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Aryl-alkyl-lysines: Membrane-active fungicides that act against biofilms of *Candida albicans*

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ABSTRACT: Mortality due to pathogenic fungi has been exacerbated by the rapid development of resistance to frontline antifungal drugs. Fungicidal compounds with novel mechanisms of action are urgently needed. Aryl-alkyl-lysines, which are membrane-active small molecules,

were earlier shown to be broad-spectrum antibacterial agents with potency *in vitro* and *in vivo*. Herein, we report the antifungal properties of aryl-alkyl-lysines. After identifying the most active compound (NCK-10), we tested its activity against a panel of clinically relevant pathogenic fungi and examined NCK-10's effect against immature and mature biofilms of *Candida albicans*. NCK-10 was capable of inhibiting growth of various species of fungi (including *Candida spp*., *Cryptococcus spp.*, and *Aspergillus fumigatus*) at concentrations similar to antifungal drugs used clinically. It was observed that polarization and permeability of the fungal cell membrane was compromised upon addition of NCK-10, indicating its mechanism is disruption of the fungal cell membrane. In addition to interfering with growth of planktonic fungi, NCK-10 demonstrated the ability to both inhibit biofilm formation and reduce the metabolic activity of cells in *C. albicans* biofilm. Additionally, our compound was capable of crossing the blood-brain-barrier in an *in vitro* model, expanding the potential antifungal applications for NCK-10. Overall, aryl-alkyl-lysines were found to be excellent compounds that warrant further investigation as novel antifungal agents.

Key words: Antimicrobial peptides, Antifungal drugs, Fungal Biofilms, Peptidomimetics

INTRODUCTION

Fungal infections afflict millions of patients annually with more than 50% mortality associated with invasive fungal infections.[1] Despite the fact that infections caused by pathogenic fungi lead to significant mortality and morbidity, the threat of these pathogens is often overlooked.[1] Only four classes of antifungal drugs are currently available to clinicians: polyenes, azoles, echinocandins and 5-fluorocytosine.[2] While azoles inhibit the biosynthesis of ergosterols,

polyenes bind to ergosterol, form pores, and induce leakage of intracellular ions.[2-3] In contrast, echinocandins and 5-fluorocytosine exert their antifungal effect by a different method. Echinocandins inhibit fungal cell wall biosynthesis while 5-fluorocytosine inhibits both protein synthesis and DNA synthesis in fungi.[4] There are reports of resistance to each antifungal agent mentioned above; this makes it imperative to design and develop novel antifungal agents.[5]

In additional to targeting fungal viability, identifying novel antifungals capable of interfering with key fungal virulence factors (such as biofilm formation) is highly desirable. Similar to bacteria, fungi are capable of forming stable communities within biofilms that are extremely difficult to treat.[6] Only recently have therapeutic agents against antifungal biofilms gained more prominence in the research community.[7-8] Thus design of novel antifungal agents which can act against both planktonic and biofilm forms of pathogenic fungi is highly desirable.

Antimicrobial peptides and their mimics have gained popularity among researchers as next generation therapeutic agents, primarily because of their broad spectrum of activity.[9-10] Several antimicrobial peptides possess notable antifungal activity. This has prompted additional research in peptidomimetic designs with antifungal properties.[11-13] Although, none of the peptidomimetic designs have progressed to preclinical studies to date, they bear much promise as potential drugs for the treatment of pathogenic fungi.

Our research group has designed several macromolecular and small molecule membrane-active agents which are potent antimicrobial agents alone and in combination with traditional antibiotics.[14-16] Interestingly, most of these agents also possess potent antibiofilm properties against both Gram-positive and Gram-negative bacteria.[17-18] Herein, we have explored the antifungal properties of one subset of bioactive molecules in our compound library, aryl-alkyl-lysines. Aryl-alkyl-lysines are compounds consisting of two hydrophobic groups (a lipid tail and

an aromatic head group) and two positive charges (contributed by L-Lysine).[19] They were earlier shown to be potent antibacterial agents both *in vitro* and *in vivo*.[17-18] Herein, we tested the activity of compounds from the NCK series, containing naphthalene rings, and the BCK series, containing benzene rings, against clinically-relevant pathogenic fungi. After identification of the most active compound (NCK-10), its antifungal mechanism of action was confirmed. Furthermore, the ability of the compound to inhibit *C. albicans* biofilms was also examined. Finally, we studied if NCK-10 was capable of passively permeating across the relatively impermeable blood-brain-barrier, using an *in vitro* model. The results garnered provide a valuable foundation for further investigation for potential clinical applications of NCK-10 as a novel antifungal agent.

RESULTS AND DISCUSSION

Synthesis: Aryl-alkyl-lysines were synthesized and characterized using a previously published protocol.[19] They were a robust ensemble of a naphthalene or a benzene moiety, a lipophilic chain and an L-Lysine moiety (Figure 1). Herein, we have studied the efficacy of representative compounds bearing the naphthalene ring (NCK series) or benzene ring (BCK series). In the NCK series, the length of the lipophilic tail was varied from hexyl to decyl group to yield compounds NCK-6, NCK-8 and NCK-10. In the BCK series, the lipid tail was varied from decyl to tetradecyl moiety to obtain compounds BCK-10, BCK-12 and BCK-14.

Antifungal activity:

The antifungal activity of the six compounds presented above were initially screened against two fungal strains, *C. albicans* SC5314 and *C. neoformans* H99, in YPD media. The MIC (minimum inhibitory concentration or the concentration at which the growth of the fungal cells are inhibited) and MFC (minimum fungicidal concentration or the concentration of the

compound at which fungal cells are killed) of the compounds is presented in Table 1. NCK-6 lacked antifungal activity against *C. albicans* SC5314. The MIC for NCK-8 was 25 μg mL^{-1} and correlated to the MFC, indicating this compound is fungicidal at this concentration. From the NCK series, NCK-10, was the most potent antifungal compound identified, as it inhibited growth of *C. albicans* SC5314 at a concentration of 12.5 μg mL^{-1}. In the BCK-series, BCK-12 showed potent activity inhibiting fungal growth at 25 μg mL^{-1} and demonstrating a fungicidal effect at 25 μg mL^{-1}. Although the decyl analogue BCK-10 was slightly active against *C. albicans* (MIC of 25 μg mL^{-1}), BCK-14 was inactive even at a concentration of 50 μg mL^{-1}. Against *C. neoformans* H99, however, all the compounds (except for NCK-6 and BCK-14) inhibited growth at 3.1 μg mL^{-1}. From this screen, NCK-10 emerged as the most active compound against both *C. albicans* and *C. neoformans*. Thus, we moved to further explore the spectrum of antifungal activity of NCK-10.

The susceptibility of cationic antimicrobial agents often vary in different media.[20] We have reported similar observations against bacteria previously.[21-22] In order to test if YPD media had an adverse effect with respect to susceptibility, we determined the MIC and MFC of NCK-10 in RPMI medium as well. Interestingly, a significant decrease in the MIC value was observed. The MIC of the compound against *C. albicans* SC5314 in RPMI media was found to be 1.56 μg mL^{-1}. Thus, we chose to carry out further susceptibility testing using RPMI medium. NCK-10 was subsequently tested against additional clinical fungal species including *C. albicans, C. parapbsilosis, C. glabrata, C. tropicalis, C. gattii, C. neoformans* and *A. fumigatus* (Table 2). Susceptibility of nine strains of *C. albicans* to NCK-10 was examined. Out of these nine strains, two strains (NR-29351 and NR-29365) were susceptible to fluconazole (MIC of 0.5 μg mL^{-1}). Against NR-29351, NCK-10 was active at 1 μg mL^{-1} while amphotericin B was active at 0.5 μg

mL^{-1}. Against NR-29365, however, NCK-10 (MIC 0.5 μg mL^{-1}) was four-fold more active than amphotericin B (MIC 2 μg mL^{-1}). Against the remaining seven strains of *C. albicans*, fluconazole was not active up to a concentration of 64 μg mL^{-1}. Against *C. albicans* NR-29448, NCK-10 matched the potency of amphotericin B, inhibiting fungal growth at a concentration of 0.5 μg mL^{-1}. Against *C. albicans* ATCC MYA A573, NCK-10 (MIC 0.5 μg mL^{-1}) was two-fold more active than amphotericin B. When examined against *C. albicans* NR-29438, NR-29366, NR-29367, ATCC 26790 and ATCC 64124, amphotericin B inhibited growth at 1 μg mL^{-1} which was similar to the MIC for NCK-10 (2 μg mL^{-1}).

