** effectively inhibited several azole-resistant fungi (*C. albicans* ATCC 10231 (strain **A**), *C. albicans* ATCC 64124 (strain **B**) that has mutation in *ERG11* sequences, *C. albicans* ATCC 90819 (strain **D**), *C. albicans* ATCC MYA-1237 (strain **F**), and *C. albicans* ATCC MYA-1003 (strain **G**)), potentially suggesting that these compounds will remain unaffected by the action of azole-resistant fungi. This is in agreement with our results showing that, as with bacteria, **3d** may also be able to delay the development of resistance by fungi (Figure S27).

We also determined the MICs of the sulfone analogues **4c** and **4d** against our collection of fungal strains and found that these values did not change much and were generally within 2-fold dilution of the MICs of their corresponding thioethers **3c** and **3d** (Table 2). This suggests that metabolic *S*-oxidation of our KANB derivatives will not affect their antifungal activity.

Azoles, such as POS, are considered first-line drugs in the treatment of candidiasis and aspergillosis in humans.⁸ POS kills fungi by inhibiting cytochrome P450-dependent enzyme sterol 14- α -demethylase involved in the synthesis of ergosterol, a key structural component of fungal membrane. Like other azoles, POS also faces many therapeutic challenges such as host side effects, drug–drug interaction, and poor absorption of the drug, resulting in limited therapeutic use.⁴⁵ The frequent report of emergence of fungal resistance against POS has further complicated the treatment of invasive fungal infections in humans, and higher drug dosage is often required. In this study, we have evaluated the in vitro interactions of POS with our best antifungal candidates **3c** and **3d** against various yeast strains (Table 3). These antifungal interactions were quantified by calculating the fractional inhibitory concentration index (FICI).⁴⁶ Both **3c** and **3d** exhibited remarkable synergistic interaction with POS against all tested fungal strains with FICI values ranging from 0.07 to 0.31, except for **3d** against *C. albicans* ATCC 10231 (strain **A**) (FICI = 0.56). It is noteworthy to mention that the MIC values of POS were reduced by 16–64-fold in the presence of **3c** and by 16–32-fold in the presence of **3d**. Also, POS lowered the MIC values of **3c** and **3d** by 4–16-fold and 2–8-fold, respectively. So far, there has only been one report of a less potent antifungal AG-derived cationic amphiphile (MIC = 15.6 $\mu\text{g/mL}$) capable of such synergy with azoles.⁴⁷ Our results thus suggest that the use of **3c** or **3d** in conjunction with POS may provide better

alternatives to treat refractory fungal infections and more importantly in the clinical cases, where low doses of POS are desirable to reduce toxicity or drug–drug interactions.

Spontaneous Frequency of Resistance Studies

To establish the spontaneous frequency of resistance of *C. albicans* ATCC 10231 (strain **A**) to the combination of compound **3c** and POS, three concentrations of POS (0.06, 0.125, or 0.25 $\mu\text{g/mL}$) were tested in combination with **3c** (8 $\mu\text{g/mL}$). Only the combination of **3c** (8 $\mu\text{g/mL}$) and POS (0.25 $\mu\text{g/mL}$) yielded few mutants (24 colonies) after 96 h incubation. This result suggests that the spontaneous frequency rate of resistance to the combination of **3c** and POS by this strain **A** is very low (2.4×10^{-7}).

Time-Kill Studies

To evaluate the antifungal activity kinetics of our KANB derivatives, we performed time-kill assays of **3c** and **3d** on *C. albicans* ATCC 64124 (strain **B**) over a period of 24 h (Figure 2). At subinhibitory concentrations (1/2 MICs), **3c** and **3d** showed fungistatic activity similarly to the known antifungal agent AmB. However, at concentrations equivalent to their MICs, **3c** and **3d** both rapidly reduced the CFU of *C. albicans* by 2 log 10 after 6 h treatment. In addition, complete fungal cell death was observed after 24 h for **3c** and after 12 h for **3d**. These results show that **3c** and **3d** exhibited a fungicidal effect on the azole-resistant strain *C. albicans* ATCC 64124, which is in agreement with our previous observation with amphiphilic TOB analogues.¹⁵

