

Characterization of Aurone X as a Potential Drug Candidate Against *Cryptococcus neoformans*

by
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A thesis presented to the Honors College of Middle Tennessee State University in partial fulfillment of the requirements for graduation from the University Honors College
Spring 2016

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Dedication

I dedicate this thesis research project, the hundreds of hours spent in lab and analyzing data, the presentations that have come from this research to my family. None of this could have been accomplished without them and of course the Grace of God!

JazakAllah hu khairan shukran Mamma, Abbu, Asfah, Alaa, and Asma for coming in with me to read plates at night, sleeping at school, listening to my endless science talk that made no sense to you, always encouraging me, and taking every one of my successes as your own, because they were due in large part to you and your never ending, unwavering support and duas (prayers).

This is also dedicated to my grandparents- those who are here physically and those who are felt in spirit.

Acknowledgements

I would like to thank Danielle Araujo for taking me under her wing and introducing me to my project, training me in the techniques I needed, and remaining patient throughout the whole thing!

I would also like to thank Dr. Erin McClelland for taking a chance on the younger, more inexperienced version of me that she met two years ago, and letting me into her lab! I cannot exaggerate the support, advice, training, and encouragement that she has extended to me over the course of this project. I would also like to thank her for pushing me to present my research, apply for grants, and believing in me enough to develop a thorough, comprehensive project I would head from start to finish. On a similar note, I would like to thank the entire McClelland lab. They created a collaborative, understanding, supportive, welcoming, and fun environment that I looked forward to being in everyday (despite having to teach me how to autoclave multiple times and exploiting my jumpy disposition when I was alone working in the hood)!

This project could not have been possible without the help and collaboration with Dr. Scott Handy and Zach Taylor. Thank you for synthesizing my aurone, even if we needed dauntingly large amounts!

I am also indebted to the Honors College. Thank you for the opportunity to conduct undergraduate research and write a thesis. Thank you for being understanding towards any delays I faced, and discussing obstacles. Ms. Laura Clippard, thank you for always having an open door, being patient, listening to what I had to say, and going out of your way to help me- every single time. Thank you Dean Vile, Dr. Phillips, and anyone else that was involved in the selecting me as a Buchanan Fellow for the Class of

2016. This has been the biggest blessing and platform for me going into my four years at MTSU! Thank you for giving me the honor of a paid education- the magnitude of which I do not take lightly and hope to build upon with every academic endeavor I embark on. A big thank you to my Buchanan classmates! There is no other group of people I would have wanted to go through our classes or navigate the first few years of college with. I am so grateful to have befriended the kindest, brightest, and genuine people at MTSU! I hope that success and happiness will follow you every step of the way in every aspect of your life.

I am also grateful to the Undergraduate Research Center and the Biology Department for awarding me with funding for my project.

Abstract

Cryptococcus neoformans (*Cn*) is an opportunistic, fungal pathogen that can spread from the lungs to the central nervous system and cause life-threatening meningitis, most commonly in immune depressed individuals. There are currently four drugs on the market that are the standard treatment; however, these have resulted in renal toxicity, liver failure, and resistant strains. The lack of safe, effective medication for treating cryptococcal meningitis is the motivation for identifying if aurone X is a potential drug candidate by characterizing the compound's inhibition. The screening of an aurone library using the A27-M2 CLSI standard micro-dilution method identified aurone X, which showed > 90% inhibition to *Cn*. Toxicity assays conducted on rat L6 fibroblasts and human THP1 macrophages showed minimal toxicity. Additional tests were performed to determine aurone x's potential as a drug candidate. We characterized the minimum inhibitory concentrations (MIC) of aurone X in different medias, at different cell concentrations, with different serotypes and strains, and tested for synergy with Fluconazole, Flucytosine, and Amphotericin B. Based on our data of aurone X's low toxicity and effectiveness at low dosages, aurone X could be a possible drug candidate against *Cn* infections.

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List of Terms

1. **Antibody:** a protein produced in response to a specific antigen, defends the immune system.
2. **Antigen:** a structure that causes the production of antibodies to attack a pathogen once it binds it.
3. **Amphotericin B:** A drug currently used to treat fungal meningitis.
4. **Asparagine Media:** selective enrichment medium that mimics the environment of the cerebral spinal fluid, and allows for slow growth of microorganisms.
5. **Aurone:** a plant flavonoid that causes yellow coloring of flowers.
6. **Library:** A collection of compounds. We screened an extract and aurone library for inhibitory qualities against *Cn*.
7. **Centrifuge:** a rapidly rotating machine that separates liquids according to density using centripetal force.
8. **Cidal agent:** when the agent is removed from cells, the cells do not recover and are killed.
9. **CLSI (Clinical Laboratory Standards Institute):** organization that develops clinical laboratory testing standards.
10. ***Cryptococcus neoformans*:** A common opportunistic, encapsulated fungus. Found in soil, and bird excrement, this fungus causes fungal meningitis in immunocompromised individuals. It is the fourth most common cause of death in AIDS patients.

- 11. *Cryptococcus gattii*:** A respiratory, encapsulated fungus that infects the meninges similar to its relative *C. neoformans*. A recent outbreak of infection has occurred over the last 10 years along the Pacific Coast of North America.
- 12. Cuvette:** clear plastic tube used to read samples in the spectrophotometer.
- 13. Dilution Plate:** A diagnostic technique that includes various concentrations of drug in conjunction to the microorganism it is being tested with. The various dilutions allow for identification of dosages of the drug at which it is effective at inhibiting a microorganism.
- 14. Dimethyl sulfoxide (DMSO):** is a polar solvent that dissolves polar and nonpolar molecules and mixes with organic solvents and water.
- 15. Edge Effect:** A discrepancy seen in the results of the outer rim of wells in micro-dilution plates.
- 16. Extract:** A compound isolated from a complex mixture.
- 17. Flavonoid:** a plant pigment important for attracting pollinators, nitrogen fixation, chemical messengers, and physiological regulators.
- 18. Fluconazole:** A drug currently used to treat fungal meningitis.
- 19. Flucytosine:** A drug currently used to treat fungal meningitis.
- 20. Hemocytometer:** A specialized chamber that allows for easier cell counting under the microscope.
- 21. H99S:** a lab-adapted serotype A strain of *Cn* most often used when conducting experiments on *Cn*.
- 22. IC50 (Half Maximal Inhibition Concentration):** a measure of effectiveness in inhibition of a certain biological function.

