Antifungal Activity of Novel Macrocycle Derivatives

Jacob Lieberman[1], Tim Hubin[2], and Karen L. Wozniak[1]
Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, OK[1]
Chemistry and Physics Department, Southwestern Oklahoma State University, Weatherford, OK[2]

Lew Wentz Foundation

Abstract

Cryptococcus neoformans is an opportunistic fungal pathogen that causes cryptococcosis. After inhalation, the organism disseminates to the brain, where it causes cryptococcal meningitis. Annually, approximately 225,000 immunocompromised individuals develop cryptococcal meningitis, resulting in over 181,000 deaths. To treat these patients, there are only four classes of antifungals currently available and these options are toxic at high concentrations and ineffective. In addition, fungal pathogens are becoming resistant to existing antifungals. In the current study, we are testing the antifungal activity of macrocycle compounds against *C. neoformans*. These compounds have been shown to be active against many other fungal pathogens, allowing us to hypothesize that these compounds would exhibit antifungal activity against *C. neoformans.* We first tested 12 macrocycle compounds against *C. neoformans* strain H99. After incubating each compound at different concentrations with H99, the minimum inhibitory concentration (MIC) was calculated. Compounds exhibiting antifungal activity were then tested for cytotoxicity using the mouse macrophage cell line J774.A. Effective, non-toxic compounds were then assayed with existing antifungal drugs in checkerboard assays to determine possible synergistic or antagonistic activity. The majority of the compounds showed antifungal activity. Of these compounds, 6 were non-toxic. Initial checkerboard assays have shown synergistic and indifferent interactions between the tested compounds and antifungal drugs. Future studies will focus on identifying the mechanism of action of these compounds. Confocal and electron microscopy will be used to identify changes in fungal cell wall & membrane morphology, and screening of mutant libraries will be used to identify mutants resistant to these compounds.

Introduction

Cryptococcus neoformans is an opportunistic fungal pathogen found all over the world, primarily in soil. Immunocompromised people become infected with cryptococcosis after inhaling the fungus. The pathogen can spread to the central nervous system, where it causes cryptococcal meningitis, which is a life-threatening illness. Yearly, over 220,000 people with HIV/AIDS are infected with the fungus and around 181,000 die. This is an extremely high mortality rate of 82%. Current antifungal drugs are often ineffective, highly toxic, and fungi are becoming resistant to them. Along with the negatives of the drugs, there are also very few of them, with only four currently approved classes. Finding new therapies is essential in combating the rapidly evolving fungi.

Methods

Strain and Media: *Cryptococcus neoformans strain* H99 was grown in yeast peptone dextrose (YPD) for 18 hours at 30˚C in a shaking incubator. After incubation, the cells were washed 3x with phosphate buffered saline (PBS). The fungal cells were then diluted with PBS and trypan blue, and cells were counted using a hemocytometer. Dilution calculations were performed and the remainder of the fungal tube was diluted in order to get the proper concentration for experimentation.

Compounds: The compounds used in this experiment were created and patented by Dr. Tim Hubin at Southwestern Oklahoma State University. The compounds are all derivatives of macrocycles (Figure 1).

Minimum Inhibitory Concentration (MIC) Assay: H99 was incubated with the macrocyclic compounds with the goal of determining if any of the compounds could inhibit growth of *C. neoformans.* H99 was diluted to a concentration of 0.5×10^3 cells/ml. In a 96-well plate, 100 µL of media was placed into each well. The final column acted as the negative control. Compounds were added to the first column at a concentration of 100 µg/mL and were serially diluted across the plate. 100µL of the diluted H99 was added to all of the experimental wells and column 11, which acted as the positive control. Plates were then incubated at 35˚C for 48 hours. Following incubation, the plates were read using a BioTek Gen5 Microplate reader. This study was conducted in triplicate with two media, YNB + Glucose and RPMI-MOPS. Each individual experiment also had triplicate wells, which resulted in a total of 9 data points per dilution for each compound.

