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Towards a clinical antifungal peptoid; Investigations into the therapeutic potential of AEC5

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Abstract

Cryptococcus neoformans is a fungal pathogen that causes cryptococcal meningitis in immunocompromised individuals. Existing antifungal treatment plans have high mammalian toxicity and increasing drug resistance, demonstrating the dire need for new, non-toxic therapeutics. Antimicrobial peptoids are one alternative to combat this issue. Our lab has recently identified a tripeptoid, AEC5, with promising efficacy and selectivity against *Cryptococcus neoformans*. Here we report studies into the broad-spectrum efficacy, killing kinetics, mechanism of action, *in-vivo* half-life, and sub-chronic toxicity of this compound. Most notably, these studies have demonstrated that AEC5 rapidly reduces fungal burden, killing all viable fungi within 3 hours. Additionally, AEC5 has an *in-vivo* half-life of 20+ hours and no observable *in vivo* toxicity following 28 days of daily injections. This research represents an important step in the characterization of AEC5 as a practical treatment option against *Cryptococcus neoformans* infections.

Graphical Abstract

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SUPPORTING INFORMATION

Additional supporting information may be found online.

Notes – The authors declare no competing financial interests.

1 | INTRODUCTION

Cryptococcus neoformans (*C. neoformans*) is a pathogenic yeast that causes cryptococcal meningitis, a life-threatening disease, via spore inhalation.¹ In healthy individuals, fully developed immune systems typically fight off any beginning infection, however in immunocompromised individuals, such as HIV/AIDS and transplant patients, that is not the case. There are approximately 220,000 cases of cryptococcal meningitis among HIV/AIDS patients each year, and 82% of those cases will be fatal.² *Cryptococcus* has now become the most common cause of meningitis in adults in sub-Saharan Africa, primarily due to the limited access to healthcare for individuals suffering from HIV/AIDS. It has been estimated that in this part of the world *C. neoformans* infections may cause more deaths than tuberculosis.³

Current therapeutic options are limited due to increasing drug resistance and high mammalian toxicity. The three antifungals most commonly used to treat *C. neoformans* infections are amphotericin B, fluconazole, and flucytosine. These three drugs, while effective at killing fungal cells, do so in a way that leaves patients with toxic side effects including liver and kidney failure.⁴ To combat this, the modern course of treatment for these infections includes a concoction of all three drugs mixed together in a medical-type cocktail, which yields its own less-than-desirable side effects.⁴ This lack of a clear concise plan of attack against this type of fungal infection demonstrates a dire need for new, nontoxic therapeutics.

One known alternative to combat toxicity levels and drug resistance is the use of antimicrobial peptides (AMP's). AMPs are generally helical, cationic, and amphipathic molecules that have been shown to act on the cell membrane.⁵ AMP's take advantage of specific differences between mammalian and fungal cells and can target accordingly. Unfortunately, peptides are quickly broken down in the body by proteases, leaving very little time to target any fungal cells, making them poor therapeutics.⁶ A solution to this problem involves the employment of peptide-mimics termed peptoids. Peptides are characterized as having an amide backbone with side chains attached to the α -carbons of the amide backbone, while peptoids have side chains attached to the nitrogen of the amide backbone. This small structural difference increases *in vivo* stability by inhibiting protease recognition.⁷ A host of studies have identified antibacterial peptoids, as summarized in a recent review by Molchanova *et al.*,⁸ however, there are limited reports of antifungal peptoids.^{9–11} By using a high-throughput screening assay we have recently identified a tripeptoid with antifungal properties against *C. neoformans* from a combinatorial peptoid library.¹¹ Termed AEC5, this antifungal peptoid has no observable cytotoxicity against human lung, liver, or red blood cells at the minimum inhibitory concentration for *C. neoformans* and is fully stable in human serum containing active proteases for at least 48 hours.¹¹ Based on these preliminary data AEC5 shows strong promise in addressing the dearth of antifungal options to combat *C. neoformans* infections. However, significant characterization of AEC5 is required before concluding that this promise translates into real therapeutic value. We therefore sought to evaluate the broad-spectrum antimicrobial activity, mechanism of action, and various pharmacological properties of AEC5 and report the results of our efforts here.