- 23. L6 Fibroblasts:** rat cells used to test compounds' toxicity in skeletal muscle.
- 24. Latent:** existing, but remaining dormant and hidden.
- 25. Media:** a liquid or gel designed to support microorganism growth.
- 26. Meningitis:** Inflammation of the meninges, the coverings of the brain and spinal cord. Results from either bacterial, viral or fungal infection. Symptoms are progressive and range from headache, neck stiffness, and death.
- 27. MIC (Minimal Inhibitory Concentration):** the lowest concentration at which a compound will inhibit the growth of a microorganism.
- 28. Minimal Media:** a selective media low in nutrients commonly used when growing microorganisms.
- 29. Multichannel:** A type of pipette which can pipette in multiples.
- 30. Optical Density (OD):** A measure of absorbance of a specific wavelength of light.
- 31. PBS- Phosphate Buffered Saline:** Used to wash cells clean of any drugs, dyes, or indicators.
- 32. Presto Blue Cell Viability Reagent:** the fastest live cell indicator reagent available, measures cellular respiration and allows you to monitor and visualize incubated plates even prior to reading them on a spectrophotometer.
- 33. RPMI+MOPS:** The standard growth media used in our experiments. Contains no protein, lipids, or growth factors leading to slower microbial growth. Mixed with MOPS to buffer the pH to 7.0, allowing *Cn* to grow.
- 34. Serotype:** a diagnostic method that distinguishes between strains of a microorganism.

- 35. Spectrophotometer:** A machine used to identify the optical density of a sample.
Used to determine the concentration of cells in any sample.
- 36. Static agent:** when the agent is removed from cells, the cells recover and grow.
- 37. Supernatant:** When a cell suspension is centrifuged it separates into a liquid portion and solid pellet. The liquid portion is called the supernatant.
- 38. Synergy:** Describes two or more compounds or mechanisms that work together to amplify results such that the sum of the parts is greater than the individual parts.
- 39. THP1 Macrophage:** a commonly used cell line of human immune cells that interacts with foreign pathogens by ingesting and chemically breaking them down. This cell line is commonly used to study macrophage interactions with pathogens.
- 40. Toxicity Assay:** test that determines how lethal a compound is to cells.
- 41. Triazole:** a compound that contains a ring of three nitrogens and two carbons.
- 42. Trypan Blue:** commonly used dye used when counting cells in a hemocytometer.
Allows for easier visualization of dead cells.
- 43. Yeast Extract Peptone Dextrose (YPD):** A nutrient rich growth media. Used as a broth and agar plates when growing *Cn*.

I. Introduction

A. Background

Cryptococcus neoformans (*Cn*) is an opportunistic, fungal pathogen that can spread from the lungs to the central nervous system and cause life-threatening meningitis (1). Infection occurs by inhalation of spores, and produces fatal effects most commonly in immune depressed individuals. *Cn* infection frequently infects the nervous system in AIDS patients, as well as transplant recipients on immunosuppressive drugs. Although less commonplace, contamination of healthy individuals with properly functioning immune systems does occur. In this case, the inhaled spores typically become latent in the respiratory tract (2).

Cryptococcal meningitis is an illness that occurs once the inhaled, microscopic fungal spores reach the central nervous system. Symptoms get progressively worse, beginning with fever, nausea and ending with confusion, neck and chest pain, blurred and light sensitive vision, and possibly death (3; 4). This opportunistic fungus has led to high mortality rates; of the approximately 957,900 annual cases, about 624,700 result in deaths (5). This alarming rate, was met with the development of a standard treatment plan: administration of Amphotericin B (Amp B) and Fluconazole. The current drugs are not without faults, however; fluconazole resistant strains are becoming widespread. Amp B is fairly toxic and has led to kidney toxicity, as well as having an impractical method of delivery. Intravenous injections are plausible solutions in the United States, but not in Sub-Saharan Africa, the region with the highest incidence rate (1; 6).

Differences in the surface protein antigens suggest the existence of five different serotypes, or subgroups, of *Cn* strains: A, B, C, D, and an AD hybrid (7). Serotypes A, D, and AD are responsible for most of the infections of immune deficient individuals, while B and C appear in cases with immunologically sound hosts. Of these, serotype A is the most prevalent in the United States, accounting for 203 of 272 of the strains isolated in the United States (8). H99S is a serotype A strain of *Cn* that was used in the majority of our experiments.

While treatments of cryptococcal infections exist, certain strains of *Cn* have demonstrated resistance to Flucytosine, Fluconazole, and Amp B. In addition, they account for failure or relapse during treatment of meningitis and cause major side effects (11). This confirms the need for further research into potential new drugs to combat *Cn*. The lack of safe, effective medication for dealing with cryptococcal meningitis is the area of interest for this research project. We determined if a specific aurone- a flavonoid responsible for the yellow pigment in flower like snapdragons and dahlias-could be used as a new therapeutic for *Cn* infections (16). These compounds are found in nature, however aurone X is a chemically synthesized molecule that was prepared as part of an aurone library in Dr. Scott Handy's lab (9). Aurone X was identified out of a preliminary screen of compounds showing > 90% inhibition of *Cn*. Toxicity assays done on human THP1 macrophages demonstrated IC50 values >100 μ M, suggesting little toxicity to mammalian cells. We characterized how aurone X inhibits *Cn*. We determined if aurone X inhibits the growth of *Cn* in other media besides the standard Roswell Park Memorial Institute medium (RPMI), if aurone X was inhibitory with higher numbers of cells of *Cn* (to mimic numbers seen in an acute infection), and if aurone X showed inhibition towards

other serotypes and strains of *Cn*. We also determined whether aurone X inhibited growth cidally or statically, if aurone X was synergistic with other drugs, and what aurone X's mechanism of action might be.

B. Thesis Statement

The deficit in safe and effective medication on the market to treat fungal meningitis was the motivating factor for characterizing aurone X's inhibitory characteristics against *Cn*. We believe that using the approach variations of the A27-M2 CLSI, standard micro-dilution method with customizable dilutions in a 96 well plate allowed us to characterize how aurone X inhibited *Cn*, as this procedure has been used in the past to target drug candidates. Overall, the purpose of this thesis project was to characterize aurone X's potential as a drug candidate against *Cn*.

