Effect of Scytovirin and testosterone on Cryptococcus neoformans

Marjan Berenji Jalaei

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EFFECT OF SCYTOVIRIN AND TESTOSTERONE ON CRYPTOCOCCUS NEOFORMANS

by

MARJAN BERENJI JALAEI

A THESIS

Submitted in partial fulfillment of the requirements for the degree of Master of Science in The Department of Biological Sciences to The School of Graduate Studies of The University of Alabama in Huntsville

HUNTSVILLE, ALABAMA

2019
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We, the undersigned members of the Graduate Faculty of The University of Alabama in Huntsville, certify that we have advised and/or supervised the candidate on the work described in this thesis. We further certify that we have reviewed the thesis manuscript and approve it in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences.

[Signatures and dates]

Committee Chair

Department Chair

College Dean

Graduate Dean
ABSTRACT
The School of Graduate Studies
The University of Alabama in Huntsville

Degree Master of Science College/Program Science / Biology
Name of Candidate Marjan Berenji Jalaei
Title Effect of Scytovirin and Testosterone on Cryptococcus neoformans

Pathogenic fungi have become increasingly important due to a wide range of fungal infections worldwide and emergence of drug resistant strains. Cryptococcus neoformans is an opportunistic fungal pathogen that causes mostly pulmonary infections, meningitis and meningoencephalitis. The rate of mortality in males is higher compared to females. Testosterone, the major steroid hormone in males, has been shown to affect the C. neoformans pathogenicity. Searching for an effective antifungal drug against C. neoformans, in particular for males, is a matter of importance. Scytovirin is a lectin with newly discovered antifungal activity associated with cell wall interaction. Scytovirin was tested against two pathogenic strains of C. neoformans, 24067 and H99S, in the presence of testosterone to determine its effects on major virulence factors (polysaccharide capsule and melanization) and budding. The results showed Scytovirin not only reduced capsule size in strain H99S in the presence of testosterone, but also induced melanization and altered the time course of budding for strain 24067. Thus, Scytovirin, with inhibitory effects on C. neoformans, is a new and potential antifungal agent with potential increased potency in the higher mortality male population.
ACKNOWLEDGMENTS

It would be laborious work to go around such an aspect without the assistance and support of a number of experts. At the first glance it would be my pleasure to convey my especial thanks to Dr. Robert McFeeters for positioning me as a member of the best lab group at the University of Alabama in Huntsville and putting me on a right track in doing my project. It is unconceivable to get around this work without constant assistance and encouragement he furnished. I will always remember the great opportunities he created for me and tolerance he put up with. I also admit my special thanks to my committee members Dr. Debra Moriarity and Dr. Luis Cruz-Vera for their invaluable insight, recommendations and encouragement all the way through. I wish you all my best. I admit my great thanks to the biology department for assisting my graduate education. I would appreciate my family and friends for their steady pursuant and support. In the absence of them I would not be able to accomplish such a difficult rout.
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CHAPTER ONE

INTRODUCTION

1.1 Antifungals

Recently, the importance of fungal infections has become noteworthy in the field of medicine because of the wide range of population susceptible to infection. Patients under treatment with immunosuppressed drugs, individuals with organ transplants and those with AIDS are the most common populations at risk for fungal infections (Frosco M, 1998). These populations have grown due, in part, to medical advances. Thus fungal infections are a growing burden for worldwide healthcare.

Polyenes, pyrimidine analogs, echinocandins, and azoles are four major classes of antifungals that have frequently been used for treatment of fungal infections over the past few decades. Consequently, the emergence of antifungal resistance has increased and antifungals therapies have become less effective (Sanglard, 2016). Also, each class of antifungal has shown toxic side effects. For instance, amphotericin B, considered the “gold standard,” is widely used against various pathogenic fungi but brings about nephrotoxicity (Dixon & Walsh, 1996). Triazoles such as fluconazole and itraconazole are more commonly used than amphotericin B due to their lower side effects.
However, their spectrum of activity is limited to use in combination with other antifungal drugs (Dixon & Walsh, 1996). Therefore, the increase in antifungal resistance coupled with the limitation in activity of existing antifungal drugs makes the need for new effective antifungals pressing.

1.2 Pathogenic Fungi

Currently fungal pathogenesis is considered one of the most serious health problems worldwide. The frequency of fungal disease is highly elevated in immunocompromised versus immunocompetent patients, with outcomes depending on the fungal species. Overall fungi require four characteristics to infect humans: high temperature tolerance, ability to invade the human host, lysis and absorption of human tissue, and resistance to the human immune system (Kohler, Casadevall, & Perfect, 2015). The spectrum of human fungal pathogens encompasses numerous diseases with afflictions extending from the outer surface of the skin and mucosal surfaces to infection of internal organs. Fungal infections kill one and a half million people worldwide every year (Kim, 2016; Reedy, Bastidas, & Heitman, 2007).

1.3 Cryptococcus

One of the major fungal pathogens is Cryptococcus neoformans. In terms of epidemiology, Cryptococcus neoformans is found globally with prevalence among untreated patients with AIDS (Antinori, 2013). It contains four distinct serotypes, A, B, C and D which are identified by two C. neoformans varieties (var). Serotypes A and D belong to C. neoformans var. neoformans and serotypes B and C belong to C.
*Cryptococcus neoformans* var. *gattii* (Dromer, Gueho, Ronin, & Dupont, 1993), now considered a separate strain referred to as *C. gattii*. Serotype A is most prevalent in North and South America while serotype D, making up only 9% of reported cases, is predominantly found in Europe (Banerjee, Datta, & Casadevall, 2004). *C. gattii* has the highest prevalence (35-100% of reported cases) in Australia, Mexico, many South American countries, Hawaii, southern California, and many Asian countries to the north of the Equator (Cambodia, Thailand, Vietnam, Nepal), as well as in central Africa. Serotype B was about five times more common than serotype C (Banerjee et al., 2004). Being a global threat with increasing incidence, new and potent antifungals are needed for effective treatment of cryptococcal infections.

