Mechanism of Lysosomal Cathepsin B Degradation of *Cryptococcus neoformans*

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ABSTRACT

Cryptococcus neoformans is an opportunistic fungal pathogen that is spread through inhalation and predominantly causes disease in immunocompromised patients. The infection begins in the lungs as pneumonia and can disseminate into the central nervous system causing a fatal brain infection called cryptococcal meningitis. Dendritic cells (DCs) in the respiratory tract can phagocytose and kill inhaled *C. neoformans* in the lysosomal compartment. In addition, DC lysosomal extract has anti-cryptococcal activity. A purified lysosomal protease, cathepsin B, exhibits significant growth inhibition of *Cryptococcus* by an unknown mechanism resulting in osmotic lysis of the organism. Due to the morphology observed after cathepsin B treatment, we hypothesize that the cathepsin B is targeting the fungal cell wall integrity pathway. For these studies, we have treated *C. neoformans* with cathepsin B and have shown that cathepsin B has anti-cryptococcal activity. We are currently examining the cell wall integrity pathway with/without treatment by Western blot, and are examining the phosphorylation of PKA (the first protein in the signaling cascade) to determine if cathepsin B affects this pathway. The minimum inhibitory concentration assay of cathepsin B is also being examined in comparison with amphotericin B, a common antifungal drug known to inhibit *C. neoformans* growth. Future studies will examine phosphorylation of PKA and additional proteins in the cell wall integrity pathway and the effects of cathepsin B on cryptococcal virulence factors to identify additional mechanisms of cathepsin B anti-cryptococcal activity.

INTRODUCTION

Cryptococcus neoformans is an opportunistic fungal pathogen that is ubiquitous in nature and can predominantly be isolated from soil containing bird guano and rotting wood (Soltani, et al., 2013). The infection is typically spread through inhalation of aerosolized organisms. When inhaled, *C. neoformans* can cause severe infection in immunocompromised patients, particularly transplant recipients and those with HIV/AIDS (Pappas, et al., 2010; Park, et al., 2009). However, healthy individuals are rarely affected. The infection manifests in the lungs causing pneumonia, but due to its propensity to invade the central nervous system, it can rapidly disseminate into the bloodstream, causing cryptococcal meningitis (Góralska, et al., 2018). This clinical manifestation is often fatal in AIDS patients. In AIDS patients alone it is estimated that there are over 220,000 cases each year, resulting in over 181,000 deaths (Rajasingham, et al., 2017). Unfortunately, there are limited treatment options. Drug toxicity and antimicrobial resistance are current challenges (Denning & Bromley, 2015). In over 30 years, there have been no new antifungal drugs with cryptococcal activity introduced for treatment, highlighting the necessity of new antifungal agents targeting *Cryptococcus* (O'Halloran, et al., 2017).

Previous studies have shown that inhaled C. *neoformans* can be phagocytosed and killed by dendritic cells (DCs) in the lysosomal compartment through the endosomal and lysosomal pathways (Wozniak & Levitz, 2008). Additionally, it has been shown that DC lysosomal extract has significant anti-cryptococcal activity against multiple cryptococcal strains (Hole, et al., 2012). Cathepsin B is a lysosomal cysteine protease that primarily functions in intracellular proteolysis and is also known to be involved in hormone activation and bone turnover (Mort & Buttle, 1998). Interestingly, purified cathepsin B was found to inhibit the growth of C. n*eoformans* using an unknown non-oxidative mechanism, resulting in the osmotic lysis of the

fungus. Using scanning electron microscopy and transmission electron microscopy structural changes of the cells when treated with cathepsin B show a crescent morphology indicative of cell death with a ruptured cell wall but an intact cell membrane (Hole, et al., 2012). Since the inhibition of C. *neoformans* by cathepsin B is concentration-dependent, minimum inhibitory concentration (MIC) tests are being conducted to determine the lowest concentration of cathepsin B to be used in the following experiments.