Against *C. parapsilosis* ATCC 22019, NCK-10 had MIC and MFC values of 0.5 μg mL^{-1} and 1 μg mL^{-1} respectively, which is comparable to the control antifungals amphotericin B (MIC of 0.5 μg mL^{-1} and MFC of 2 μg mL^{-1}) and superior to fluconazole (MIC and MFC of 2 μg mL^{-1}). Against two strains of *C. glabrata* (ATCC MYA-2950 and ATCC 66032), which were resistant to fluconazole (MFC >64 μg mL^{-1}), NCK-10 was active at 1 μg mL^{-1} (MIC and MFC). These strains were susceptible to amphotericin B (MIC 0.5-1 μg mL^{-1}). We tested the activity of NCK-10 against two species of *C. tropicalis* ATCC 1369 and ATCC 13803. Against both strains, amphotericin was active at 0.5 μg mL^{-1}. NCK-10 was also active at 0.5 μg mL^{-1} against ATCC 1369 while fluconazole was active at 2 μg mL^{-1}. Against ATCC 13803, fluconazole was active at 0.5 μg mL^{-1} while NCK-10 was active at 1 μg mL^{-1}. Overall, the potency of NCK-10 against *Candida* species was similar to, and against several strains better than, the clinical antifungals amphotericin B and fluconazole.

Against two strains of *C. gattii* (NR-43208 and NR-43209), NCK-10 and amphotericin B exhibited identical MIC values of 1 μg mL^{-1}, whereas the MIC of fluconazole was 2 μg mL^{-1} and 8 μg mL^{-1} respectively. Against four strains of *C. neoformans* (NR-41292, NR-41294, NR-41295

and NR-41296), the MIC of fluconazole varied from 4 μg mL^{-1} to 16 μg mL^{-1}. The antifungal activity of NCK-10 against these same strains was superior (MIC ranged from 1 μg mL^{-1} to 2 μg mL^{-1}) and was comparable to the activity of amphotericin B (MIC ranged from 0.5 μg mL^{-1} to 1 μg mL^{-1}).

Similar to fungal pathogens like *C. neoformans*, *Aspergillus fumigatus* severely affects immunocompromised individuals including patients with leukemia, cystic fibrosis, human immunodeficiency virus, and patients undergoing organ and stem cell transplants; mortality rates associated with this pathogen are as high as 90%.[23] We tested NCK-10 against five strains of *A. fumigatus* (NR 35301, NR 35302, NR 35303, NR 35304 and NR 35308) which were all resistant to fluconazole (MIC >64) but susceptible to amphotericin B (MIC 0.5 μg mL^{-1} or less). Against all strains of *A. fumigatus* that were tested, NCK-10 was fungicidal at 4 μg mL^{-1}.

Fungicidal time-kill kinetics: Per the MFC data, NCK-10 appears to be a fungicidal agent against all fungal species tested. We moved to confirm this observation via a standard time-kill assay. The growth of *C. albicans* SC5314 in the presence of NCK-10 (at concentrations equivalent to the MIC and 4×MIC) was observed over a five-hour period. As depicted in Figure 2, NCK-10 was effective in diminishing the fungal count within the first hour. At the end of three hours >90% was observed. Although, a few colonies were observed thereafter, the ability of the compound to lyse fungal cells was evident.

Mechanism of action: As mentioned earlier, most antibiotics require very high concentrations to have sufficient activity against stationary phase bacteria.[24] NCK-10 when treated against *E. coli* cells in stationary phase, showed complete killing even at its MIC. While in the control, the bacterial cell count was maintained at 5 log CFU, no colonies were observed in NCK-10 treated cultures (the limit of detection in this experiment is 50 CFU mL^{-1}.

We previously confirmed NCK-10 exerted its antibacterial effect by infiltrating the cell membrane.[17-18] Thus, we postulated that NCK-10 would behave in a similar manner against fungi. To confirm this, the compound's ability to depolarize and permeabilize the fungal cell membrane of *C. albicans* (SC5314) was examined (Figure 3). The ability of NCK-10 to perturb the polarization across fungal membranes was studied using the membrane potential sensitive probe, $DiSC_3(5)$. Although the fluorescence of the dye is initially quenched upon being taken up by fungal cells, the fluorescence intensity of the dye is enhanced upon perturbation of the membrane potential. NCK-10 at concentrations equivalent to its MIC and 2 × MIC was able to depolarize the fungal membrane within one minute (Figure 3A), in a dose-dependent manner.

We then checked if the compound was able to compromise the membrane to permit influx of external substances. The external agent used was propidium iodide (PI), which cannot enter the cell unless the cell membrane is compromised. Upon binding to nucleic acids, there is an enhancement in the fluorescence signal emitted. Upon addition of the compound, PI found rapid entry inside the fungal cell as presented in Figure 3B. These results support our hypothesis that NCK-10 exerts its antifungal effect by rapidly permeabilizing the fungal cell membrane.

Microscopic observation of cell viability

In order to further confirm the proposed antifungal mechanism of action, NCK-10's ability to compromise the fungal cell membrane was observed microscopically. In this experiment, two dyes SYTO-9 and PI were used to differentiate between live and dead/membrane-compromised cells. SYTO-9 gains entry both in intact and membrane-compromised cells. However, as mentioned earlier, PI can only enter membrane-compromised cells. As can be seen from Figure 4, in the untreated control, only uptake of SYTO-9 by *C. albicans* SC5314 was observed, indicating that the cell membrane was not compromised. However, upon treatment of *C. albicans*

SC5314 cells with NCK-10 (at its MIC)), PI entered the cells easily. In the merged image, one can see the predominance of the red dye (PI) in comparison to the untreated control, further confirming the membrane-disrupting ability of NCK-10.

Anti-biofilm properties

The ability of the compound to both prevent and disrupt biofilms of *C. albicans* SC5314 was tested. To examine inhibition of biofilm formation, we incubated NCK-10 at different sub-MIC concentrations (ranging from MIC to MIC/32) and allowed the fungal biofilms to grow for three days. Subsequently, the metabolic activity of biofilm that was formed was quantified using the MTT assay. As presented in Figure 5A, with respect to the control, the compound was able to inhibit formation of biofilms in a concentration-dependent manner, At MIC/32, more than 30% inhibition of fungal biofilm formation was observed. At MIC/4 the compound produced an 80% inhibition of biofilm formation, relative to the untreated control. The effect was most pronounced at MIC/2 and MIC/4 concentrations.

We next investigated the efficacy of the compound to disrupt pre-formed fungal biofilm. *C. albicans* SC5314 biofilms were grown for 48 hours before subjecting them to compound treatment. The concentration of the compound tested ranged from MIC/4 to 4 × MIC. The metabolic activity of the biofilm was significantly diminished by 25% even at MIC/2. No antibiofilm activity was observed for NCK-10 at MIC/4 (Figure 5B). At concentrations equal to the MIC and beyond, more than 80% reduction in the metabolic activity of cells in the biofilm was observed, relative to the untreated control.