We note that these efforts have been concomitant with efforts to improve the potency and toxicity profile of AEC5 through structure activity relationship (SAR) studies.¹² However, we sought to complete detailed analysis of AEC5 alongside SAR to contribute to the limited literature on antifungal peptoid mechanism of action and *in vivo* characteristics. Recent studies have confirmed that, much like peptides, peptoids are often antibacterial through cell membrane disruption and rely on membrane composition differences to be selective for the pathogen over native cells.^{13,14} Antifungal peptides also generally act through membrane disruption, but may also cause fungal cell death through cell wall disruption or through intracellular targets that result in ROS generation or programmed cell death.¹⁵ Reports of peptoids *in vivo* indicate that they have poor oral bioavailability, excellent tissue absorption, slow elimination through the feces, long half-lives, and limited toxicity, at least when tested acutely.^{13,16,17} One of these studies demonstrates the *in vivo* antibacterial efficacy of a peptoid, with an average two log order reduction in bacterial counts in mice compared to saline controls.¹³ We report here that AEC5 works quickly against *C. neoformans*, with nearly 50% reduction in fungal growth after 30 minutes and complete elimination of viable fungi after 3 hours. Furthermore, AEC5 is effective against *C. neoformans* infected macrophages, exhibits an *in vivo* murine half-life of 20+ hours, and shows no sub-chronic toxicity in a mouse model up to 50 mg/kg over 28 days. Thus, the data suggest that AEC5 may be a promising new therapeutic against *C. neoformans* infections.

2 | MATERIALS AND METHODS

2.1 | Minimum Inhibitory Concentration (MIC).

The minimum inhibitory concentration of AEC5 was determined against all seven of the ESKAPE pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Enterococcus faecium*) as previously described.¹⁸ Two-fold serial dilutions of AEC5 ranging from 100 to 3.13 µg/mL were evaluated by broth microdilution assay (CLSI, M07-Ed11).¹⁹ Vehicle and tetracycline were used as negative and positive controls, respectively. Cell viability was determined spectrophotometrically following PrestoBlue addition.

The MIC was also determined against *Cryptococcus gattii* strains R265 and R272 as done previously.¹¹ Additionally, *Candida albicans* (*C. albicans*) lab strain SC5134 susceptibility to AEC5 was evaluated following a procedure modified from *C. neoformans* MIC testing using the broth microdilution method (CLSI, M27-A3).²⁰ Briefly, *C. albicans* was streaked on a potato dextrose agar (PDA) plate from frozen stock. The plate was incubated at 35 °C for 24–48 hours. 1–2 colonies were resuspended in sterile 0.85% saline. The optical density at 600 nm (OD₆₀₀) was acquired on a spectrophotometer and adjusted by adding saline or cells to achieve an OD₆₀₀ of 0.15–0.25. Cells were vortexed and diluted 1:100 in Roswell Park Memorial Institute (RPMI) media containing 3-morpholinopropane sulfonic acid (MOPS; 0.1 M). Subsequently, the 1:100 solution was vortexed and diluted further to give a 1:20 solution in RPMI + MOPS (final dilution of 1:2000 from saline plus cells). Two-fold serial dilutions of AEC5 were prepared in water at 100x the final desired test concentrations. In a 96-well plate, 198 µL of the 1:20 solution of media + cells were plated with 2 µL of 100x AEC5 in triplicate. Vehicle, media, and amphotericin B (2 µg/mL) controls were plated as

well. Plates were incubated at 35 °C overnight, a shorter incubation time than with *C. neoformans* due to the robust growth observed for *C. albicans* in RPMI + MOPS media. To test cell viability, 20 µL of PrestoBlue was added to each well, incubated at 35 °C for 2 hours, and fluorescent intensity of each well tested (Ex. 555 nm; Em. 585 nm). All triplicate assays were repeated at least twice on different days.

2.2 | Killing Kinetics.

To determine the killing kinetics of AEC5, 50 mL of yeast extract-peptone-dextrose (YPD) was inoculated with *C. neoformans* lab strain H99S and incubated at 37 °C for approximately 32 hours. Cultures were spun down (600 x g 5 min) and washed with phosphate buffered saline (PBS). This was repeated three times. Cells were counted and two 1×10^5 cells/mL aliquots were prepared in 20 mL YPD. Vehicle control or AEC5 at 4x the MIC (25 µg/mL) was then added to each flask. After the addition of AEC5, flasks were incubated for 24 hours at 37 °C. OD₆₀₀ was recorded and dilution series aliquots (10^0 - 10^{-5}) were spotted on YPD plates every 30 minutes for the first three hours and every three hours thereafter. The plates were incubated for approximately 36 hours and then colonies were counted.

2.3 | Synergy Testing.

