Mechanism of Action of Antifungal Peptoids

by

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Abstract

Due to the rise of drug resistant strains of fungal pathogens such as *Cryptococcus neoformans* and *Candida albicans*, there has been a need to identify new antifungal agents. In comparison to naturally produced antifungal peptides, antifungal peptoids, sequence-specific oligo-N-substituted glycines, mainly differ in structure, which prevents protease recognition giving higher bioavailability. Previous studies have shown that peptoids are effective fungicides. RMG8-8 and RMG9-11, two peptoids recently discovered in the Bicker Lab, have proven to be effective antifungal agents against *C. neoformans* and *C. albicans*, respectively. Reported here will be studies to determine the mechanism of action and other vital therapeutic properties of RMG8-8 and RMG9-11 using various biochemical and microbiological assays. Preliminary results of critical micelle concentration, the minimum concentration of a compound needed to form micelles, testing indicate that RMG8-8 as well as RMG9-11 do not exist as micelles at their minimum inhibitory concentrations, but rather function unimolecularly. Using a PAMPA assay, it was found that RMG8-8 is likely unable to penetrate the blood brain barrier (BBB). However, RMG9-11 demonstrated good permeability, indicating that it may be able to penetrate the BBB to treat dangerous neurological infections of fungi. Through a cytoplasmic membrane depolarization assay of RMG9-11 against *C. albicans*, it was discovered that the peptoid was able to depolarize the cell membrane in a concentration dependent manner. In future

work, assays will be conducted to further understand the mechanism of action of both peptoid compounds to address the rising concern of drug resistant strains of fungal pathogens.

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Terms and Definitions

1. Peptide: a molecule composed of a short chain of amino acids connected through peptide bonds.
2. Peptoid: a peptide mimic that differs by having side chains attached to the nitrogen atoms rather than the α-carbon atoms.
3. *In vivo*: tested within a whole living organism.
4. Minimum Inhibitory Concentration (MIC): the minimum concentration of a compound required to prevent 90% growth of a microbe.
5. Micelle: a collection of molecules that form a colloidal particle.

Introduction

Individuals can acquire a plethora of infections from other organisms. One specific type of organism that can cause infections in humans are fungi, which exist freely in nature. Fungi are eukaryotic organisms that can come in many forms such as yeast and mushrooms.[1] The fungi that cause diseases in organisms including humans are known as fungal pathogens. There are several types of fungal pathogens such as *Cryptococcus neoformans* and *Candida albicans*. *C. neoformans*, an intracellular pathogenic fungus, is present in the environment in bird feces and a major cause of pulmonary cryptococcosis.[1] *C. neoformans* mainly infects individuals that are immunocompromised such as people with HIV/AIDS, an immunodeficiency virus that attacks the human immune system, and can cause death in many cases.[2] By the same token, *Candida* species are the leading cause of fungal nosocomial infections with a mortality rate of 33%.[3] *C. albicans* is commonly found in the gastrointestinal tract of humans and can spread infections through direct or indirect contact between individuals. This fungal pathogen causes many harmful diseases including thrush and candidemia. The infections caused by both of these fungal pathogens, as well as, others give rise to serious health risks in humans.[2]

Due to fungal pathogens having high mortality rates and causing serious health issues in humans, there is a dire need for antifungal drugs that can improve the outcome of a patient. However, there are a few concerns with antifungal treatments. For one, there are only a few classes of antifungals used clinically.[4] With such a high mortality rate in humans caused by fungal pathogens, it is difficult to treat these infections and diseases using antifungal treatments since they are limited in availability. Second, these high mortality

rates have caused the limited number of available antifungal agents to be used extensively. This widespread use has created a rise in drug resistant strains among fungal pathogens.[4] Thus, alternative strategies are required for treatment.

Naturally occurring antimicrobial peptides (AMPs) are relied on by many organisms as the first line of defense against microbial infections.[4] Unfortunately, antimicrobial agents such as AMPs are not as effective when used clinically for treating fungal pathogens. This is due in part to the poor bioavailability of AMPs which actually limits their clinical use primarily to topical applications with only a few peptides being targeted for systemic delivery.[5] AMPs are also more useful in mechanisms against bacteria than fungi because, while bacterial and mammalian cell membranes are very different, fungal and mammalian cell membranes can be quite similar. One promising class of antifungals are peptoids, which are sequence-specific oligo-N-substituted glycines.[6] One of the main differences among peptides and peptoids is their structure. Peptoids differ in that the side chains are on the amide-nitrogen instead of the alpha-carbon as peptides.[6] Peptoids are not recognized by proteases in our bodies, which will prevent these peptidomimetics from breaking down.[6] This makes peptoids stable *in vivo* giving them excellent half-lives. Additionally, peptoids are more hydrophobic than peptides giving them better bioavailability and cell permeability.[5] Therefore, peptoids have the potential to be new, effective, and safe antifungals to use in clinical settings.

Two specific peptoids recently discovered in the Bicker lab are termed RMG8-8 and RMG9-11.[2,7] These two peptoids possess promising antifungal properties against *C. neoformans* and *C. albicans*, respectively, including excellent potency, low toxicity, and a rapid rate of fungal killing. (**Figure 1**). The structure of RMG8-8, a 5-mer, is smaller and

simpler than RMG9-11, which is a 7-mer. RMG8-8 has also been shown to be minimally toxic along with selectivity ratios ranging from 43 to 121 whereas RMG9-11 ranged from 29 to 114. The selectivity ratio is mathematically determined by dividing the value for a compound's toxicity by the value for a compound's antimicrobial efficacy, making a higher selectivity ratio advantageous. RMG8-8 had a minimum inhibitory concentration (MIC), the compound concentration needed to kill 90% of a microbe, of 1.56 μg/mL against *C. neoformans* and an efficacy of 25 μg/mL for *C. albicans*. By the same token, RMG9-11 was more effective against *C. neoformans* with an MIC of 3.13 μg/mL compared to 6.25 μg/mL against *C. albicans*. This is a strong MIC against *C. albicans* and RMG9-11 is being developed as an anti-*Candida* peptoid.

