Synthesis and Characterization of the Therapeutic Potential of Antifungal Peptoid β-5

by

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Abstract

*Cryptococcus neoformans* is a pathogenic yeast species that is one of the leading causes of Cryptococcal meningitis. This form of meningitis, which begins with the inhalation of yeast spores, has a significant mortality rate of 81% percent, with high incidence in those who are immunocompromised. Current antifungal treatments such as fluconazole and amphotericin B have detrimental side effects, leaving a significant need for better alternative treatments. Peptoids, which are mimics of the natural peptides found in living organisms, exhibit beneficial characteristics such as protease degradation evasion and therefore longer half-lives, offer an alternative route for antifungal compound development. Peptoid compounds discovered in our own lab, such as β-5, must be characterized by determining efficacy against pathogenic species such as *C. neoformans* as well as the toxicity of the compounds in the presence of mammalian cells. Herein, assays for determining these factors have shown that β-5 has low toxicity in several mammalian cell lines and significant and rapid inhibition of *C. neoformans*. These characteristics, which are linked to the compound’s structure, suggest that future investigation can focus on working to further enhance the compound’s overall efficacy through structural modification.
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Terms and Definitions

1. Peptide: a molecule made up of smaller units of amino acids that contain stereogenic centers.
2. Peptoid: a peptide mimic that differs by having no stereogenic centers with substituent groups attached to nitrogen atoms rather than α-carbon atoms.
3. Minimum Inhibitory Concentration (MIC₉₀): the minimum concentration of a compound to prevent 90% of growth in a tested sample.
4. Optical Density: used to measure absorbance and is equivalent to the log base 10 of the ratio of light entering a substance and light transmitted (exiting) the material.
5. Protection: a general term for a chemical reaction that adds a chemical moiety to a functional group in order to block the reactivity of that particular functional group.
6. Deprotection: the removal by chemical reaction of a protecting group, in order to return the functional group to its original more reactive structure.
7. TD₅₀: The concentration of compound necessary to cause a 50% decrease in cells.
Introduction

*Cryptococcus neoformans* is a yeast species that has proven to be pathogenic and is one of the primary causes of cryptococcal meningitis. Inhalation of the fungal spores can cause an infection of the lungs which can spread to the central nervous system (CNS) allowing for the onset of meningitis.\(^1\) Although *C. neoformans* is known to have a widespread prevalence, cryptococcal meningitis is most commonly exhibited in immunocompromised individuals, including those with HIV/AIDS, and transplant patients. Those with weakened immune systems are unable to fight off the primary infection of the lungs before it spreads through the bloodstream.\(^2\) While uncommon in healthy individuals, cryptococcal meningitis according to the World Health Organization accounts for approximately 15% of all HIV related deaths and has an estimated 81% mortality rate.\(^3\)

Cryptococcal meningitis has only three commonly used treatments: amphotericin B, fluconazole, and flucytosine, all of which are potent antifungals.\(^4\) Although these medications are effective for treating the infection, they are extremely toxic and are associated with severe side effects including organ failure of the liver and kidneys.\(^5\) The use of antimicrobial peptides (AMPs), which can differentiate between fungal and mammalian cells, was an alternative developed to offer a solution to toxicity and antifungal resistance.\(^6\) AMPs are peptides that have antimicrobial properties but have a backbone structure which is susceptible to proteases when administered, resulting in short and sometimes ineffective half-lives that average shorter than one hour.\(^7\) In an effort to bypass degradation due to proteases, peptide mimics known as peptoids have been developed to
offer a new route to antimicrobial treatment. Peptoids, also known as N-substituted glycine oligomers, have an amide backbone structure similar to peptides, but differ in the fact that they are substituted at the nitrogen whereas peptides are substituted at the α-carbon of the backbone. This slight difference gives peptoids greater structural flexibility, no stereogenic centers, and overall results in significantly greater bioavailability and lower toxicity to mammalian cells. Peptoids also have longer half-lives because they can evade proteases due to their distinct structure.