Cytotoxicity Assay: Compounds that displayed a low MIC concentration in either of the media were selected for analysis via the cytotoxicity assay. Compounds were incubated with mouse macrophage line J774 for 24 hours and analyzed via an XTT assay. The optical density was measured to determine the percent of viable cells. This measurement was based on the positive control wells (100% living cells) and the negative control wells (0%). Percent viability was subtracted from 100% in order to determine the percent of cells killed (cytotoxicity %). This study was conducted in triplicate for each compound.

Checkerboard Assay: Compounds with low cytotoxicity were selected for this study. Each compound was added to and serially diluted across a plate. A second dilution going down the plate was performed with Amphotericin B. After measuring the optical density, a formula was used to calculate the fractional inhibitory concentration index (FICI) to determine if the compound and Amp. B displayed an antagonistic (FICI > 4), indifferent (.5 ≤ FICI ≤ 4), or synergistic (FICI < .5) relationship with each other.

Results

Figure 1. Some Macrocycle Compounds Tested for Antifungal Activity

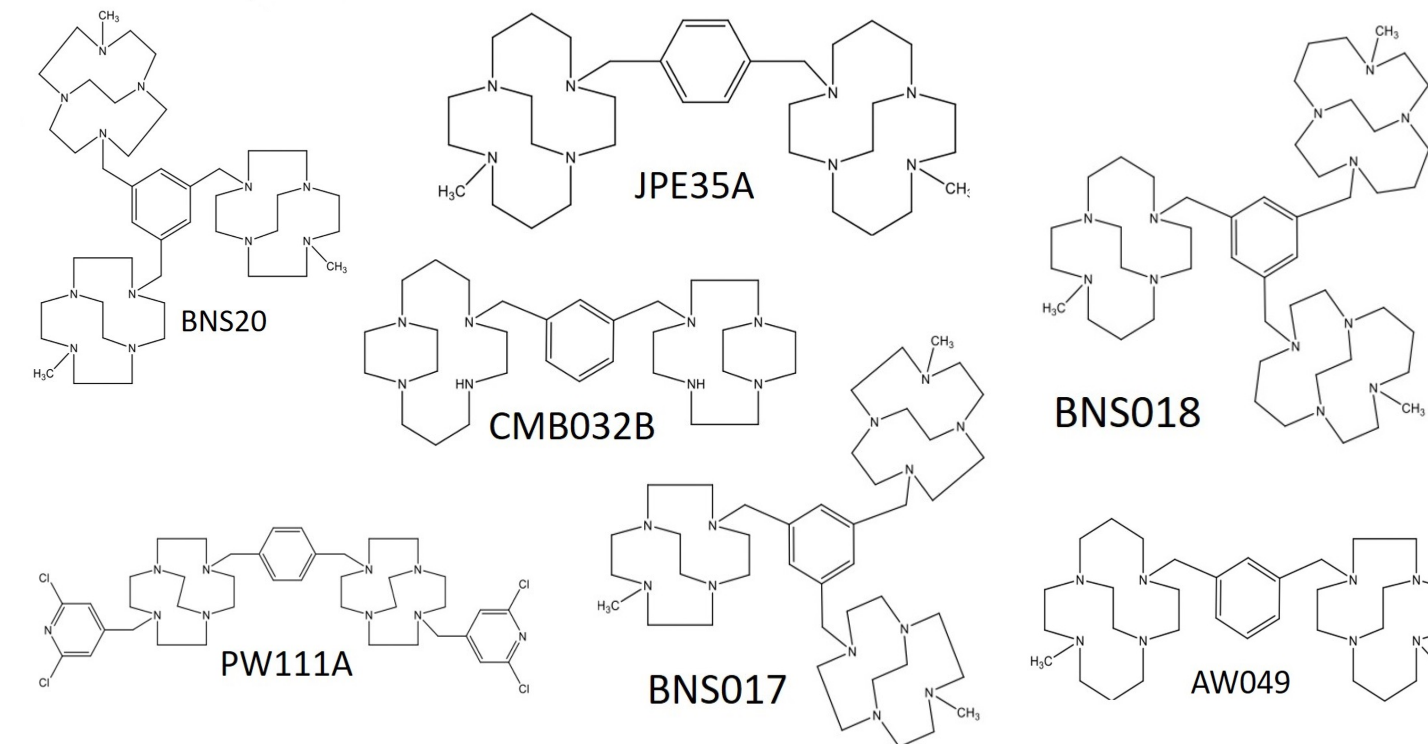
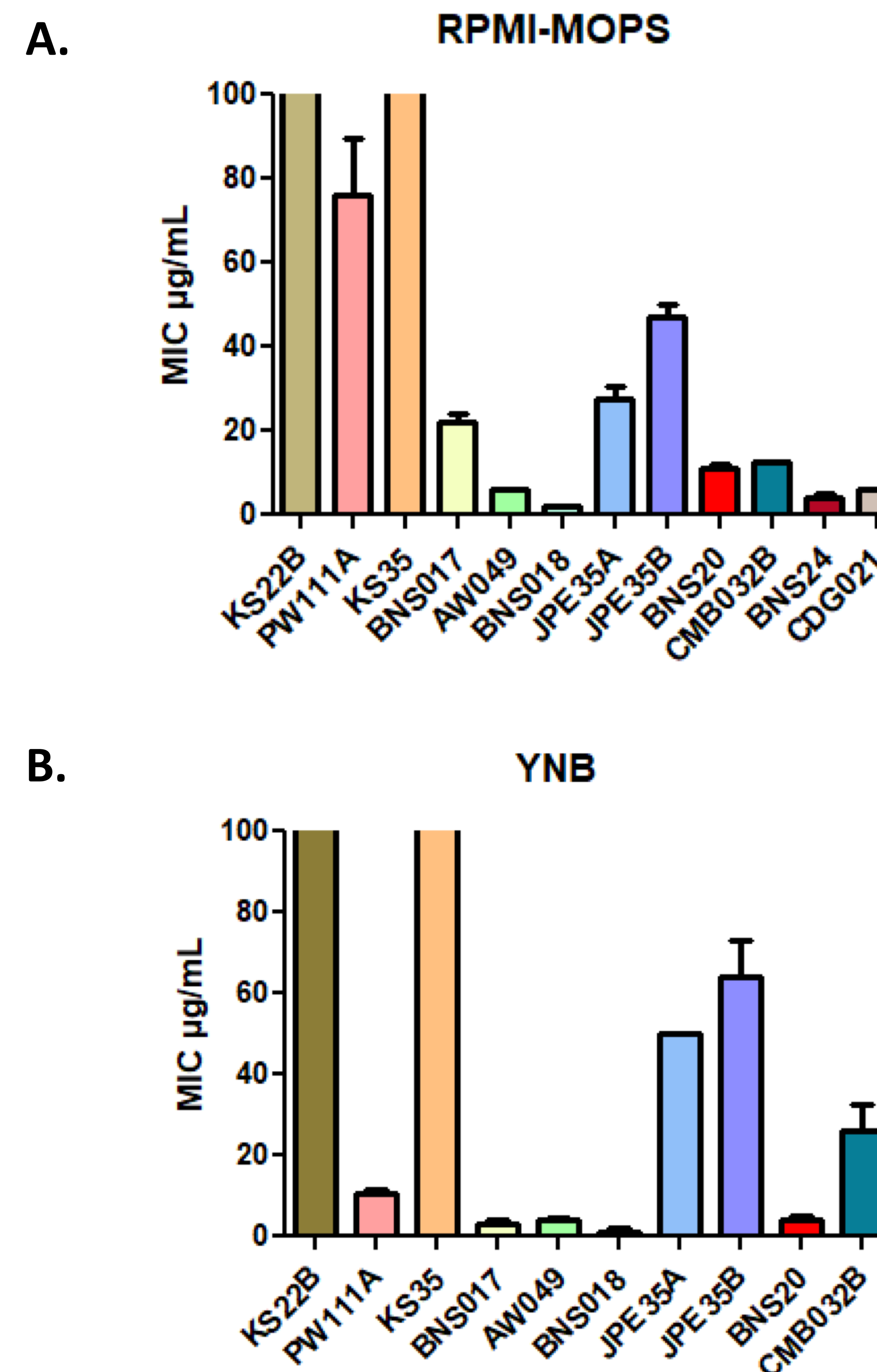
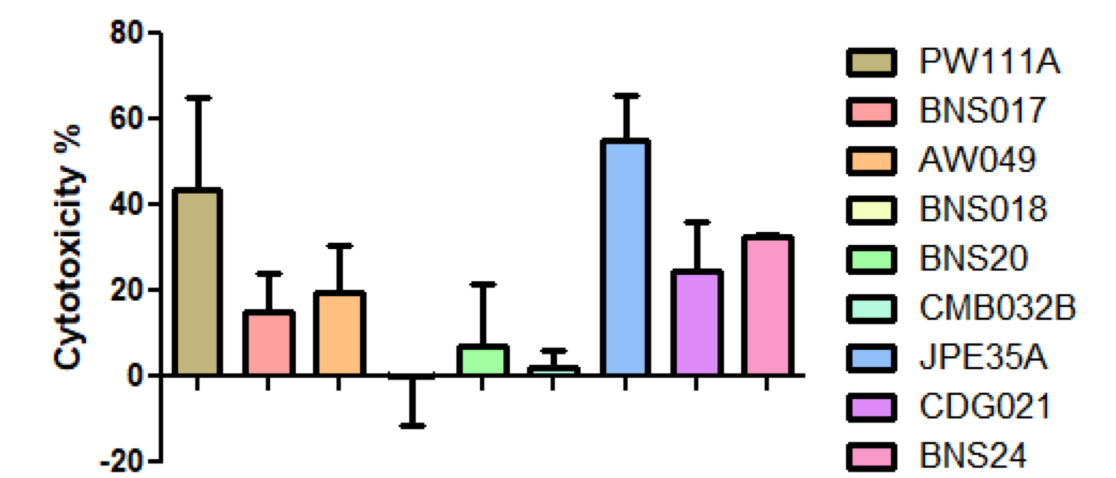