Possible synergy was evaluated for AEC5 in combination with three known antifungal drugs, amphotericin B, fluconazole, and flucytosine. This testing followed closely our own previous procedures and CLSI guidelines for testing against *C. neoformans*.^{11,20} Briefly, *C. neoformans* strain H99S was grown on yeast extract peptone dextrose (YPD) agar at 35 °C. After 96 hours, 2–3 colonies were transferred to 5 mL of 0.85% saline solution. The optical density (OD) at 600 nm was acquired on a spectrophotometer and adjusted by adding saline or cells to achieve an OD₆₀₀ of 0.15–0.25. A total of 100 µL of the saline + cells solution was added to 9.9 mL RPMI + MOPS to give a 1:100 solution. Subsequently, the 1:100 solution was vortexed and diluted further to give a 1:20 solution in RPMI + MOPS (final dilution of 1:2000 from saline plus cells). Amphotericin B, fluconazole, and flucytosine stocks (100x desired test concentrations) were prepared in 100% DMSO while the AEC5 stocks (100x desired test concentrations) were prepared in lipopolysaccharide (LPS) free PBS. Each plate contained one known drug and doses of AEC5 ranging from 50 µg/mL to 1.06 µg/mL. Ranges of known antifungals were tested as follows; amphotericin B (0.06–0.002 µg/mL); fluconazole (16–0.5 µg/mL); flucytosine (8–0.25 µg/mL). Drugs were added individually to each well and mixed gently. After 72 hour at 35 °C, 20 µL of PrestoBlue was added to each well. The plate was incubated at 35 °C for another 8 hours followed by fluorescent analysis using a spectrophotometer. All assays were done in triplicate for each concentration and repeated at least twice on different days. Using these data, we calculated the fractional inhibitory concentration index (FIC_i) of each combination to determine if the combinations of drugs were synergistic, implying they would have different mechanisms of action. FIC_i values were calculated as follows:

$$\text{FIC}_i = (\text{MIC of drug A in combination} / \text{MIC of drug A alone}) + (\text{MIC of drug B in combination} / \text{MIC of drug B alone})$$

FICI values were interpreted as follows: FICI < 0.5 synergistic; 0.5 FICI 4 indifferent; FICI >4 antagonist.^{21,22}

2.4 | Sorbitol Susceptibility.

Sorbitol susceptibility assays were completed per previously reported MIC protocols with or without 0.8 M sorbitol.¹¹ A total of 198 μ L of media plus cells was combined with 2 μ L of 2-fold serially diluted peptoid or known antifungal (100x stock) in a 96-well plate. The plate was incubated at 35 °C for 72 hours before adding 20 μ L of PrestoBlue and incubating again for 8 hours. The fluorescence was collected (excitation 555nm; emission 585nm). Data were collected in triplicate and repeated on two different days. Linear regression analysis was used to compare the difference with or without sorbitol addition. Analysis and p-value calculations were completed in JMP statistical software (version 14; SAS Institute).

2.5 | Media and Cell Concentration Effect on AEC5 Efficacy.

The minimum inhibitory concentration of AEC5 was determined against *C. neoformans* in YPD and RPMI + MOPS at 2×10^3 , 2×10^4 , and 2×10^5 cells as previously described (Figure S1).¹⁸ *C. neoformans* lab strain H99S was streaked onto YPD plate from frozen stock and incubated for 96 hours at 35 °C. After 96 hours, 3–5 colonies were added to 10 mL 0.85% saline solution and counted. Appropriate amounts of cells were added to media resulting in one of each 2×10^3 , 2×10^4 , and 2×10^5 cell concentrations in both YPD and RPMI + MOPS. Two fold serial dilutions of AEC5 were prepared in LPS-free PBS at 100x concentrations. In a 96-well plate 198 μ L of media concentrations were combined with 2 μ L of AEC5 dilution in triplicate. The plate was incubated at 35 °C for 72 hours. PrestoBlue was added, the plate incubated for an additional 8 hours, and the fluorescence was collected (excitation 555nm; emission 585nm).

2.6 | Efficacy in Infected Macrophages.