Antifungal Mechanism of Action

Cell membrane disruption has been shown to be a mode of action peculiar to antifungal AG-derived cationic amphiphiles.¹⁵ Using propidium iodide (PI), a dye that fluoresces upon penetrating membrane-compromised cells and binding to nucleic acids, we were able to investigate the effects of our best antifungal candidates on the fungal membrane integrity of the azole-resistant yeast *C. albicans* ATCC 64124 (strain **B**). After exposing these yeast cells to various concentrations of **3c** and **3d** (1 $\times$ MIC or 2 $\times$ MIC), we observed 44% or 90%, and 49% or 84% of cell staining with PI, respectively. However, cells treated with KANB and untreated showed minimal staining (5% and <2%, respectively) (Figure 3). This shows that **3c** and **3d** affect membrane permeability and are both more effective than KANB in causing PI permeation in the azole-resistant *C. albicans* ATCC 64124 cells. Furthermore, **3c** and **3d** showed equal rate of permeability of fungal membrane.

Hemolysis

AG-derived cationic amphiphiles have previously been shown to trigger membrane disruption. To evaluate the selectivity of our KANB derivatives **3a–e** toward bacterial or fungal membranes, hemolytic assay was performed using murine red blood cells (mRBCs) (Figure 4). As observed with their antifungal activity, KANB derivatives also showed chain-length-dependent effect on RBCs in the order **3a** (C₈) < **3b** (C₁₀) < **3c** (C₁₂) < **3d** (C₁₄) < **3e** (C₁₆). Compound **3c** lysed 40% of mRBCs at 125 $\mu\text{g/mL}$, which is a 4-fold higher concentration than its antifungal MIC. Compound **3d**, on the other hand, lysed 15% of mRBCs at 7.8 $\mu\text{g/mL}$ (**3d**'s antifungal MIC) and 73% of mRBCs at 500 $\mu\text{g/mL}$ (32-fold

higher than **3d**'s antifungal MIC). Compound **3e** also lysed 16% of mRBCs at 7.8 $\mu\text{g/mL}$ (twice its antifungal MIC) and 81% of mRBCs at 62.5 $\mu\text{g/mL}$ (16-fold higher than its antifungal MIC). However, KANB and compounds **3a** and **3b** caused little to no hemolysis at higher concentrations. We also observed that **3d** and **3e** reach a plateau a lot quicker than **3c**, and do not attain 100% (Figure 4). This is probably due to aggregation as the length of the lipophilic alkyl chain increases. In comparison to the known antifungal AmB, KANB, **3a**, **3b**, **3c**, and **3d** all showed relatively lower hemolytic activities. **3e**, on the contrary, displayed hemolytic activity similar to that of AmB. Similarly, KANB, **3a**, **3b**, **3c**, and **3d** displayed lower hemolytic activities than the known membrane targeting antibiotic gramicidin, while **3e** was more hemolytic than gramicidin. Therefore, even though **3e** was the most active compound against fungi, it appears to rapidly turn hemolytic, leaving us with **3c** and **3d** as our best antifungal agent candidates. Importantly, when used in conjunction with POS, as less of **3c** and **3d** was required to display antifungal activity, the hemolytic activity resulting from using this lesser amount would greatly decrease the hemolytic activity of these KANB derivatives. With the MIC value of **3c** dropping from 31.2 to 1–8 $\mu\text{g/mL}$ when used alone versus in combination with POS, 50 $\times$ MIC would be needed to lyse 60% mRBCs, rendering **3c** ~30% less hemolytic than AmB and ~20% less hemolytic than gramicidin. Therefore, it is predicted that our compounds, especially when used in combination with POS, may not display problematic hemolysis of red blood cells.