Figure 2. Macrocycle Compounds Have Antifungal Activity against *C. neoformans*



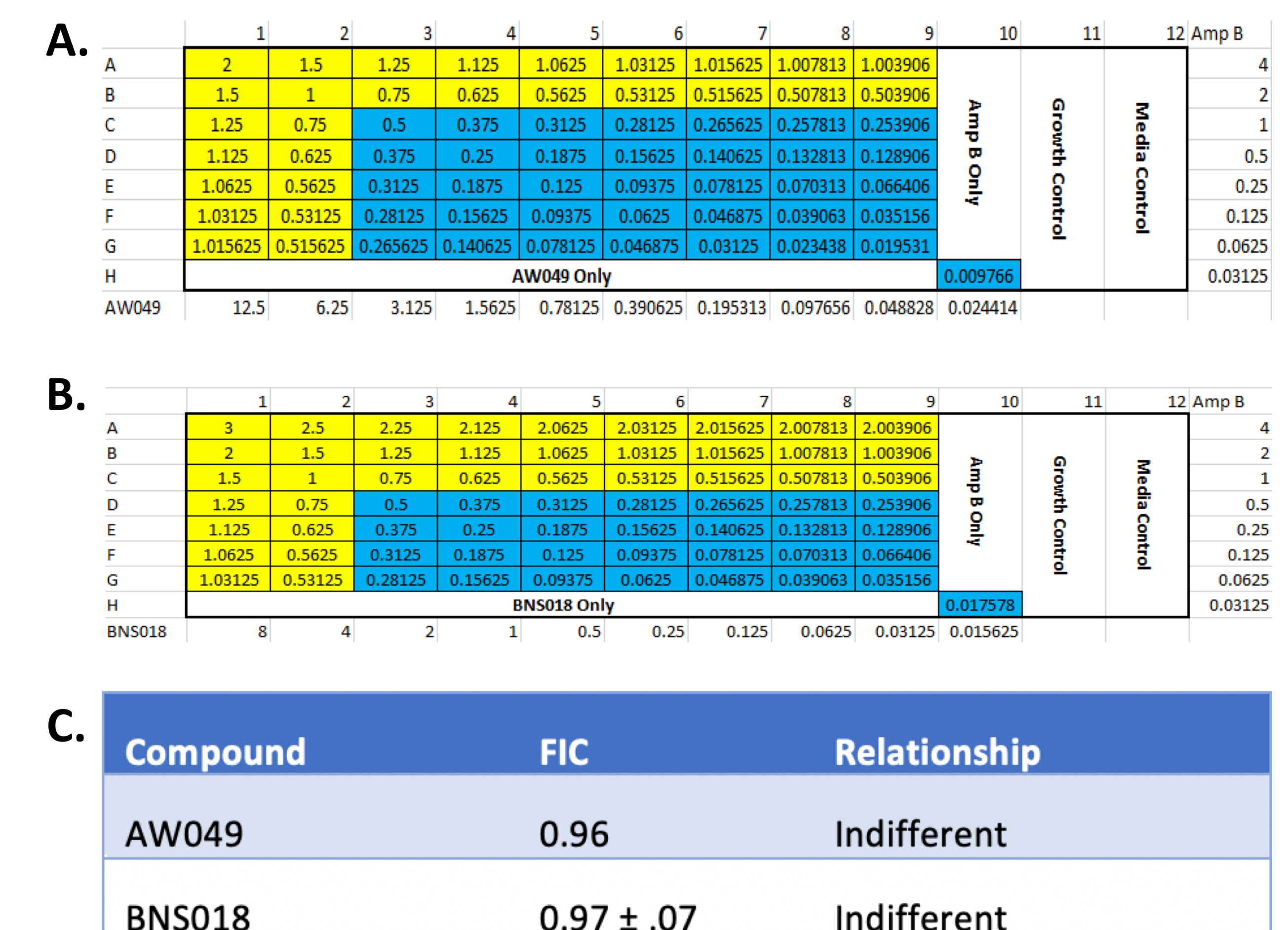
Conclusion: MIC graphs for all tested compounds against *C. neoformans* strain H99 in (A.) RPMI-MOPS or (B.) Yeast Nitrogen Base plus 2% glucose (YNB-Glucose). Compounds high antifungal activity in RPMI-MOPS included BNS017 with an MIC of 22.22 µg/mL, AW049 with an MIC of 6.25 µg/mL, BNS018 with an MIC of1.91 µg/mL, JPE35A with an MIC of 27.78 µg/mL, BNS20 with an MIC of 11.11 µg/mL, CMB032B with an MIC of 12.5 µg/mL, BNS24 with an MIC of 4.17 µg/mL, and CDG021 with an MIC of 6.25 µg/mL. Compounds with high antifungal activity in YNB-Glucose included PW111A with an MIC of 10.42 µg/mL, BNS017 with an MIC of 3.3 µg/mL, AW049 with an MIC of 4.17 µg/mL, BNS018 with an MIC of 1.3 µg/mL, BNS20 with an MIC of 4.28 µg/mL, and CMB032B with an MIC of 26.04 µg/mL.

Figure 3. Macrocycle Compounds Have a Range of Cytotoxicity



Conclusion: Cytotoxicity assays indicate compounds BNS017, AW049, BNS018, BNS20, CMB032B, and CDG021 are non-toxic to J774 mouse macrophages because the cytotoxicity measurements are < 30%.