In the development of antimicrobial peptoids, the focus lies on finding an optimum balance between maximizing effectiveness against fungal growth and minimizing toxicity to mammalian cells. Thus, the process of synthesizing a peptoid must go hand in hand with the characterization of that peptoid, to better understand its potential as an antimicrobial therapeutic. Since all peptoids share the same backbone structure, the unique properties of a peptoid result from the character of each substituent, termed R-groups, attached at the nitrogen atoms of the backbone. The development of the Peptoid Library Agar Diffusion (PLAD) assay, which allows for the high-throughput screening of combinatorial peptoid libraries with different R group combinations, gave rise to the discovery of the peptoid AEC5 (**Figure 1**). AEC5, which is a tripeptoid, has three R groups including a tridecyl alkyl carbon tail at position 1, a primary cationic amine in position 2, and an aromatic heterocyclic furan ring at position 3. Characterization of AEC5 identified the tripeptoid as effective against *C. neoformans* while also showing minimal toxicity towards several different types of mammalian cells.

In order to further investigate the properties of each R-group and its association with AEC5’s overall effectiveness, each position was explored sequentially, using AEC5
derived peptoids with alternative groups at position 1, 3, and then 2.\textsuperscript{5} During testing of different aromatic heterocycles in position 3, it was discovered that a thiophene in this position instead of a furan both improved antifungal efficacy and decreased mammalian toxicity. This compound was termed \( \gamma \)-2. While testing various cationic amines at position 2 of \( \gamma \)-2, it was discovered that replacing the primary cationic amine with a trimethylated cationic amine, resulted in a peptoid with maintained efficacy against \textit{C. neoformans} as well as lower toxicity against certain mammalian cell lines.\textsuperscript{5} While showing initial promise as an antimicrobial peptoid, this peptoid, now known as \( \beta \)-5 (\textbf{Figure 1}), remains uncharacterized in terms of its effectiveness against other pathogens including the ESKAPE bacteria and \textit{Candida albicans}, toxicity against other mammalian cell lines, stability against protease degradation, rate of fungal killing, and other therapeutic parameters that must be evaluated before concluding whether \( \beta \)-5 is a good candidate for pre-clinical testing.

\textbf{Figure 1.} Structure of AEC5, which was optimized by an iterative structure activity relationship (SAR) study into \( \beta \)-5 with improved antifungal efficacy and lower mammalian cytotoxicity.
Methodology

Phase I: Synthesis of β-5 using Solid Phase Synthesis

N-Monomethoxytrityl-1,4-diaminobutane (Mmt-diaminobutane) was first synthesized to serve as a mono-protected diamine that is used in the synthesis of β-5 at position 2 (Figure 1). Once added to the peptoid chain, the Mmt-diaminobutane is deprotected and trimethylated to form the final R group structure at position 2. First, 155 mmol of 1,4-diaminobutane was stirred and slowly added to 15 mL of dichloromethane (CH$_2$Cl$_2$) for 10 minutes on ice. Using a separate flask, 17.0 mmol of monomethoxytrityl chloride (Mmt-Cl) was dissolved in 100 mL of CH$_2$Cl$_2$. This solution was then added dropwise to the 1,4-diaminobutane solution on ice using an addition funnel over a span of 1 hour. Following addition, the reaction was removed from ice and stirred for 4 hours allowing it to reach room temperature. Next, the solution was dried in vacuo to remove solvent. The remaining solid was then dissolved in 1:1 0.5 M aqueous sodium bicarbonate:dichloromethane (aq NaHCO$_3$:CH$_2$Cl$_2$). This mixture was extracted twice using CH$_2$Cl$_2$. The organic layer was collected and dried on calcium chloride, and solvent was removed in vacuo to yield 1.8967 g of product (yield >100%).