C. neoformans H99S was grown in YPD until log phase at 37 °C. The next day, 5×10^5 cells/mL of murine J774.16 macrophage-like cells were seeded into a 96-well plate and incubated overnight at 37 °C + 5% CO₂. Log phase *C. neoformans* cells were then washed 3X and resuspended with phosphate buffered saline (PBS). 3.6×10^6 cells were incubated with 1 μ g/mL lipopolysaccharide (LPS), 200 units IFN- γ and 10 μ g/mL anti-GXM mAb 18B7 (a kind gift of Arturo Casadevall) to opsonize the *C. neoformans* cells. Old media was then removed from the 96-well plate and fresh media containing *C. neoformans* was added to the macrophages. The plate was then incubated for an hour at 37 °C + 5% CO₂ so that phagocytosis of *C. neoformans* by the macrophages could occur. After incubation, J774 media was removed and the cells were gently washed 3X with 300 μ L PBS to remove the extracellular *C. neoformans*. 200 μ L of media containing a range of concentrations of AEC5: 0 μ g/mL, 6 μ g/mL, or 24.4 μ g/mL AEC5 was added to the macrophages + *C. neoformans* in the appropriate wells. All concentrations were tested in quadruplicate. The plate was incubated at 37 °C + 5% CO₂ for 24 hours, after which, the macrophages were lysed with 0.1% sodium dodecyl sulfate (SDS) for the experiments in which extracellular (EC) and intracellular (IC) pools were combined. For the experiments in which EC and IC pools were separated, macrophages were lysed as above after removing the extracellular *C. neoformans* cells for plating. One well of the quadruplicate of both EC and IC cells were counted by

hemocytometer to determine the plating dilutions. *C. neoformans* cells were plated on YPD agar (two plates/well) and incubated at 37 °C for 2 days. The colonies from the different treatment groups were counted and the results analyzed.

2.7 | *In-Vivo* Half-life.

An *in-vivo* half-life study in a mouse model was performed with AEC5 by Frontage Laboratories, Inc (Exton, Pennsylvania). For this study 6 male CD-1 (3 per group) mice were injected intraperitoneally or intravenously with 5 mg/kg or 1 mg/kg doses of AEC5, respectively. We note that this study was meant as an initial exploration of the AEC5 half-life and that, although 3 mice per group is a relatively small sample size, there is minimal variation observed between mice in the terminal phase of compound clearance, giving a reasonably confident half-life estimation. Blood samples were collected from the mice at 2, 15, and 30 minutes and 1, 2, 4, 6, 8, and 24 hours after the AEC5 was administered and diluted into an EDTA solution. An aliquot (20 µL) of each sample was transferred to a 96-well plate before adding 200 µL of a Warfarin internal standard in acetonitrile. This plate was vortexed for 10 min, centrifuged (10 min, 4000 rpm), and 100 µL of each sample transferred to a new 96-well plate and diluted with water. This plate was vortexed briefly before analysis by LC/MS/MS on a Shimadzu LC-30AD with autosampler. Samples were separated on an ACE C8 column (2.1 × 50 mm, 5 µm) using a linear gradient of 0.1% formic acid in water to 0.1% formic acid in acetonitrile over 3.6 min. Eluted samples were detected on an Applied Biosystems API 5000 MS/MS under positive mode. A standard curve of AEC5 concentrations (1–2000 ng/mL) was generated and confirmed by quality control samples using the same analytical technique (Figure S2). Pharmacokinetic properties were calculated by a non-compartmental analysis approach using PhoenixTM WinNonlin® (version 6.3.0, Pharsight Corp., St. Louis, MO). Blood compound concentrations in the terminal phase of compound clearance (>4–6 h) were used for pharmacokinetic analysis to provide a terminal phase half-life. It is important to note that the last blood collection time (T_{last} ; 24 h) was less than twice the estimated half-life, so PK parameters are based on data extrapolated from the linear regression analysis of the terminal phase time points.

2.8 | *In-Vivo* Sub-Chronic Toxicity.

Ethics statement.—All animal use complied with the standards described in the NIH Guide for the Care and Use of Laboratory Animals, The US Animal Welfare Act, PHS Policy on Humane Care and Use of Laboratory Animals and Middle Tennessee State University Institutional Animal Care and Use Committee guidelines. The protocol was approved by the IACUC of Middle Tennessee State University (protocol #16–3004). Experiments were not randomized or blinded and were done once. For euthanasia, carbon dioxide overdose was used.

Six 6-week old male C57Bl/6 mice (Jackson Labs, Bar Harbor, ME) were injected intraperitoneally, once a day for 28 days. The mice were injected, 2 per group, with 100 µL of LPS-free PBS or 100 µL of AEC5 solution at 10 mg/kg or 50 mg/kg AEC5 (3 groups total). The mice were weighed daily before injection. On day 28 the mice were sacrificed and the brain, kidneys, liver, heart, and lungs were collected for each animal. These organs

were examined for histopathology by the Vanderbilt Translational Pathology Shared Resource Core (Nashville, TN).