II. Methods

In order to obtain the data necessary to create IC₅₀ curves of H99S and aurone X in different growth medias, we used a variation of the A27-M2 CLSI, standard micro-dilution method in 96 well plates for each experiment we tested. Each of the experiments, with the exception of Experiment G and H, were conducted following this general protocol, with few variations. The variations are listed under the experiment itself.

General Method

A YPD plate was streaked for isolation of H99S, and incubated for 96 hours at 37° C. After this time we set up a dilution plate. Five mLs of saline were pipetted into a 15 mL conical test tube, and 1 mL of saline was pipetted into a cuvette.

Approximately 3 small colonies of *Cn* from the YPD plate were added into the 15 mL test tube, and vortexed for 1.5 minutes. One mL of the saline + cell solution was pipetted into another cuvette and measured for an optical density (OD) on the spectrophotometer. The desired range for the OD is 0.17-0.19 to get the desired concentration of *Cn* cells per mL of the solution. These steps were common to every experiment we conducted. What follows may have some variation according to the experiment.

Two dilutions of the media used in the experiment, usually RPMI+MOPS, were made in 15 mL conical test tubes: a 1:100 dilution was made from 9.9 mL media + 100 μ L initial solution, and a 1:20 dilution of the 1:100 dilution was made from 9.5 mL media + 500 μ L of the 1:100 dilution. The 1:20 dilution was made twice, and then poured into a sterilized tray. Three mL of pure media was poured into a second sterile tray.

Seven hundred μ L of 6 individual dilutions of the desired concentrations of aurone X were made up in 2 mL test tubes. Each of these dilutions was then plated in triplicate for a total of 200 μ L/ well in a black microdilution plate. Our controls were pipetted into Row H: 1-3 had 180 μ L RPMI+MOPS with *Cn* cells + 18 μ L pure RPMI+MOPS + 2 μ L Amp B. Wells 4-6 μ L had 180 μ L RPMI+MOPS with *Cn* cells + 20 μ L 1% DMSO in RPMI+MOPS. Wells 7-9 had 180 μ L RPMI+MOPS with *Cn* cells + 20 μ L pure RPMI+MOPS. Wells 10-12 had 200 μ L pure RPMI+MOPS. A saline border of 200 μ L/well was pipetted into Row A, and wells A-G of Column 1 and 12. The plate was then covered with aluminum foil to prevent dessication and incubated at 35° C for 66 hours. After

66 hours, 20 μ L of Presto Blue was added to every well, and returned to the incubator for 8 hours. The absorbance of the plate was read using SoftProMax software at 530 nm in order to quantify the number of viable cells in each well.

The number of cells/mL was calculated by counting cells under a microscope after a 1:2 dilution of Trypan Blue + cell dilution was made. After the cells were counted in each quadrant of the hemocytometer counting chamber the concentration of cells was calculated by:

$$\text{Total cells} \div 4 = \text{Average cells} / \text{quadrant}$$

$$(\text{Average cells} / \text{quadrants} \times 2) (10^4) = \text{cells} / \text{mL}$$

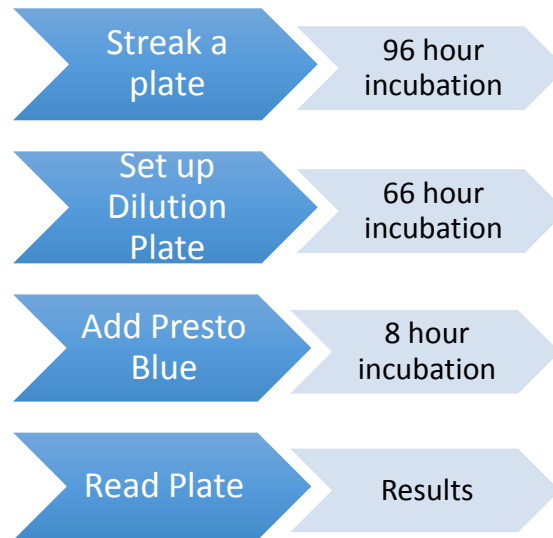


Figure 1: General A27-M2-CLSI Method Diagram

A. Screening the Aurone Library

In order to screen the aurone library for inhibition against C. neoformans, each extract was tested at 6 concentrations. Extract A, B, and C are general representatives of the three extracts from the library that were tested on a plate.

This experiment followed the general method with the one exception of the dilutions that were plated and tested. Instead of testing aurone X at 6 different concentrations, we tested 18 different aurones and plant extracts on a plate, which were each at a concentration of 100 $\mu\text{g/mL}$.

B. Testing if Aurone X Binds to Specific Plastics

In order to test for suspected affinity to certain plastics, we tested aurone X's MIC in different plastics.

When the initial screening performed by the graduate student before me was done, she identified the MIC of aurone X to be 25 $\mu\text{g/mL}$. Once we began testing aurone X in different medias, we saw different MIC values than what had previously been detected. Because nothing else had been changed, we suspected that aurone X was binding to the plastic. In this experiment we tried to identify if this was true.

The general method was followed through making the dilutions of RPMI+MOPS dilutions. In a clear micro-dilution plate, 140 μL of aurone X at 200 $\mu\text{g/mL}$ was pipetted into well A. Seventy μL of RPMI+MOPS was pipetted into wells A-G of Column 1. A 1:2 dilution was carried down the plate through Row H by pipetting 70 μL from consecutive rows. One hundred eighty μL of RPMI+MOPS with the cells was pipetted into A2-G2, A3-G3, and A4-G4. Twenty μL from Row A1-G1 of the clear plate was pipetted in triplicate into the black plate again.

The next steps were duplicated in polystyrene 5 mL round bottom tubes and polypropylene 2 mL microcentrifuge tubes. Six tubes were used to individually

create the dilutions of aurone X, 200-6.25 $\mu\text{g}/\text{mL}$. The appropriate quantities of aurone X and RPMI+MOPS with cells was calculated using the equation:

$$C_1V_1 = C_2V_2$$

The individual dilutions were created and pipetted in triplicate as normal. The polystyrene and polypropylene tubes were plated in columns 5-7 and 8-10, respectively. The saline border, controls, and incubations were completed identically to the general method.