The peptoid was synthesized using the well-established submonomer method.$^{11}$ The peptoid synthesis scheme follows a general pattern of two repeated reactions, a bromoacylation reaction, followed by an amination step involving a nucleophilic SN2
substitution reaction where the desired R group containing an amine replaces bromine on the monomer (Figure 2). As a trimeric peptoid, β-5 was synthesized one monomeric unit at a time by building the structure on Rink amide resin. Termed solid phase synthesis, the peptoid was synthesized from C to N terminus adding R groups in the sequence of R₃, then R₂, and R₁ last. The Rink amide resin (loading capacity: 0.75 mmol/g) was first swelled by adding dimethylformamide (DMF) and rocking for 20 minutes. The resin was then treated twice with 20% piperidine in DMF and agitated on a rocker for 10 minutes to deprotect the Fmoc group on the amines on the resin. The resin was washed with 5-7 mL of DMF, shaken, and drained 3 times. To confirm deprotection, a Kaiser test was run using 75 µL of ninhydrin and a small sample of washed resin, with heat added briefly. A purple color change indicates a positive test and successful deprotection as ninhydrin reacts with primary amines. After confirming deprotection, 1.5 mL of 2 M bromoacetic acid in anhydrous DMF was added. Next 1.5 mL of 3.2 M N,N'-diisopropylcarbodiimide (DIC) in anhydrous DMF was added to the resin. The mixture was then microwaved at 10% power for two 15-second intervals and rocked for 15 minutes. Three cycles of washing with DMF, shaking, and draining were completed to remove unreacted materials followed by a Kaiser test. At this stage a negative Kaiser test (no change in color) was expected as the bromoacetic acid has attached to the resin at the primary amine, eliminating the reactive terminal amine which causes a color change in a positive test. Next the second reaction stage involves adding 3 mL of 2 M 2-thiophenemethylamine in anhydrous DMF to the resin to install the amine group for the R₃ position. The same two reaction methods were used to add the second monomeric unit with R₂ (Mmt-diaminobutane) and the third monomeric unit with R₁ (aminotridecane). To check the synthesis between units a small
A sample of resin was added to a 1.5 mL microcentrifuge tube with 500 µL of trifluoroacetic acid (TFA). The solution was then bubbled for 2-3 minutes to evaporate the acid. Once evaporated, 500 µL of deionized water was added to prepare the sample for Electrospray Ionization Mass Spectrometry (ESI-MS) analysis. This process allowed for quick verification that the addition of units had been successful after each monomer was synthesized on the growing peptoid.

After the third amination reaction (with aminotridecane) was completed, a Boc-protection reaction was completed to protect the primary amine on the N-terminus of the peptoid before deprotecting Mmt-diaminobutane. A solution of 10 molar equivalents of Boc-anhydride and 3 mL of 5% N-methylmorpholine (NMM) in DMF was added to the resin which was then rocked for one hour. This reaction notably forms CO₂ gas as a by-product, and therefore requires venting every 10 minutes. Next the Mmt-diaminobutane at position R₂ was deprotected using 15 mL of 1% TFA with CH₂Cl₂. The acid solution was added to the resin in 3 mL aliquots, which was then rocked for 10 minutes and washed with CH₂Cl₂ then drained. After 5 cycles of added solution, rocking, and draining, a Kaiser test was run to confirm the removal of the Mmt group and deprotection of the position two amine.

The process of free-basing was then used to deprotonate the newly deprotected amine group at position 2. The reaction involves using a mild base, 5% NMM in DMF, which was added to the resin and rocked for 5 minutes. This serves as a preparation reaction for the trimethylation of the amine at position 2 to achieve the final structure of β-5. Trimethylation was carried out using methyl iodide (10 molar equivalents), cesium carbonate (Cs₂CO₃; 10 molar equivalents), and DMF. Cs₂CO₃, a base, was necessary to
prevent the formation of hydrogen iodide (HI) which forms as a by-product and to deprotonate the amine to keep the reaction progressing. The three reactants were added to the resin in the specific order listed and then rocked for 24 hours. After rocking was completed, the resin was washed 3x with DMF, 3x water, 3x DMF, and finally 3x with CH₂Cl₂.