Cytotoxicity

In order to justify the selectivity of the compounds towards fungal cells in comparison to mammalian cells, we conducted the cytotoxicity of the most active compound, NCK-10 against

human colorectal cells (HRT-18). Till 20 μg mL^{-1} the compound was not at all toxic to the cells (Figure 6). This represents a 40-fold difference between the MIC values obtained against some *Candida albicans* strains for the compound under same conditions. The assay showed that the effective concentration at which 50 % of the cells were killed by the compound was 42 μg mL^{-1}. This experiment proved that the compound was selectively toxic toward fungal cells over mammalian cells.

Parallel artificial membrane permeability assay (PAMPA) for ability to cross the blood brain barrier (BBB)

Certain fungal infections, particularly Cryptococcal meningitis and Rhinocerebral mucormycosis, necessitate that a viable therapeutic agent be capable of crossing the BBB in order to reach the target site of infection. Thus we evaluated the ability of compounds NCK-10 to penetrate the BBB utilizing PAMPA-BBB (Table 3). Although the value obtained for NCK-10 was not as high as that of the highly permeable drug verapamil, it was found to be superior to the low permeability control drug, atenolol, indicating NCK-10 is capable of passively traversing across the barrier imposed by the blood brain barrier. Traditionally, compounds possessing a mean permeability coefficient less than 2×10^{-6} cm sec^{-1} will experience difficulty in effectively crossing the BBB. As NCK-10, possesses a mean permeability coefficient (Table 3) of 2.87×10^{-6} cm sec^{-1}, this indicates that NCK-10 would be a good candidate for investigation of treatment of fungal infections impacting the brain (namely meningitis).

DISCUSSION

Pathogenic fungi are notorious for causing both superficial and life-threatening infections both in healthy and immunocompromised individuals.[25] More than 300 million individuals worldwide are afflicted with a serious fungal infection. Although infections caused by bacteria, viruses and

parasites are often highlighted, infections caused by pathogenic fungi have largely remained out of limelight. The emergence of resistance to front-line antifungal drugs (including fluconazole) has further exacerbated the issue. This necessitates the identification and development of new antifungal agents to address this significant public health challenge. Membrane-active agents have shown much promise as broad-spectrum antimicrobial agents.[26] However, few synthetic membrane-active agents have been investigated against pathogenic fungi. Aryl-alkyl-lysines have been shown to be safe and effective antibacterial compounds with potent activity in murine models of infection.[17-18] Given their membrane-active nature we surmised that aryl-alky-lysines would be potent antifungal agents capable of treating fungal infections as well.

Initially we screened six compounds from our library against the most common fungi encountered in clinical infections, *C. albicans* and *C. neoformans*. Though most of the compounds possessed antifungal activity, NCK-10 was found to be the most active compound (similar to previous studies involving pathogenic bacteria). We moved to conduct a more robust analysis by examining the antifungal activity of NCK-10 against a panel of fungal clinical isolates that encompasses most of the pathogenic fungi encountered in infections in hospitals. *Candida parapsilosis* has been gaining notoriety as an invasive fungus responsible for the most infections caused by *Candida* spp. after *C. albicans*.[27] Furthermore, excessive use of immunosuppressive agents and broad-spectrum antimycotic agents has led to emergence of pathogenic strains of *C. glabrata*, especially for patients suffering from HIV and diabetes.[28] A major problem with this microorganism is that some of the strains are inherently resistant to fluconazole therapy. *Candida tropicalis* is another major problem in India, wherein it is the most dominant contributor to candidiasis caused by Candida-non-albicans (CAN) species. Cryptococcosis and cryptococcal meningitis are potentially fatal diseases caused by

Cryptococcus spp. Although *Cryptococcus neoformans* mostly affects immunocompromised individuals (particularly patients infected with HIV), *Cryptococcus gattii* has been reported to cause illness in immunocompetent individuals.[29] Drugs against such pathogens are urgently needed, particularly in Sub-Saharan Africa where the largest population of AIDS patients resides.

Notably, several strains were resistant to antifungal drugs including fluconazole. It is evident from MIC and MFC values disclosed in Tables 2 that the efficacy of the compound was comparable to that of the marketed drugs amphotericin B and fluconazole against *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. neoformans*, *C. gatti*, and *A. fumigatus*. Against certain isolates, NCK-10 was more active than both fluconazole and amphotericin B.

Previously we demonstrated in bacteria that NCK-10 exerts its antibacterial effect by targeting the cell membrane. In the present study, we confirmed that the antifungal mechanism of action of NCK-10 is similar, as the fluorescence intensity of the membrane impermeable dye PI significantly increased in the presence of *C. albicans* cells treated with NCK-10, within one minute. This observation was confirmed visually using fluorescence microscopy as a significant increase of PI was observed inside *C. albicans* cells exposed to NCK-10, in contrast to untreated cells. Our preliminary investigation into the antifungal mechanism of action of NCK-10, however does not preclude the possibility that NCK-10 also exerts its effect by targeting other cellular processes. Indeed, a chemical proteomics study with the aryl-alkyl-lysine compounds may indeed shed light into their ability to bind to important fungal proteins. A detailed investigation in relation to the mechanism of action of these compounds against fungi is currently underway. It is also clear though, that the rapid fungicidal action of the compounds can be attributed to its membrane-active nature. NCK-10 was shown to be not toxic to mice at

therapeutically relevant concentrations and herein we have shown that there is substantial selectivity against fungal cells over HRT cells (Figure 6). [17-19] This selectivity also emphasizes on the potential of the compound as a template for design of future drugs.

PAMPA is a valuable tool in early-stage drug discovery to evaluate the ability of compounds to passively permeate across biological membranes (such as the BBB). The PAMPA-BBB model mimics the rigid, hydrophobic nature of the BBB membrane and has been shown to correlate well with the more laborious *in-situ* brain perfusion assay. [30-32] One of the most interesting characteristics discovered in this study is NCK-10 appears to be capable of passively permeating across the BBB. Though further *in vivo* studies need to be conducted, this observation potentially opens the door to use NCK-10 for treating both fungal and bacterial infections impacting the brain (such as meningitis). One caveat of the PAMPA-BBB method is it does not account for potential interaction of compounds with membrane proteins that help transport drugs across the BBB. Thus, further investigation to evaluate the impact of membrane transporters to enhance permeation of NCK-10 across the BBB needs to be conducted.

In addition to the challenge posed by free-floating, planktonic fungal cells, fungal biofilms pose a significant additional concern. These biofilms are capable of forming on medical devices (such as indwelling catheters) leading to recurring infections that are challenging to treat.[33] Understandably, several recent studies have focused on identifying agents that are effective at attacking biofilms of pathogenic fungi.[7-8, 34-35] The present study revealed that our compound, NCK-10, was capable of both inhibiting biofilm formation (at subinhibitory concentrations) and reducing the metabolic activity of fungal cells encased in a biofilm. Although the ability of NCK-10 to inhibit and reduce the metabolic activity of pre-formed biofilms was investigated against *C. albicans* only, the antibiofilm activity of NCK-10 has

potential to be extended to other pathogenic fungi as well. This is indeed an important contribution to the field, as it is imperative to have drugs which can act against both planktonic cells and biofilms of pathogenic fungi.

CONCLUSION

Overall, present study demonstrates the promise of aryl-alkyl-lysines, specifically NCK-10, as prospective antifungal agents. NCK-10 possesses excellent efficacy in vitro against various species of clinically-relevant fungi and is also capable of interfering with biofilms formed by C. albicans. The compound exerts its antifungal effect by targeting the cell membrane of fungi, inducing rapid lysis. The next step towards validating the therapeutic potential of these compounds as antifungal agents is testing their efficacy in suitable animal models of infection. Given fungi are responsible for various infections of the body, it will be worthwhile to investigate whether NCK-10 is able to treat invasive fungal infections (e.g. candidiasis), brain infections (caused by Cryptococcal meningitis), skin infections (onchomycosis) and pulmonary infections. At present, NCK-10 represents a good lead for design of a new generation of antifungal agents.