3 | RESULTS AND DISCUSSION

3.1 | Broad spectrum testing

Given the efficacy of AEC5 against *C. neoformans* (MIC 6.3 µg/mL), we initially investigated the broad-spectrum antimicrobial activity of AEC5 against *Cryptococcus gattii*, *Candida albicans*, and the ESKAPE bacterial pathogens (Figure 1A). Like *C. neoformans*, *C. gattii* often results in cryptococcal meningitis.²³ However, unlike *C. neoformans*, *C. gattii* is capable of infecting immunocompetent individuals and although outbreaks of *C. gattii* are rare, infection by *C. gattii* is on average fatal in roughly a quarter of those infected regardless of immune system status.²⁴ *C. albicans* is another yeast like pathogenic fungus primarily responsible for hospital borne fungal infections and is often isolated from dangerous cross-kingdom biofilm infections.²⁵ The ESKAPE bacteria, an acronym for *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Enterococcus faecium*, represent a group of pathogens most often responsible for drug-resistant bacterial infections.²⁶ AEC5 demonstrated only modest efficacy against Gram-positive *S. aureus*, *E. faecalis*, and Gram-negative *P. aeruginosa*. Poor efficacy was observed against the other Gram-negative bacteria as well as *E. faecium*. AEC5 was also poorly efficacious against *C. albicans* with an MIC of 100 µg/mL, possibly due to differences in the composition of the cell wall as *C. albicans* contains more β-glucans while *C. neoformans* contains more α-glucans.²⁷ Alternatively, differences in the glycolipids in the cell membrane may be contributing to the observed difference in MIC.²⁸ Promisingly, AEC5 was very effective against two different strains of *C. gattii*, R265 and R272. These strains of *C. gattii* were isolated from a 2001 outbreak in Vancouver, Canada.²⁹ AEC5 has an MIC of 3.13 µg/mL against both R272 and the more virulent R265,³⁰ making it even more potent against *C. gattii* than *C. neoformans* and suggesting that AEC5 may be a promising antifungal therapeutic option for all cryptococcal infections.

3.2 | Killing Kinetics

AEC5 kills *C. neoformans* quickly, with no viable fungi present 3 hours following treatment. Compounds that exert antifungal activity quickly are a necessity in the clinic to improve patient outcome. Reports for the killing kinetics of fluconazole indicate that this compound is primarily fungistatic against *C. neoformans* and only becomes fungicidal at elevated concentrations (>8x MIC), with 50% reductions in fungal growth taking 48–72 hours.³¹ We note that we have shown AEC5 to be fungicidal at the MIC against *C. neoformans* previously.¹¹ Killing kinetic reports of amphotericin B are mixed, but indicate that against clinical isolates from HIV/AIDS patients, this compound may require 10 or more hours to reduce fungal growth by 50%.³² Against other clinical isolates, amphotericin B has been reported to kill at a rate similar to the rate reported here for AEC5, although at some concentrations regrowth was observed after 48 hours.³¹ This may be due to amphotericin B fungistatic activity below the MIC or resistance development by *C. neoformans*. The killing kinetics of AEC5 were evaluated using a growth inhibition assay (Figure 1B). *C.*

neoformans was incubated with either vehicle control or AEC5 at 4x the MIC (25 µg/mL) to account for increased cell count and more robust growth media compared to traditional MIC conditions, as determined experimentally (Figure S1). At various time intervals over 30 hours OD₆₀₀ was taken, and aliquots of cells were plated to quantify colony forming units (CFUs). The data collected showed nearly 50% fungicidal inhibition of fungal growth after just 30 minutes and 99% inhibition at 2 hours, with complete fungal growth inhibition after 3 hours. Furthermore, unlike previous reports with amphotericin B, no fungal recovery and regrowth was observed over the 24-hour study. More exhaustive studies are underway to evaluate the ability of *C. neoformans* to develop resistance to AEC5, but the data presented here indicate that AEC5 eliminates *C. neoformans* fungicidally, quickly, and with no immediately observed resistance or regrowth.