Once synthesis was complete, the peptoid was cleaved from the resin. To cleave the peptoid a total volume of 7 mL of 95% TFA 2.5% H₂O and 2.5% triisopropylsilane (TIS) solution was added carefully to the resin, followed by rocking for 1 hour. This reaction not only cleaves the peptoid from the resin but also served as a deprotection to remove the Boc group from the N-terminus. The solution was collected then bubbled for 30 minutes to remove TFA, before it was resuspended in a 1:1 solution of acetonitrile and water. To separate and purify the sample, reverse phase High Performance Liquid Chromatography (RP-HPLC) was run using a Varian HPLC with photodiode array detector, Supelco C-18 column, and a solvent gradient of 100% water to 100% acetonitrile containing 0.05% TFA. The samples collected from RP-HPLC were then analyzed using ESI-MS (Appendix A, Figure 1) to verify the correct compound was collected. The samples were then dried down using a rotary evaporator for 5-10 minutes to remove acetonitrile. Peptoid sample was then frozen with liquid nitrogen and placed on a lyophilizer to sublimate all water. These procedures were carried out successfully to yield 10.1 mg of β-5 (yield = 9.28%) as shown in Figure 3.
Phase II: Broad Spectrum Analysis

The Minimum Inhibitory Concentration (MIC) assay against *Cryptococcus neoformans* lab strain H99S was first completed following CLSI guidelines\(^\text{12}\) to verify and compare the synthesis of β-5 against the previously established β-5 MIC against *C. neoformans*.\(^\text{5}\) The MIC\(_{90}\) of a compound is defined as the minimum concentration of a compound required to inhibit at least 90% of growth. Determining the MIC is fundamental for characterizing the properties of an antimicrobial compound as it is an important factor in the compound’s ability to fight off infection. For the MIC against *C. neoformans*, a yeast peptone dextrose (YPD) plate was streaked with a frozen stock of *C. neoformans* and then incubated at 37 °C for 96 hours. A series of 100x compound concentrations were prepared in sterile water, using two-fold serial dilutions from a stock solution of β-5. Colonies from the incubated plate were then collected and used to inoculate 5 mL of 0.85% saline solution. The optical density of the solution was checked at a wavelength of 530 nm (OD\(_{530}\)) and adjusted to fall within the range of 0.15-0.25. A 100 μL aliquot of this solution was then added to 9.9 mL of Roswell Park Memorial Institute (RPMI) cell media containing 3-(N-morpholino)propanesulfonic acid (MOPS) buffer to form a 1:100 solution. A 500 μL aliquot of this solution was lastly added to 9.5 mL of RPMI-MOPS to form a final 1:20 cell solution. In a 96-well-plate, 198 μL of the cell solution was added to each well, followed by 2 μL of each 100x stock dilution in triplicate. Amphotericin B at a concentration of 2 μg/mL was used as a positive control, and vehicle (sterile water) acted as the negative control. The cell-containing wells were then surrounded by a border of
wells containing 200 µL phosphate-buffered saline (PBS) to minimize distortion due to evaporation during incubation. The 96-well plate was then incubated for 72 hours, and manually read by observation of fungal growth to determine MIC. This assay was completed and verified that the MIC of the β-5 I synthesized matches the previously reported MIC of 3.13 µg/mL.\(^5\)

In order to determine the MIC against \textit{C. albicans}, the preceding methods were again followed, with the exception of shortened incubation times and the use of PrestoBlue Cell Viability Reagent to quantitatively determine MIC. A YPD plate streaked with a frozen stock of \textit{C. albicans} was incubated at 37˚C for 24 hours. Using a 96-well plate for fluorescence-based assays instead, the plate was prepared in an identical procedure with the same positive and negative controls. Media only wells were also included to serve as fluorescence controls. After preparation, the plate was incubated for only 24 hours at 37˚C. Afterwards, 20 µL of PrestoBlue was added to each well, and the plate was further incubated for 1 hour. A SpectraMax M5 Plate Reader was used to read fluorescence (excitation 555 nm; emission 585) which served as an indicator for cell viability. The greater the fluorescence measured, the greater number of viable fungal cells present in the well. The procedure was then repeated in biological triplicate on three different days to verify MIC.