Recent studies, as well as the morphology of the cells after cathepsin B treatment, suggest that the antifungal mechanism may be targeting the cell wall integrity (CWI) pathway. The CWI pathway is primarily responsible for the maintenance and biosynthesis of the fungal cell wall (Free, 2013). The target of this study is Pkc-1, which is the first protein in the signaling cascade. Studies will use western blot analysis to examine the phosphorylation of PKC-1 and additional proteins in the signal cascade before and after the treatment of cathepsin B.

MATERIAL & METHODS

Strains & Media. *Cryptococcus. neoformans* strain H99 (serotype A) was recovered from a 15% glycerol stock stored at -80°C prior to use in these experiments. The strain was maintained on yeast extract/peptone/dextrose (YPD) agar plates. Yeast cells were grown for 18 hours at 30°C with shaking in YPD broth. Cells were washed three times with sterile phosphate-buffered saline (PBS). Cells were quantified using trypan blue dye exclusion in a hemacytometer.

Incubation of lysosomal extracts with *Cryptococcus*. Following culture for 18 hours at 30°C, organisms were washed 3X with sterile PBS and resuspended in 10mM phosphate buffer with 2% RPMI, pH 5.5 (phosphate buffer) and adjusted to the concentration needed for the

experiment. For each well, 50 µl ($2.5x10^5$/ml) of *Cryptococcus* were added to a 96-well plate. Phosphate buffer (50 µl) was added to control wells, and 50 µl of lysosomal extract was added to the test wells, to a total volume of 100 µl. Plates were then incubated for 24 hours at 37°C. Following incubation, organisms were diluted in sterile PBS and plated on YPD agar to determine CFU. Killing of *Cryptococcus* was defined as CFU below the initial inoculum and inhibition was defined as CFU below the growth in media alone.

Incubation of purified cathepsin B with *Cryptococcus*. Purified enzyme killing of *Cryptococcus* was tested using cathepsin B at 50 µg/ml in phosphate buffer with *Cryptococcus*, as described above.

Electron Microscopy. *Cryptococcus neoformans* strain H99 (serotype A) was incubated with cathepsin B and/or calpain as described above for 12 hours. Cells were then fixed with 4% formaldehyde and 1% glutaraldehyde and processed for both scanning and transmission electron microscopy.

Protein Purification. Proteins were purified from *C. neoformans* using both the Allprep Fungal DNA/RNA/Protein Kit (Qiagen) kit and the Zymo Research Yeast Protein Kit using manufacturer's instructions for Western Blot Analysis. Protein was quantified using the Take3™ plate and reading on the Synergy HTX multi-mode plate reader (BioTek, Winooski, VT).

Gel Electrophoresis. Protein samples were combined with Lammeli Sample Buffer and heated for 5 mins before loading. A pre-cast 8% mini gel was used in surelock gel and running buffer was poured in. Samples and Benchmark™ pre-stained ladder were loaded in wells and the gel was run at 150V for 1 hour. The gel was stained with Coomassie blue to visualize protein bands.

Western Blot. The membrane transfer was run at 30V for 2 hours and proteins were transferred to a PVDF membrane. The membrane was washed in TBST for 5 mins three times. The membrane was covered with blocking buffer and incubated for 1 hour on a shaker. F-actin Antibody (NH3)(1:500) invitrogen was diluted in 10mL of blocking buffer and and incubated with the membrane for 1 hour on a shaker. The membrane was washed again in TBST. Goat anti-mouse IgG (H+L), horseradish peroxidase conjugate (1:10000) invitrogen was diluted in 10mL of blocking buffer and incubated with the membrane for 1 hour on a shaker. TBST was used to wash the membrane. PKA C-alpha Rabbit Antibody and Anti-rabbit IgG, HRP-linked antibodies were used at varying concentrations and need further troubleshooting.

Chemiluminescence Imaging. SuperSignal West Dura Extended Duration Substrate Kit was used to detect F-actin on the membrane. 1 ml of each substrate was mixed by inversion and allowed to incubate on the membrane for 1 minute before imaging using the Protein Simple Fluorochem E (Protein Simple, San Jose, CA).