MATERIALS AND METHODS

Materials and Media:

Yeast extract peptone dextrose (YPD) was obtained from HIMEDIA, India. RPMI purchased from GIBCO, Amphotericin B, fluconazole, atenolol, verapamil, HEPES, DiSC3 (5), MTT and Propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO). SYTO 9 dye was purchased from Invitrogen. For optical density (OD) and fluorescence measurement, Tecan Infinite Pro series M200 Microplate Reader was employed. An Olympus microscope (Model BX51) and Olympus DP71 camera were used for the fungal imaging.

Synthesis

The synthesis of the compounds was performed using a previously published protocol.[19] Briefly, in the first step, aromatic aldehydes (1 eq) and alkyl amines (1 eq) were reacted in a 1:1 mixture of dry chloroform and methanol for 6 h. Following this, reductive amination was achieved by adding Sodium borohydride (1.8 eq) to it in ice cold condition. Upon completion of the reaction the solvents were evaporated under reduced pressure (not to dryness) and diluted with diethyl ether. Subsequently, 2N NaOH was added to the mixture and stirred for 15 minutes. The organic layer was separated, washed with water, brine and dried over $MgSO_4$. To this 4N HCl was added to obtain precipitates was observed. The solvent was completely removed and the precipitate was dissolved in minimum volume of ethyl acetate (a few drops of methanol was added to dissolve completely). To this hexane was added to obtain pure crystals of the target compound.

In the second step, amide coupling reaction was performed using HBTU. Briefly, to Boc-Lys(Boc)-OH (1.2 eq) in 5:2 DMF/$CHCl_3$, *N,N*-Diisopropylethylamine (DIPEA, 2.5 eq) and HBTU (1 eq) was added at 0°C. Subsequently the secondary amines (obtained from the first step) were added (1 eq). The mixture was stirred at 0°C for 30 minutes and subsequently at RT for 24 h typically. Upon completion of the reaction organic solvents were evaporated under reduced pressure and the contents were dissolved in ethyl acetate. This was then washed with 0.5 M $KHSO_4$, H_2O (thrice) and brine. After passage through anhydrous Na_2SO_4, the organic layer was evaporated under reduced pressure and the residue was purified using column chromatography (only $CHCl_3$) to obtain the product.

In the final step compounds obtained in the second step were dissolved in DCM and were deprotected by stirring with CF_3COOH (50% by volume) at RT. All the volatile components

were removed, and the product is purified by reverse phase HPLC using 0.1% Trifluoroacetic acid (TFA) in water/acetonitrile (0-100%) as mobile phase to more than 95% purity. C_{18} column (10mm diameter, 250 mm length) and UV detector (at 270 nm wavelength) were used. Subequently, the compounds were dried and characterized by ^{1}H NMR, ^{13}C NMR, IR and mass spectrometry.

Initial fungal susceptibility assay

The antifungal activity of the compounds was initially tested against *Candida albicans* SC5314 and *Cryptococcus neoformans* H99 using a previously described 96-well plate assay, with few modifications.[16] Briefly, the fungal strains were grown overnight in 5 mL YPD (1% yeast extract, 2% peptone and 2% dextrose). Fungal growth was measured via a spectrophotometer (OD_{600}) and cells were diluted in fresh media (either in YPD or RPMI) to get the required concentration of 1.3×10^5 cells mL^{-1}. An aliquot (150 µL) of the culture dilution (equivalent to 2×10^4 cells) was added to wells containing 50 µL of water or the compounds, at different concentrations. The plates were incubated at 30 °C for 20 hours with shaking (180 rpm) to allow sufficient growth of fungal cells. The growth of the fungi was measured (OD_{600}) using media alone (YPD/RPMI) as a blank. Simultaneously, 3 µL of the culture from each well was taken and spotted onto a YPD agar plate to determine the minimum fungicidal concentration (MFC). The concentration where the optical density of the test well was close to the value of the blank well value was categorized as the minimum inhibitory concentration (MIC).

Antifungal activity against clinical isolates

The minimum inhibitory concentration (MIC) of the most potent compound, NCK-10, was determined against several clinical strains of *Candida species*, *Cryptococcus species* and one

strain of *Aspergillus fumigatus*. *C. albicans*, *Cryptococcus spp.*, *A. fumigatus* (tested from 64 μg mL^{-1} down to 0.5 μg mL^{-1}) were transferred to a 96-well plate and incubated at 37 ºC for 48 hours (or 72 hours for *C. neoformans*) .The MICs reported represent the lowest concentration of each compound necessary to inhibit fungal growth, by visual inspection. For determination of the minimum fungicidal concentration, aliquots (5 μL) were transferred from wells with no growth onto YPD agar plates. Plates were incubated at 37 ºC for 48 hours before MFC results were recorded. Amphotericin B and fluconazole were used as control antifungals.

Kinetics study

In order to confirm if NCK-10 was fungistatic or fungicidal, a time-kill experiment was conducted. Fungal cells were grown overnight and added to a 96-well plate as described above. Starting at the initial time point, a small aliquot (3 μL) of cells was taken and spotted onto a YPD plate every hour (for five hours). The plates were then incubated at 30 °C for 24 hours to score for viable cells.

Mechanism of action

Depolarization of fungal cell membrane

Briefly, 10^7 CFU ml^{-1} of *C. albicans* SC5314 was washed and resuspended in 5 mM glucose, 5 mM HEPES buffer and 100 mM KCl solution in a 1:1:1 ratio. To this solution $DiSC_3$ (5) dye (obtained from Sigma-Aldrich) was added to a final concentration of 2 μM. The fungal suspension containing the dye (200 μL) was preincubated for 20 min in a black 96-well plate with a transparent bottom. The fluorescence of the fungal suspension was measured (excitation wavelength: 622 nm; emission wavelength: 670 nm) and allowed to stabilize at room temperature before the addition of 2 μL of NCK-10 (final concentration was equivalent to the

MIC (12.5 μg mL^{-1}) or 2 × MIC (25 μg mL^{-1})). After addition, the fluorescence intensity was measured every minute for 20 minutes.

Permeabilization of fungal cell membrane

Propidium iodide (PI) dye (15 μM, final concentration) (obtained from Sigma-Aldrich) was added to a fungal suspension containing *C. albicans* SC5314 (~10^7 cells mL^{-1}) in 5 mM HEPES and 5 mM glucose (pH 7.4). The suspension containing the dye (200 μL) was then added to the well of a 96-well, clear-bottom black plate. After four minutes, NCK-10 (2 μL) was added to the solution to a final concentration equivalent to the MIC (12.5 μg mL^{-1}) or 2 × MIC (25 μg mL^{-1}). The fluorescence intensity was measured at excitation wavelength of 535 nm (slit width: 10 nm) and emission wavelength of 617 nm (slit width: 5 nm). The uptake of PI was detected by the increase in fluorescence for 10 minutes; this correlates to permeabilization of the inner cell membrane.

Microscopy assay for viability

C. albicans SC5314 was grown in a 96-well plate for six hours in the presence or absence of NCK-10 (12.5 μg mL^{-1}). The cells were harvested, washed once with sterile water, and resuspended in 50 μL of sterile water. The cell suspension was incubated with propidium iodide (PI, 15 μM final concentration) and SYTO9 (2 μM final concentration) at room temperature for 30 minutes. The cells were then observed under a fluorescence microscope. A bright-field filter was used for differential interference conference (DIC) images whereas green and red emission filters were used to capture SYTO 9 and PI fluorescence, respectively. The images were captured using an Olympus microscope (Model BX51) and an Olympus DP71 camera. The images were further processed using Image Pro-Plus software and ImageJ.