Among the various fungal infections, cryptococcosis has become increasingly important (Kwon-chung et al., 2014). Cryptococcosis is caused by the two etiological agents, *Cryptococcus neoformans* and *Cryptococcus gattii*, although both *C. neoformans* and *C. gattii* belong to the phylum Basidiomycota, each has its own ecological niche and prevalence of infection. Pigeon guano and higher plants are two common habitats occupied by *C. neoformans* and *C. gattii*, respectively. Lungs and the central nervous system are two main affected sites of cryptococcosis, and infection with *C. neoformans* widely occurs in immunocompromised patients. Conversely *C. gattii* mostly targets immunocompetent patients with significantly lower occurrence in immunocompromised individuals (Gibson & Johnston, 2015; Nielsen, Obaldia, & Heitman, 2007).

Cryptococcal infections take place through breathing in spores and dehydrated yeast cells (Buchanan & Murphy, 1998; Chun & Madhani, 2010). Once inhaled and settled in the lung tissue, the infection proceeds to initiation of pulmonary infection or
spread over influencing other organs and finally invasion of the central nervous system (Guerra, Seabra, Souza, & Rozental, 2014). This pathogenic yeast causes an estimated 1 million cases of pulmonary and cryptococcal meningitis including more than 600,000 deaths every year (Janbon et al., 2014). 30% of AIDS patients in sub-Saharan Africa suffer from cryptococcal meningitis, which can be a cause of death among those without access to antiretroviral therapy (Archibald et al., 2004). Figure 1 shows global burden of cryptococcal meningitis in patients with low CD4 count in different parts of the world.

![Global burden of cryptococcal meningitis](adapted_from_Rajasingham_et_al.,_2017)

**Figure 1** Annual incidence of cryptococcal meningities worldwide in patients with low CD4 count in 2014 (Adapted from Rajasingham et al., 2017).
1.4 Gender Based Infection of *C. neoformans*

*C. neoformans* epidemiology shows a large number of differences in infections in male versus female patients. Most notably, there is a higher incidence of *C. neoformans* infection in males than females (McClelland et al., 2013). Evidence about the population of cryptococcal infected male and female populations before the prevalence of HIV worldwide showed that susceptibility to *C. neoformans* is higher in males compared to females. Also, the *C. neoformans* infected population during the HIV epidemic still showed an increased rate of mortality in males than females. In terms of gender based differences in infection, it has been found that estrogen and testosterone have different effects on *C. neoformans* and reactivity of *C. neoformans* with testosterone results in a decreased immune response (Guess, Rosen, & McClelland, 2018). Therefore it can be concluded that *C. neoformans* infection is hormone related. Increased mortality in males compared to females occurs and common antifungal agents are without increased efficacy on infected males. Therefore searching for new antifungal agents, in particular one that can effectively decrease the severity of infection in male patients, is a matter of importance.

1.5 Capsule of *C. neoformans*

*C. neoformans* is exclusive among fungal pathogens since it is encapsulated by a polysaccharide capsule (Kim, 2016). Pathogenicity of *C. neoformans* depends upon several virulence factors (Li, Rautengarten, Heazlewood, & Doering, 2018; Pontes & Frases, 2015). The polysaccharide (PS) capsule was found to be the major virulence factor of *C. neoformans*. Studies demonstrated that the acapsular mutants fail to infect
the host or they only slightly induce virulence (Buchanan & Murphy, 1998; Pontes & Frases, 2015; Rocha et al., 2015). The PS capsule is physically attached to the cell wall and is a barrier for macrophages to carry out phagocytosis (S. Frases et al., 2009; Li et al., 2018; Vecchiarelli et al., 2013). During infection, capsular PS is released to the extracellular space increasing the effective size of the cryptococcal cell. Getting larger reduces the ability of the host immune system phagocyte to destroy or inactivate the capsular cryptococci effectively (Li et al., 2018; Zaragoza, Telzak, Bryan, Dadachova, & Casadevall, 2006).

The cryptococcal capsule is comprised mainly of two polysaccharides, glucuronoxylomannan (GXM) and galactoxylomannan (GalXM) (Doering, 2009). It also contains mannoproteins as a minor component (Zaragoza et al., 2009). The hypothesized secretory pathway of capsular PS suggests that it is synthesized inside the cell and transported to the cell wall by vesicles through exocytosis (Doering, 2009; Rodrigues, Nimrichter, Oliveira, Nosanchuk, & Casadevall, 2008; Yoneda & Doering, 2006) (Figure 2).
Figure 2 Synthesis of polysaccharide capsule in *C. neoformans*. The polysaccharide capsule (C) is synthesized within the cell in Golgi apparatus (G), packed in multivesicular body (MVB) inside the cell, transported across the cell wall (CW) via exocytosis and physically attached to the cell wall (Adapted from Zaragoza et al., 2009).

Capsule morphology is the result of the arrangement of these three molecules so that they make a filamentous network (Araújo et al., 2016) with size ranges from 0.5-1.0 μm from the cell wall to 10-80 μm, depending on their existing condition (McFadden & Casadevall, 2001). The plentiful capsule component, GXM, is a linear mannose (Man) polymer including single glucuronic acid and xylose side chains (Li et al., 2018) (Figure 3). Pathogenicity of *C. neoformans* mostly depends upon GXM as it contains ~90% of the capsular PS structure (Yoneda & Doering, 2006). The second polysaccharide, GalXM, is comprised of a galactose structure with single galactofuranose (GaLf) and galactomannan side chains making a different number of glucuronic acid and xylose residues (Li et al., 2018).
Figure 3 Repeating structure of GXM as a major component of polysaccharide capsule in *Cryptococcus neoformans* (Adapted from Jones, 2017).

Assembly of capsular PS on the cell surface requires connection of the capsular components to the cell wall (Susana Frases, Nimrichter, Viana, Nakouzi, & Casadevall, 2008). Carbohydrate structures of the cell wall containing glucan and chitin, together with the complex polysaccharide capsule, enables pathogens to withstand host defenses and bring about disease (Li et al., 2018; Yoneda & Doering, 2006) (Figure 4).

Figure 4 Demonstration of polysaccharide capsule, three major components of the cell wall (Mannan, β Glucan and Chitin) and membrane in *C. neoformans* (Adapted from Jones, 2017).
Cryptococcal cell wall contains α- and β-glucans (Mukaremera et al., 2018).