Cytotoxicity

An important parameter in the development of drugs is their ability to selectively target microbial cells without affecting mammalian cells. This is especially the case for antifungal agents because fungi and humans are both eukaryotes and their cells would thus be similar on a biological level. To evaluate the toxicity of our KANB derivatives **3a–d**, cytotoxicity assays were conducted against two nucleated mammalian cell lines, A549 and BEAS-2B (Figure 5). While compounds **3a** and **3b** displayed little to no toxicity against these cell lines like KANB itself, **3c** had an IC_{50} value $>62.5 \mu\text{g/mL}$ against A549 (Figure 5A) and an IC_{50} value $>125 \mu\text{g/mL}$ against BEAS-2B (Figure 5B). These values were 4–64-fold higher than the antifungal MIC values. Likewise, the IC_{50} values of **3d** against both cell lines were $>62.5 \mu\text{g/mL}$, which correspond to 8–32 times its antifungal MICs. These results indicate that our KANB derivatives display a low toxicity profile against mammalian cell lines. The abundance of lipid-like components in the fungal membranes (phosphatidylcholine, L- α -phosphatidylethanolamine, L- α -phosphatidylinositol, ergosterol)^{16,48} as compared to the mammalian membranes (phosphatidylcholine, cholesterol)^{16,48} could account for this selectivity. Indeed, our KANB derivatives being cationic and rich in amino and hydroxyl groups are capable of hydrogen and ionic interactions with the polar and negatively charged head groups present on the glycerophospholipid and sterols constituents of fungal membranes, and as such should have a higher preference for fungal membranes than mammalian membranes. Compounds **3c** and **3d** thus appear attractive as potential candidates for the development as antifungal agents.