Inhibition of *C. albicans* biofilm formation

Candida albicans SC5314 cells were grown overnight in YPD, pelleted and washed with 1 × phosphate buffered saline (PBS). Washed cells were resuspended in RPMI media to reach a final concentration of 6.7×10^5 cells mL^{-1}. Compound NCK-10 (50 µL; corresponding to its MIC value of 12.5 µg mL^{-1} and further dilutions) and 150 µL of cell suspension were mixed together and added into wells of a 96-well plate. Wells lacking either compound or cell suspension were utilized as positive and negative controls. The plates were incubated at 37 °C for 72 hours in stationary condition. Inhibition of biofilm was then quantified using the MTT assay, as follows. The wells were washed twice with 1 × PBS. MTT solution (100 µL of 1 mg mL^{-1} in 1× PBS, main stock concentration 5 mg mL^{-1}) was subsequently added to wells and incubated at 37 °C for four hours. The MTT solution was discarded and wells were washed once with 1 × PBS. DMSO (100 µL) was added into wells and further incubated at 37 °C in the dark for an hour. The solution was then transferred to a fresh plate and the optical density was measured at 590 nm to quantify the extent of biofilm inhibition compared to untreated wells.

Disruption of pre-formed *C. albicans* biofilms

Candida albicans SC5314 cells were grown overnight in YPD, pelleted and washed with 1 × PBS. Washed cells were resuspended in RPMI medium to a final concentration of 1×10^6 cells mL^{-1}. An aliquot of cell suspension (100 µL) was added into wells and incubated at 37 °C for 24 hours. The non-adhered cells were removed from the wells and 50 µL of fresh RPMI medium along with 50 µL of varying concentrations of the compound (MIC equals to 12.5 µg mL^{-1}) were added into the cells. As a control, 100 µL of RPMI media alone was added. These plates were further incubated at 37 °C for 48 hours. Inhibition or disruption of biofilm was then quantified using the MTT assay, as described above.

Compound NCK-10 were assayed (at different concentrations) against a human colorectal (HRT-18) cell line to determine the potential toxic effect to mammalian cells *in vitro*. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal horse serum at 37 °C with CO_2 (5%). Control cells received DMSO alone at a concentration equal to that in drug-treated cell samples. The cells were incubated with the compound (in triplicate) in a 96-well plate at 37 ºC with CO_2 (5%) for two hours. The assay reagent MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) (Promega, Madison, WI, USA) was subsequently added and the plate was incubated for four hours. Absorbance readings (at OD_{490}) were taken using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA). The quantity of viable cells after treatment with the compounds was expressed as a percentage of the viability of DMSO-treated control cells (average of triplicate wells ± standard deviation). The toxicity data was analyzed via a two-way ANOVA, with post hoc Dunnet's multiple comparisons test ($P < 0.05$), utilizing GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

Parallel artificial membrane permeability assay (PAMPA) for examining ability to cross the blood brain barrier (BBB)

NCK-10 and control drugs (atenolol and verapamil) were dissolved in transport buffer (pH 7.4) to a final concentration of 10 μM. The filter membrane was coated with 4 μL of a 20 mg mL^{-1} porcine brain lipid in dodecane. An aliquot (300 μL) of the compound solution was added to the donor well. The acceptor well was filled with 200 μL of transport buffer (Dulbecco's Phosphate Buffered Saline). The acceptor filter plate was carefully placed on to the donor plate and was left undisturbed for 18 hours. Samples of the donor and acceptor wells were analyzed, in quadruplicate, by LC-MS/MS and the effective permeability (P_e) was calculated as follows:

$$log\ P_e = log\left\{-\frac{V_D V_A}{(V_D + V_A)At} ln\left(1 - \frac{[drug]_A}{[drug]_E}\right)\right\}$$

Where P_e is the permeability, V_D and V_A are the volumes of the donor and acceptor compartments, A is the area of the membrane, t is the incubation time, and A and E subscripts on the drug concentration terms refer to the acceptor and equilibrium concentrations, respectively.

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Notes

The authors declare no competing financial interest. JNCASR has filed a patent application based on the work described in the manuscript.

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REFERENCES

1) Brown, G. D.; Denning, D. W.; Gow, N. A. R.; Levitz, S. M.; Netea, M. G.; White, T. C., Hidden killers: Human fungal infections. *Sci Transl Med* **2012,** *4* (165), 165rv13.
2) Ghannoum, M. A.; Rice, L. B., Antifungal agents: Mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin. Microbiol. Rev.* **1999,** *12* (4), 501-17.
3) Sheehan, D. J.; Hitchcock, C. A.; Sibley, C. M., Current and emerging azole antifungal agents. *Clin. Microbiol. Rev.* **1999,** *12* (1), 40-79.
4) Denning, D. W., Echinocandins: a new class of antifungal. *J. Antimicrob. Chemother.* **2002,** *49* (6), 889-891.

5) Kanafani, Z. A.; Perfect, J. R., Resistance to antifungal agents: Mechanisms and clinical impact. *Clin. Infect. Dis.* **2008,** *46* (1), 120-128.
6) Ramage, G.; Rajendran, R.; Sherry, L.; Williams, C., Fungal biofilm resistance. *Int. J. Microbiol.* **2012,** *2012*, 528521.
7) Liu, R. H.; Chen, X. Y.; Falk, S. P.; Masters, K. S.; Weisblum, B.; Gellman, S. H., Nylon-3 polymers active against drug-resistant *Candida albicans* biofilms. *J. Am. Chem. Soc.* **2015,** *137* (6), 2183-2186.
8) Karlsson, A. J.; Pomerantz, W. C.; Neilsen, K. J.; Gellman, S. H.; Palecek, S. P., Effect of sequence and structural properties on 14-helical beta-peptide activity against *Candida albicans* planktonic cells and biofilms. *ACS Chem Biol* **2009,** *4* (7), 567-579.
9) Hancock, R. E. W.; Sahl, H. G., Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **2006,** *24* (12), 1551-1557.
10) Zasloff, M., Antimicrobial peptides of multicellular organisms. *Nature* **2002,** *415* (6870), 389-395.
11) De Lucca, A. J.; Walsh, T. J., Antifungal peptides: novel therapeutic compounds against emerging pathogens. *Antimicrob. Agents Chemother.* **1999,** *43* (1), 1-11.
12) Larsen, C. E.; Larsen, C. J.; Franzyk, H.; Regenberg, B., Antifungal properties of peptidomimetics with an arginine-[beta-(2,5,7-tri-tert-butylindol-3-yl)alanine]-arginine motif against *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*. *FEMS Yeast Res* **2015,** *15* (3), fov011.
13) Bojsen, R.; Torbensen, R.; Larsen, C. E.; Folkesson, A.; Regenberg, B., The synthetic amphipathic peptidomimetic LTX109 is a potent fungicide that disturbs plasma membrane integrity in a sphingolipid dependent manner. *PLoS One* **2013,** *8* (7), e69483.
14) Uppu, D. S.; Akkapeddi, P.; Manjunath, G. B.; Yarlagadda, V.; Hoque, J.; Haldar, J., Polymers with tunable side-chain amphiphilicity as non-hemolytic antibacterial agents. *Chem. Commun.* **2013,** *49* (82), 9389-9391.
15) Hoque, J.; Konai, M. M.; Samaddar, S.; Gonuguntala, S.; Manjunath, G. B.; Ghosh, C.; Haldar, J., Selective and broad spectrum amphiphilic small molecules to combat bacterial resistance and eradicate biofilms. *Chem. Commun.* **2015,** *51* (71), 13670-13673.
16) Hoque, J.; Akkapeddi, P.; Yadav, V.; Manjunath, G. B.; Uppu, D. S.; Konai, M. M.; Yarlagadda, V.; Sanyal, K.; Haldar, J., Broad spectrum antibacterial and antifungal polymeric paint materials: synthesis, structure-activity relationship, and membrane-active mode of action. *ACS Appl Mater Interfaces* **2015,** *7* (3), 1804-1815.
17) Ghosh, C.; Manjunath, G. B.; Konai, M. M.; Uppu, D. S. S. M.; Paramanandham, K.; Shome, B. R.; Ravikumar, R.; Haldar, J., Aryl-alkyl-lysines: Membrane-active small molecules active against murine model of burn infection. *ACS Infectious Diseases* **2016,** *2* (2), 111-122.
18) Ghosh, C.; Manjunath, G. B.; Konai, M. M.; Uppu, D. S.; Hoque, J.; Paramanandham, K.; Shome, B. R.; Haldar, J., Aryl-Alkyl-Lysines: Agents that kill planktonic cells, persister cells, biofilms of MRSA and protect mice from skin-infection. *PLoS One* **2015,** *10* (12), e0144094.
19) Ghosh, C.; Manjunath, G. B.; Akkapeddi, P.; Yarlagadda, V.; Hoque, J.; Uppu, D. S.; Konai, M. M.; Haldar, J., Small molecular antibacterial peptoid mimics: the simpler the better! *J. Med. Chem.* **2014,** *57* (4), 1428-1436.
20) Choi, H.; Chakraborty, S.; Liu, R.; Gellman, S. H.; Weisshaar, J. C., Medium effects on minimum inhibitory concentrations of nylon-3 polymers against *E. coli*. *PLoS One* **2014,** *9* (8), e104500.