Minimum Inhibitory Concentration. Minimum Inhibitory Concentration (MIC) assays were conducted as described by the Clinical Laboratory Standards Institute (CLSI, 2008). Briefly, in a 96-well plate, 100µl of media was added to rows 1-11. In row 12, 100µl of Amphotericin B (200µg/ml) or cathepsin B (451µg/ml) was added. After adding the drug, 100µl was taken from row 12 and added to row 11. This 1:2 dilution was repeated until row 2. 100µl was taken from row 2 and discarded, and row 1 remained as media alone (as a growth control row). 100µl of *C. neoformans* was added to every well. The plate was incubated at 35°C for two days. The plate was analyzed using the TAKE3^{TM} plate and read on the Synergy HTX multi-mode plate reader (BioTek, Winooski, VT). For the Amphotericin B MIC assay, RPMI-MOPS media was used and

$0.5x10^3$/ml of *C. neoformans* was added to the plate. For the Cathepsin B MIC assay, phosphate buffer media (0.1M potassium phosphate, plus 2% RPMI, pH 5.5) was used and $10x10^7$/ml of *C. neoformans* was added to the plate.

RESULTS

Anti-cryptococcal activity of DC lysosomal extract and Cathepsin B. Previous research concludes that DC lysosomal extract and Cathepsin B exhibit anti-cryptococcal activity (Hole, et al., 2012; Wozniak & Levitz, 2008). To confirm these results an in-vitro killing assay was performed. Results showed there was significant cryptococcal killing and inhibition of *C. neoformans* when treated with the substances (Figure 1). There was significant killing of the *C. neoformans* when added to DC lysosomal extract with CFU/ml levels close to zero. The purified lysosomal protease, cathepsin B (50μg/ml) exhibited significant growth inhibition of *C. neoformans* with CFU/ml levels four times lower than untreated fungi.

The results of another study indicated that cathepsin B kills the fungus by an unknown mechanism using osmotic lysis (Hole, et al., 2012). Electron microscopy of *C. neoformans* treated with Cathepsin B revealed a ruptured cell wall and an intact cell membrane (Figure 2). The published information regarding the organization of the *C. neoformans* cell wall reveals there are no substrates for cathepsin B to bind (Free, 2013, p.33-82). The cathepsin B may be targeting the cell wall integrity pathway resulting in osmotic lysis of the cell (Figure 3). Because of the nature of the cell wall in maintaining osmolality, in addition to the CWI pathway being a common antifungal target, it is likely that the cathepsin B mechanism of action involves the CWI pathway specifically the mitogen-activated protein (MAP) kinase cascade (Levin, 2005). The MAP kinase cascade begins with the phosphorylation of Pkc-1, which is our target protein.

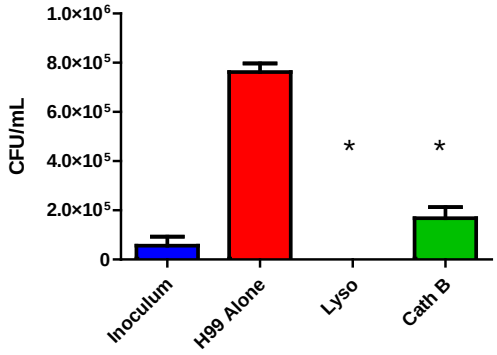


Figure 1. Anti-cryptococcal Activity of DC Lysosomal Extract and Lysosomal Protein Cathepsin B. *Cryptococcus* is killed by the lysosomal extracts. Purified lysosomal enzyme cathepsin B significantly inhibits the growth of *Cryptococcus* at 50 µg/ml concentration. Data shown are means (+/-) SEM for 3 independent experiments.

A.

B.