CONCLUSIONS

We have synthesized five cationic amphiphilic KANB derivatives, **3a–e**, by attaching linear alkyl chains of various length (C₈, C₁₀, C₁₂, C₁₄, and C₁₆) at the 6''-position of parental KANB through a thioether linkage. Biological evaluation revealed that **3a** (C₈) and **3b** (C₁₀) exhibited little to no antimicrobial activities, whereas **3c** (C₁₂) and **3d** (C₁₄) showed promising antibacterial and antifungal potency in vitro, and **3e** (C₁₆) was the most active against fungi and with no potency on bacteria. Compound **3c** was the most effective at inhibiting the growth of Gram-positive bacteria, closely followed by **3d**. Also, both of these compounds were the poorest substrates of AMEs and could evade the action of these bacterial resistance enzymes better than KANB. Compound **3d** in particular was the most effective at delaying the development of bacterial resistance. Our KANB derivatives also exhibited chain length-dependent antifungal activities against fungi in the order **3a** (C₈) < **3b** (C₁₀) < **3c** (C₁₂) < **3d** (C₁₄) < **3e** (C₁₆). Compounds **3c**, **3d**, and **3e** were 4, 16, and 32 times more effective than KANB, respectively, against several of the tested yeast strains. Interestingly, these KANB derivatives showed comparable or, in most cases, enhanced antifungal activity against all yeast strains when compared to the commercial antifungal drugs FLC, ITC, and POS. Against the filamentous fungus *Aspergillus nidulans* (strain **H**), our KANB derivatives **3a–d** were more active than their TOB counterparts previously reported.¹⁵ Compounds **3c**, **3d**, and **3e** were 64 times better than KANB and 32 times better than FLC. In vitro antifungal combination studies of POS with **3c** or **3d** also revealed remarkable synergistic interactions, with the MIC values of POS dropping by 16–64-fold in the presence of **3c** and by 16–32-fold in the presence of **3d**. POS also lowered the MIC values of **3c** and **3d** by 4–16-fold and 2–8-fold, respectively. Finally, although **3e** was the most active against yeasts, it appeared to be quite hemolytic to murine red blood cells. Compounds **3c** and **3d**, on the contrary, showed low hemolytic activities and mammalian cell toxicities. By synthesizing their sulfone analogues **4c** and **4d**, we were also able to show that their biological activities will not change much under the effect of metabolic *S*-oxidation. As compared to our previous reports of TOB variants that investigated their ribosome-targeting capabilities³⁰ or fungicidal activity,¹⁵ this present report further expands the study to include the development of resistance in bacteria and fungi, along with the exciting synergistic effects of two of our KANB derivatives to azoles. It thus appears that **3c** and **3d** could be useful lead compounds for the development of antifungal agents. Furthermore, extensive in vivo study of these KANB derivatives with azole antifungal drugs will be an encouraging research avenue to be pursued, as this may offer interesting alternatives in the treatment of human fungal infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Fosso MY, Li Y, Garneau-Tsodikova S. New trends in aminoglycosides use. *MedChemComm*. 2014; 5:1075–1091. [PubMed: 25071928]
2. Houghton JL, Green KD, Chen W, Garneau-Tsodikova S. The future of aminoglycosides: the end or renaissance? *ChemBioChem*. 2010; 11:880–902. [PubMed: 20397253]
3. Lee HB, Kim Y, Kim JC, Choi GJ, Park SH, Kim CJ, Jung HS. Activity of some aminoglycoside antibiotics against true fungi, *Phytophthora* and *Pythium* species. *J Appl Microbiol*. 2005; 99:836–843. [PubMed: 16162234]
4. Mahl DL, de Jesus FP, Loreto E, Zanette RA, Ferreiro L, Pilotto MB, Alves SH, Santurio JM. *In vitro* susceptibility of *Pythium insidiosum* isolates to aminoglycoside antibiotics and tigecycline. *Antimicrob Agents Chemother*. 2012; 56:4021–4023. [PubMed: 22508303]
5. Knight SC, Anthony VM, Brady AM, Greenland AJ, Heaney SP, Murray DC, Powell KA, Schulz MA, Spinks CA, Worthington PA, Youle D. Rationale and perspectives on the development of fungicides. *Annu Rev Phytopathol*. 1997; 35:349–372. [PubMed: 15012528]
6. Strange RN, Scott PR. Plant disease: a threat to global food security. *Annu Rev Phytopathol*. 2005; 43:83–116. [PubMed: 16078878]
7. Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, McCraw SL, Gurr SJ. Emerging fungal threats to animal, plant and ecosystem health. *Nature*. 2012; 484:186–194. [PubMed: 22498624]
8. Falci DR, Pasqualotto AC. Profile of isavuconazole and its potential in the treatment of severe invasive fungal infections. *Infect Drug Resist*. 2013; 6:163–174. [PubMed: 24187505]
9. Sheehan DJ, Hitchcock CA, Sibley CM. Current and emerging azole antifungal agents. *Clin Microbiol Rev*. 1999; 12:40–79. [PubMed: 9880474]
10. Dolton MJ, McLachlan AJ. Optimizing azole antifungal therapy in the prophylaxis and treatment of fungal infections. *Curr Opin Infect Dis*. 2014; 27:493–500. [PubMed: 25229352]
11. Chang CW, Fosso M, Kawasaki Y, Shrestha S, Bensaci MF, Wang J, Evans CK, Takemoto JY. Antibacterial to antifungal conversion of neamine aminoglycosides through alkyl modification. Strategy for reviving old drugs into agrofungicides. *J Antibiot*. 2010; 63:667–672. [PubMed: 20924381]
12. Fosso M, AlFindie MN, Zhang Q, de Nziko VP, Kawasaki Y, Shrestha SK, Bearss J, Gregory R, Takemoto JY, Chang CW. Structure-activity relationships for antibacterial to antifungal conversion of kanamycin to amphiphilic analogues. *J Org Chem*. 2015; 80:4398–4411. [PubMed: 25826012]
13. Chang CW, Takemoto JY. Antifungal amphiphilic aminoglycosides. *MedChemComm*. 2014; 5:1048–1057. [PubMed: 25110571]
14. Shrestha SK, Chang CW, Meissner N, Oblad J, Shrestha JP, Sorensen KN, Grilley MM, Takemoto JY. Antifungal amphiphilic aminoglycoside K20: bioactivities and mechanism of action. *Front Microbiol*. 2014; 5:671. [PubMed: 25538692]
15. Shrestha SK, Fosso MY, Green KD, Garneau-Tsodikova S. Amphiphilic tobramycin analogues as antibacterial and antifungal agents. *Antimicrob Agents Chemother*. 2015; 59:1565–1570.
16. Shrestha S, Grilley M, Fosso MY, Chang CW, Takemoto JY. Membrane lipid-modulated mechanism of action and noncytotoxicity of novel fungicide aminoglycoside FG08. *PLoS One*. 2013; 8:e73843. [PubMed: 24040088]
17. Corcoran, JW.; Hahn, FE. Mechanism of Action of Antimicrobial and Antitumor Agents. Springer Science & Business Media; New York: 2012. p. 744
18. Zhang J, Chiang FI, Wu L, Czyryca PG, Li D, Chang CW. Surprising alteration of antibacterial activity of 5''-modified neomycin against resistant bacteria. *J Med Chem*. 2008; 51:7563–7573. [PubMed: 19012394]
19. Bera S, Zhanel GG, Schweizer F. Design, synthesis, and antibacterial activities of neomycin-lipid conjugates: polycationic lipids with potent Gram-positive activity. *J Med Chem*. 2008; 51:6160–6164. [PubMed: 18778047]
20. Hanessian S, Szychowski J, Adhikari SS, Vasquez G, Kandasamy P, Swayze EE, Migawa MT, Ranken R, Francois B, Wirmer-Bartoschek J, Kondo J, Westhof E. Structure-based design,