21) Hoque, J.; Konai, M. M.; Gonuguntla, S.; Manjunath, G. B.; Samaddar, S.; Yarlagadda, V.; Haldar, J., Membrane active small molecules show selective broad spectrum antibacterial activity with no detectable resistance and eradicate biofilms. *J. Med. Chem.* **2015,** *58* (14), 5486-5500.
22) Uppu, D. S.; Samaddar, S.; Ghosh, C.; Paramanandham, K.; Shome, B. R.; Haldar, J., Amide side chain amphiphilic polymers disrupt surface established bacterial bio-films and protect mice from chronic *Acinetobacter baumannii* infection. *Biomaterials* **2016,** *74*, 131-143.
23) Latge, J. P., *Aspergillus fumigatus* and aspergillosis. *Clin. Microbiol. Rev.* **1999,** *12* (2), 310-350.
24) Mascio, C. T.; Alder, J. D.; Silverman, J. A., Bactericidal action of daptomycin against stationary-phase and nondividing *Staphylococcus aureus* cells. *Antimicrob. Agents Chemother.* **2007,** *51* (12), 4255-4260.
25) Roemer, T.; Krysan, D. J., Antifungal drug development: challenges, unmet clinical needs, and new approaches. *CSH Perspect. Med.* **2014,** *4* (5), a019703
26) Ghosh, C.; Haldar, J., Membrane-active small molecules: Designs inspired by antimicrobial peptides. *ChemMedChem* **2015,** *10* (10), 1606-1624.
27) Trofa, D.; Gacser, A.; Nosanchuk, J. D., *Candida parapsilosis*, an emerging fungal pathogen. *Clin. Microbiol. Rev.* **2008,** *21* (4), 606-625.
28) Fidel, P. L.; Vazquez, J. A.; Sobel, J. D., *Candida glabrata*: Review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. *Clin. Microbiol. Rev.* **1999,** *12* (1), 80-96.
29) Kwon-Chung, K. J.; Fraser, J. A.; Doering, T. L.; Wang, Z. A.; Janbon, G.; Idnurm, A.; Bahn, Y. S., *Cryptococcus neoformans* and *Cryptococcus gattii*, the etiologic agents of cryptococcosis. *CSH Perspect. Med.* **2014,** *4* (7), a019760.
30) Wang, Q.; Rager, J. D.; Weinstein, K.; Kardos, P. S.; Dobson, G. L.; Li, J.; Hidalgo, I. J., Evaluation of the MDR-MDCK cell line as a permeability screen for the blood-brain barrier. *Int. J. Pharm.* **2005,** *288* (2), 349-359.
31) Tsinman, O.; Tsinman, K.; Sun, N.; Avdeef, A., Physicochemical selectivity of the BBB microenvironment governing passive diffusion--matching with a porcine brain lipid extract artificial membrane permeability model. *Pharm. Res.* **2011,** *28* (2), 337-363.
32) Di, L.; Kerns, E. H.; Bezar, I. F.; Petusky, S. L.; Huang, Y., Comparison of blood-brain barrier permeability assays: in situ brain perfusion, MDR1-MDCKII and PAMPA-BBB. *J. Pharm. Sci.* **2009,** *98* (6), 1980-1991.
33) http://wwwnc.cdc.gov/eid/article/10/1/03-0119_article
34) You, J.; Du, L.; King, J. B.; Hall, B. E.; Cichewicz, R. H., Small-molecule suppressors of *Candida albicans* biofilm formation synergistically enhance the antifungal activity of amphotericin B against clinical *Candida* isolates. *ACS Chem. Biol.* **2013,** *8* (4), 840-848.
35) Wang, S.; Wang, Y.; Liu, W.; Liu, N.; Zhang, Y.; Dong, G.; Liu, Y.; Li, Z.; He, X.; Miao, Z.; Yao, J.; Li, J.; Zhang, W.; Sheng, C., Novel carboline derivatives as potent antifungal lead compounds: design, synthesis, and biological evaluation. *ACS Med. Chem. Lett.* **2014,** *5* (5), 506-511.

Table 1. Antifungal activity of BCK and NCK compounds against *Candida albicans* and *Cryptococcus neoformans*.

Compounds	*C. albicans* SC5314		*C. neoformans* H99	
	MIC ($\mu g\ mL^{-1}$)	MFC ($\mu g\ mL^{-1}$)	MIC ($\mu g\ mL^{-1}$)	MFC ($\mu g\ mL^{-1}$)
NCK-6	>50	>50	25	25
NCK-8	25	25	3.1	3.1
NCK-10	12.5	25	3.1	3.1
BCK-10	25	50	3.1	3.1
BCK-12	25	25	3.1	3.1
BCK-14	>50	>50	6.2	6.2

Results are an average of at least two independent experiments and each experiment was performed in triplicates.