The MIC against the ESKAPE bacteria (\textit{Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, Enterococcus faecalis, and Escherichia coli}) was determined as published previously following CLSI guidelines.\(^7,13\) Briefly, this MIC assay began with streaking a Lysogeny Broth (LB) plate with a frozen stock of one of the bacteria and then incubated at 37˚C for
24 hours. Serial 2-fold dilutions of 10x compound concentrations of a stock β-5 solution were prepared in sterile water. Colonies were then added to Tryptic Soy Broth (TSB) and tested for optical density at OD$_{600}$ to achieve a value between 0.08-0.15. The TSB inoculant was then diluted to a 1:200 solution with Cation Adjustment Muller-Hinton Broth (CAMHB), for a final concentration of 5x10$^5$ CFU/mL. A 96-well plate for fluorescence-based assays was used, and 10 µL of the 10x dilutions were added in triplicate to the wells after addition of 90 µL of the cell inoculant for a total well volume of 100 µL at 1x peptoid dilution. Tetracycline at a concentration of 20 µg/mL was used as the positive control, vehicle (sterile water) acted as the negative control, and media only wells served as fluorescence controls. The plate was incubated for 24 hours at 37 °C, after which 10 µL of PrestoBlue was added to each well, and then incubated an additional 1-2 hours. Using a SpectraMax M5 Plate Reader, fluorescence (excitation 555 nm; emission 585) was read and used to determine MIC. The procedure was repeated in biological triplicate on three different days.

Toxicity of β-5 against HepG2 liver cells, 3T3 mouse fibroblasts, and human red blood cells has been reported previously.$^5$ We further evaluated β-5 cytotoxicity by testing against HPL1A peripheral lung epithelial cells and A549 adenocarcinoma lung cells, since the lung is the primary site of C. neoformans infection. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% penicillin, streptomycin, and glutamine (PSG), at 37 °C in a 5% CO$_2$ atmosphere. Cells were then seeded into a 96-well plate in phenol red free DMEM containing 10% FBS. After 3 hours of incubation, the cells were treated with 2- fold serially diluted peptoid in PBS from 200 – 3.125 µg/mL. Wells were included with PBS only (vehicle) to serve as a
negative control and 1% Triton X-100 as a positive control. The plate was then incubated for 72 hours, after which 20 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and allowed to incubate for 3.5 hours. Media was removed from the wells and replaced with 100 µL of dimethyl sulfoxide (DMSO) to lyse cells and solubilize metabolized MTT. The plate was then incubated at 37 °C for 15 minutes, before absorbance was recorded. Using a SpectraMax M5 Plate Reader set at 570 nm, the absorbance was recorded and used to calculate percent inhibition. In this assay, absorbance is proportional to the number of live cells, as the metabolism of MTT into a purple dye in live cells is what produces a measurable absorbance in the sample. The half maximal inhibitory concentration (IC50) is also calculated using GraFit and represents the concentration of peptoid at which there is a 50% decrease in viable cells (reported as toxicity dose 50% or TD50). This assay was repeated two more times on different days.