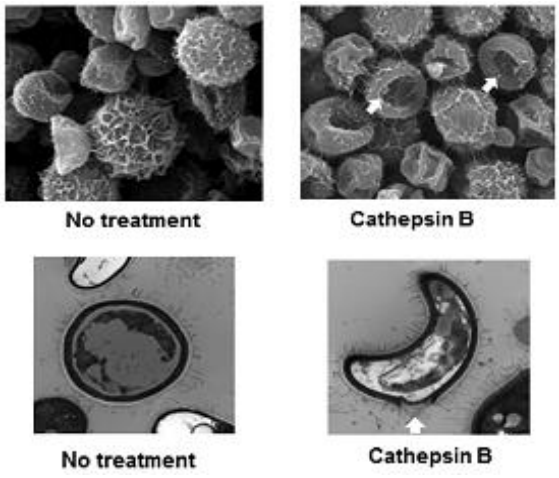
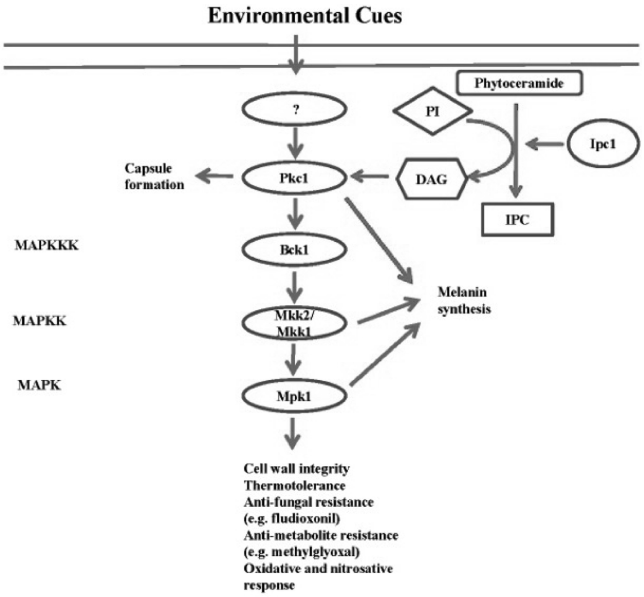


Figure 2. Cathepsin B Structural Effects on *Cryptococcus*. Cathepsin B causes structural changes to *Cryptococcus* following 12-hr incubation by (A) scanning electron microscopy and (B) transmission electron microscopy.

Hole, et al., 2012



F**igure 3. Fungal Cell Wall Integrity Pathway.** Diagram of the CWI MAP kinase cascade.

Jung and Bahn, *Mycobiology*, 2009

Protein Purification using Zymo Research Protein Kit is needed for analysis of proteins in Western Blot. Two protein purification kits, Allprep Fungal DNA/RNA/Protein Kit (Qiagen) kit and the Zymo Research Yeast Protein Kit, were compared in order to determine which would produce visible bands using the least amount of cells. *C. neoformans* proteins from different cell amounts (1million, 3 million,10 million, and 30 million) were purified from the Qiagen kit and quantified (Table 1). The proteins were separated using gel electrophoresis and analyzed through coomassie staining and western blot (Figure 4A and 4B). *C. neoformans* proteins from higher cell amounts (10 million, 15 million, 20 million, and 25 million) were purified from the Zymo kit and analyzed similar to the proteins purified from the Qiagen kit, excluding coomassie staining (Table 2 and Figure 5A). The results show the purified protein amounts using the Zymo kit was higher than the amounts using the Qiagen kit. Using the Zymo kit, the 42kDa F-actin band can be visualized at all concentrations tested. In comparison, using the Qiagen kit, the band can only be visualized at the concentration containing 30 million cells.

Sample Read#	Well ID	Name	Location	280 Raw	260 Raw	320 Raw	280	260	260/280	µg/mL
1	SPL1	$1x10^6$Cells	B2	0.046	0.049	0.042	0.001	0.002	1.875	16.444
1	SPL2	$3x10^6$ Cells	C2	0.043	0.046	0.04	0.001	0.001	2	12.479
1	SPL3	$10x10^6$ Cells	D2	0.047	0.049	0.04	0.004	0.004	0.837	89.156
1	SPL4	$30x10^6$Cells	E2	0.066	0.061	0.041	0.024	0.016	0.659	474.316
1	SPL5	BSA	F2	0.106	0.087	0.039	0.066	0.045	0.684	1319.86

Table 1. Quantification of proteins purified from Qiagen kit. The quantification of *Cryptococcus* proteins purified from Qiagen kit using cell counts of 1 million, 3 million, 10 million, and 30 million cells.