Table 2. Activity of NCK-10 against clinical isolates of pathogenic fungi in comparison to amphotericin B and fluconazole.-

Fungal strains	NCK-10		Amphotericin B		Fluconazole	
	MIC ($\mu g\ mL^{-1}$)	MFC ($\mu g\ mL^{-1}$)	MIC ($\mu g\ mL^{-1}$)	MFC ($\mu g\ mL^{-1}$)	MIC ($\mu g\ mL^{-1}$)	MFC ($\mu g\ mL^{-1}$)
Candida albicans NR-29448	0.5	1	0.5	1	>64	ND*
Candida albicans NR-29351	1	2	0.5	0.5	0.5	8
Candida albicans NR29438	2	2	1	2	>64	ND
Candida albicans NR-29366	2	2	1	1	>64	ND
Candida albicans NR-29367	2	2	1	2	>64	ND
Candida albicans ATCC *26790*	2	2	1	1	>64	ND
Candida albicans NR-29365	0.5	0.5	2	2	0.5	>64
Candida albicans ATCC MYA573	0.5	0.5	1	1	>64	ND
Candida albicans ATCC 64124	2	2	1	1	>64	ND
Candida parapbsilosis ATCC22019	0.5	1	0.5	2	2	2
Candida glabrata ATCC MYA-2950	1	1	1	1	32	>64

Candida glabrata ATCC 66032	1	1	0.5	2	16	>64
Candida tropicalis ATCC 1369	0.5	0.5	0.5	0.5	2	>64
Candida tropicalis ATCC 13803	1	1	0.5	2	0.5	>64
Cryptococcus gattii NR-43208	1	1	1	2	2	2
Cryptococcus gattii NR-43209	1	1	1	4	8	8
Cryptococcus neoformans NR41294	2	2	1	1	4	8
Cryptococcus neoformans NR-41296	2	2	1	1	4	8
Cryptococcus neoformans NR-41295	2	2	0.5	0.5	16	32
Cryptococcus neoformans NR-41292	1	1	1	16	8	8
Aspergillus fumigatus NR-35308	4	4	0.25	0.025	>64	ND
Aspergillus fumigatus NR-35304	4	4	≤0.0625	0.25	>64	ND
Aspergillus fumigatus NR-35303	4	4	≤0.0625	0.125	>64	ND
Aspergillus fumigatus NR-35301	4	4	0.25	0.25	>64	ND
Aspergillus fumigatus NR-35302	2	2	0.5	0.5	>64	ND*

*ND stands for not detectable

Table 3. Effective permeability coefficient of NCK-10 and control drugs obtained from the PAMPA-BBB assay.

Test Agent	Mean P_e (cm/sec)	Inference
NCK-10	2.87×10^{-6}	Good permeability
Atenolol	0.000013×10^{-6}	Low permeability control
Verapamil	14.6×10^{-6}	High permeability control

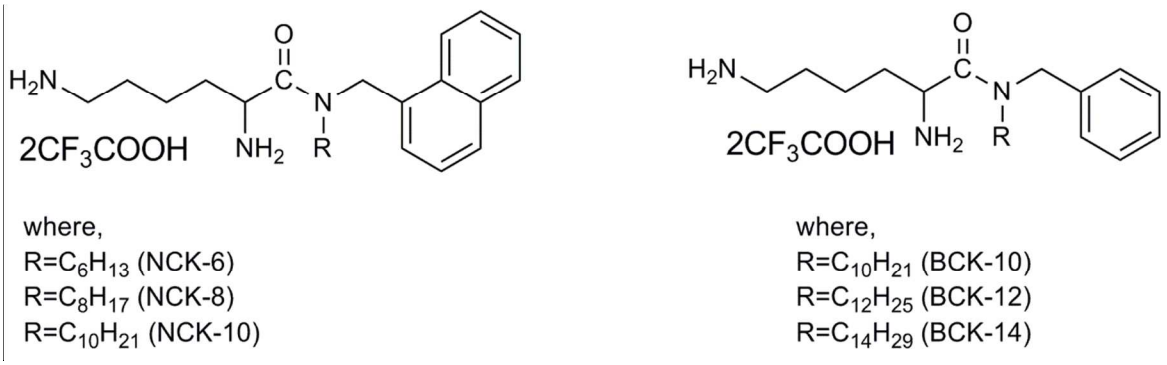

Figure 1. Chemical structures of compounds presented in this study.

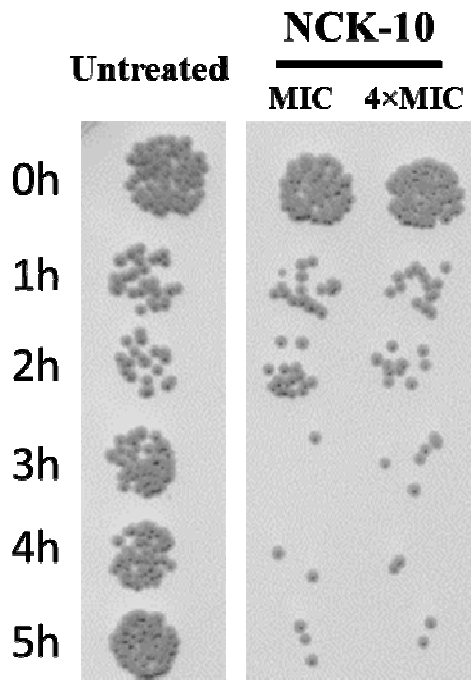

Figure 2. Kinetics of fungicidal action of NCK-10.

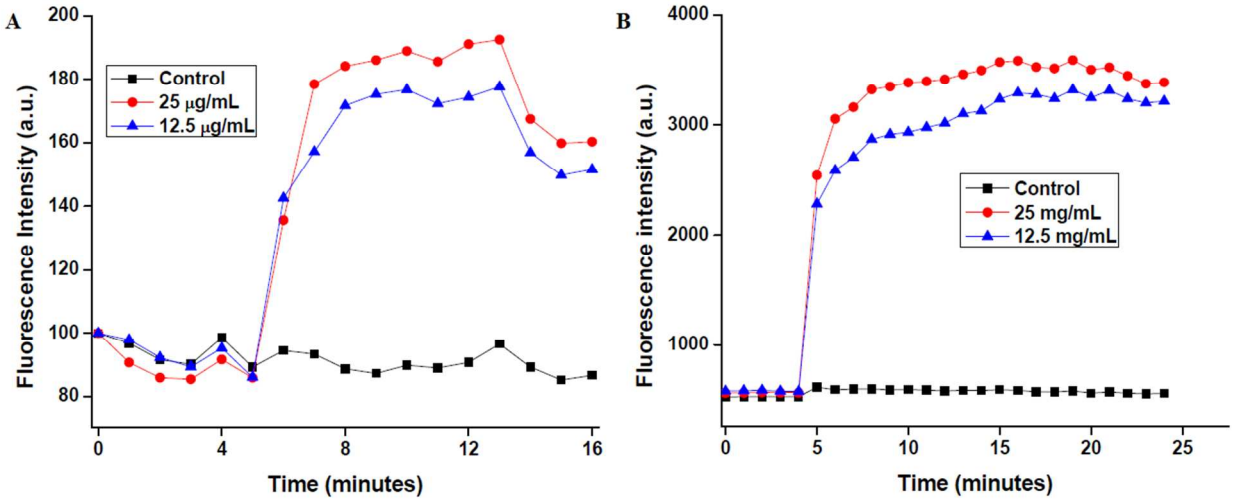

Figure 3. Ability of NCK-10 to infiltrate the cell membrane of *C. albicans* A) Depolarization of fungal cell membrane B) Permeabilization of the cell membrane. (a.u. stands for arbitrary units)