Phase III: In vitro Therapeutic Development

Two important metrics of an antifungal’s therapeutic potential are whether it arrests growth (fungistatic) or kills fungi (fungicidal) and how rapidly it arrests growth or kills a pathogen. Both of these things can be determined through a time-to-kill assay, also known as a killing kinetics assay. The time-to-kill assay began by inoculating 50 mL of YPD with a frozen stock of *C. neoformans* lab strain H99S and incubating at 37 °C for 14-20 hours. The cells were then pelleted by centrifugation at 600 g for 5 minutes, followed by washing
with PBS. This process was repeated two more times. Next the cells were resuspended in YPD, counted, and separated into two 1x10^5 cells/ mL aliquots with 20 mL of YPD. A vehicle control or β-5 at 4x the MIC (12.5 µg/mL) was then added to each flask with cell aliquots. The flasks were then incubated at 37 °C for 4 hours with shaking. At various time points 1 mL aliquots were collected, serially diluted (10^0-10^5), and spotted on YPD plates. Aliquots were taken at 6, 12, 18, 30, and 60 minutes and then every hour after 60 minutes past the start of the experiment at 120 and finally 180 minutes. Spotted YPD plates were incubated for 36 hours and colonies counted to determine viable cells remaining at that time point (CFU/mL). By observing whether fungi were able to grow again on YPD plates after β-5 was removed allowed us to conclude whether β-5 is fungistatic or fungicidal. By plotting CFU/mL versus time, the rate of fungal killing was determined. The assay was repeated three times on separate days to give appropriate biological replicates. We expected β-5 to be fungicidal and kill *C. neoformans* rapidly, as we have shown with another anti-Cryptococcal peptoids.4

**Results**

**R.1 Compound verification**

The trimeric peptoid β-5 was synthesized beginning with the synthesis of the protected submonomer Mmt-diaminobutane (Figure 2). Monomethoxytrityl chloride dissolved in CH₂Cl₂ was added dropwise to a solution of 1,4 diaminobutane in CH₂Cl₂ and
subsequently purified to yield 1.897 g of N-Monomethoxytrityl – 1,4-diaminobutane (yield >100%). Excessive yield likely resulted from the incomplete removal of solvent or diamine starting material. This submonomer was made in order to carry out selective deprotection after incorporation into the peptoid structure which allows for the trimethylation of β-5 on resin at position 2 alone, while the N-terminus remains Boc protected until the final cleave of the compound from the resin.

**Figure 2.** Synthesis of N-Monomethoxytrityl – 1,4-diaminobutane.
Figure 3. Reaction Scheme for Solid Phase Synthesis of β-5. The scheme shows the repeated steps of bromoacylation followed by an S_N2 substitution where an amine is reacted to replace bromine. Methylation is achieved towards the end of synthesis by protecting the N-terminus with Boc, selectively deprotecting the Mmt group with a dilute 1% TFA solution and methylating with methyl iodide. Treatment with a strong 95% TFA solution removes the Boc group and cleaves peptoid from the solid phase, yielding β-5.
Once the Mmt-diaminobutane compound was synthesized, the structure of β-5 was synthesized using a two-step cycle of bromoacylation and S_N2 substitution using the amine of interest for each submonomer added to the resin (Figure 3). The amines used in the synthesis of β-5 were thiophenemethylamine, Mmt-diaminobutane, and tridecylamine. To achieve the complete structure of β-5, once all three submonomer had been synthesized on resin the amine at position 1 was Boc protected, followed by the removal of the Mmt protection group at position 2. Position 2 was then trimethylated using methyl iodide and cesium carbonate before the compound was fully cleaved from the resin and purified, yielding 10.1 mg of β-5 (yield = 9.28%).

The structure of β-5 was verified using ESI-MS (Figure 4) prior to moving forward with broad spectrum analysis and in vitro therapeutic testing. MS analysis confirmed the expected mass of 580.1 g/mol.
Figure 4. Mass spectra of β-5 after cleavage from resin. The spectra shows a representation of relative intensity versus mass to charge ratio of the sample. The molecular weight of β-5 is predicted to be 580.8965 g/mol, and is therefore represented by the indicated base peak at 580.1 m/z.