3.3 | Synergy

We hypothesized that AEC5 is antifungal through cell membrane disruption, like other antimicrobial peptides and peptoids, and completed the following studies to evaluate that hypothesis. AEC5 shows here a synergistic relationship with flucytosine, but is indifferent to fluconazole or amphotericin B (Table 1 and Figure S3). Evaluating new antimicrobial agents in combination with established antimicrobials to determine whether the combined compounds are synergistic, antagonistic, or indifferent can provide information regarding antifungal mechanism of action.^{33,34} These studies also indicate which synergistic combinations of compounds may have clinical value. Current treatment plans for patients with *Cryptococcal* infections rely on initial treatment with amphotericin B in combination with flucytosine before switching to an extended regimen of fluconazole.³⁵ Amphotericin B kills fungal cells by binding to ergosterol in the cell membrane, causing pores in the membrane that result in leakage of cytoplasmic components and cell death.³⁶ Flucytosine blocks pyrimidine metabolism in the cell, inhibiting DNA synthesis.³⁷ Fluconazole prevents lanosterol conversion to ergosterol, effectively preventing cell membrane formation.³⁸ Through our synergy testing we found a synergistic effect on potency when AEC5 was combined with flucytosine, with a fractional inhibitory concentration index (FIC_i) value of 0.23. An indifferent effect was observed when AEC5 was combined with fluconazole or amphotericin B, with FIC_i values of 0.75 and 1.50, respectively. FIC_i values were interpreted as follows: FIC_i < 0.5 synergistic; 0.5 ≤ FIC_i ≤ 4 indifferent; FIC_i > 4 antagonist.²¹ If two drugs work via different mechanisms synergistically then the drug combination should be more potent than the sum of the single drug's individual effects. Alternatively, reports indicate that non-synergistic combinations of drug (indifferent or antagonistic) may or may not function by the same antimicrobial mechanism.^{33,34} It is noteworthy to point out that the MIC of amphotericin B does not change when combined with AEC5, while the MIC of AEC5 drops by two-fold. While this could be standard variation, the method by which we measured synergy is more robust than the standard checkerboard assay in that, every drug concentration was done in triplicate for each experiment. Similarly, the MIC of fluconazole drops by four-fold when combined with AEC5, though the MIC of AEC5 only drops by two-fold. These data suggest that AEC5 has a different mechanism of action compared to flucytosine and does not nullify nor fully confirm the current hypothesis that AEC5 works through membrane disruption. Using a synergistic mechanism similar to the one between amphotericin B and flucytosine,³⁹ AEC5 may improve flucytosine potency by simply

permeabilizing the membrane, allowing flucytosine to easily traverse into the cell and exert its effect on its intracellular target. Flucytosine monotherapy is ineffective in the majority of cryptococcosis cases and is most often used in combination with amphotericin B, which presents complicating side-effects.⁴⁰ Therefore, AEC5 may serve as a valuable amphotericin B alternative for flucytosine combination antifungal therapy. Especially given the favorable toxicity profile of AEC5 presented below.

3.4 | Sorbitol Susceptibility

As previously stated, it is reported that antifungal peptides kill cells via membrane disruption, but can also elicit cell death through cell wall disruption or intracellular targets.¹⁵ Given that synergy data do not dispute the hypothesis that AEC5 works through cell membrane disruption, the possibility that AEC5 works through cell wall disruption was investigated by looking at the effects of sorbitol on MIC. Sorbitol acts as an osmotic protectant through stabilization of fungal protoplasts that form during cell wall disruption by cell wall targeting antifungals.^{41,42} Thus, if AEC5 works through cell wall disruption instead of cell membrane permeabilization, then the presence of sorbitol should increase the MIC of AEC5. To complete this study *C. neoformans* cells were treated with serial dilutions of either AEC5, amphotericin B, fluconazole, or flucytosine in media with or without 0.8 M sorbitol (Figure 2 and Figure S4). Linear regression analysis was used to compare drug efficacy with our without sorbitol and calculate statistical significance between the two group. Surprisingly, the addition of sorbitol significantly increased the susceptibility of *C. neoformans* cells to AEC5 by two-fold ($p < 0.0001$). The MIC without sorbitol was 12.5 $\mu\text{g/mL}$ while the MIC with sorbitol was 6.25 $\mu\text{g/mL}$. This change is small, but was consistent across multiple replicates. The addition of sorbitol also significantly increased the susceptibility of *C. neoformans* to fluconazole ($p = 0.0029$), but not amphotericin B ($p = 0.5053$) or flucytosine ($p = 0.8980$). These data suggest that AEC5 does not inhibit *C. neoformans* through cell wall disruption, however, reports indicate that sorbitol can act as a cell stressor towards an antifungal compound, as it does here, if that compound targets the high-osmolarity glycerol (HOG) response pathway.^{41,43,44} The mitogen-activated protein kinase (MAPK) Hog1 is at the heart of this pathway, which modulates a host of *C. neoformans* traits including stress-response, virulence, ergosterol biosynthesis, and fungal growth.^{43,44} This pathway and its regulatory components are complex and not fully understood. Significant further study will be required to confirm if and how AEC5 is targeting some component of the HOG pathway, leading to *C. neoformans* cell death.