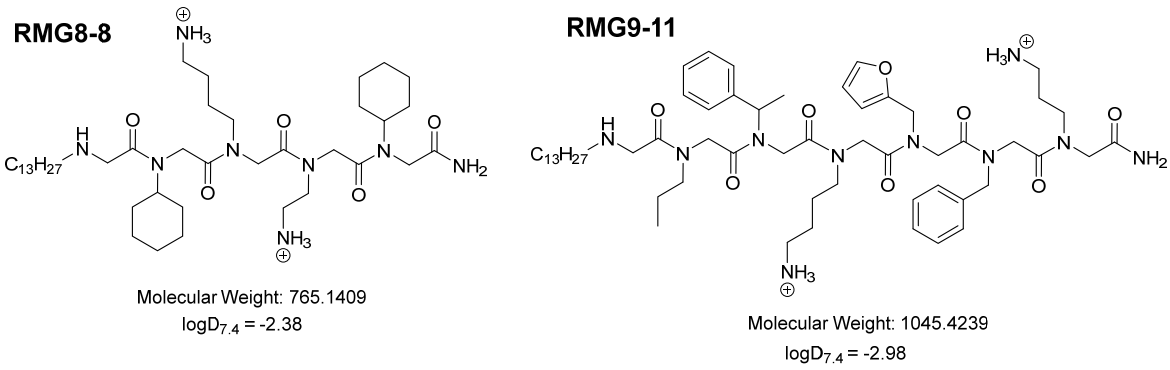


Figure 1. Structure of antifungal peptoids RMG8-8 and RMG9-11.

Previous literature stresses the mechanism of action of various antimicrobial peptoids in order to provide another option to treat antifungal infections. It has been previously reported by Barron et al. that lipophilic peptoids, similar to those discovered by our own group, primarily exist as micelles at their MIC, which directly affects how they

work to kill microbes although the exact mechanism is unknown.[6] If they exist as micelles, they are more readily disrupted when in the human body rather than if they existed as unimolecular compounds. Certain self-assembled morphologies are more promising towards antibacterial activity against ESKAPE pathogens such as peptoids exhibiting a high fraction of long, worm-like micelles rather than ellipsoidal assemblies.[6] Lipopeptoids are known to form micellular particles and the nanostructure formed is directly related to their activity and mechanism of action.[6]

Also contributing to the mechanism of action of antimicrobial peptides, previous literature suggests the induction of apoptosis.[8] According to Dong et al., an antimicrobial peptide, Bac8c, indicates a membrane-targeted mechanism of action via its ability to induce membrane perturbations.[9] Additionally, the mechanism of action could be further explained by the binding of antifungal peptoids to DNA, as it has been shown previously to cause mass flocculation of anionic biomolecules, leading to cell death.[10] Concerning the therapeutic properties of compounds, a neuroregenerative peptoid from amphibian neuropeptide exhibits the ability to cross the blood-brain barrier (BBB) while maintaining healthy morphology of rat primary cortical neurons.[11]

With promising antifungal properties, it is important to characterize the *in vitro* therapeutic properties of both RMG8-8 and RMG9-11. Using various biochemical and microbiological assays, detailed throughout this paper, the mechanism of action as well as other important pre-clinical properties of RMG8-8 and RMG9-11 against *C. neoformans* and *C. albicans* were explored. Herein we report that both peptoids do not exist as micelles at their minimum inhibitory concentrations, but rather function unimolecularly as indicated through a critical micelle concentration assay. Using a parallel artificial membrane

permeability assay (PAMPA), it was found that RMG8-8 is likely unable to penetrate the BBB. However, RMG9-11 demonstrated good permeability, indicating that it may be able to penetrate the BBB to treat dangerous neurological infections of fungi. Moreover, RMG9-11 can depolarize the cell membrane in a concentration dependent manner as exhibited through a cytoplasmic membrane depolarization assay against *C. albicans*. The successful completion of this research provides information that will continue the progress of developing peptoids as new, effective, and safe antifungals in clinical settings for treatment against fungal pathogens.

Methodology

Phase I: Synthesis and confirmation of RMG8-8 and RMG9-11

To begin, RMG8-8 and RMG9-11 were synthesized using the well-established submonomer method.[2] These molecules were synthesized on small beads made of polystyrene (resin). Fmoc deprotection is the first step to expose the free amine. Each building block of solid-phase peptoid synthesis was done using two reactions: bromoacylation and amination. Bromoacylaction activates bromoacetic acid with diisopropylcarbodiimide (DIC) to make the OH of the carboxylic acid a good leaving group. To complete the bromoacylation the activated carboxylic acid reacts with an amine on the resin to form a new amide by nucleophilic acyl substitution. Amination was then completed by adding in an amine with the desired side chain, allowing the amine to displace the bromine attached to the resin by nucleophilic substitution. These two reactions were repeated for each building block of RMG8-8 and RMG9-11 along with a colorimetric test for amines, known as a Kaiser test, to confirm each synthetic step. Once all building blocks were added, trifluoroacetic acid (TFA) was added to cleave the peptoid from the resin. Next, the peptoids were purified using reverse-phase high performance liquid chromatography (RP-HPLC). Mass spectrometry was then done to confirm the structure of the peptoid. The peptoid was then dried down using the rotary evaporator and the lyophilizer. Both peptoid syntheses were successful. The percent yield of RMG8-8 was 27.4% (36.1 mg) and the yield of RMG9-11 was 35.1% (58.9 mg).

Moreover, MIC testing was conducted to confirm the antifungal activity of both peptoids against *C. albicans*. To begin, a yeast peptone dextrose (YPD) plate was streaked with a frozen stock of *C. albicans* for isolation and incubated for 48 hours. A 20 mg/mL stock solution was made for RMG9-11 and RMG8-8. A total of 7 two-fold serial dilutions of both peptoids were made in water at 100x concentration starting at 50 mg/mL and ending at 0.78 mg/mL in addition to a blank control consisting of only sterile water. A 5 mL solution of 0.85% saline was inoculated with 1-2 colonies and vortexed for 30 seconds. The optical density of the solution was checked at 530 nm to ensure we received an OD_{530} of 0.15 to 0.25. Next, a 1:100 solution of Roswell Park Memorial Institute-3-(N-Morpholino) propanesulfonic acid (RPMI-MOPS), a basic medium buffer, and cells were prepared by adding 0.100 mL of saline + cells to 9.9 mL of RMPI-MOPS and vortexed briefly. Then, a 1:20 solution was prepared by adding 0.5 mL of the 1:100 solution to 9.5 mL of RPMI-MOPS and immediately vortexing before use. Using a 96-well plate, 198 µL of 1:20 RPMI-MOPS in addition to cells in each well. Next, 2 µL of 100x peptoids compounds, the vehicle control, RPMI-MOPS, and a 2 µg/mL concentration of the positive control, amphotericin B (AmpB), were added in triplicate. The plate was then incubated at 37 °C for 24 hr. Once incubated, the plate was read manually for visible growth. RMG9-11 indicated an MIC of 25 µg/mL and RMG8-8 exhibited 12.5 µg/mL against *C. albicans*.

Phase II: Critical micelle concentration assay

A critical micelle concentration assay using 8-anilino-1-naphthalene sulfonic acid (ANS) was designed and utilized in order to determine the concentration with which RMG8-8 and RMG9-11 form micelles in relation to their MIC levels against *C. albicans*.