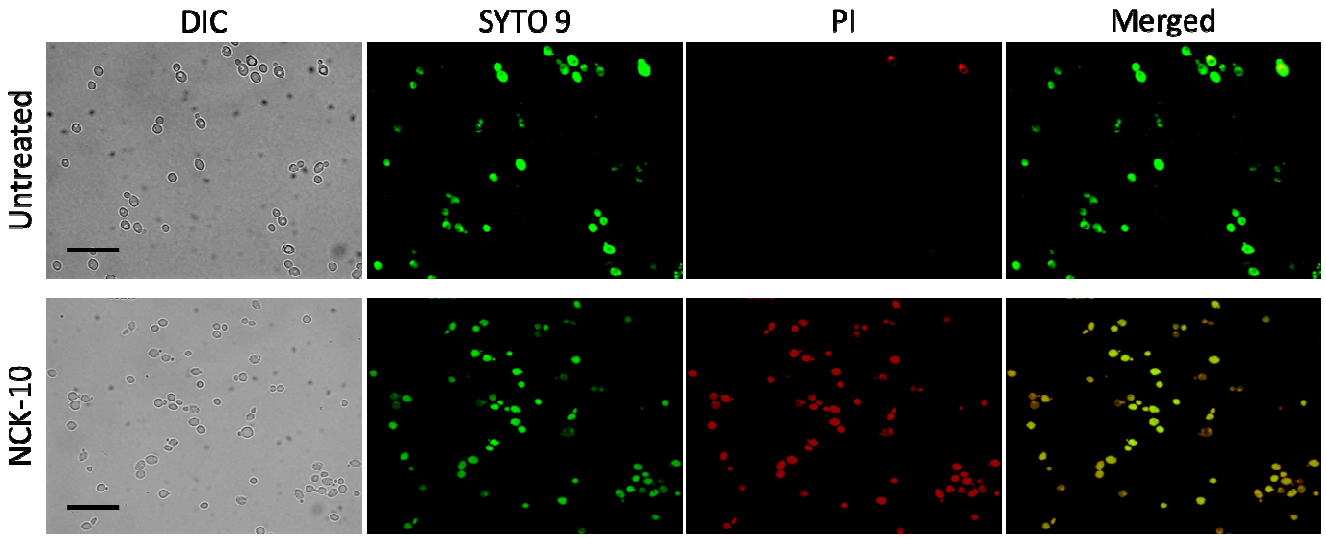

Figure 4. Fluorescence microscopy images of *C. albicans* after staining with SYTO 9 and PI in absence (control) or presence of NCK-10 (2 × MIC). Scale bar, 5 μm

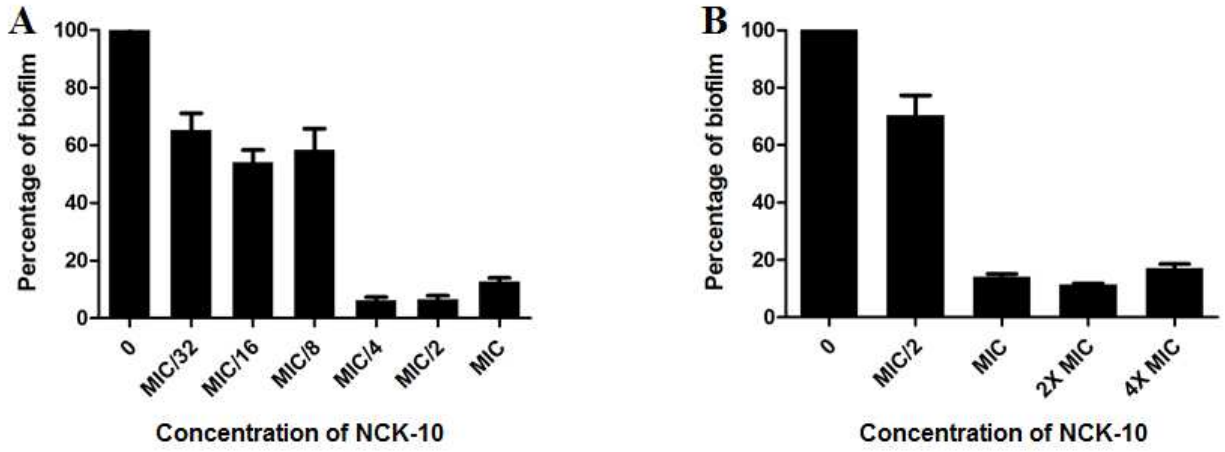

Figure 5. Ability of the compound NCK-10 to A) inhibit the formation of *C. albicans* biofilm and B) Disruption of pre-formed *C. albicans* biofilm; MIC equal to 12.5 μg mL^{-1}. The experiment was repeated at least twice with three independent replicates in each experiment. Error bars represent standard error of mean.

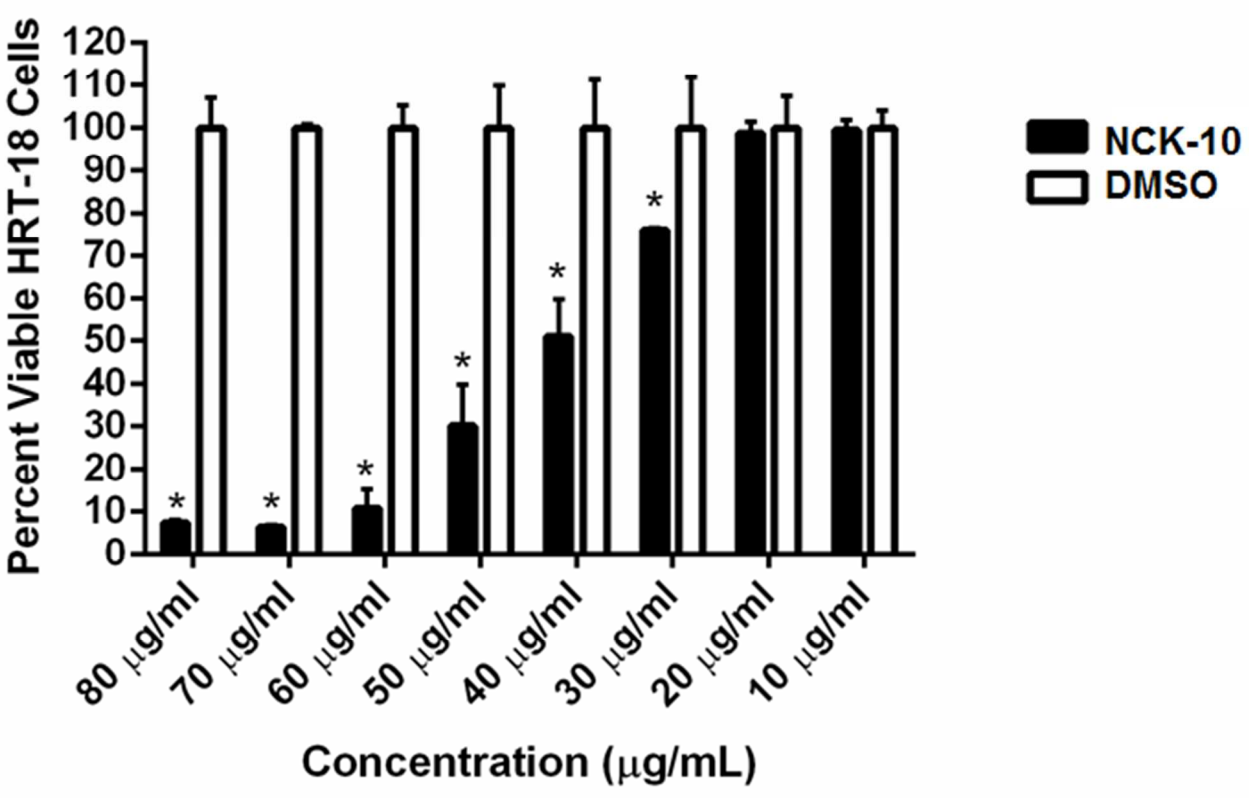

Figure 6. Toxicity analysis of NCK-10 against human colorectal cells (HRT-18). Percent viable mammalian cells (measured as average absorbance ratio (test agent relative to DMSO)) for cytotoxicity analysis of NCK-10 (tested in triplicate) at different concentrations against HRT-18 cells using the MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. Dimethyl sulfoxide (DMSO) was used as a negative control to determine a baseline measurement for the cytotoxic impact of the compounds. The absorbance values represent an average of a minimum of three samples analyzed for the compound. Error bars represent standard deviation values for the absorbance values. A two-way ANOVA, with

post hoc Dunnet's multiple comparisons test, determined statistical difference between the values obtained for the compound and DMSO (denoted by the asterisk) ($P < 0.05$).

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Aryl-alkyl-lysines: Membrane-active fungicides that act against biofilms of *Candida albicans*

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