- synthesis, and A-site rRNA cocrystal complexes of functionally novel aminoglycoside antibiotics: C2'' ether analogues of paromomycin. *J Med Chem.* 2007; 50:2352–2369. [PubMed: 17458946]
21. Zhang J, Keller K, Takemoto JY, Bensaci M, Litke A, Czyryca PG, Chang CW. Synthesis and combinational antibacterial study of 5''-modified neomycin. *J Antibiot.* 2009; 62:539–544. [PubMed: 19629142]
 22. Hanessian S, Pachamuthu K, Szychowski J, Giguere A, Swayze EE, Migawa MT, Francois B, Kondo J, Westhof E. Structure-based design, synthesis and A-site rRNA co-crystal complexes of novel amphiphilic aminoglycoside antibiotics with new binding modes: a synergistic hydrophobic effect against resistant bacteria. *Bioorg Med Chem Lett.* 2010; 20:7097–7101. [PubMed: 20970332]
 23. Bera S, Zhanel GG, Schweizer F. Antibacterial activities of aminoglycoside antibiotics-derived cationic amphiphiles. Polyol-modified neomycin B-, kanamycin A-, amikacin-, and neamine-based amphiphiles with potent broad spectrum antibacterial activity. *J Med Chem.* 2010; 53:3626–3631. [PubMed: 20373816]
 24. Ouberaï M, El Garch F, Bussiere A, Riou M, Alsteens D, Lins L, Baussanne I, Dufrene YF, Brasseur R, Decout JL, Mingeot-Leclercq MP. The *Pseudomonas aeruginosa* membranes: a target for a new amphiphilic aminoglycoside derivative? *Biochim Biophys Acta, Biomembr.* 2011; 1808:1716–1727.
 25. Szychowski J, Kondo J, Zahr O, Auclair K, Westhof E, Hanessian S, Keillor JW. Inhibition of aminoglycoside-deactivating enzymes APH(3'-IIIa) and AAC(6')-II by amphiphilic paromomycin O2''-ether analogues. *ChemMedChem.* 2011; 6:1961–1966. [PubMed: 21905229]
 26. Herzog IM, Green KD, Berkov-Zrihen Y, Feldman M, Vidavski RR, Eldar-Boock A, Satchi-Fainaro R, Eldar A, Garneau-Tsodikova S, Fridman M. 6''-Thioether tobramycin analogues: towards selective targeting of bacterial membranes. *Angew Chem, Int Ed.* 2012; 51:5652–5656.
 27. Dhondikubeer R, Bera S, Zhanel GG, Schweizer F. Antibacterial activity of amphiphilic tobramycin. *J Antibiot.* 2012; 65:495–498. [PubMed: 22781280]
 28. Zimmermann L, Bussiere A, Ouberaï M, Baussanne I, Jolival C, Mingeot-Leclercq MP, Decout JL. Tuning the antibacterial activity of amphiphilic neamine derivatives and comparison to paromamine homologues. *J Med Chem.* 2013; 56:7691–7705. [PubMed: 24083676]
 29. Van Schepdael A, Delcourt J, Mulier M, Busson R, Verbist L, Vanderhaeghe HJ, Mingeot-Leclercq MP, Tulkens PM, Claes PJ. New derivatives of kanamycin B obtained by modifications and substitutions in position 6''. 1. Synthesis and microbiological evaluation. *J Med Chem.* 1991; 34:1468–1475. [PubMed: 2016724]
 30. Fosso MY, Zhu H, Green KD, Fredrick K, Garneau-Tsodikova S. Tobramycin variants with enhanced ribosome-targeting activity. *ChemBioChem.* 2015; 16:1565–1570. [PubMed: 26033429]
 31. Berkov-Zrihen Y, Herzog IM, Benhamou RI, Feldman M, Steinbuch KB, Shaul P, Lerer S, Eldar A, Fridman M. Tobramycin and nebramine as pseudo-oligosaccharide scaffolds for the development of antimicrobial cationic amphiphiles. *Chem - Eur J.* 2015; 21:4340–4349. [PubMed: 25652188]
 32. Pokrovskaya V, Belakhov V, Hainrichson M, Yaron S, Baasov T. Design, synthesis, and evaluation of novel fluoroquinolone-aminoglycoside hybrid antibiotics. *J Med Chem.* 2009; 52:2243–2254. [PubMed: 19301822]
 33. Ramirez MS, Tolmasky ME. Aminoglycoside modifying enzymes. *Drug Resist Updates.* 2010; 13:151–171.
 34. Green KD, Chen W, Garneau-Tsodikova S. Effects of altering aminoglycoside structures on bacterial resistance enzyme activities. *Antimicrob Agents Chemother.* 2011; 55:3207–3213. [PubMed: 21537023]
 35. Green KD, Chen W, Houghton JL, Fridman M, Garneau-Tsodikova S. Exploring the substrate promiscuity of drug-modifying enzymes for the chemoenzymatic generation of *N*-acylated aminoglycosides. *ChemBioChem.* 2010; 11:119–126. [PubMed: 19899089]
 36. Green KD, Garneau-Tsodikova S. Domain dissection and characterization of the aminoglycoside resistance enzyme ANT(3'')-II/AAC(6'')-IId from *Serratia marcescens*. *Biochimie.* 2013; 95:1319–1325. [PubMed: 23485681]