Among glucan compositions, polysaccharide α-1,3-glucan is one of the essential factors for connection of the capsule to the cell wall (Fonseca et al., 2009; Reese & Doering, 2003). Inhibition of α-1,3-glucan expression or removing it by α-1,3-glucanase can remodel the cell wall in a way that the capsule fails to connect to the cell wall appropriately, making the cryptococci cell avirulent or less virulent (Reese & Doering, 2003; Reese et al., 2007). In fungal cells N-acetylglucosamine (GlcNAc) is a monomeric unit of chitin and plays an important structural role on the cell surface. In the course of infection with C. neoformans, synthesis of GlcNAc-containing molecules and capsular polysaccharides increases in parallel and it has been found that GXM, the major capsular component, has affinity for the molecules containing GlcNAc (Fonseca et al., 2009). Deacetylation of chitin results in the formation of chitosan which is a linear polysaccharide with different proportions of D-glucosamine and N-acetyl-D-glucosamine in its structure (Zakaria, Zakaria, Musa, Hamilin, & Zulkifly, 2012). Chitosan is one of the other important molecules in the cryptococcal cell wall. Cells lacking chitin and its deacetylated derivatives show impaired cell wall-capsule connection and changes in the regular formation of capsule on the cell surface (Baker, Specht, Donlin, & Lodge, 2007; Fonseca et al., 2009). Pathogenicity of C. neoformans can be increased by its capsule enlargement under stress conditions such as period of in vivo infection or growth in the existence of low iron, mammalian serum, and physiological concentration of carbon dioxide in vitro (Movahed et al., 2015).
1.6 Melanization of *C. neoformans*

Another virulence factor that contributes to the pathogenicity of *C. neoformans* is melanin formation. *C. neoformans* produces melanin to protect itself against stress conditions and immune responses such as oxidative stress and invasion of macrophages (Mednick, Nosanchuk, & Casadevall, 2005; Pereira, Bueno, Dias, Paula, & Siqueira, 2009). Formation of melanin tightly depends on the expression of laccase enzyme and the presence of melanin precursors (Waterman et al., 2007). Data from immuno-electron microscopy has shown that laccase is localized to the carbohydrate cell wall. Therefore, peripheral localization of laccase on the *C. neoformans* cell wall allows this enzyme to activate melanin production through oxidation of various phenolic compounds (Zhu & Williamson, 2004). Once melanin is formed it is deposited in the cell wall (Eisenman, Frases, Nicola, Rodrigues, & Casadevall, 2009). Melanization of *C. neoformans* is of particular interest to this research because it can be investigated as one of *C. neoformans* defense mechanisms against antifungals.

1.7 Scytovirin

Scytovirin is considered to be a strong antiviral lectin which is found in the Cyanobacterium called *Scytonema varium*. It has been produced as a recombinant protein in *E. coli* (Moulaei et al., 2007). Scytovirin has significant inhibitory activity against human immunodeficiency virus (HIV) (Garrison et al., 2014; Moulaei et al., 2007). Thus Scytovirin has the potential to become a pharmaceutical agent for prevention of this viral infection (Moulaei et al., 2007). The molecular weight of Scytovirin is 9.7 KDa (Xiong, O’Keefe, Botos, Wlodawer, & Mcmahon, 2006) and it is a
single polypeptide chain of 95 amino acids, with a conserved internal 39 amino acid repeat (McFeeters et al., 2013; Moulaei et al., 2007). Structural analysis of Scytovirin has shown two domains for this protein, SD1 (residue 1-48) and SD2 (residue 49-95). Scytovirin contains one inter-domain disulfide bond which is formed by Cys7 of SD1 and Cys55 of SD2 (Moulaei et al., 2010). It has been reported that Scytovirin does not have regular secondary structure (Moulaei et al., 2007) (Figure 5).

Figure 5 Two nearly identical domains of Scytovirin, red represents SD1 and blue represents SD2. Disulfide bonds are shown in yellow. Gray boxes show different residues between SD1 and SD2.

It has been found that Scytovirin has antifungal activity against different serotypes of *C. neoformans* and exerts its inhibitory effect through the cell wall (Jones, Mcclelland, Mcfeeters, & Mcfeeters, 2017). Biochemical analysis of *C. neoformans* cell wall shows chitin, glycoproteins and glucans as the main components (Muszewska, Pilsyk, Perlinska-Lenart, & Kruszewska, 2018). Although *C. neoformans* contains a large amount of mannose in its polysaccharide capsule, Scytovirin binds high mannose oligomers of N-linked fungal glycans very specifically (W.Adams et al., 2004). The localization of Scytovirin on the cryptococci cell wall was shown by fluorescence confocal microscopy (Jones et al., 2017). Binding properties of Scytovirin to *C.
*neoformans* and its antiviral activity creates an insight into its antifungal properties. It has been found that Scytovirin has antifungal activity against pathogenic fungi *C. neoformans* and *C. gattii* with different degrees of inhibition for each cryptococcus variety so that serotype D from *C. neoformans* and serotypes B and C from *C. gattii* are more inhibited by Scytovirin. Additionally, combination of Scytovirin and other antifungals such as amphotericin B, flucytosine and fluconazole revealed synergy (Jones et al., 2017). Thus further investigation of the antifungal properties of Scytovirin is warranted.

### 1.8 Effect of Scytovirin and Testosterone on *C. neoformans*

Scytovirin, a novel antifungal lectin, was used in this study to determine whether it has significant impact on *C. neoformans* cells in the presence of testosterone. Because the occurrence of *C. neoformans* infections caused by serotypes A and D is more frequent worldwide than other serotypes, this study has focused on the strains 24067 and H99s which belongs to serotypes D and A, respectively.