C. Testing Aurone X in Various Medias

When characterizing aurone X's inhibition, it was important to identify the MIC of aurone in various medias- especially ones that best mimicked the environment of the cerebral spinal fluid where *Cn* replicates in a real infection. A YPD plate was streaked, OD was measured, and 1:100 and 1:20 dilutions were made using the same technique, but used different medias. A total of 3 medias were tested on one plate, either YPD, Asparagine, or Minimal media. Each of the 1:20 dilutions was poured into a sterile tray. One mL of corresponding pure media were poured into separate trays.

Five individual dilutions were created in 2 mL microcentrifuge tubes for each media type, and then pipetted in triplicate into a black micro-dilution plate. Saline, controls, and incubations followed the general method.

Furthermore, the MIC of aurone X in YPD, Asparagine, RPMI+MOPS, and Minimal Media was more accurately identified by doing a 15-point dilution. Expanding to a broader range of dilutions allowed us to more specifically identify the MIC, because of the customizability of the concentration of aurone X we

selected. Fifteen individual dilutions were created in 2 mL test tubes of a single media (ie: only Asparagine), beginning at 1000 $\mu\text{g/mL}$ and ending at 3.125 $\mu\text{g/mL}$. Each dilution was plated in triplicate. The controls and saline border were added, and the plate was incubated as above. The experiment was repeated for each media three separate times.

D. Testing Aurone X for Inhibition Against Various Strains and Serotypes of *Cn*

There are many different serotypes and strains of *Cn* that infect and cause illness in individuals. Identifying whether aurone X inhibits the growth of other clinical and laboratory strains allows us to have a more comprehensive picture of the scope of aurone X's effectiveness.

A YPD plate was streaked, OD was measured, and 1:100 and 1:20 dilutions of RPMI+MOPS were created identical to the general method.

Six individual dilutions, 200-6.25 $\mu\text{g/mL}$ were created of aurone X and H99S *Cn*, and two other strains from either Serotype A, D, or *C. gattii* with aurone X. A total of 2 strains (from the serotype being tested) + the standard H99S (which served as a control) fit onto a plate. Each dilution was plated in triplicate. The saline border, controls, and incubation was identical to the general method. The experiment was repeated three separate times for each strain of each serotype.

E. Testing Aurone X at Varying *Cn* Cell Concentrations

In a real cryptococcal infection, we have upwards of 10^8 *Cn* cells in our body. The A27-M2 CLSI protocol we followed has an average of about 2,000 cells/well. This is not a realistic model for a real infection. For this reason, we characterized aurone X's behavior in increasing numbers of *Cn* cells which more closely replicated a realistic infection.

A YPD plate was streaked, OD was measured, and the cells were counted on the hemocytometer following the general method, but in a different order than normally done. The number of cells/mL was calculated to be: $y \times 10^6$. A 1:20 dilution with RPMI+MOPS was created to yield a concentration of 10^5 cells/mL. 1:10 and 1:100 dilutions of the 10^5 test tube were performed to yield 10^4 and 10^3 cells/mL concentrations, respectively. Each of the concentrations were poured into sterile trays to be used to create the dilutions of aurone X.

Six dilutions of aurone X, 200-6.25 $\mu\text{g/mL}$, were created for each *Cn* cell concentration in 2 mL test tubes. Dilutions were plated in triplicate, saline and controls were added, and the plate was incubated according to the general method. This experiment was repeated three times.

F. Testing Aurone X for Synergy with Current Drugs

When testing for synergy between aurone X and current drugs, we used two different techniques. The checkerboard assay, is the test recognized by the scientific community and was done first. We then repeated the experiments using

a procedure that was more aligned with the set up of the other experiments we conducted for this project.

There are four drugs that are currently used to treat cryptococcal meningitis infections, using three different pathways. Drugs with different mechanisms are often administered together. Assessing if aurone X acts synergistically with drugs with known mechanisms implies that aurone X acts via a different pathway.

Checkerboard Assay:

Using the general method: a YPD plate was streaked, the OD was measured, and the cells were counted on the hemocytometer. After calculating the number of cells/mL in the solution, the amount of pure RPMI+MOPS and *Cn* solution needed to get 10^6 cells was calculated, where y was the number calculated previously.

$$C_1 V_1 = C_2 V_2$$

$$(5 \times 10^3 \text{ cells/mL}) (9000 \mu\text{L}) = (y \times 10^6 \text{ cells/mL}) (V_2)$$

The calculated dilution was made up in a conical test tube. In the microdilution plate, a 200 μL saline border was pipetted and 50 μL of pure RPMI+MOPS was added to every well in Columns 3-11, excluding the controls, and a total of 100 μL of RPMI+MOPS was added into Rows F and G. In 2 mL test tubes, we created dilutions of either Amp B, Fluconazole, or Flucytosine at concentrations 4 times greater than the desired concentration, because it was diluted fourfold once in the plate. One hundred μL of the highest concentration was pipetted vertically into wells B-G of Column 2. The next dilution was

pipetted into Column 3, etc. Afterwards, we took a multichannel and pipetted 50 μL out of Column 2 and into Column 3. We continued this down the plate and discarded the 50 μL out of Column 11. Next, we made up 700 μL of 4 different aurone X dilutions in 2 mL test tubes- at four times higher than the desired concentration. We then pipetted 50 μL of the highest dilution into Row B; Fifty μL of the next dilution was pipetted into Row C, continuing through Row E. Finally, 100 μL of the RPMI+MOPS + *Cn* solution was pipetted into every well, and the controls were pipetted into Row H before incubation. This experiment was repeated twice for each drug.

A27-M2 CLSI Variation:

A YPD plate was streaked, the OD was measured, and 1:100 and 1:20 dilutions of RPMI+MOPS were created identical to the general method.