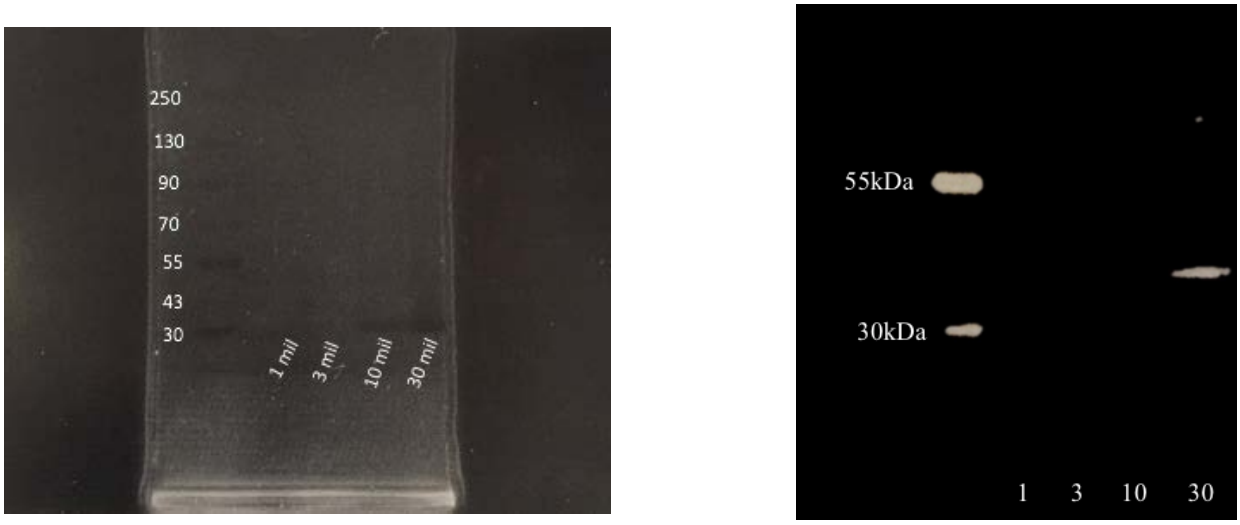


Figure 4. Analysis of Qiagen Protein Purification. (A) Coomassie Stain of gel indicates that protein bands can be visualized from 30 million cells. Bands are faintly seen from 10 million cell and cannot be seen at all from 3 million and 1 million cells. (B) Through western blot analysis, 42kDa F-actin was only identified at the concentration of 30 million cells.

Sample Read #	Well ID	Name	Location	280 Raw	260 Raw	320 Raw	280	260	260/280	µg/mL
1	SPL1	$10x10^6$ Cells	B2	0.173	0.21	0.089	0.068	0.1	1.469	1366.906
1	SPL2	$15x10^6$ Cells	C2	0.238	0.289	0.12	0.105	0.152	1.45	2098.586
1	SPL3	$20x10^6$ Cells	D2	0.366	0.448	0.187	0.168	0.248	1.478	3350.612
1	SPL4	$25x10^6$ Cells	E2	0.467	0.577	0.243	0.214	0.323	1.506	4283.347

Table 2. Quantification of proteins purified from Zymo kit. The quantification of *Cryptococcus* proteins purified from Zymo kit using cell counts of 10 million, 15 million, 20 million, and 25 million cells.