37. Chen W, Biswas T, Porter VR, Tsodikov OV, Garneau-Tsodikova S. Unusual regioversatility of acetyltransferase Eis, a cause of drug resistance in XDR-TB. *Proc Natl Acad Sci U S A*. 2011; 108:9804–9808. [PubMed: 21628583]
38. Green KD, Pricer RE, Stewart MN, Garneau-Tsodikova S. Comparative study of Eis-like enzymes from pathogenic and nonpathogenic bacteria. *ACS Infect Dis*. 2015; 1:272–283. [PubMed: 27622743]
39. Houghton JL, Biswas T, Chen W, Tsodikov OV, Garneau-Tsodikova S. Chemical and structural insights into the regioversatility of the aminoglycoside acetyltransferase Eis. *ChemBioChem*. 2013; 14:2127–2135. [PubMed: 24106131]
40. Chen W, Green KD, Tsodikov OV, Garneau-Tsodikova S. Aminoglycoside multiacetylating activity of the enhanced intracellular survival protein from *Mycobacterium smegmatis* and its inhibition. *Biochemistry*. 2012; 51:4959–4967. [PubMed: 22646013]
41. Groll AH, Walsh TJ. Uncommon opportunistic fungi: new nosocomial threats. *Clin Microbiol Infect*. 2001; 7:8–24.
42. Xie JL, Polvi EJ, Shekhar-Guturja T, Cowen LE. Elucidating drug resistance in human fungal pathogens. *Future Microbiol*. 2014; 9:523–542. [PubMed: 24810351]
43. Gullo A. Invasive fungal infections: the challenge continues. *Drugs*. 2009; 69(Suppl 1):65–73.
44. Chowdhary A, Sharma C, Hagen F, Meis JF. Exploring azole antifungal drug resistance in *Aspergillus fumigatus* with special reference to resistance mechanisms. *Future Microbiol*. 2014; 9:697–711. [PubMed: 24957095]
45. Dolton MJ, Ray JE, Chen SC, Ng K, Pont L, McLachlan AJ. Multicenter study of posaconazole therapeutic drug monitoring: exposure-response relationship and factors affecting concentration. *Antimicrob Agents Chemother*. 2012; 56:5503–5510. [PubMed: 22890761]
46. Meletiadis J, Mouton JW, Meis JF, Verweij PE. *In vitro* drug interaction modeling of combinations of azoles with terbinafine against clinical *Scedosporium prolificans* isolates. *Antimicrob Agents Chemother*. 2003; 47:106–117. [PubMed: 12499177]
47. Shrestha SK, Grilley M, Anderson T, Dhiman C, Oblad J, Chang CT, Sorensen KN, Takemoto JY. In vitro antifungal synergy between amphiphilic aminoglycoside K20 and azoles against *Candida* species and *Cryptococcus neoformans*. *Med Mycol*. 2015; 53:837. [PubMed: 26260746]
48. Makovitzki A, Avrahami D, Shai Y. Ultrashort antibacterial and antifungal lipopeptides. *Proc Natl Acad Sci U S A*. 2006; 103:15997–16002. [PubMed: 17038500]

Figure 1.