Each peptoid was also compared to a CMC test of a control surfactant, Tween-20, that has indicated high fluorescence with ANS.[12] The CMC is the minimum concentration of a compound needed to form micelles. These experiments were run in triplicate on different days using a nonsterile black 96-well plate. Two-fold serial dilutions of all compounds were created in sterile water at 13 different concentrations starting at 800 μM and ending at 0.39 μM. A blank control was created consisting of only solvent, sterile water. Each peptoid and control were pipetted as technical duplicates of 97 μL in sterile water and 3 μL of ANS was added followed by a brief incubation, one hour, at room temperature before measuring the fluorescence of each well using a plate reader. The excitation was set at 370 nm, and the emission was used at 490 nm for the plate reader. Fluorescence is directly related to the formation of micelles, which are cage-like structures with pockets consisting of a hydrophobic environment. ANS fluoresces when inserted into the hydrophobic environment of a micelle but does not fluoresce in aqueous solutions. CMC is essentially determined by the point which fluorescence rises from the baseline of the plotted data.

Phase III: Parallel artificial membrane permeability assay.

Using the parallel artificial membrane permeability assay kit (Bioassay Systems, PAMPA-096), the PAMPA assay was carried out in order to determine if both peptoids are capable of crossing the BBB, which is mimicked.[13] A BBB lipid solution in dodecane was prepared by suspending 5 μL of dried brain lipids in 600 μL of dodecane before adding this lipid mixture to the 96-well plate to form the simulated BBB membrane. Stock solutions of peptoid or manufacturer control compounds were made in dimethyl sulfoxide (DMSO) at 10 mM, which allowed testing of both peptoids. The three stock solutions of

RMG8-8 and RMG9-11 were made as two-fold serial dilutions starting at 0.5 mg/μL and tested in triplicate. Using a 96-well plate, 500 μL of RMG8-8 and RMG9-11 stock solutions were pipetted in addition to 200 μM of equilibrium standards and the blank control, phosphate buffered saline (PBS), in the donor plate. These experimental variables were tested in duplicate to ensure the activity is consistent with both concentrations' replicates. The plate was placed into an acceptor plate containing 300 μL of PBS and then incubated for 18 hours at 37 °C. Subsequently, the liquid from a second 96-well plate, known as the acceptor plate, was collected, and analyzed to determine the amount of peptoid that crossed the simulated BBB membrane. Using a UV-Vis spectrophotometer and 300 μL quartz microcuvettes, absorbance was determined at 214 nm, the maximum absorbance of the peptoids, in the acceptor solution. The high and medium controls' absorbance was measured at 250 nm, whereas the low control's absorbance was measured at 270 nm. The blank control, PBS, was measured at 214, 250, and 270 nm. The permeability rate was determined mathematically by using the following equation: $P_e = C\,x - \ln\left(1 - \frac{OD_a}{OD_e}\right)$ cm/s. OD_A refers to the absorbance of acceptor solution minus the blank, OD_E is the absorbance of the equilibrium standard minus the blank, and C= 7.72 $x10^{-6}$ using an 18-hour incubation. The P_{app} (cm/s) values of each peptoid, PBS, high, medium, and low controls were determined. This assay will ultimately quantify the amount of RMG8- 8 and RMG9-11 that can cross the membrane in a solution.

Phase IV: Cytoplasmic membrane depolarization assay of RMG9-11 against *C albicans*.

A cytoplasmic membrane depolarization assay of RMG9-11 was carried out using DiSC3(5) dye also known as 3,3'-Dipropylthiadicarbocyanine Iodide. This dye can be used to monitor cell membrane potential and determine if the membrane has been depolarized by antifungal peptoids, contributing to our knowledge of the peptoids' mechanism of action.[10] To begin, a 5 mL culture of *C. albicans* was prepared in YPD and left in a rocking incubator overnight at 35 °C. Then, the overnight culture was used to prepare 10 mL of $1.6x10^7$ cells/mL in supplemented PBS. Supplemented PBS was made using 0.4 g NaCl, 0.77 g NA_2HPO_4, 0.12 g KH_2PO_4, 1.8 g dextrose, 3.8 g KCl, and 500 mL deionized water. The supplemented PBS had a pH of 7.4. After overnight incubation, the OD_{530} of 1 mL overnight culture using a spectrophotometer set to 500 nm. The goal was an OD_{530} that was within the range of 0.8-0.9 to give the desired cell concentration. Next, the overnight culture was centrifuged for 5 mins at a speed of 4000 rpm to pellet the cells. Then, the YPD supernatant was removed and supplemented PBS was added to give a solution of $1.6x10^7$ cells/mL. Using a black 96-well plate, 89 μL of cell suspension was added in addition to 1 μL of a 50 μM stock of DiSc3(5) dye to yield a final concentration of 0.5 μM dye. The plate was covered in aluminum foil and left to incubate for 30 mins at room temperature in order to equilibrate the dye in the membrane. After the incubation, 10 μL of each peptoid was added in two-fold serial dilutions starting at 100 μg/mL at 10x stock into appropriate wells. Additionally, 10 μL of vehicle control, melittin, and positive control were added to the wells in triplicate. The positive control, Triton X-100, was used at 1% concentration. Melittin was tested at three two-fold serial dilutions starting at 100 μg/mL. Once all

samples were added to the wells, the plate was incubated at room temperature for 30 mins to equilibrate. Subsequently, the plate's fluorescence was measured with an excitation of 622 nm and an emission of 670 nm continually using a plate reader. The fluorescence intensity exhibited by each compound was directly related to the depolarization or permeability of the cell membrane.

Results and Discussion

R.1 Compound verification

RMG8-8 and RMG 9-11 were each synthesized using the solid-phase submonomer approach as outlined in **Figure 2** and **Figure 4.**, respectively the structure was confirmed using mass spectrometry. The expected mass of RMG 8-8 was 763 Da. As shown in **Figure 3**, the M+H signal for RMG8-8 that was synthesized came out to be 764 Da. The percent yield of RMG8-8 was 27.4% (36.1 mg). According to **Figure 5**, RMG9-11 had an expected mass of 1043 Da, which was confirmed by the M+H signal on the mass spectra of 1044 da. Both peptoid syntheses were successful. The percent yield of RMG9-11 was 35.1% (58.9 mg).