Six individual dilutions of *Cn* and aurone X, ranging from 200-6.25 $\mu\text{g/mL}$, were created. The drug, either Fluconazole, Flucytosine, or Amphotericin B, we selected to test for synergy was then used to create 6 dilutions of *Cn* and drug. When testing Fluconazole and Flucytosine, 1:2 dilutions ranging from 16-0.5 $\mu\text{g/mL}$ were created. When testing Amphotericin B dilutions of 2-0.0625 $\mu\text{g/mL}$ were made. Finally, six test tubes contained mixtures of aurone X and a current drug with *Cn* (ie: 200 $\mu\text{g/mL}$ of aurone X and 2 $\mu\text{g/mL}$ Amphotericin B). The aurone dilutions were pipetted in triplicate in Rows 2-4, the mixture in 5-7, and the drug in Row 8-10. The saline border, controls, and incubation were all done according to the general method. The experiment was repeated three times in addition to the Checkerboard assay.

G. Testing if Aurone X Inhibits Statically or Cidally

In order to test how aurone X inhibits the growth of *Cn*, we tested whether it behaves cidally, killing the *Cn* cells, or statically, pausing cell growth when it is present.

After reading the results of a plate testing different cell concentrations, we pipetted 200 μ L out of one well of each dilution on the plate into 2 mL test tubes, including the DMSO control. One mL of PBS was added to the test tubes. The tubes were centrifuged for 5 minutes at 2000 rpm and the supernatant was disposed of. The pellet was resuspended in 1 mL of PBS, and the process was repeated twice more, concluding with a final resuspension. Ninety μ L of pure PBS was pipetted into Columns 2-6 wells A1- F1. One hundred μ L of the resuspended pellets were added into Column 1. Ten μ L were removed from Column 1 and mixed into Columns 2 through 6. Ten μ L of each individual dilution concentration was then removed and spotted on a YPD plate in a grid pattern. The plates were given time to dry before incubating at 35° C for 48 hours.

After the incubation period, the presence of any colonies implied a static pathway. No cells implied cidal killing of *Cn*. This experiment was repeated three times for all strains and serotypes tested.

H. Growth Curve With and Without Aurone X

The H99S *Cn* culture was grown for 25 hours at 37 °C in 50 mL of YPD in an Erlenmeyer flask. The next day, the YPD cultures were washed by pouring

the H99S culture into a 50 mL conical tube and centrifuging it at 2000 rpm for 5 minutes. The supernatant was disposed of in bleach and the pellet resuspended in 10 mL of PBS, centrifuged at 2000 rpm for 5 minutes, and the supernatant discarded. Two more PBS washes were done, and then the pellet was resuspended in 3 mL of PBS. A 1:500 dilution in a 2 mL test tube was created by adding 2 μ L of the PBS + *Cn* solution to 998 μ L of PBS. A 1:2 dilution of Trypan Blue and the 1:500 dilution was used to count the cells on the hemocytometer. After counting 50-200 cells, we calculated the cells/ mL (*y*) and how much of the 3 mL PBS + *Cn* solution was needed to have 10^3 cells in each YPD culture using:

$$C_1 V_1 = C_2 V_2$$

$$(1 \times 10^3 \text{ cells/ mL}) (10,000 \mu\text{L}) = (y \times 10^8 \text{ cells/ mL}) (V_2)$$

The calculated amount was added into six 250 μ L Erlenmeyer Flasks. The calculated the amount of aurone X that needed to be added to the flasks, that would have aurone X at its MIC and $\frac{1}{2}$ MIC in YPD, were added. *Note: 11,300 μ g/mL is the concentration of our aurone X stock.*

MIC:

$$C_1 V_1 = C_2 V_2$$

$$(11,300 \mu\text{g/mL}) (V_1) = (10,000 \mu\text{L}) (200 \mu\text{g/mL})$$

$$V_1 = 176.99 \mu\text{L}$$

$\frac{1}{2}$ MIC:

$$C_1 V_1 = C_2 V_2$$

$$(11,300 \mu\text{g/mL}) (V_1) = (10,000 \mu\text{L}) (100 \mu\text{g/mL})$$

$$V_1 = 88.5 \mu\text{L}$$

These quantities were added to two corresponding Erlenmeyer Flasks each, labeled as YPD + MIC or YPD + ½ MIC. The last two Erlenmeyer Flasks were labeled as YPD Only, and 176.99 µL of DMSO were added in order to have equal volumes of 10 mL. The 6 flasks were then incubated at 37° C at 150 rpm for 23 hours. Beginning at 23 hours, we measured the OD of 1 mL from each flask every 3 hours. One mL of pure YPD was used as the blank before reading the cuvettes at 530 nm in the spectrophotometer. After this, the cuvettes were returned to the incubator. At each time point we also spotted a plate using Procedure G, without washing the cells in PBS, to determine cell presence or cell death, as well as cidal or static inhibition. This process of measuring the OD and spotting a YPD plate was repeated over the course of 60 hours.

III. Results

A. Thirty-four extracts and aurones showed > 90% inhibition of *Cn* at 100 µM.

After conducting the screening three times, we concluded that there were 34 extracts and 2 aurones in the library of compounds provided from the Guanxi Botanical Garden in China, and purified in Dr. Scott Handy's lab in the chemistry department, that inhibited *Cn* at > 90% inhibition of *Cn* at 100 µM. Specific compounds were then selected to test their toxicity to THP1 macrophages and L6 fibroblasts in Dr. Newsome's lab. Aurone X was found to have a low toxicity (>100 µM) to THP1 macrophages and L6 fibroblasts, and a low minimum inhibitory concentration (MIC = 25 µg/mL) against *Cn* (Figure 3 and Table 2).

B. Aurone X binds to polystyrene and tissue culture treated polystyrene

Previous experiments conducted by Danielle Araujo, the graduate student on the project before me, identified aurone X as having an MIC of 25.0 µg/mL. However, when techniques and assays utilized alternative types of plastics, we saw significantly different MIC values. When using 5 mL polystyrene round bottom tubes the MIC was 50.0 µg/mL. Using 96 Well Tissue Culture Polystyrene plates treated with Vacuum Gas Plasma produced an MIC of 200 µg/mL. While using 2 mL Polypropylene microcentrifuge tubes yielded an MIC of 25.0 µg/mL (Table 1).

C. Aurone X's MIC's in RPMI+MOPS, Asparagine, YPD, and Minimal Media

We identified the MIC of aurone X in several medias. In RPMI+MOPS and Asparagine medias the MIC was 25.0 µg/mL. YPD had an MIC > 200 µg/mL. Finally, Minimal Media had an MIC of >1000 µg/mL (Table 2).