Since the polysaccharide capsule is the most important virulence factor for *C. neoformans* pathogenicity, this study focused on the effect of Scytovirin and testosterone on capsule size of the cells. Also melanization was investigated visually based on morphological features of the cell wall in cells exposed to Scytovirin. Lastly, the budding stages of cells were classified based on their morphological shape to determine the effect of Scytovirin on the budding process.
CHAPTER TWO

MATERIALS AND METHODS

2.1 Growth of *C. neoformans* Strains 24067 and H99S

In this study, cells are used initially from frozen stock and grown in yeast extract peptone dextrose (YEPD or YPD) broth media. To prepare broth culture, 10 μl of 24067 or 50 μl of H99S frozen stock were added to 3 mL of YPD broth media and incubated at 37 °C in a rotary shaker with moderate shaking of 150 rpm for 3-4 days. These cultures were streaked on the YPD agar plates and incubated at 37 °C for 4 days until colonies with creamy and butyrous (buttery) appearance show up on the plate. Cells from a colony on the YPD plate were checked for their capsule size by staining the cells with India ink and visualizing them using 100x magnification power of the light microscope. A single colony from the YPD plate was used to inoculate 5 mL of 100% Sabouraud dextrose medium which was then incubated at 37 °C in rotary shaker at 150 rpm for ~18h. The culture was grown to an OD_{600} of ~0.67 (mid-log phase), equivalent to the concentration of ~ 2 x 10^7 cells/mL. The media used in this section was made based on the following recopies:
**Table 1** YPD Broth (YEPD Broth).

<table>
<thead>
<tr>
<th>Ingredients</th>
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<td>20</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>10</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20</td>
</tr>
<tr>
<td>Final PH (at 25 °C)</td>
<td>6.5±0.2</td>
</tr>
</tbody>
</table>

**Table 2** YPD Agar.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Grams / Liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>20</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>10</td>
</tr>
<tr>
<td>Glucose</td>
<td>20</td>
</tr>
<tr>
<td>Agar</td>
<td>20</td>
</tr>
</tbody>
</table>

**Table 3** 100% Sabouraud Dextrose Broth.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Grams / Liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic Digest of Casein</td>
<td>5</td>
</tr>
<tr>
<td>Enzymatic Digest of Animal Tissue</td>
<td>5</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20</td>
</tr>
<tr>
<td>Final PH (at 25 °C)</td>
<td>5.6±0.2</td>
</tr>
</tbody>
</table>
2.2 Broth Macrodilution Assay

Broth macrodilution was performed in two different media, RPMI/MOPS and 10% Sabouraud dextrose broth to determine whether there is a change in minimum inhibitory concentration (MIC) of Scytovirin using different media. For broth macrodilution MIC testing, Scytovirin with initial concentration of 400 μM was serially diluted by half in PBS and added to 5 mL snap cap tubes in 100 μl aliquots. 100% Sabouraud dextrose broth culture was diluted 1:100 for cell count using Bright-Line™ hemocytometer. Cells were then diluted to a concentration of 2.22 x 10^3 cells/mL in the media. Aliquots of 900 μl of 2.22 x 10^3 cells/mL in the media was added to the tubes containing Scytovirin for total volume of 1 mL and final cell concentration of 2000 cells/mL. Final concentrations of Scytovirin in each culture were 0, 0.1, 0.3, 0.6, 1.3, 2.5, 5.0, 10, 20 and 40 μM. The cultures were incubated at 37 °C in rotary shaker with moderate shaking of 150 rpm for 72 hours. The culture without Scytovirin and a culture containing 1 μM final concentration of amphotericin B were used as the positive and negative controls, respectively.

2.3 Capsule Induction of the Cryptococcus 24067 and H99S

Capsule growth can be induced by several factors including low nutrient conditions, serum and CO₂ exposure. In this experiment, the low nutrient condition was used for capsule induction. A single colony of C. neoformans on the YPD agar plate was used to inoculate 100% liquid Sabouraud dextrose medium which was then incubated at 37 °C in rotary shaker at 150 rpm. Once the culture reached an OD₆₀₀ of ~ 0.67, it was diluted 1:100 in 10% Sabouraud dextrose broth medium buffered to pH 7.3 with 50 mM
MOPS, incubated at 37 °C in rotary shaker with moderate shaking of 150 rpm to induce the capsule.

Table 4 10% Sabouraud Dextrose Broth.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>500 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM MOPS</td>
<td>4.69 grams</td>
</tr>
<tr>
<td>100% Sabouraud Dextrose Broth</td>
<td>50 mL</td>
</tr>
<tr>
<td>Final PH (at 25 °C)</td>
<td>7.3</td>
</tr>
</tbody>
</table>

2.4 Cryptococcal Cell Staining for Microscopic View

The 10% Sabouraud dextrose broth cell cultures were pelleted at 870 g for 5 minutes. The supernatant was discarded and the pellet was washed 3 times. Each time the supernatant was discarded, the pellet was resuspended in the same volume of PBS before re-pelleting the culture. After the last wash, 5-10 μl of the supernatant was kept at the bottom of the tube and the rest of it decanted and the pellet resuspended in the remaining supernatant. 4 μl of suspension was dropped on the glass slide and mixed with 2 μl of 0.9% saline and 1.4 μl India ink. A coverslip was put on the mixture without foam formation under it. The prepared sample was visualized with 100x magnification on a light microscope.

2.5 Capsule Size Measurement

Because the prepared sample may contain cells with different capsule size at budding stages, a field of view representative for all types of the cells was selected.
At least 50 cells from the selected field of view were pictured using AmScope software v3.7. The diameter of the capsular space in each cell was determined by measuring the diameter of the spherical cell body without capsule and subtracting it from the diameter of the spherical volume of the entire cell (including cell body and capsule around it) (McFadden & Casadevall, 2001). The unit of measurement in AmScope software is a pixel. Thus, in order to convert pixel to micron the calibration slide, containing 100 divisions of 0.01 mm, was visualized microscopically by 100x magnification power. 10 Divisions were measured in terms of pixels and the average of them was used to generate the conversion of 446 pixels = 10 μm. Diameter were measured in pixels and then converted to microns using the above equation.