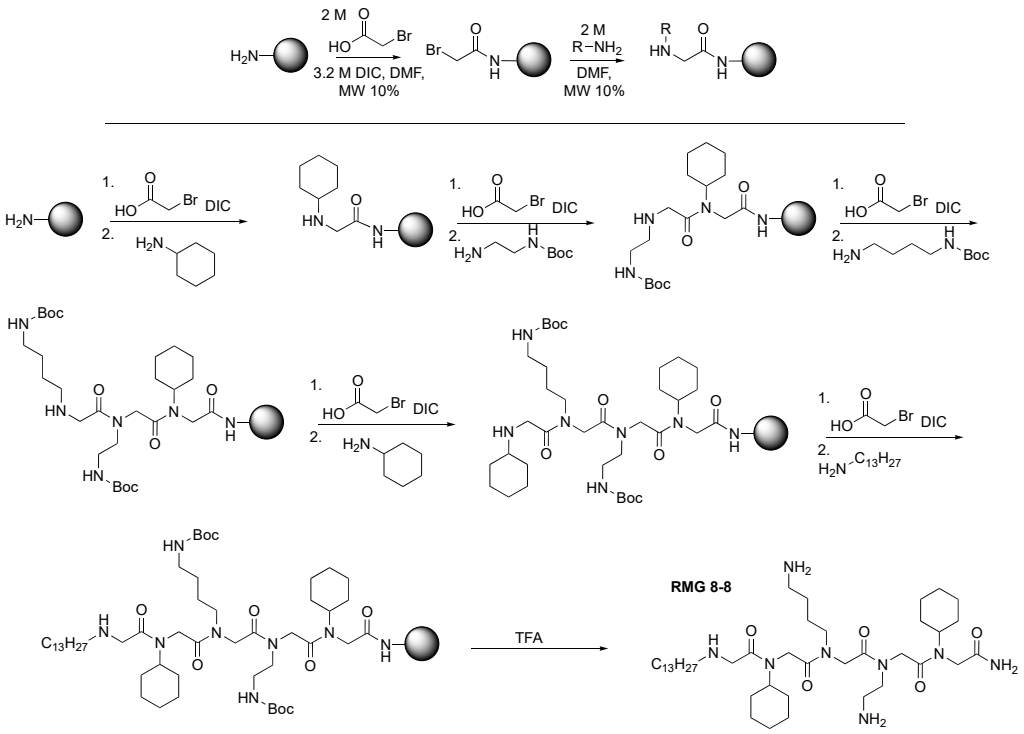


Figure 2. Solid-phase synthesis of antifungal peptoid, RMG8-8, using the submonomer method.

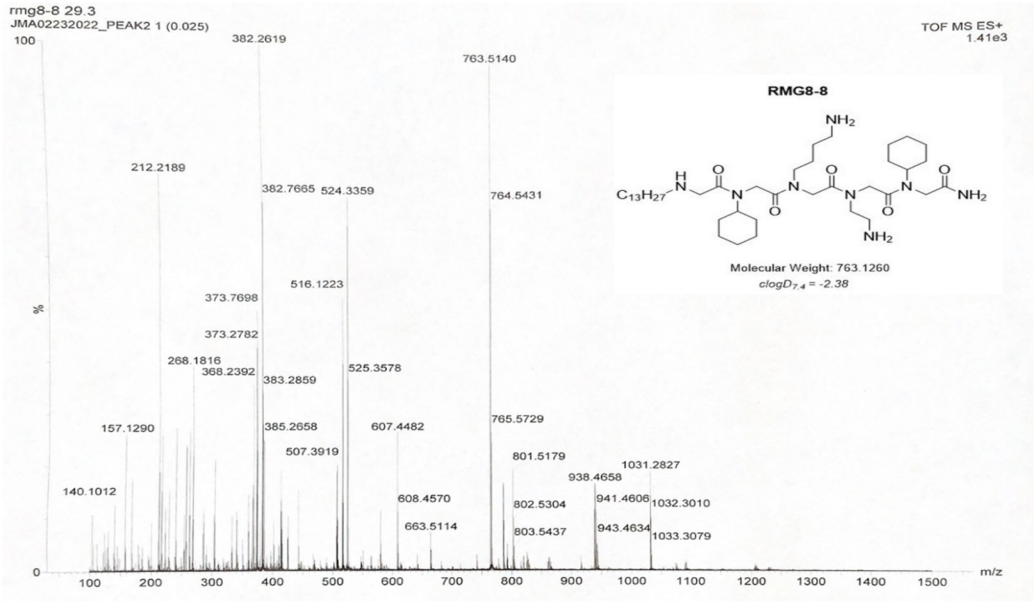


Figure 3. Mass spectra of RMG8-8 peptoid showing the expected mass of 763 Da. Other signals are attributed to fragmentation or carryover from previous MS analysis.