D. Aurone X's inhibition against Serotype A and D *Cn* strains, and *C. gattii*

We tested four serotype A strains, three of them, B18, B45, and B58, were clinical isolates of patients in Botswana. H99S, B18, and B45 all had MIC's of 25.0 µg/mL. B58 had an MIC of 12.5 µg/mL. The three serotype D isolates we tested, 24067, JEC21, and B3501, all had an MIC of 25.0 µg/mL. Similarly, the two strains of *C. gattii* we tested, R265 and R272, also had MIC's of 25.0 µg/mL (Table 3) (Figure 2 B).

E. Aurone X's inhibition in 10^3 , 10^4 , and 10^5 cells

When testing the MIC of aurone X at increasing cell concentrations, we determined that the MIC doubled when cell count was increased by a log. The MIC's were 25, 50, and 100 $\mu\text{g/mL}$ for 10^3 , 10^4 , and 10^5 cells respectively (Table 4) (Figure 2 A).

F. Aurone X behaves synergistically with Fluconazole and Amphotericin B, and had no interaction with Flucytosine.

We found the same results when using the Checkerboard Assay and the A27-M2 CLSI Variation.

When testing aurone X for synergy with Amphotericin B, we determined that the MIC for Amp B was 0.25 $\mu\text{g/mL}$, for aurone X was 25.0 $\mu\text{g/mL}$, and when mixed was 6.25 $\mu\text{g/mL}$ Aurone X and 0.0312 $\mu\text{g/mL}$ Amp B (Table 5).

When testing Flucytosine for synergy, we identified the MIC of Flucytosine as 8.0 $\mu\text{g/mL}$, aurone X as 25.0 $\mu\text{g/mL}$, and the mixture as 25.0 $\mu\text{g/mL}$ aurone X and 8.0 $\mu\text{g/mL}$ Flucytosine (Table 5).

When testing aurone X for synergy with Fluconazole, we determined that the MIC for Fluconazole was 8.0 $\mu\text{g/mL}$, for aurone X was 25.0 $\mu\text{g/mL}$, and when mixed was 6.25 $\mu\text{g/mL}$ for aurone X and 2.0 $\mu\text{g/mL}$ for Fluconazole (Table 5).

G. Aurone X inhibits *Cn* cidally until its MIC, and statically above its MIC.

We tested whether aurone X inhibited *Cn* cidally or statically, after reading results for our cell concentration plates. We found that the aurone

suppressed *Cn* growth tidally through the MIC for the respective cell concentration identified in the varying cell concentrations. Aurone X inhibited *Cn* statically at lower concentrations (Figure 4).

H. Aurone X Inhibits *Cn* Growth from the Beginning

We conducted a growth curve to determine when aurone X begins to inhibit *Cn* during its replication cycle. We found that aurone X suppressed *Cn* growth from the initial time point through the end of the 60 hour time point (Figure 5).



Figure 2 A and B: Completed dilution plates for cell concentrations and serotypes.

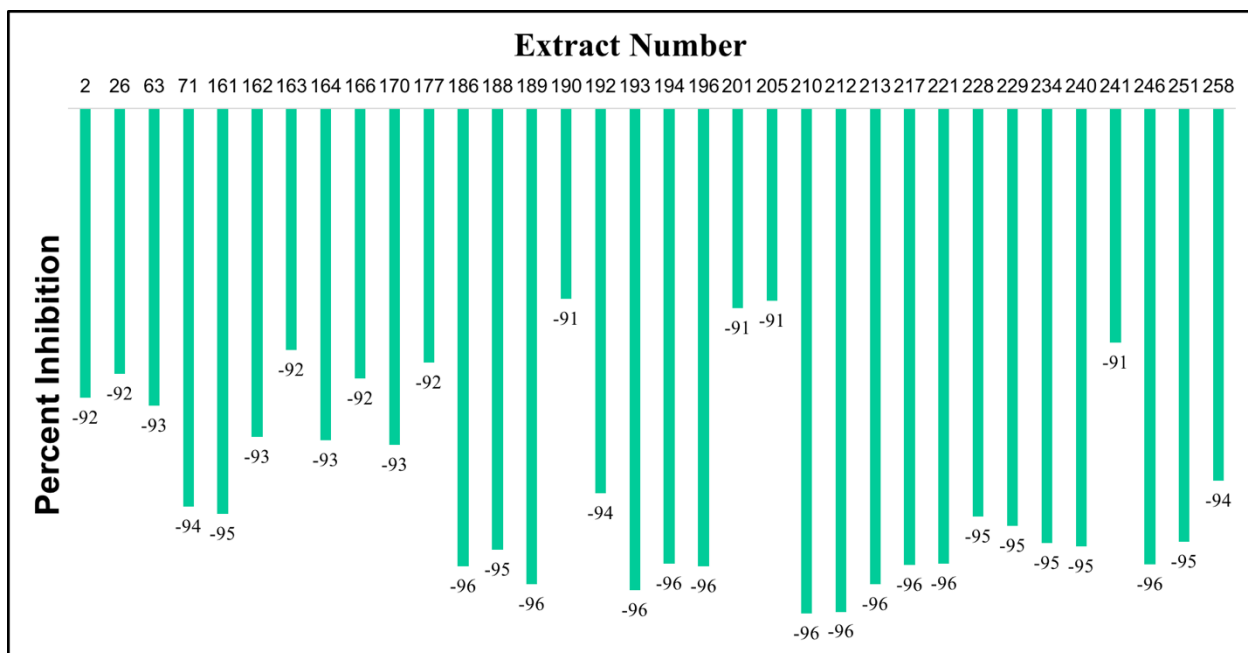


Figure 3: This figure shows the inhibition of each plant extract from the Guanxi Botanical Garden that was screened against *Cn* at a concentration of 100 $\mu\text{g/mL}$. Aurone X is extract #??

Table 1: MIC of *Cn* and Aurone X Binding to Various Plastics.

Plastic	Minimum Inhibitory Concentration ($\mu\text{g/mL}$)
96 Well Tissue Culture Treated Polystyrene**	200
Polystyrene 5mL Round Bottom Tube	50
Polypropylene 2 mL microcentrifuge tubes	25

** 96 Well Tissue Culture Treated by Vacuum Gas Plasma Polystyrene

Table 2: MIC values of *Cn* and aurone X in various medias.