3.5 | Efficacy in Infected Macrophages

To determine if AEC5 was efficacious in treating *C. neoformans*-infected macrophages, fungal burden assays in J774 macrophages were conducted with three different doses of AEC5: 0, 6 and 24 $\mu\text{g/mL}$. In one set of experiments where macrophages were lysed after 24 hours, there was less CFU/mL in macrophages treated with 24 $\mu\text{g/mL}$ vs. no AEC5 (Figure 3, $p < 0.005$). To determine if AEC5 was penetrating macrophage intracellular compartments, fungal burden assays were set up as above, but the extracellular supernatant was removed prior to lysing the macrophages. CFU/mL were then determined separately for both the extracellular and intracellular pools. In these experiments, there was a significant decrease in extracellular CFU/mL for both doses of AEC5 compared to no AEC5 (Figure 3,

0 vs. 24 $\mu\text{g/mL}$, $p < 0.005$ and 0 vs. 6 $\mu\text{g/mL}$, $p < 0.005$), but, there was no significant difference in the intracellular CFU/mL for any dose of AEC5 ($p = 0.9579$), suggesting that AEC5 is not effectively penetrating the intracellular compartments of the macrophages. We note that the observed trend in intracellular data makes it appear that AEC5 is reducing fungal burden within these cells, but the fact that this reduction is not significant likely means that this reduction in intracellular fungal burden is due to a reduction in extracellular fungal burden, thereby reducing the number of fungi available to infect macrophages.

3.6 | Half-Life

AEC5 has a long *in vivo* half-life of 20+ hours by multiple routes of injection. As part of the body's natural function, peptides are readily recognized and degraded by proteases,⁷ with average *in vivo* half-lives of less than 30 minutes, limiting their therapeutic value.⁴⁵ Extended half-lives *in vivo* have been reported for peptoids compared to peptides due to protease inability to recognize, bind to, and degrade peptoids.^{7,16,17,46} The *in vitro* stability of AEC5 to proteases in human serum was previously determined with essentially no degradation observed after 48 hours.¹¹ We therefore sought to evaluate the *in vivo* half-life of AEC5 in a murine model with the assistance of Frontage Laboratories, Inc (Figure 4). Two groups of mice were injected intraperitoneally or intravenously with 5 mg/kg or 1 mg/kg doses of AEC5, respectively. AEC5 content in blood draws at various time points was quantified by LC/MS/MS against a standard curve of peptoid. These data indicate that by intravenous injection AEC5 has a half-life of 25.3 hours, which we note was extrapolated beyond the final 24-hour time point. Data indicate that a moderate approximated volume distribution (3.7 L/kg) and low rate of clearance (2 mL/min/kg) (Table S1), in addition to the inherent proteolytic stability of peptoids is responsible for the observed long half-life. AEC5 administered by intraperitoneal injection yielded a half-life of 20.2 hours. Since peptoid was administered into the peritoneal cavity and measured in the blood, other valuable parameters provide information regarding AEC5 absorption through the peritoneum. Maximum absorption ($C_{\text{max}} = 1771 \text{ ng/mL}$) from the peritoneum was achieved at 1 hour ($t_{\text{max}} = 1 \text{ h}$) with a total drug exposure over time ($\text{AUC}_{\text{inf}} = 14,517 \text{ h} \cdot \text{ng/mL}$) (Table S2). This yielded a bioavailability (F) of 42%. Overall, these data indicate that AEC5 has excellent absorption out of the peritoneal cavity and a very long half-life regardless of the route of administration. Previous reports indicate that peptoids have very low oral bioavailability,¹⁶ however, given the promising pharmacokinetics of AEC5 future efforts will evaluate orally administered pharmacokinetics of this peptoid.