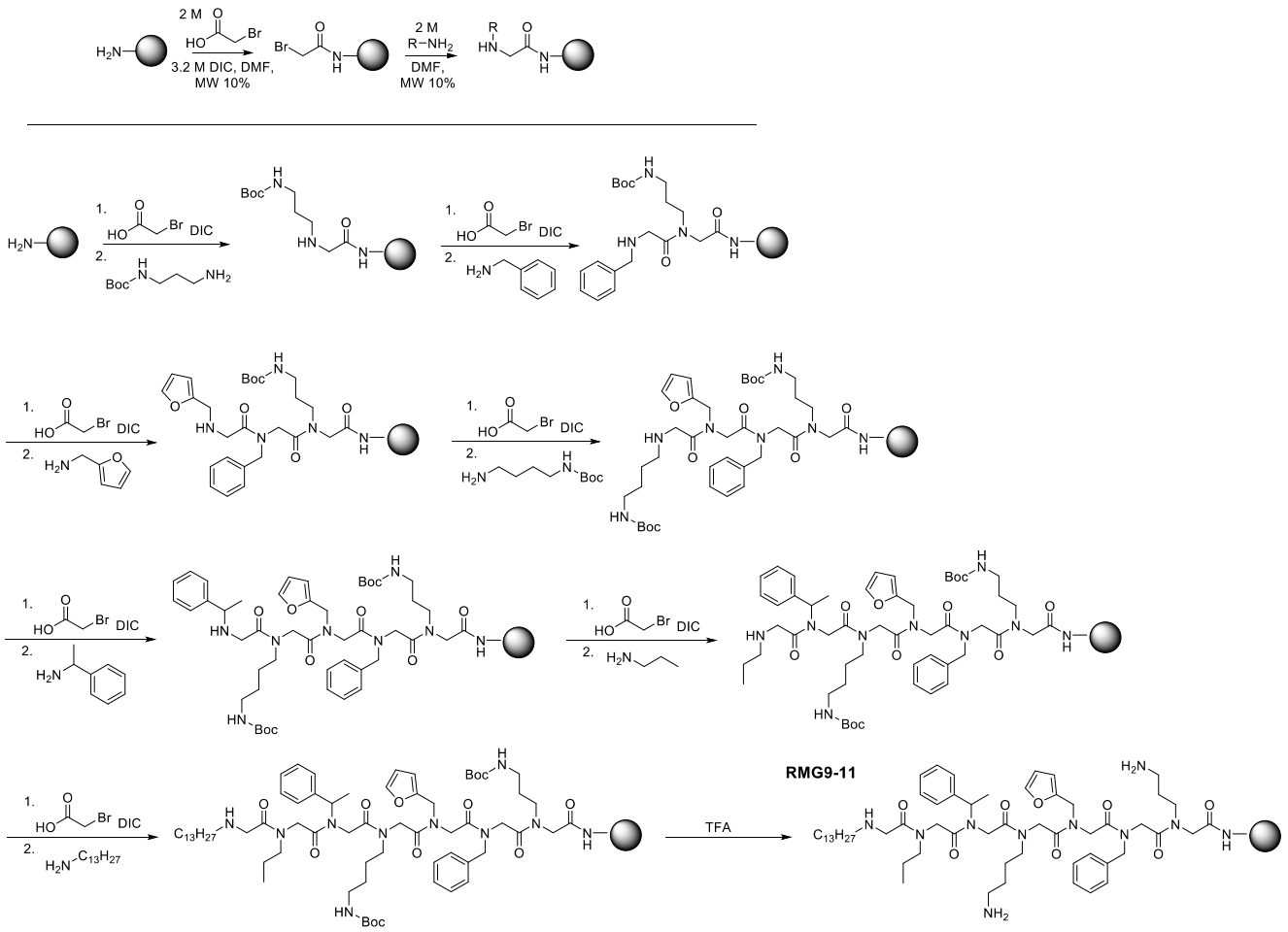


Figure 4. Solid-phase synthesis of antifungal peptoid, RMG9-11, using the submonomer method.

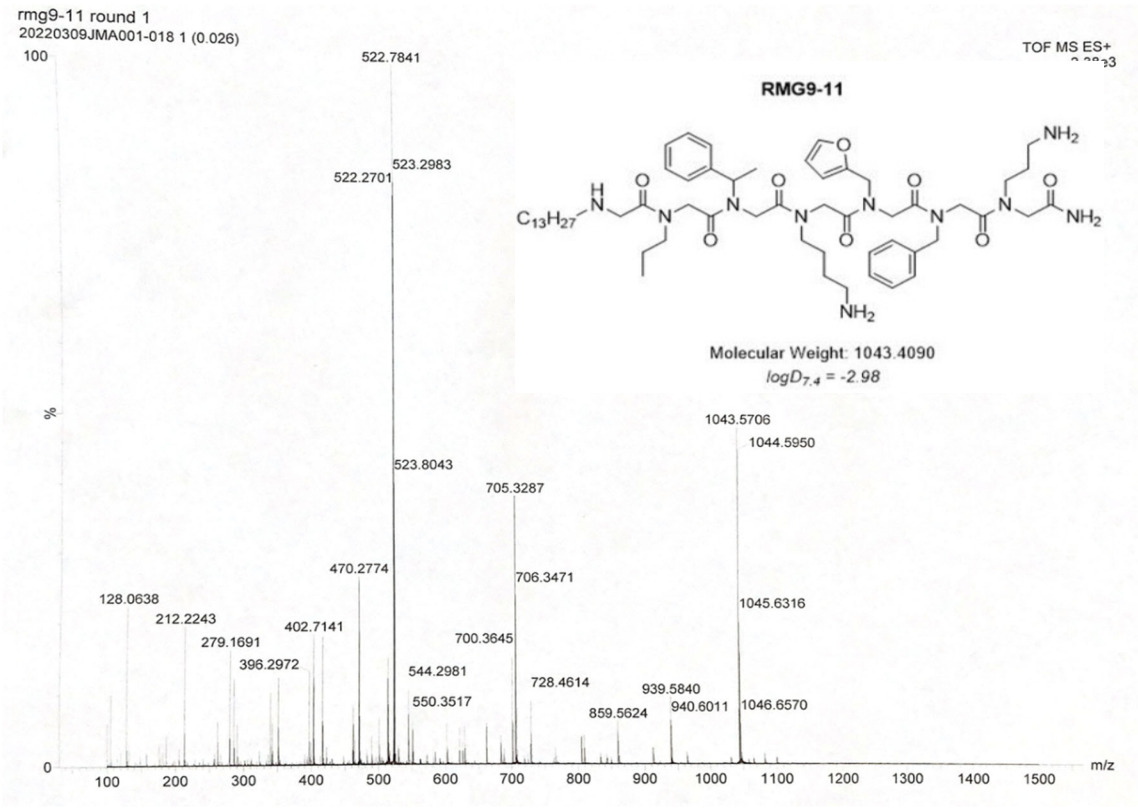


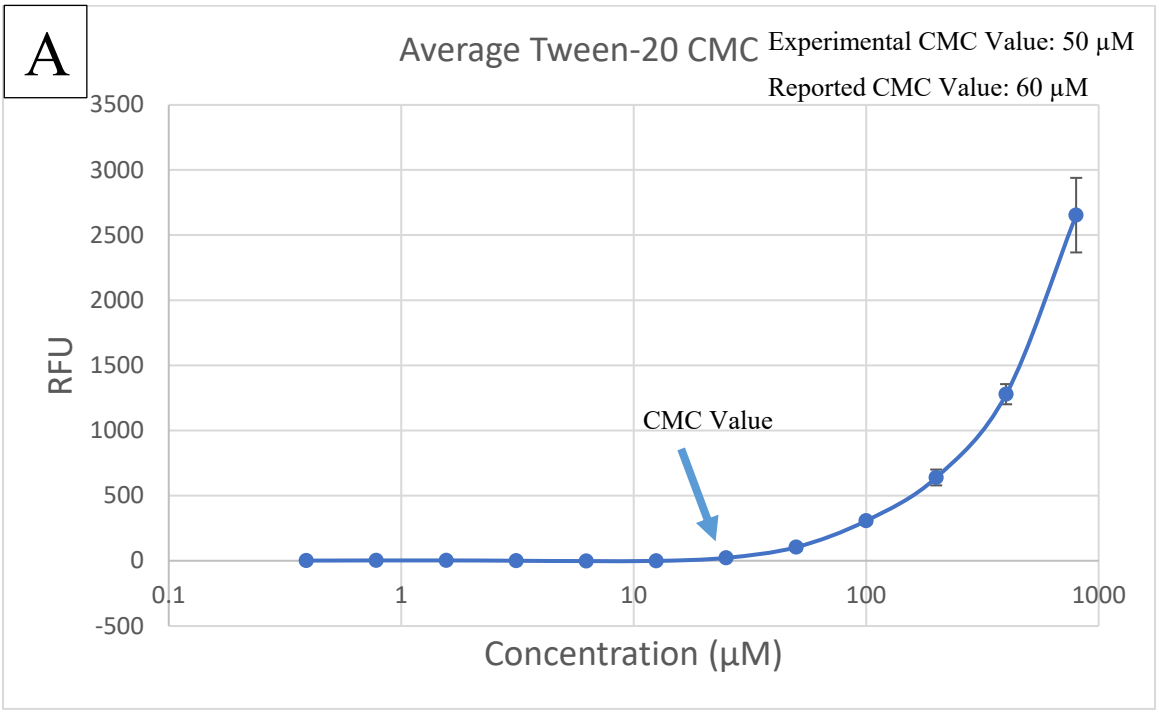
Figure 5. Mass spectra of RMG9-11 peptoid showing the expected mass of 1043 Da. Other signals are attributed to fragmentation or carryover from previous MS analysis.