Bar graph displaying the relative initial rates of reactions of the various AMEs with KANB and its derivatives **3a–e** and **4c, d**. Rates are normalized to KANB.

Figure 2.

Representative time-kill studies of KANB derivatives **3c** and **3d** against azole-resistant *C. albicans* ATCC 64124 (strain **B**). (A) Cultures were exposed to **3c** at 8 µg/mL (○), 16 µg/mL (▼), and 32 µg/mL (△). (B) Cultures were exposed to **3d** at 2 µg/mL (○), 4 µg/mL (▼), and 8 µg/mL (△). In both panels, cultures were exposed to AmB at 1 µg/mL (■) or to a no drug control (●).

Figure 3.

(A) Representative dose-dependent membrane permeabilization effects of KANB and its derivatives **3c** and **3d** on azole-resistant *C. albicans* ATCC 64124 (B). From top to bottom: Propidium iodine (PI) dye uptake by yeast cells without drug, with KANB (62.5 $\mu\text{g/mL}$), with **3c** (1 $\times$ and 2 $\times$ MIC), and with **3d** (1 $\times$ and 2 $\times$ MIC). (B) Quantitative representation of the images shown in panel A.

Figure 4. Hemolytic activity of KANB, gramicidin, amphotericin B (AmB), and **3a–e** on mouse red blood cells.

Figure 5. Mammalian cell cytotoxicity of KANB and its derivatives **3a–d** against (A) A549 cell line and (B) BEAS-2B cell line.

Scheme 1. Structure of Kanamycin B (KANB) and Its Derivatives Utilized in This Study^a

^aReagents and conditions for the synthesis: (i) RSH , Cs_2CO_3 , DMF, 67–78%; (ii) neat TFA, 82%–quantitative; (iii) *m*-CPBA/ CHCl_3 then neat TFA, 88–98%.

Table 1

MIC Values (mg/mL)^a of KANA, KANB, 3a–e, and 4c, d against Various Gram-Positive and Gram-Negative Bacterial Strains

bacterial strains	KANA	KANB	3a	3b	3c	3d	3e	4c	4d
Gram-Positive									
<i>B. subtilis</i> 168 (I)	2–4	4	>128	128	>128	>128	>128	>128	>128
<i>L. monocytogenes</i> ATCC 19115 (III)	>128	0.25	8	2	1	0.5–1	16	4–8	2–4
MRSA ^b (III)	>128	>128	128	32	32	32–64	>128	64	128
<i>M. smegmatis</i> MC2-155 (IV)	0.25	0.25	64	8	2	>128	8	4	2
<i>S. aureus</i> ATCC 29213 (V)	>128	0.25	64	16–32	8	16	>128	128	128
<i>S. aureus</i> NRS382 (USA 100) (VI)	>128	>128	128	32	8	32	128	64	64
<i>S. aureus</i> NRS383 (USA 200) (VII)	>128	128	64	16	4	8–16	>128	32	128
<i>S. aureus</i> NRS385 (USA 300) (VIII)	>128	32	128	32–64	16	16–32	>128	64	128
<i>S. aureus</i> NRS22 (USA 600) (IX)	>128	32	64	16	16	16	128	32	64
<i>S. epidermidis</i> ATCC 12228 (X)	1	0.5	>128	>128	>128	>128	>128	>128	>128
VRE ^c (XI)	>128	>128	128	32	16	16	128	32	32
Gram-Negative									
<i>A. baumannii</i> ATCC 19606 (XII)	4	4	>128	>128	>128	>128	>128	128	>128
<i>E. cloacae</i> ATCC 13047 (XIII)	2	0.5	>128	>128	>128	128	>128	>128	>128
<i>E. coli</i> MC1061 (XIV)	8–16	2	>128	>128	>128	>128	>128	>128	>128
<i>K. pneumoniae</i> ATCC 27736 (XV)	16–32	4	>128	>128	>128	>128	>128	128	>128
<i>P. aeruginosa</i> ATCC 27853 (XVI)	>128	16	64	32	32	64	>128	16	32
<i>S. enterica</i> ATCC 14028 (XVII)	16	2	>128	>128	>128	32	>128	64	128

^a All experiments were performed at least in duplicate.^b MRSA = methicillin-resistant *Staphylococcus aureus*.^c VRE = vancomycin-resistant *enterococci*.