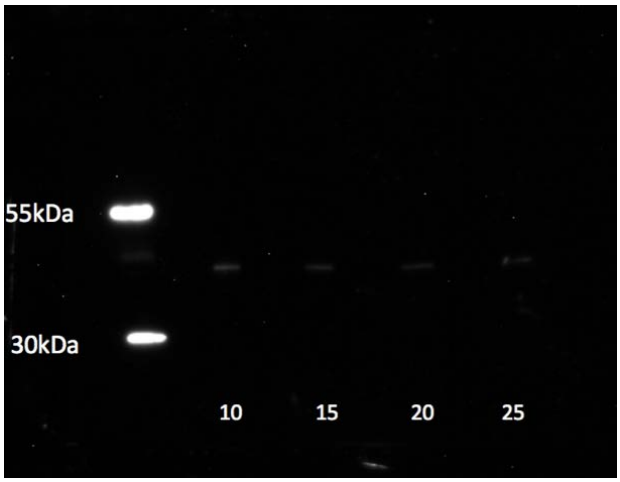


Figure 5. Analysis of Zymo Protein Purification. Quantification of purified *Cryptococcus* proteins. 42kDa F-actin was successfully identified at all concentrations through chemiluminescence imaging.

Un-phosphorylated PKA-C antibody can not be visualized at tested concentrations.

To begin experiments to determine if the cell wall integrity pathway of *C. neoformans* is activated when treated with cathepsin B, control experiments using untreated fungi must be performed and antibody concentration must be optimized. Three different antibody concentration combinations have been tested: primary antibody concentration of 1:1000 and secondary antibody concentration of 1:1000, primary antibody concentration of 1:500 and secondary antibody concentration of 1:1000, and primary antibody concentration of 1:500 and secondary antibody concentration of 1:500. The 42kDa PKA-C antibody was not identified in any of the three combination (Figure 6-8).

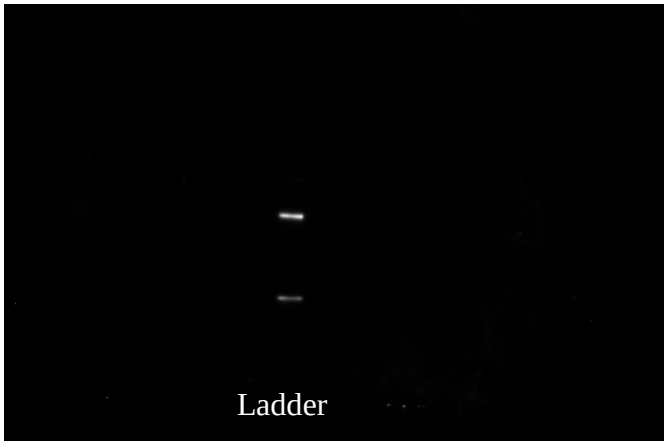


Figure 6. Imaging of western blot using primary antibody concentration of 1:1000 and secondary antibody concentration of 1:1000. 42KDa PKA-C was not identified at these antibody concentrations.

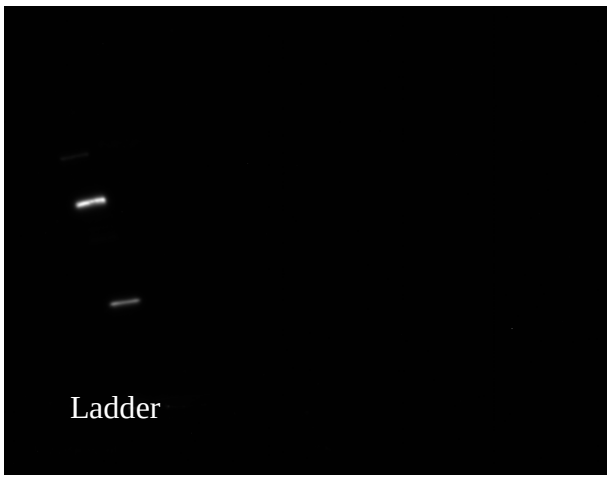


Figure 7. Imaging of western blot using primary antibody concentration of 1:500 and secondary antibody concentration of 1:1000. 42KDa PKA-C was not identified at these antibody concentrations.