3.7 | Sub-Chronic Toxicity

A host of articles by our group and others can be found that report the hemolytic and *in vitro* toxicity of antimicrobial peptoids, which is generally quite low.^{11,18,47–49} However, there are only limited reports on the *in vivo* toxicity of peptoids, both of which evaluate 24-hour acute toxicity.^{13,50} We therefore sought to evaluate, to our knowledge for the first time, the longer term 28-day sub-chronic toxicity of a peptoid, namely AEC5. Given the AEC5 half-life results, we decided to inject three groups of mice intraperitoneally daily for 28 consecutive days with either 0, 10, or 50 mg/kg AEC5. A dose of 10 mg/kg was meant to mimic the *in vitro* MIC of AEC5 while 50 mg/kg was chosen because we hypothesized that this dose would likely be toxic to mice. Mice given this higher dose exhibited relatively immediate

agitation and lethargy that subsided within 1 hour. No other gross toxicity effects were observed for any of the dosing groups throughout the course of the study. After 28 days, mice were sacrificed, several organs (heart, lungs, liver, kidneys, and brain) collected and fixed, and histopathological analysis performed at the Vanderbilt Translational Pathology Shared Resource Core. AEC5 exhibited no observable histopathological toxic effects at either dose in the liver, kidney, heart, lungs and brain (Figure 5; Table S3). These data help to advance the characterization of AEC5 as a viable therapeutic option.

4 | CONCLUSIONS

AEC5 has proven to be a potent antifungal agent against *C. neoformans* and *C. gattii*, with modest efficacy against other microbial pathogens. Here we sought to advance the idea that AEC5 and perhaps similar antifungal peptoids identified in our lab can serve as viable therapeutic options for those dealing with deadly cryptococcal infections. AEC5 kills *C. neoformans* quickly with a dramatic reduction in viable fungi and complete killing within only a few hours. Additionally, the favorable absorption profile following intraperitoneal injection, long *in vivo* half-life, and non-existent sub-chronic toxicity of AEC5 further support the therapeutic tractability of this antifungal peptoid. We undertook several experiments in an attempt to elucidate the mechanism of action for AEC5, which we believe still remains relatively unknown, although data presented here do not nullify the hypothesis that AEC5 may work through membrane disruption. Other therapeutic properties currently being tested include the ability of *C. neoformans* to develop resistance to AEC5, permeability across the blood-brain barrier, and the ability of AEC5 to reduce fungal burden and extend survival in an animal model of *C. neoformans* infection. Should these data prove favorable, AEC5 would be poised for clinical evaluation as a therapeutic option for those dealing with cryptococcal infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Broad-spectrum efficacy against *C. gattii*, *C. albicans*, and the ESKAPE bacterial pathogens. (B) Killing kinetics of AEC5 against *C. neoformans* H99S indicate that this peptoid reduces fungal growth by nearly 50% in the first 30 minutes with elimination of viable fungi after 3 hours. Inset graph provides a more accurate view of the initial 3 hours of the complete 24 hour experiment. MIC = minimum inhibitory concentration; CFU = colony forming units.

Figure 2. Linear regression analysis of the dose response efficacy of AEC5, amphotericin B, fluconazole, and flucytosine against *C. neoformans* with or without 0.8 M sorbitol to evaluate the possible effect of AEC5 on the fungal cell wall. P-values calculated from linear regression analysis using JMP statistical software. Data for each drug concentration is represented by six data points (two biological replicates of technical triplicates). R² values are provided as a measure of line fit accuracy.

Figure 3.

In cellulo efficacy of AEC5 in *C. neoformans* infected J774 macrophages. AEC5 significantly reduces fungal burden in pooled macrophages and extracellularly, but not intracellularly. **p < 0.005. Data represent the average of two experiments each measuring eight replicates per test group. Error bars present the standard deviation.

Figure 4.
In vivo analysis of AEC5 stability by intravenous (IV; blue) or intraperitoneal (IP; orange) injection in three mice per group. Data indicate good absorption from the peritoneal cavity and long terminal half-lives regardless of injection route.

Figure 5. Histopathological tissue analysis following daily AEC5 injections over 28 days. Data indicate no observable sub-chronic tissue toxicity at either 10 or 50 mg/kg compared to mice treated with vehicle control.

Table 1.

Synergistic studies of AEC5 indicate that this peptoid is synergistic with flucytosine, but indifferent to fluconazole and the membrane disrupting antifungal, amphotericin B, suggesting that AEC5 (Drug A) has a different mechanism of action than flucytosine. Data depicted are the average of three independent experiments. MIC = minimum inhibitory concentration ($\mu\text{g/mL}$); FIC = fractional inhibitory concentration.

Drug Alone			Combination		
Drug B	MIC-A	MIC-B	MIC-A	MIC-B	FIC index
Amphotericin B	6.25	0.0156	3.13	0.0156	1.50
Fluconazole	3.13	8	1.56	2	0.75
Flucytosine	6.25	4	1.06	0.25	0.23