Figure 4. Macrocycle Compounds Have Indifferent Interactions with Amphotericin B



A.

	1	2	3	4	5	6	7	8	9	10	11	12	Amp B
A	2	1.5	1.25	1.125	1.0625	1.03125	1.015625	1.007813	1.003906	Amp B Only	Growth Control	Media Control	4
B	1.5	1	0.75	0.625	0.5625	0.53125	0.515625	0.507813	0.503906				2
C	1.25	0.75	0.5	0.375	0.3125	0.28125	0.265625	0.257813	0.253906				1
D	1.125	0.625	0.375	0.25	0.1875	0.15625	0.140625	0.132813	0.128906				0.5
E	1.0625	0.5625	0.3125	0.1875	0.125	0.09375	0.078125	0.070313	0.066406				0.25
F	1.03125	0.53125	0.28125	0.15625	0.09375	0.0625	0.046875	0.039063	0.035156				0.125
G	1.015625	0.515625	0.265625	0.140625	0.078125	0.046875	0.03125	0.023438	0.019531				0.0625
H	AW049 Only									0.009766			0.03125
AW049	12.5	6.25	3.125	1.5625	0.78125	0.390625	0.195313	0.097656	0.048828	0.024414			

B.

	1	2	3	4	5	6	7	8	9	10	11	12	Amp B
A	3	2.5	2.25	2.125	2.0625	2.03125	2.015625	2.007813	2.003906	Amp B Only	Growth Control	Media Control	4
B	2	1.5	1.25	1.125	1.0625	1.03125	1.015625	1.007813	1.003906				2
C	1.5	1	0.75	0.625	0.5625	0.53125	0.515625	0.507813	0.503906				1
D	1.25	0.75	0.5	0.375	0.3125	0.28125	0.265625	0.257813	0.253906				0.5
E	1.125	0.625	0.375	0.25	0.1875	0.15625	0.140625	0.132813	0.128906				0.25
F	1.0625	0.5625	0.3125	0.1875	0.125	0.09375	0.078125	0.070313	0.066406				0.125
G	1.03125	0.53125	0.28125	0.15625	0.09375	0.0625	0.046875	0.039063	0.035156				0.0625
H	BNS018 Only									0.017578			0.03125
BNS018	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125	0.015625			

C.

Compound	FIC	Relationship
AW049	0.96	Indifferent
BNS018	0.97 ± .07	Indifferent

Conclusion: AW049 (Figure 4A) has indifferent activity with Amphotericin B and has an FICI of 0.96. BNS018 (Figure 4B) also has indifferent activity with Amphotericin B and has an FICI of 0.97 ± .07.

Conclusions & Future Directions

Conclusions:

- Compounds PW111A, BNS017, AW049, BNS018, JPE35A, BNS20, CMB032B, BNS24, and CDG021 were effective at inhibiting the growth of *C. neoformans* strain H99 at a lower concentration than the other compounds in either YNB-glucose and/or RPMI-MOPS.
- Compounds BNS017, AW049, BNS018, BNS20, CMB032B, and CDG021 are non-toxic to tested mammalian cells.
- Both AW049 and BNS018 have an indifferent relationship with Amphotericin B.

Future Studies:

Minimum Fungicidal Concentration (MFC) Assay

- *C. neoformans* and antifungal compounds will be incubated at the MIC dilution and two previous dilutions above the MIC concentration for 48 hours. Following plating for growth, fungicidal activity will be calculated.

Checkerboard Assays

- Checkerboard assays will be repeated on compounds in Fig. 4 and on all of the other compounds that had a low MIC and cytotoxicity.

Mutant Library Assays

- Using a mutant library of *C. neoformans*, the compounds will be tested to determine if specific genetic mutations result in resistance to these antifungal compounds. Mutations that lead to resistance could indicate the mechanism of action of these antifungal compounds.

Confocal and Electron Microscopy

- Confocal and electron microscopy will be used to determine structural changes caused by the compounds.