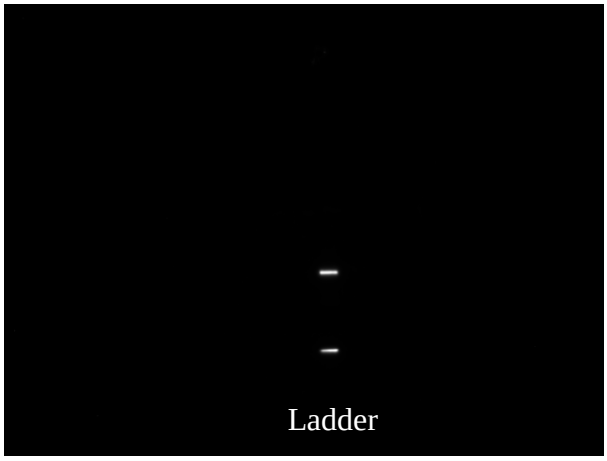


Figure 8. Imaging of western blot using primary antibody concentration of 1:500 and secondary antibody concentration of 1:500. 42KDa PKA-C was not identified at these antibody concentrations.

Minimum Inhibitory Concentration Assay. A MIC assay was performed on known antifungal drug Amphotericin B. The MIC was found to be in a normal range of 1.56-3.125

µg/ml (data not shown). Another assay was performed using cathepsin B and 10 million cryptococcal cells (the minimum number identified by Western Blot for F-actin, Figure 5) though there was no MIC due to the concentration of drug not starting high enough to inhibit the *Cryptococcus* (Table 3). Additional MIC assays will need to be performed using cathepsin B starting at a higher concentration.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.765	0.76	0.762	0.76	0.769	0.754	0.754	0.753	0.756	0.775	0.786	0.743
B	0.773	0.765	0.756	0.752	0.753	0.759	0.754	0.75	0.761	0.76	0.776	0.701

Table 3: MIC assay results for cathepsin B. There was no inhibition of *Cryptococcus* in any of the wells.

DISCUSSION

There are many modes of action of antifungal components but the increased incidence of resistance has led to the exploration of new drug targets. New approaches to antifungal drug development include targeting the components in the fungal cell wall pathways (Hasim & Coleman, 2019). Cathepsin B has anti-*cryptococcal* activity but the mechanism of this activity is still unknown. The structural changes of cryptococcal cells when treated with cathepsin B suggests interferences with the cell wall pointing to the targeting of the cell wall integrity pathway.

These preliminary studies contain results to be used in future experiments. Cathepsin B was confirmed to have anti-fungal activity against *C. neoformans*. This anti-cryptococcal

mechanism is in the process of being identified by western blot analysis. The Zymo protein kit was found to purify more proteins than the Qiagen protein kit, making it the best option for the western blot experiments. The bands binding to the un-phosphorylated PKA-C antibody should be visualized in western bot analysis of untreated *Cryptococcus* since the pathway should not be activated and there should be un-phosphorylated PKA-C proteins. The inability to visualize the antibody could be due to the concentration conditions or a higher number of Cryptococcus cells need to be purified. Finding the MIC of cathepsin B could help in determining how much to scale up the cell count in regards to the on-hand access of cathepsin B.

Future studies will include testing other antibody concentration combinations to visualize the un-phosphorylated PKA-C band. Once optimization is complete, both the phosphorylated and un-phosphorylated PKA-C antibody will need to be analyzed using western blots analysis of *cryptococcal* cells treated with and without cathepsin B to determine if the cell wall integrity pathway is being activated by cathepsin B. If the phosphorylated PKA-C band is visualized in the treated cells, the next proteins in the CWI MAP kinase cascade will need to be analyzed such as Bck1, Mkk2, Mkk1, and Mpk1. To determine the minimum concentration of cathepsin B needed to inhibit Cryptococcus to use in future experiments. MIC assays will need to be performed starting at a higher concentration. These future studies will allow us to determine the role of the CWI pathway in the anti-cryptococcal activity of cathepsin B.

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