Types of Media	
Media	Minimum Inhibitory Concentration ($\mu\text{g/mL}$)
RPMI + MOPS	25
Asparagine	25
YPD	> 200
Minimal	> 1,000

Table 3: MIC values of *Cn* and *C. gattii* strains with aurone X.

Strains and Serotypes	
Strains	Minimum Inhibitory Concentration ($\mu\text{g/mL}$)
Serotype A	
H99S	25
B18	25
B45	25
B58	12.5
Serotype D	
24067	25
JEC21	25
B3501	25
<i>C. gattii</i>	
R265	25
R272	25

Table 4: MIC of *Cn* and aurone X at various cell concentrations.

Cell Concentrations	
Cell Concentrations (per mL)	Minimum Inhibitory Concentration ($\mu\text{g/mL}$)
10^3	25
10^4	50
10^5	100

Table 5: MIC values of synergy experiments with aurone x and current fungal meningitis drugs.

Strains and Serotypes	
Strains	Minimum Inhibitory Concentration ($\mu\text{g/mL}$)
Amphotericin B	
Amphotericin B	0.25
Aurone X	25.0
Mix	6.25 Aurone X + 0.0312 Amp B
Flucytosine	
Flucytosine	8.0
Aurone X	25.0.
Mix	25 Aurone X + 8.0 Flucytosine
Fluconazole	
Fluconazole	8.0
Aurone X	25.0
Mix	6.25 Aurone X + 2.0 Fluconazole

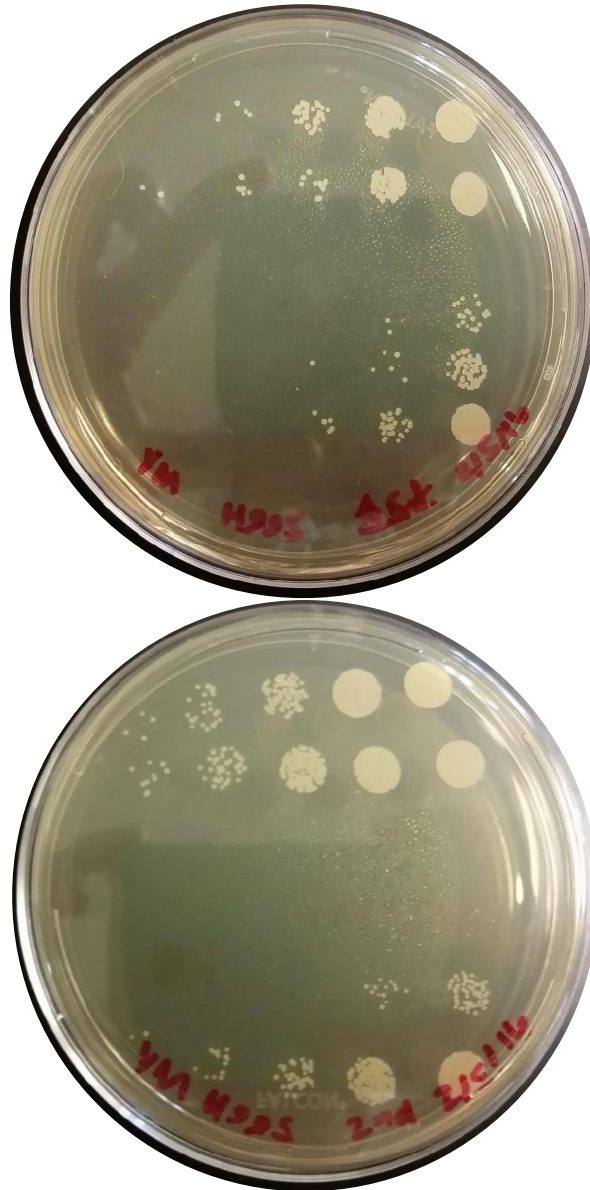


Figure 4 A and B: These plates demonstrate the aurone X's timeline of inhibition against *Cn* H99S. When the drug was at the MIC concentration, it inhibited statically until 29 hrs. After this time point, there were no colonies implying that aurone X was behaving cidally.

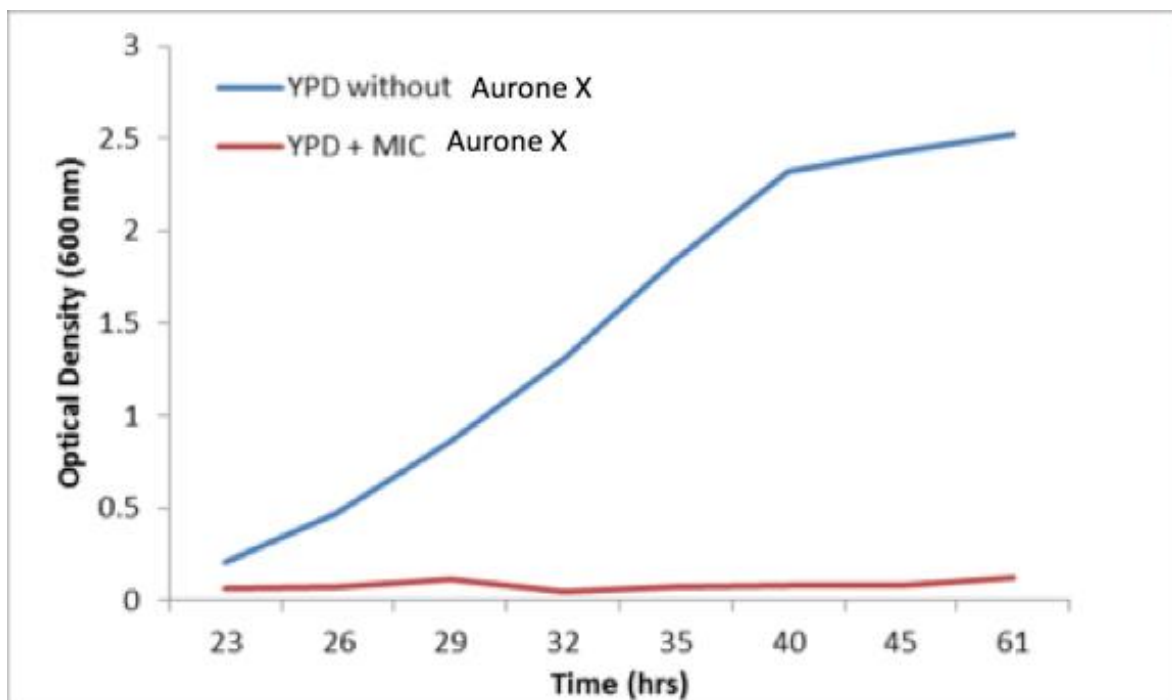


Figure 5: This graph depicts the data gathered from the growth curve experiment. The growth of *Cn* was greatly inhibited in the presence of aurone X.