Furthermore, MIC testing was done to confirm the antifungal activity of RMG8-8 and RMG9-11 against *C. albicans*. This test ultimately measures the lowest compound concentration resulting in greater than 90% inhibition of fungal growth consistently on multiple days. Two-fold serial dilutions of each peptoid were prepared in triplicate along with vehicle (negative) and AmpB (positive) controls. The MIC of RMG8-8 and RMG9-11 was 25 µg/mL and 12.5 µg/mL, respectively, which is similar to the reported MIC values for these compounds against *C. albicans*, validating the compounds that were synthesized.[2,7]

R.2 Critical Micelle Concentration Assay

Testing was done to determine the critical micelle concentration (CMC) against both RMG8-8 and RMG9-11 and a control surfactant (Tween-20). This tested for the minimum concentration at which micelles form, which may affect how peptoids exert antimicrobial activity.[12] When analyzing CMC graphs (**Figure 6**), the CMC is defined as the concentration where fluorescence rises from the baseline. To measure the concentration level at which each peptoid formed micelles, ANS was added into each well of the 96-well plate. ANS is a hydrophobic dye that is non-fluorescent in aqueous solutions but fluoresces when inserted into the hydrophobic environment of a micelle. Tween-20 had a CMC of between 50 and 100 μM, similar to the reported CMC value for Tween-20 of 50 μM (**Figure 6A**).[12] RMG8-8 had a CMC of 12.5 μg/mL (**Figure 6B**). RMG9-11 had a CMC of 25 μg/mL (**Figure 6C**). Additionally, RMG8-8 and RMG9-11 had a much greater fluorescence intensity when compared to the Tween-20 by ~10 fold. This increase in fluorescence for the antifungal peptoids could be due to increased hydrophobicity in the micelles for RMG8-8 and RMG9-11, compared to Tween-20. Tween-20 has a shorter tail than RMG8-8 and RMG9-11, which could explain why they have a much larger fluorescence compared to the control. The average CMC results from three trials completed with Tween-20 (control) and antifungal compounds RMG8-8 and RMG9-11 are illustrated in **Figure 6**. When compared to the MIC concentrations, the concentration at which the antifungal peptoids form micelles are actually higher than the concentration at which they

inhibit growth of *C. albicans* or *C. neoformans* although the concentrations are fairly close. This indicates that the peptoids are likely in a non-micellular, unimolecular state when interacting with fungi at their MIC concentration. This contradicts some previous studies performed by Barron et al. as they suggested that lipohilic peptoids, such as RMG8-8 and RMG9-11, primarily exist as micelles at their MIC value.[6] This led these researchers to conclude that forming micelles aids in antimicrobial activity.[6] However, both of the peptoids studied here exist as unimolecular structures at their MIC, which could potentially provide more promising therapeutic properties *in vivo* where micelles could be readily disrupted by biological lipids or other conditions.



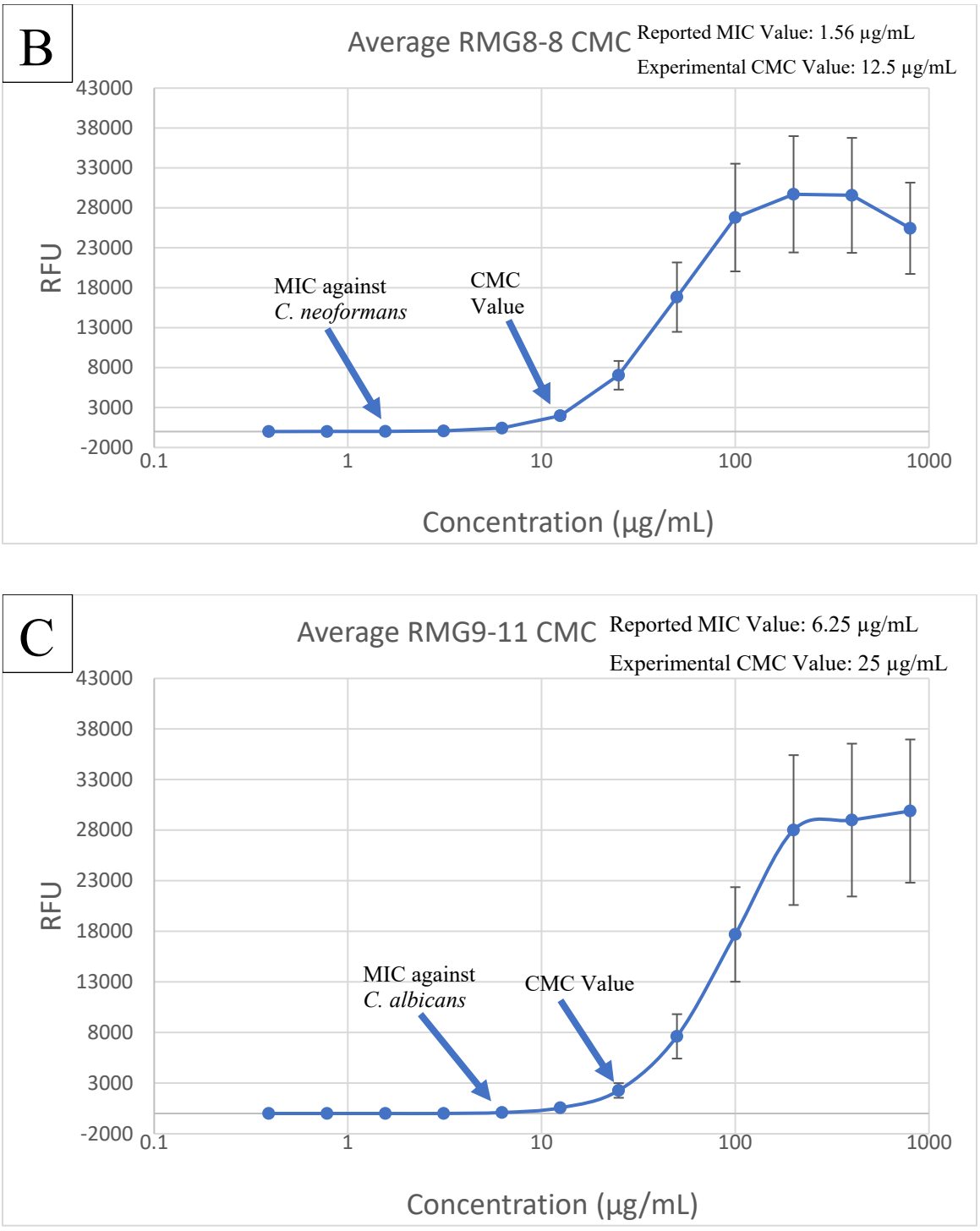


Figure 6. Data plotted are average fluorescence from the ANS assay versus compound concentration. Error bars represent standard deviation between the trials. Arrows indicate CMC and MIC value. A) Tween-20 data. B) RMG8-8 data. C) RMG9-11 data.