R.2 Minimum Inhibitory Concentration (MIC)

The MIC of the newly synthesized β-5 was assessed against several fungal and bacterial pathogens. The MIC of the compound was first determined against *C. neoformans* lab strain H99S to be 3.13 µg/mL (Table 1), verifying its reproducibility with the
previously tested and published value.\textsuperscript{5} The MIC for β-5 against \textit{C. albicans} was completed to further analyze the efficacy against fungal pathogens and was determined to be 50 µg/mL (\textbf{Table 1}). This is comparable to the MIC reported for AEC5\textsuperscript{4} and indicates that this series of peptoids does not have strong anti-Candida activity. Given the health risks associated with the \textit{ESKAPE} bacteria, we tested the MIC of β-5 against each bacterium (\textbf{Table 1}). The resulting values for minimum inhibition suggest that β-5 exhibits higher efficacy against gram positive \textit{ESKAPE} bacteria (\textit{E. faecium}, \textit{E. faecalis}, and \textit{S. aureus}) with values ranging from 12.5-25 µg/mL versus the gram-negative strains tested (\textit{E. coli}, \textit{K. pneumoniae}, \textit{A. baumannii}, and \textit{P. aeruginosa}) which resulted in values ranging from 25 µg/mL to values beyond the tested range of 100 µg/mL. This trend is comparable to previously tested compounds which show similar difficulty disrupting the growth of double membrane bacteria. Overall, β-5 showed the most inhibition against the fungal pathogen \textit{C. neoformans}. 
Table 1. Minimum inhibitory concentration (MIC₉₀) values of β-5 against fungal and bacterial pathogens.

<table>
<thead>
<tr>
<th>Organism</th>
<th>MIC₉₀ (µg/mL)</th>
<th>β-5</th>
<th>AEC5</th>
<th>RMG8-8</th>
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<tr>
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<td><strong>C. neoformans</strong> (H99S)</td>
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**R.3 Cell Toxicity against HPL1A and A549 Cell Lines**

As an antifungal target for *C. neoformans* related infections it was important to further assess the toxicity of β-5 in mammalian cells. In the case of *C. neoformans* the site of primary infection is often in the lungs and therefore the focus of assessing toxicity using mammalian lung cells. Using two lung cell lines - HPL1A genetically modified cells and A549 immortalized cancer cells, toxicity of β-5 was evaluated to determine the
concentration which decreases the population of viable mammalian cells by 50% (TD\textsubscript{50}). The cell toxicity of $\beta$-5 against HPL1A cells was determined to be 40.4 $\mu$g/mL (Table 2). This value is slightly improved, though still within error of the HPL1A toxicity of AEC5 (36.3 $\mu$g/mL), the previously reported parent compound of $\beta$-5. It was also of interest to determine and compare the toxicity $\beta$-5 in HPL1A cells to the toxicity of another compound discovered in the lab, RMG8-8, which has shown greater inhibition towards \textit{C. neoformans} than $\beta$-5. While greater inhibition is often linked to higher toxicity, the data shows that RMG8-8 is less toxic than $\beta$-5, having a TD50 that is nearly two fold greater than $\beta$-5 (74 $\mu$g/mL; Table 2).\textsuperscript{10,14} Further support of this trend in compound efficacy was seen in our results when testing toxicity in an immortalized lung cell line, A549 adenocarcinoma lung cells. All three compounds, $\beta$-5, AEC5, and RMG8-8 were tested to more accurately assess the toxicity of the compound of interest ($\beta$-5). Testing the additional compounds provided necessary comparative data previously undetermined for AEC5 and RMG8-8 in A549 cells. These data indicate that AEC5 is quite toxicity to A549 cells (TD\textsubscript{50} = 21.9 $\mu$g/mL) while $\beta$-5 is about half as toxic (TD\textsubscript{50} = 45.9 $\mu$g/mL) and RMG8-8 is less toxicity still (TD\textsubscript{50} = 68.6 $\mu$g/mL).
Table 2. TD₅₀ values for β-5, AEC5, and RMG8-8 in HPL1A and A549 cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cell Line</th>
<th>TD₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEC5</td>
<td>HPL1A</td>
<td>36.3 ± 11.1</td>
</tr>
<tr>
<td></td>
<td>A549</td>
<td>21.9 ± 10.2</td>
</tr>
<tr>
<td>β-5</td>
<td>HPL1A</td>
<td>40.4 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>A549</td>
<td>45.9 ± 5.1</td>
</tr>
<tr>
<td>RMG8-8</td>
<td>HPL1A</td>
<td>74.5 ± 7.8</td>
</tr>
<tr>
<td></td>
<td>A549</td>
<td>68.6 ± 6.4</td>
</tr>
</tbody>
</table>