### 2.6 Preparation of Testosterone Solution

A testosterone solution was made from its powder with the molecular formula of C_{19}H_{28}O_{2} and molecular weight of 288.42 g/mol. Testosterone is soluble in both ethanol and DMSO with maximum concentration of 25 mM and 100 mM, respectively (www.tocris.com). Because DMSO has an inhibitory effect on growth of cryptococcal cells, absolute ethanol was used as a solvent to make the testosterone solution in which 5 mg of testosterone powder was measured with analytical balance and added to 17.34 mL ethanol to make 1 mM solution. The 1 mM solution was diluted 1:1,000 in 100% ethanol to get the concentration of 1 μM. In order to fully dissolve the testosterone in ethanol, a rapid stirring method was used.
2.7 Effect of Scytovirin and Testosterone on the Cryptococcal Capsule

To measure the effects on capsule, 10% Sabouraud dextrose broth cell culture was tested with Scytovirin, the mixture of Scytovirin and testosterone, and testosterone itself in parallel. The effect of testosterone on the Cryptococcal capsule, with or without Scytovirin, was tested based on its physiological concentration of 10 nM (Kelsey et al., 2014). The concentration of Scytovirin was determined based on the MIC value in 10% Sabouraud dextrose broth medium. To investigate the range of changes in the cryptococcal capsule size that may occur in the presence of high and low concentrations of Scytovirin, twice, equal, half and one quarter of the MIC value were selected.

Scytovirin with an initial concentration of 13.3 μM was serially diluted by half in PBS and added to 5 mL snap cap tubes in 90 μl aliquots. 10 μl ethanol was added to each tube and the solution brought up to 100 μl. Final concentrations of Scytovirin in each tube were 1.19, 0.6, 0.3 and 0.14 μM. To determine the effect of Scytovirin and testosterone on the capsule size of the cells, 10 μl of 1 μM testosterone was added to each tube instead of ethanol for final physiological concentration of 10 nM. For testing the effect of testosterone with physiological concentration of 10 nM on the capsule size, 90 μl PBS was mixed with 10 μl of 1 μM testosterone in a snap cap tube. Also, a control sample was made by combining 10 μl ethanol (solvent for testosterone) and 90 μl PBS (solvent for Scytovirin) in a tube. Lastly, in order to keep the cells in a stable capsule inducing condition, 900 μl of 10% Sabouraud dextrose broth cell culture at OD_{600} of 0.22, equivalent to the concentration of 6.2 x 10^6 cells/mL, was added to the all tubes for total volume of 1 mL and a final concentration of 5.58 x 10^6 cells/mL. All of the cultures were incubated at 37 °C in rotary shaker with moderate shaking of 150 rpm.
The same experimental procedure was followed as explained above for strain H99S with the exception of Scytovirin concentrations which were prepared based on the MIC value for H99S. To make twice, equal, half and one quarter of the MIC value, Scytovirin was serially diluted by half in PBS for final concentrations of 10, 5, 2.5 and 1.2 μM. Lastly, 900 μl of 10% Sabouraud dextrose broth cell culture at OD₆₀₀ of 0.26, equivalent to the concentration of 6.15 x 10⁶ cells/mL, was added to the all tubes explained above for total volume of 1 mL and final concentration of 5.53 x 10⁶ cells/mL.

Cells from each sample were visualized at 24, 48 and 72 hours (for both 24067 and H99S strains). At each time point, 100 μl of each sample was transferred to an Eppendorf tube, and cells were prepared for staining as described previously.
CHAPTER THREE

RESULTS

The novel antifungal lectin, Scytovirin, was tested in combination with testosterone to determine the effect on cryptococcal capsule size. This combination was also tested to examine the effects on growth, distribution of the cells based on their budding stages, and melanization. These together can determine how Scytovirin and testosterone can effectively reduce the pathogenicity of Cryptococcus neoformans, which is globally more frequent in males compared to females. For this purpose, a range of Scytovirin concentrations were used to inhibit the growth of C. neoformans in relation to the MIC determined from a macrodilution assay. Scytovirin was combined with a physiological concentration of testosterone (10 nM) to determine any significant changes in cryptococcal cells. The capsule size of the cells in each sample was compared to the control containing cells in PBS and ethanol, the solvents for Scytovirin and testosterone, respectively. Cells were categorized as normal, early budding, mid budding and late budding cells based on the different budding stages in the cryptococcal cell cycle to determine whether Scytovirin interferes with budding. The effect of different concentrations of Scytovirin on melanization was investigated by following the
increasing or decreasing trend of the percentage of cells with a thick cell wall versus concentrations of Scytovirin.

Collectively, ten samples were tested for this experimental procedure in which only 10 nM testosterone was used with various concentrations of Scytovirin for their range of effect on the cryptococcal capsule size. Classification of the cells as normal, early budding, mid budding, late budding, melanized and non-melanized was done visually based on cell morphology. Two experimental strains of *C. neoformans*, 24067 (serotype D) and H99S (serotype A), were used for this experiment, treated identically to compare their statistical results.

### 3.1 Broth Macrodilution MIC

MIC values of Scytovirin for broth macrodilution in two different media (Table 5), RPMI/MOPS and 10% Sabouraud dextrose broth, were found to be more effective at a lower concentration in 10% Sabouraud dextrose broth for both cryptococcal strains.

<table>
<thead>
<tr>
<th>Media</th>
<th>*C. neoformans strain</th>
<th>MIC [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% Sabouraud dextrose Broth</td>
<td>24067</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>H99S</td>
<td>5</td>
</tr>
<tr>
<td>RPMI/MOPS</td>
<td>24067</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>H99S</td>
<td>&gt;40</td>
</tr>
</tbody>
</table>
3.2 Induction of *C. neoformans* Capsule

Cell capsules were induced and measured at 24-hour intervals up to 72 hours in order to determine the appropriate time point to add Scytovirin and testosterone to the cells. Morphological features of un-induced and induced cells are shown in Figure 6. While measuring the capsule size of the cells, budding cells were separated from individual cells to compare their capsule size under the stress conditions. The measured result revealed that the mean of the capsule diameter was slightly higher in budding cells in comparison to individual cells. Consequently, all cells were added together for final measurement. In Figure 7 and 8, time 0 represents the capsule size of the cells immediately after transition into the induction media. Capsule enlargement continued and reached to its maximum size at 48 hour and was repressed while getting close to 72 hours.

![Un-induced and Induced *C. neoformans*](image)

**Figure 6** Un-Induced and Induced *C. neoformans*. 
Figure 7 Capsule size as a function of time for strain 24067. Error bars represent standard error of the mean from 3 measurements.