MIC Values ($\mu\text{g/mL}$) Determined for KANB, Its Derivatives 3a-e and 4c, d, and Three Control Antifungal Agents (POS, ITC, and FLC) against Various Yeast Strains and Filamentous Fungi^a

Table 2

yeast strains	KANB	3a	3b	3c	3d	3e	4c	4d	POS	ITC	FLC
<i>C. albicans</i> ATCC 10231 (A) ^b	>125	>125	125	31.2	7.8	3.9	31.2	3.9	0.5	0.5	62.5
<i>C. albicans</i> ATCC 64124 (B) ^b	>125	>125	125	31.2	7.8	3.9	31.2	3.9	>62.5	>62.5	>125
<i>C. albicans</i> ATCC MYA-2876 (C) ^c	>125	>125	125	31.2	7.8	3.9	31.2	3.9	7.8	7.8	15.6
<i>C. albicans</i> ATCC 90819 (D) ^b	>125	>125	125	31.2	15.6	3.9	62.5	7.8	31.2	31.2	>125
<i>C. albicans</i> ATCC MYA-2310 (E) ^c	>125	>125	62.5	7.8	7.8	3.9	62.5	7.8	31.2	31.2	>125
<i>C. albicans</i> ATCC MYA-1237 (F) ^b	>125	>125	125	31.2	7.8	3.9	62.5	7.8	15.6	31.2	62.5
<i>C. albicans</i> ATCC MYA-1003 (G) ^b	>125	>125	125	31.2	7.8	3.9	62.5	7.8	15.6	31.2	62.5
Filamentous Fungi											
<i>Aspergillus nidulans</i> ATCC 38163 (H)	>125	15.6	1.95	1.95	1.95	1.95	3.9	1.95	1.95	1.95	>62.5

^a All experiments were performed in duplicate. For yeast strains, MIC-0 values (complete inhibition) are reported for KANB and 3a-e, whereas MIC-2 values (50% inhibition) are reported for azoles. For filamentous fungi, MIC-0 values are reported for all compounds.

^b Strains resistant to azoles.

^c Strains susceptible to azoles.

Table 3

In Vitro Susceptibility of *C. albicans* Species to 3c (C₁₂) and 3d (C₁₄) Alone and in Combination with POS^a

yeast strains	MIC values (μg/mL) for 3c					MIC values (μg/mL) for 3d				
	drug alone		combination			drug alone		combination		
	POS	3c	POS	3c	FICIs	interpretation	POS	3d	POS	FICIs
<i>C. albicans</i> ATCC 10231 (A) ^b	1	32	0.06	8	0.31	SYN	1	8	0.06	4
<i>C. albicans</i> ATCC 64124 (B) ^b	>25	32	0.78	2	0.09	SYN	>25	8	1.56	1
<i>C. albicans</i> ATCC MYA-2876 (C) ^c	25	16	0.78	2	0.15	SYN	25	8	1.56	1
<i>C. albicans</i> ATCC 90819 (D) ^b	>25	32	0.78	4	0.15	SYN	>25	8	0.78	1
<i>C. albicans</i> ATCC MYA-2310 (E) ^c	>25	16	0.39	1	0.07	SYN	>25	4	1.56	1
<i>C. albicans</i> ATCC MYA-1237 (F) ^b	>25	32	0.78	2	0.09	SYN	>25	8	0.78	1
<i>C. albicans</i> ATCC MYA-1003 (G) ^b	>25	32	0.78	2	0.09	SYN	>25	8	1.56	2

^a All experiments were performed in duplicate. FICI (fractional inhibitory concentration index) was determined to assess the in vitro antifungal interactions. It is calculated using the formula (MIC of drug A combination/MIC of drug A alone) + (MIC of drug B combination/MIC of drug B alone). When FICI = 0.5, there is synergy (SYN). If 0.5 < FICI < 4, there is no interaction between the drugs (IND). However, when FICI = 4, there is antagonism (ANT).⁴⁶

^b Strains resistant to azoles.

^c Strains susceptible to azoles.