IV. Discussion

Cryptococcal meningitis is the fourth most common cause of death in AIDS patients. An illness that often leads to disastrous or fatal symptoms and outcomes requires a treatment. The current challenge facing this field is the lack of a safe and effective drug. In this study we were interested in characterizing aurone X as a potential drug candidate. We were motivated by the rise of side effects associated with the current drugs. Aurone X's low toxicity in L6 fibroblasts and human THP1, and high inhibition of *Cn* in our initial library screening were the basis on which this project was based.

We further investigated aurone X's behavior in various conditions to better ascertain the compound's capabilities and limits. One of the initial tests was identifying whether the compound binds to certain plastics. We found that it bound significantly less to the 2 mL polypropylene microcentrifuge tubes, than the 96 Well Tissue Culture Polystyrene treated plates we had been using in our earliest experiments.

We identified the MIC's of aurone X in YPD, minimal media, asparagine, and RPMI+MOPS. From this, we saw that the MIC was lower in RPMI+MOPS and Asparagine medias in which *Cn* replicates much slower. This is encouraging because these medias-especially Asparagine- closely mimic the cerebral spinal fluid, where *Cn* grows and replicates in a real infection. The results we obtained for the MIC in minimal media were a significant contrast from the results found with the other medias. This was very intriguing because both Asparagine and Minimal media have comparable amounts of nutrients. For this reason we expected aurone X to behave similarly in both types of media, but there was a large discrepancy. We hypothesize that this is because one of the components of the media is somehow inhibiting the action of aurone X. Further testing could be done to determine what in the media is causing this to occur since Asparagine and Minimal media are defined. What is different between the medias? Also, are there any other drugs that show differences in the MICs between these medias? If so, that could be discussed.

We also tested if aurone X inhibits other strains and serotypes of *Cn* other than H99S. The other Serotype A strains we tested were isolates from patients

with *Cn* in Botswana. We found that those as well as Serotype D strains were inhibited by aurone X. Perhaps even more significantly, aurone X inhibited *C. gattii* as well. This is encouraging in light of the recent *C. gattii* outbreak that has been occurring in the Pacific Northwest and because *C. gattii* primarily infects immunocompetent hosts (17).

We tested for the MIC in varying cell concentrations of *Cn*, and found that for every log increase in *Cn* cells, the MIC doubled (Table 4). These tests were conducted in order to determine aurone X's inhibition against *Cn* at numbers that more realistically mimic a real cryptococcal meningitis infection, about 1×10^8 cells, and suggest that higher doses of aurone X would be needed to counteract an acute *Cn* infection.

Determining the pathway of inhibition of aurone X was very important because it allowed us to conclude whether the compound acts in a different pathway than current drugs on the market. Today, drugs with different mechanisms, oftentimes Amp B and Flucytosine, are usually coupled together to target separate structures in *Cn* and treat patients. If aurone X appeared to be synergistic with Fluconazole it most likely did not target the ergosterol biosynthesis pathway that Fluconazole targets in *Cn* (11; 12). If aurone X appeared to be synergistic with Amp B, it most likely did not form pores in the cell membrane and weaken the integrity of the cell membrane. If aurone X appeared to be synergistic with Flucytosine, it would not inhibit protein and RNA synthesis and nuclear division in *Cn* (12). When conducting tests to identify synergy, we began using the widely accepted, standard Checkerboard Assay.

However, because this assay is not done in triplicate, we repeated these experiments using modifications of the A27-M2 CLSI method and found identical results. Because of this, the complicated procedure of the Checkerboard Assay, and in order to parallel our other experiments, we repeated these experiments using the A27-M2 CLSI technique. Our data showed that aurone X behaved synergistically with Amp B and Fluconazole, resulting in more inhibition of *Cn* when combined versus when given separately (Table 5). Our results also showed that Flucytosine and aurone X had similar MIC's when combined and when alone, implying that they do not interact to inhibit *Cn* and that they may have a similar mechanism of action. Which is what? Could this proposed mechanism of action explain the growth curve data?

We also determined how exactly aurone X was inhibiting *Cn*'s growth: cidally or statically. This is important, because if the fungal cells are "paused" patients run the risk of an infection re-emerging or resistant strains arising (18). On the other hand, if a drug kills the cells, there is no threat of reinfection. We discovered that aurone X acts cidally at its MIC, and statically below that value.

The final essential experiment we conducted was the growth curve. We conducted this experiment to determine when in the *Cn* replication cycle aurone X acts to inhibit growth. This was important because it can give us an idea of when in the cycle aurone X goes into effect. We found that there was a small lag time before aurone X started to take effect. We concluded this from the spotting we did on YPD plates for each timepoint. At the first three time points, 23, 26, and 29 hours, there was evidence of *Cn* growth in the YPD + MIC flasks, because we

observed colonies. After this time point there was no evidence of *Cn*, so we concluded that there was a lag time before aurone X took effect. This paired with the synergy experiments, especially the results from the flucytosine experiment, suggest that aurone X may work by inhibiting DNA and RNA synthesis, therefore having a significant impact on *Cn* and its proliferation (15).

This multi-faceted research project has demonstrated aurone X's effectiveness as an inhibitor of *Cn* growth. Overall, my research and data have found that aurone X is a potential drug candidate for treating *Cn* infections. To better characterize the compound's applicability in a real infection, we are currently conducting a toxicity experiment in mice to determine if aurone X is toxic. This series of experiments is the next step in my research to determine whether aurone X will be beneficial in treating disease caused by *Cn*.

V. References

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