Figure 8 Capsule size as a function of time for strains H99S. Error bars represent standard error of the mean.
3.3 Effect of Scytovirin and Testosterone on the Cryptococcal Capsule

Based on the preliminary data, the 24 hour time point after capsule induction was selected as the starting point to add Scytovirin and testosterone to the culture. This was chosen because at 24 hours cells in the culture with \( \text{OD}_{600} \) of 0.16 and concentration of 6 \( \times 10^6 \) cells/mL are in the middle of capsule enlargement and possible repression of capsule growth by Scytovirin and testosterone prevents cells from reaching their maximum capsule size. In this experiment, cells were tested with Scytovirin and testosterone separately and together to determine if any change in capsule size is the result of cells being exposed to only Scytovirin, testosterone or both. Each sample was compared to the control containing cells in ethanol and PBS, the solvents for testosterone and Scytovirin, respectively.

3.3.1 Effect of Scytovirin and Testosterone on the Capsule of Strain 24067

Strain 24067 was exposed to Scytovirin and testosterone and were imaged after 24, 48 and 72 hours to measure the effects on capsule size. The data showed that Scytovirin and testosterone separately do not have an effect on capsule size when compared to the control (Figures 9 and 10). Also, capsule size of the cells did not significantly change in the presence of Scytovirin and testosterone together (Figure 11). Thus it does not appear that Scytovirin and testosterone have any effect on the capsule production in 24067 cells.
**Figure 9** Capsule diameter of *C. neoformans* 24067 as a function of time with various concentrations of Scytovirin (Sc2). Scytovirin was added to the cells at 24*. Error bars represent standard error of the mean.

**Figure 10** Capsule diameter of *C. neoformans* 24067 as a function of time with testosterone 10 nM. Testosterone 10 nM was added to the cells at 24*. Error bars represent standard error of the mean.
Figure 11  Capsule diameter of *C. neoformans* 24067 as a function of time with various concentrations of Scytovirin (Sc2) along with testosterone (T) 10 nM. Scytovirin and testosterone were added to the cells at 24*. Error bars represent standard error of the mean.

3.3.2 Effect of Scytovirin and Testosterone on the Capsule of Strain H99S

Strain H99S was exposed to Scytovirin and testosterone at OD<sub>600</sub> of 0.26, equivalent to the concentration of 6.15 x 10<sup>6</sup> cells/mL, and cells from each sample were imaged after 24, 48 and 72 hours to measure the capsule size. The data revealed that Scytovirin and testosterone separately do not have effect on capsule size when compared to the control (Figures 12 and 13), whereas Scytovirin with 10 nM testosterone decreases capsule size. The effect is most pronounced at 72 hours and at the highest concentration of Scytovirin. At lower Scytovirin concentrations, no change in capsule size is observed (Figure 14).
Figure 12 Capsule diameter of *C. neoformans* H99S as a function of time with various concentrations of Scytovirin (Sc2). Scytovirin was added to the cells at 24*. Error bars represent standard error of the mean.

Figure 13 Capsule diameter of *C. neoformans* H99S as a function of time with testosterone 10 nM. Testosterone 10 nM was added to the cells at 24*. Error bars represent standard error of the mean.
3.4 Effect of Scytovirin on Cell Distribution Based on Budding Stages

Cells in each sample were classified as normal, early-budding, mid-budding and late-budding cells based on their morphology to determine the effect of Scytovirin and testosterone on duration of cell cycle phases (Figure 15). Different budding stages occur at the different phases of the cell cycle. Normal, early-budding and late-budding cells are grouped into interphase; however, referring each budding stage to its exact phase of cell cycle is dependent on DNA content inside the cells (Yamaguchi, Ohkusu, Biswas, & Kawamoto, 2007). Mid-budding cells can be grouped into mitotic phase or interphase depending on the formation of mitotic spindles. In this study, the distribution of normal, early-budding, mid-budding and late-budding cells in each sample was compared to the control at time points of 24, 48 and 72 hours for strains 24067 and H99S. Results revealed that the rate of early-budding and mid-budding cells increased at each time point.
by adding 1.2 μM Scytovirin to the 24067 cells (Figure 16-18), while the rate of budding stages in strain H99S was changed slightly by adding 10 μM Scytovirin to the cells (Figure 19-21).

![Budding stages of C. neoformans.](image)

**Figure 15** Budding stages of *C. neoformans.*
Figure 16  Budding stages of *C. neoformans* 24067 with 1.2 μM Scytovirin (Sc2) compared to control at 24 hours.
Figure 17  Budding stages of *C. neoformans* 24067 with 1.2 μM Scytovirin (Sc2) compared to control at 48 hours.
**Figure 18** Budding stages of *C. neoformans* 24067 with 1.2 μM Scytovirin (Sc2) compared to control at 72 hours.
Figure 19 Budding stages of *C. neoformans* H99S with 10 μM Scytovirin (Sc2) compared to control at 24 hours.
Figure 20  Budding stages of *C. neoformans* H99S with 10 μM Scytovirin (Sc2) compared to control at 48 hours.
Figure 21 Bud stages of *C. neoformans* H99S with 10 μM Scytovirin (Sc2) compared to control at 72 hours.
3.5 Effect of Scytovirin on Melanization of *C. neoformans*

Melanization is a defense mechanism in *C. neoformans* in which melanin is collected in the cell wall making it thicker and chromatic. The population of melanized cells in the samples containing Scytovirin was visually separated from non-melanized cells so that cells with thick cell walls were considered as melanized cells (Figure 22). The distribution of melanized cells with various concentrations of Scytovirin were compared to each other and finally compared to control in order to determine the effect of Scytovirin on melanin production. The number of melanized cells in strain 24067 increased upon exposure to 1.2 μM Scytovirin and a declining trend was seen with decreasing the concentration of Scytovirin to 0.6 μM and 0.3 μM at time points of 24, 48 and 72 hours (Figure 23). For strain H99S, the declining trend in melanization with different concentrations of Scytovirin was only seen at the 24 hour time point (Figure 24).