**R.3 Kinetic Time to Kill Assay against C. neoformans**

The rate of fungal killing for β-5 and whether this compound is fungistatic or fungicidal towards C. neoformans lab strain H99S was determined using a kinetic time to kill assay. The assay was performed by comparing the number of viable cells in control and peptoid-treated C. neoformans culture over a period of 3 hours. Aliquots taken at various times from both the control and peptoid-treated cell cultures were, serially diluted, and spotted onto YPD plates. Analyzing these plates for colonies after incubation to quantify inhibition of growth in terms of CFU/mL as a function of time revealed that β-5 begins to exhibit fungicidal effects on C. neoformans after 18 minutes compared to control (Figure 5A) with complete inhibition of growth by 120 minutes. This conclusion is
supported by the decrease in colonies present per mL after just 18 minutes and the complete absence of colony growth on plates streaked with aliquots drawn at and past 120 minutes. This result is consistent with the total inhibition of growth exhibited by previously published compound data for AEC5.\textsuperscript{4} Plotting remaining percent growth versus time for β-5 treated fungi allowed for the calculation of the time needed to reduce viable fungi by 50\% (t₁/₂) which was 22 minutes (Figure 5B). This suggests that β-5 begins significantly inhibiting the growth of \textit{C. neoformans} faster than AEC5 which had a reported t₁/₂ of 30 minutes.\textsuperscript{4} Additionally, AEC5 showed complete inhibition of growth only after 3 hours, whereas β-5 does so after only 2 hours.\textsuperscript{4} Overall, the kinetic time to kill assay shows that β-5 performs more rapidly as an antifungal in the presence of \textit{C. neoformans}. 
Figure 5. Colony-forming unit versus time of each aliquot is graphed showing growth in terms of colony concentration in β-5 (4x the MIC: 12.5 µg/mL) treated cells versus control (YPD).
Conclusions and Future Plans

The intention of this project was to explore the efficacy of the antifungal peptoid β-5 through broad-spectrum analysis and *in-vitro* therapeutic characterization. Compiling comparative data was necessary for the purpose of analyzing the structural benefits or detriments of β-5 versus its origin compound, AEC5. Ultimately, the purpose of this comparison was to assess whether β-5 should be further pursued for pre-clinical testing as an antifungal. In addition, we expected analyzing the efficacy of β-5 to provide insight for future compound development. Overall, the structural adaptations made to AEC5 to achieve the structure of β-5 are consistent with improved properties of efficacy including a lower MIC value against *C. neoformans*, lower cell toxicity in both HPL1A and A549 cells, and faster inhibition of growth against *C. neoformans*. In assessing the toxicity of β-5 in A549 cells, we also determined the toxicity of a second compound RMG8-8 in A549 cells. RMG8-8, a more recent compound from our lab, continues to show lower toxicity than both β-5 and subsequently AEC5.

A quantitative analysis of several efficacy parameters for β-5 has provided substantial data to support the assertion that β-5 shows greater promise than AEC5 as an antifungal targeting *C. neoformans*. Unfortunately, β-5 showed little efficacy towards bacterial pathogens, as well as the fungal strain *C. albicans* which continues to be a highly resistant yeast pathogen. While the efficacy of β-5 offers promise, the overall structure has shown to have limited impact on reducing toxicity compared to AEC5 in the cell lines tested here. Further, compound RMG8-8 from a different peptoid series, has performed exceptionally against *C. neoformans* and will likely be the focus of development for future
antifungal compounds. In conclusion, the project provided crucial comparative data, that can be utilized in future structural assessments, for developing future peptoids based on the structure to efficacy relationship that can be ascertained from evaluating the toxicity and inhibition properties of AEC5 versus β-5, and now even RMG8-8.
References


9. Zhang, Y. et al. Amphiphilic Polypeptoids Rupture Vesicle Bilayers To Form