R.3 Parallel Artificial Membrane Permeability Assay

The Parallel Artificial Membrane Permeability Assay (PAMPA) was utilized to evaluate blood brain barrier (BBB) permeability with RMG8-8 and RMG9-11. This assay aided in determining if these peptoids will be useful as potential drug candidates against chronic brain infections by quantifying the amount of each peptoid that could cross an artificial membrane mimicking the BBB. When beginning this stage of research, it proved challenging to determine a method to detect and quantify peptoids in solution given that they only absorb light in the UV region. Initially, a 96-well plate was used to determine the absorbance of each peptoid. However, the plastic of the plate absorbed UV light when placed into a UV-Vis plate reader, which made it difficult to accurately see the absorbance of each peptoid. Thus, 300 μL quartz microcuvettes were used because they do not absorb UV light, allowing the measured absorption to be derived from solely the peptoids. Peptoid absorbances were measured at 214 nm using a UV-Vis spectrophotometer and absorptions subtracted from values. Standard curves were generated with varying concentrations of each peptoid in triplicate, resulting in the expected linear relationship between absorbance and peptoid concentration that will allow us to quantify the peptoid in the PAMPA assay (**Figure 7**). According to **Figure 7**, 0.5 mg/mL of RMG8-8 indicates an apparent permeability constant (P_{app}) value of 3.42×10^{7} cm/s. On the other hand, 0.5 mg/mL of RMG9-11 shows a P_{app} value of 6.99×10^{6} cm/s. Generally speaking, compounds with P_{app} values between 1.1×10^{-6} cm/s and 1.0×10^{-5} cm/s are regarded to have greater pharmakokinetic characteristics.[13] The other concentrations tested did not signify any activity indicating that peptoid permeability across the BBB is concentration dependent.

This is beneficial as drugs often need to cross cell membranes to reach their target of action. *C. neoformans* and *C. albicans* infections can progress on to meningitis or other infections of brain tissue. Therefore, it is important to study if these peptoids could cross the BBB. Subsequently, RMG8-8 did not exhibit an ability to cross the BBB, which could be due to the specific side chains on the peptoids. However, RMG9-11 indicated that it can cross the mimicked membrane, which could indicate that its side chains allow for more membrane permeability. This data both supports and contradicts previous literature. A neurogenerative peptoid from amphibian neuropeptide (sequence SLKP) has shown the ability to penetrate the BBB, much like RMG9-11.[11] This peptoids exhibited few structural similarities to RMG8-8 and RMG9-11 indicating it is difficult to correlate structural features to BBB permeability. On the other hand, RMG8-8 was not able to cross the BBB as previous peptoids have indicated. This allows us to better grasp the therapeutic potential of both RMG8-8 and RMG9-11 to treat chronic brain infections.

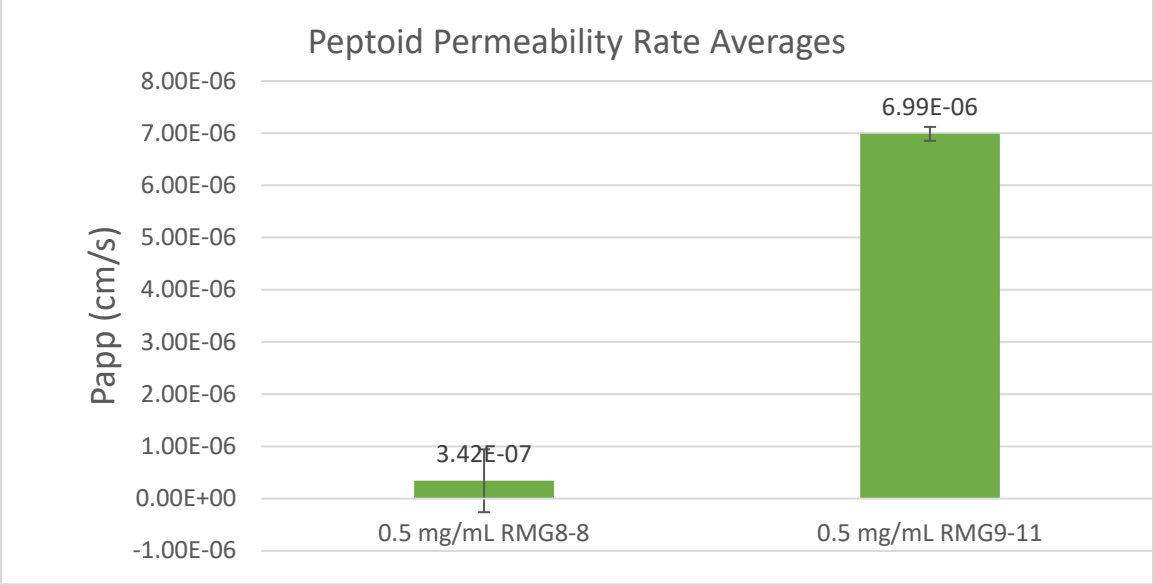


Figure 7. Peptoid permeability rate averages at the greatest concentration tested, 0.5 mg/mL, measured in Papp (cm/s). Bars represent both peptoids, RMG8-8 and RMG9-11, alone with error bars to represent standard deviation between the trials.