![Non-Melanized Cell and Melanized Cell](image)

**Figure 22** Morphological feature of melanized and non-melanized cells of strains 24067 and H99S.
Figure 23 Melanization of *C. neoformans* strain 24067 with various concentrations of Scytovirin.

Figure 24 Melanization of *C. neoformans* strain H99S with various concentrations of Scytovirin.
CHAPTER FOUR

DISCUSSION

4.1 Inhibitory Effect of Scytovirin and Testosterone on Cryptococcal Cells

The main purpose of this study was to determine the effect of Scytovirin and testosterone on *C. neoformans*. It has been found that testosterone induces the release of GXM from the capsule of *C. neoformans* in male patients with cryptococcal infection. This can result in an increase of pathogenicity and depends on type of strain causing infection (McClelland et al., 2013).

To determine whether the MIC value of Scytovirin against *C. neoformans* is changed based on their growth condition, an MIC assay was performed in capsule induction media, 10% Sabouraud dextrose broth, and the results were compared to regular media, RPMI/MOPS. By determining MIC values of Scytovirin against these strains, concentrations below and above the MIC value in 10% Sabouraud dextrose broth media were selected and tested with and without testosterone for their efficacy on cryptococcal capsule size. The broth macrodilution assay revealed a higher degree of susceptibility to Scytovirin for strain 24067 than H99S in both regular and stressed conditions, and it was found that Scytovirin is more effective in inhibiting both strains with larger capsule size under stressed conditions.
Although the polysaccharide capsule of strain 24067 is larger than H99S in stressed conditions, Scytovirin inhibits strain 24067 more effectively than H99S regardless of its capsule size.

It can be concluded from MIC values in stressed conditions and the localization of Scytovirin to the cryptococcal cell wall (Jones et al., 2017) that Scytovirin does not physically interfere with polysaccharide capsule, but instead affects the cell wall. Despite Scytovirin showing antifungal properties against *C. neoformans* serotypes A and D, data showed that capsule size of strains 24067 and H99S was not changed by exposure to Scytovirin. Additionally, strain H99S but not 24067 showed susceptibility to the combination of Scytovirin and testosterone in terms of decrease in capsule size. Although Scytovirin by itself does not change the size of cryptococcal capsule, it is in some degree effective in combination with testosterone to decrease capsule size in some, but not all, cryptococcal strains. Also, testosterone showed close similarity to the control in terms of not being effective in reducing capsule size of strains 24067 and H99S. So, antifungal properties of Scytovirin against strain H99S can be improved by using Scytovirin together with testosterone to increase the effect on capsule size of the cells.

Scytovirin, which is localized to the cryptococcal cell wall, showed a greater effect on capsule size of strain H99S at high concentration in combination with physiological concentration of testosterone. Although there is data available about the structural properties of Scytovirin and its localization on *C. neoformans*, its mechanism of action is a matter of consideration for future studies. The combined effect of Scytovirin and testosterone on capsule size can be a result of their interaction to induce changes in capsule size so that testosterone may have a significant impact on Scytovirin.
or vice versa. The same experimental procedure was provided for both strains H99S and 24067, but their response to the external stimuli was not the same as each other. This shows that *C. neoformans* serotypes A and D have distinguished intracellular and extracellular properties. Noise in the trend lines of capsule size as a function of time (Figures 9-14 in the Result section) can be investigated by repeating the experiment and doing the cell count on a larger scale. For further research about significant level of change in capsule size of strain H99S, concentrations of Scytovirin and testosterone tested in this study can be increased in various proportions. The efficacy of Scytovirin and testosterone together to reduce capsule size of strain H99S may be changed when they are exposed to the cells in different induced conditions such as CO$_2$ exposure, mammalian serum, or low nutrient condition.

### 4.2 Effect of Scytovirin on Melanization of Cryptococcal Cells

In this study, melanization as a morphological change of cell wall was recognized visually in population of cells exposed to Scytovirin, specifically at the highest concentration used in this experiment. Cells with thicker and chromatic cell walls were considered melanized cells. Melanization is another virulence factor of *C. neoformans* and occurs when cryptococcal cells need to be protected from attack of immune effector cells (Wang, Aisen, & Casadevall, 1995). Since Scytovirin acts as an antifungal substance to inhibit cryptococcal cells, the cells produce melanin as a defense mechanism. Although cells with a thicker, chromatic cell wall were visualized in both strains 24067 and H99S, a greater population of melanized cells was found in strain 24067. This suggests that each of these strains reacts to stressed conditions differently.
The expression of laccase enzyme is a key factor for melanization in cryptococcal cells. It is directly affected by the amount of glucose available for cells. Low levels of glucose result in more laccase enzyme localized to the cell wall (Waterman et al., 2007).

In this study, cells were placed in starvation conditions, 10% Sabouraud dextrose broth, and incubated for 72 hours. An upward trend in melanization as a function of time was observed in cultures with various concentrations of Scytovirin. This is consistent with the fact that the expression of laccase enzyme increases in the presence of glucose deprivation. In strain H99S melanization mainly occurred in cultures containing Scytovirin 2.5 and 1.2 μM, while in strain 24067 an upward trend was seen in cultures with Scytovirin 1.2, 0.6 and 0.1 μM. On the other hand, in strain 24067 melanization as a function of Scytovirin concentration showed downward trend from 1.2 μM to 0.3 μM at each time point. Strain H99S, in contrast, only shows downward trend from 10 μM to 1.2 μM at 24 hour. Data from strain 24067 indicates that Scytovirin initiates the response of melanization during glucose deprivation. Also melanization is increased in strain 24067 which is more susceptible, so more stressed. These data suggest that strain, level of glucose, and concentration of Scytovirin contribute to the outcome of C. neoformans melanization. Since the data about melanization was established based on visualizing cell wall appearance, further methods can be used to improve these findings. This experiment can be performed with higher concentrations of Scytovirin, greater than 1.2 μM for strain 24067 and greater than 10 μM for strain H99S, and investigated for higher populations of melanized cells compared to lower concentrations. Also, glucose rich conditions can be replaced the starvation conditions in this experiment as a control for cultures containing melanized and non-melanized cells. Transmission electron
microscopy can be used for microscopic view of cell wall architecture of melanized cells and non-melanized cells.

**4.3 Effect of Scytovirin on Cryptococcal Budding Stages**

In strain 24067 and H99S, cells containing the highest concentration of Scytovirin were separated into different budding stages based on visual observation of budding morphology to determine the effect of Scytovirin on budding state population. Strain 24067 showed consistent results of increased level of early-budding and mid-budding cells at each time point, while no consistency was visualized in budding stages of strain H99S in response to Scytovirin. Also, it was observed that, in strain H99S, the population of normal cells was significantly higher than the rest of budding stages either with or without Scytovirin at each time point. The data suggests that the mechanism of action of Scytovirin may have a different effect on budding stages of strains 24067 and H99S. Scytovirin can possibly arrest cryptococcal cells in early-budding and mid-budding stages in strain 24067 and impair the cell cycle, while it has less or no effect on the cell cycle of strain H99S. It has been found that the amount of DNA content in each budding stage represents the appropriate phase of the cell cycle (Ohkusu, Hata, & Takeo, 2001; Yamaguchi et al., 2007). Finding the amount of DNA of each budding stage in this study may give a new category for budding stages. This study can be improved by larger scale of cells for classification and reclassifying budding stages based on DNA content rather than morphological features.
CHAPTER FIVE

CONCLUSION

Infection with Cryptococcus neoformans is considered one of the serious diseases worldwide specifically among immunocompromised patients with HIV/AIDS. Pathogenicity of C. neoformans depends on several virulence factors among which polysaccharide capsule plays crucial role. One of the considerable points about cryptococcal infection is that mortality rates differ in males compared to females so that males are infected more frequently than females. Recently, testosterone and estrogen, two major hormones in males and females, respectively, are found to affect cryptococcal infection differentially. Therefore, cryptococcal infection is considered hormone related. Although there are several antifungal drugs with different mechanisms to inhibit C. neoformans and prevent cryptococcal infection, none of them have shown significant effects on the male population compared to female population.

In this study, the novel antifungal lectin, Scytovirin, was tested together with testosterone to detect the effect on C. neoformans capsule strains 24067 and H99S. The findings showed that Scytovirin and testosterone do not change the capsule size of strain 24067; however, they change the capsule of H99S. Scytovirin inhibits the growth of C. neoformans strains 24067 and H99S at their MIC values of 0.6 μM and 5 μM, respectively.
There is an effect on the capsule size of H99S, but not 24067 with exposure to high concentration of Scytovirin in the presence of testosterone. Testosterone at the physiological concentration of 10 nM does not inhibit the growth of either strain of *C. neoformans* nor does it affect capsule size. Therefore, the impact of Scytovirin in the presence of testosterone to decrease the capsule size of strain H99S makes it a better antifungal agent in males compared to females.

For future research, Scytovirin and testosterone can be added to the cells at time point 0, right after transferring the culture to 10% Sabouraud dextrose broth medium when capsule of the cells are first induced. This test can determine whether Scytovirin and testosterone can inhibit the initial capsule growth of the cells. The results of this test can be compared to the preliminary data showed in result section, Figures 7 and 8, to evaluate the effect of Scytovirin and testosterone on inhibition of capsule induction at 24, 48 and 72 hours. Furthermore, the capsule growth inhibition experiment can be performed using CO$_2$ exposure as a stressed condition to induce capsule of the cells. The range of changes in capsule size can then be determined in the presence of Scytovirin and testosterone.

Also, MIC assay can be performed with Testosterone itself and combined Scytovirin and testosterone to determine their MIC value against *C. neoformans*, and subsequently define whether testosterone can change the MIC value of Scytovirin. This experiment is important because it helps to understand if significant effect of combined Scytovirin and testosterone on Strain H99S in terms of diminishing its capsule size is due to inhibitory effect of Scytovirin and testosterone or it directly affects the capsule size without increasing inhibitory effect on the cells.
Testosterone is a steroid hormone made from cholesterol and found in human and animal cells. Ergosterol is a sterol, a subgroup of steroids. It is a compound mainly found in the membrane of fungi. For this reason, it would be interesting to determine the combined effect of ergosterol with Scytovirin on the capsule size of *C. neoformans* and to compare the results to the effect of combined Scytovirin and testosterone. This experiment may help to determine the affected sites by Scytovirin and testosterone in *C. neoformans*, as well as to find other compounds that improve the effect of Scytovirin on *C. neoformans* on reducing capsule size.

In this study, budding stages of strains 24067 and H99S was determined based on the morphological feature of their buds. Based on our visual investigation through budding stages of both strains it can be concluded that Scytovirin can impair the budding process of strain 24067 by increasing the population of early-budding and mid-budding cells, while no consistent change was seen in the budding process of strain H99S after cells were exposed to Scytovirin. Cells arrested in early-budding and mid-budding stages can take longer to double. Therefore for future study, CFUs (colony forming unit) can be used to estimate the population of cells exposed to Scytovirin. To study the effect of Scytovirin on the cell cycle of *C. neoformans*, each phase of the cell cycle can be inhibited by an appropriate inhibitor. By inhibiting each phase, population of the budding stages can be determined based on their morphological features and compared to those exposed to Scytovirin. Performing this test with different strains of *C. neoformans* can reveal the response of each strain to Scytovirin in terms of change in their cell cycle.

Melanization is a defense mechanism in *C. neoformans* strains 24067 and H99S. Scytovirin triggers greater melanin production in strain 24067 than H99S under
starvation conditions. The degree of melanization showed inverse correlation with the nutrient level of growth environment so that as the level of glucose went down melanization increased. Further experiments are needed to establish the role of Scytovirin in this melanization process. For this purpose, an experiment can be performed with higher concentrations of Scytovirin, greater than 1.2 μM for strain 24067 and greater than 10 μM for strain H99S, to compare the population of melanized cells to that of lower concentrations of Scytovirin. Also, starvation conditions can be replaced by glucose rich conditions in this experiment to characterize glucose effect on Scytovirin induced melanization.

These future studies will continue to provide understanding into the antifungal mechanism of Scytovirin and its affects in combination with testosterone. Greater understanding of the mechanism and cellular impacts in different conditions furthers antifungal development of this emerging new approach.
REFERENCES