R.3 Cytoplasmic Membrane Permeability Assay

In order to monitor cell membrane potential, a cytoplasmic membrane depolarization assay was used. The cell membrane contains a charged gradient of sodium channels. Depolarization is due to a noticeable increase in membrane potential opening of sodium channels in the cell membrane.[10] Depolarization of these sodium channels indicates a disturbance in the membrane, which is indicated by increased fluorescence in this assay by utilizing DiSC3(5) dye. This dye is particularly sensitive to membrane depolarization. Testing began on RMG9-11 using this assay against *C. albicans* because this peptoid has been proven more effective against this fungal agent. No work was done here with *C. neoformans* because the slow growth and large capsule of this microorganism would have presented logistical challenges to generating robust, confident results. As shown in **Figure 8**, RMG9-11 depolarized the cell membrane significantly in a concentration dependent manner. The peptoid's fluorescence ranged from 33.1% at 6.25 mg/mL to 69.8% at 100 mg/mL against *C. albicans*. The detergent Triton X-100 was used as a positive control and was set as 100% membrane depolarization. The bee venom peptide melittin is a known membrane disruptor/depolarizer and was used as an additional positive control. Similar to RMG9-11, melittin indicated membrane depolarization in a concentration dependent manner, although not as much as the peptoid. Melittin's percent depolarization ranged from 40.3% at 25 mg/mL to 50.9% at 100 mg/mL. The vehicle control contained 1 μL of

DiSC3(5) dye in addition to 89 μL of supplemented PBS. The vehicle control exhibited a percent depolarization of 10.8%, which was significantly lower than the Triton X-100, melittin, or RMG9-11. The average percent depolarization results from three trials completed with Triton X-100 (detergent) and RMG9-11 are illustrated in **Figure 8**. This indicates that the antifungal peptoid, RMG9-11, does in fact disturb the cell membrane in a concentration dependent manner as shown by its greater fluorescence when compared to the vehicle control as well melittin. This aligns with unpublished data from our lab showing that RMG9-11 disrupts calcein loaded liposomes designed to mimic fungal cell membranes in a concentration dependent manner. Previously, it has been reported that some antimicrobial peptides, such as Bac8c, have a mechanism of action that specifically targets membranes.[9] This compares to the results of this assay indicating that RMG9-11 was able to disturb the cell membrane in its activity. This assay has aided in determining an antifungal mechanism of action for RMG9-11 by adding in DiSC3(5) dye.

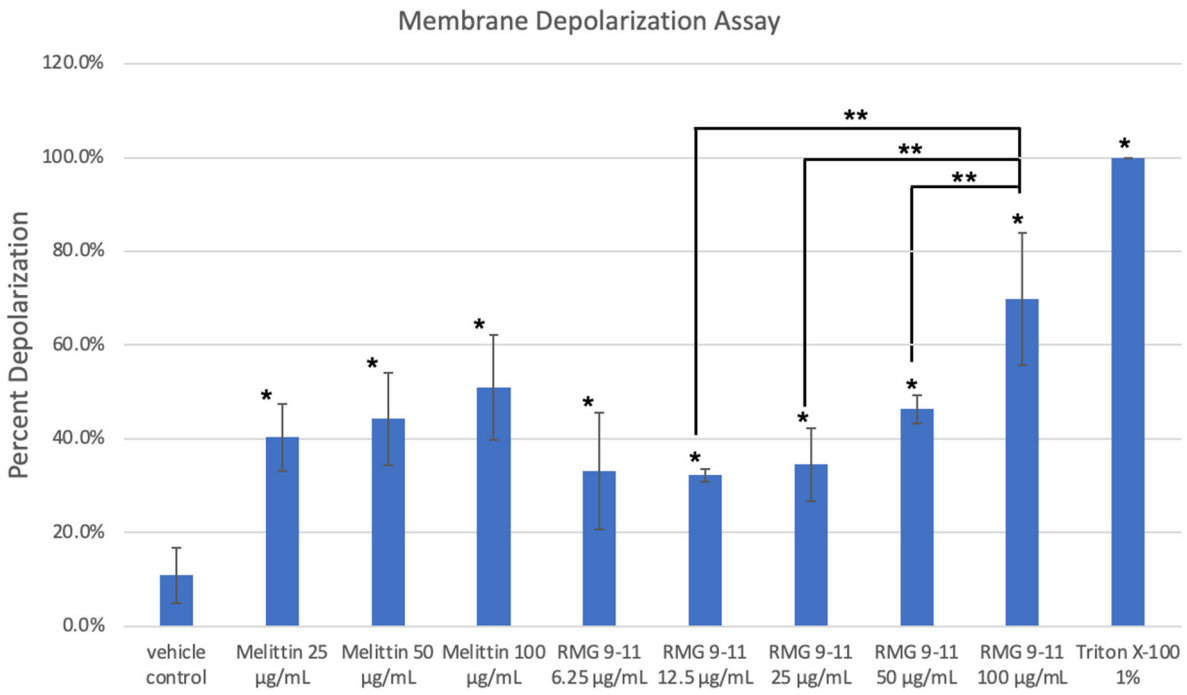


Figure 8. Data indicates membrane depolarization assay data averages using DiSC3(5) as percent depoarlization versus compound concentration. Error bars represent standard deviation between the replicates of melittin, controls, RMG8-8 and RMG9-11. *P-value < 0.5 relative to vehicle control. **P-value <0.5 between peptoid treatment groups.

Conclusions and Future Plans

The purpose of this project was to further explore the mechanism of action and therapeutic properties of two antifungal peptoids discovered in the Bicker Lab, RMG8-8 and RMG9-11, using various biochemical and microbiological assays. Characterizing the *in vitro* properties of these two antifungal agents has aided in determining if they should advance to pre-clinical testing as antifungal agents to treat patients with various fungal pathogens such as *C. albicans* and *C. neoformans*. Both peptoids initially exhibited promising antifungal properties against a particular fungal pathogen. RMG9-11 was more effective against *C. albicans*, whereas RMG8-8 was more effective against *C. neoformans*.

It was determined that neither RMG8-8 nor RMG9-11 existed as micelles in their MIC values through a critical micelle concentration assay. This points to both peptoids functioning unimolecularly rather than existing as micelles when killing fungi. This will allow future studies to grasp how these molecules exist within certain environments within the human body. Through a PAMPA assay, it was discovered that RMG8-8 was unable to penetrate the mimicked BBB. However, RMG9-11 did exhibit the ability to penetrate the BBB. With this characteristic, RMG9-11 has the potential to treat cryptococcal meningitis, chronic *C. albicans* in the brain, or other brain infections. Moreover, RMG9-11 was able to effectively depolarize the cell membrane in a concentration dependent manner as shown through a cytoplasmic membrane permeability assay against *C. albicans*. This will allow it to potentially disrupt the cell membrane effectively if used to treat fungal diseases with *C. albicans*.

Furthermore, these studies as well as future work on the mechanism of action of these two antifungal peptoids will aid in discovering new, safe, and effective antifungal

agents to treat various fungal diseases. Due to the rapid increase of drug resistant strains of fungal pathogens, this could potentially save numerous lives by implementing RMG8-8 as well as RMG9-11 in clinical settings. In conclusion, this project has provided valuable data concerning the mechanism of action and therapeutic properties of both RMG8-8 and RMG9-11 that can be implemented in pre-clinical trials to develop treatments for fungal pathogens such as *C. albicans* and *C. neoformans*.

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