Anti-Fungal Activity of Lysosomal Proteins and their Effects on *Cryptococcus neoformans*

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Abstract

Cryptococcus neoformans is an opportunistic fungal infection spread through airborne means. It affects immune compromised individuals and increases their susceptibility to the disease. Previous studies showed that dendritic cells (DCs) can kill *Cryptococcus* through phagocytosis and lysosomal killing from within the DC. The lysosomal extract from these DCs has anti-cryptococcal activity, and we now have mass spectrometry data identifying its contents. We hypothesized that DC lysosomal proteins nostrin, calmodulin, and coronin 1a have anti-fungal activity against *C. neoformans*. For these studies, we incubated lysosomal extract or these individual proteins with *C. neoformans* to measure anti-fungal activity. Our results showed nostrin and coronin-1A had significant antifungal activity, while calmodulin significantly increased cryptococcal growth. Cytotoxicity was tested in nostrin and it was not toxic to mammalian cells. Because calmodulin increased cryptococcal growth, we hypothesized that following incubation with *C. neoformans*, the media contained growth-enhancing nutrients. For this, we examined the media for macronutrients, amino acids, and metals following incubation of *C. neoformans* with calmodulin and other growth-enhancing compounds S100A6, cystatin B, calnexin, striatin, and CRISP-1. We found that incubation of *C. neoformans* with calmodulin, cystatin B, and CRISP-1 led to increased biotin in the media, and trace elements including Cd, Fe, Mn were also increased following incubation of *C. neoformans* with calmodulin. Interestingly, some compounds alone increased trace metals, but incubation with *C. neoformans* brought those levels back to baseline amounts. Future studies will compare these components in the media to those from incubations with anti-cryptococcal molecules.

Introduction

Cryptococcus neoformans is an airborne fungal pathogen, which left untreated causes meningitis in immune compromised individuals and can be fatal. There are many species of *Cryptococcus* in the wild, but *C. gattii* and *C. neoformans* are two of the major pathogenic species. Trees and the decay in tree matter and soil can cause someone to come in contact with *C. gattii* and become infected. Infection with *C. neoformans* begins with small birds such as pigeons, and their feces carries the fungal organism which can then be inhaled into the lungs. And from there the organism can make its way into the brain and cause deadly meningitis. *C. neoformans* mainly causes disease in immune compromised individuals, but other species of *Cryptococcus* such as *C. gattii* can cause disease in healthy people. Most forms of *Cryptococcus* can be transmitted through airborne means, and this organism causes disease in about 250,000 individuals world-wide each year.

When *C. neoformans* enters the lungs, macrophages and dendritic cells (DCs) recognize the fungi and can work to fight the infection. Once inside a macrophage or DC, the fungi undergoes a gradual acidification as the phagosome becomes a phagolysosome where the lysosome releases its proteins and enzymes out onto the fungal organism. However, *C. neoformans* can survive and replicate in the phagolysosome of some types of macrophages. The lysosomes from DCs have been shown to have anti-cryptococcal activity. There are more than 3,000 different proteins found in the DC lysosome that we identified by mass spectrometry. We have observed that some of the lysosomal proteins inhibit the growth of *C. neoformans*, while others actually enhance *C. neoformans* growth. Therefore, we were interested in identifying the mechanisms of this enhanced growth and identifying nutrients that lysosomal proteins may make more available to *C. neoformans* that can enhance its growth. These findings may be important to understand how the organism can replicate within the phagolysosome of some macrophages.

The goal and purpose in studying cryptococcal interactions with macrophages and dendritic cells is to understand how immune cells can kill the fungus or how the fungus may use host factors to promote its growth, potentially creating new targets for therapies. By developing methods that function in aiding mechanisms already in place to destroy *Cryptococcus* or down-regulating host factors that the fungus may use for growth, we could create effective, nontoxic drugs that could potentially save many people.

Methods

Strains and Media: *C. neoformans* strain H99 (serotype A) was grown for 18 hours at 30°C with shaking in YPD broth. Then cells were collected by centrifugation and washed three times with sterile phosphate-buffered saline (PBS). Viable yeasts were quantified using trypan blue dye exclusion in a hemacytometer. The amount needed for the inoculum was calculated from the hemacytometer counts.

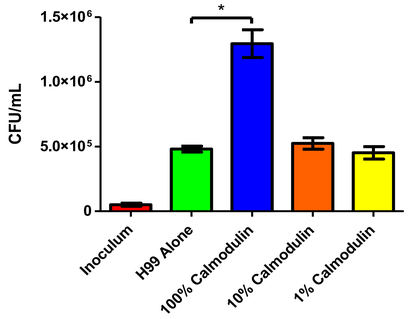
Incubation of DC lysosomal extracts with *Cryptococcus*: Lysosomal extracts were incubated with *Cryptococcus* to test killing. After cultures were grown for 18 hours at 30°C, organisms were washed 3X with sterile PBS and resuspended in 10mM phosphate buffer with 2% RPMI, pH 5.5. For each well, 50 µl ($2.5x10^5$/ml) of fungi were added to a 96-well plate in triplicate. 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, 5% CO_2. Following incubation, organisms were diluted 1:10 in sterile PBS and plated on YPD agar to determine viable colony-forming units (CFU). Killing of *Cryptococcus* was defined as CFU below the initial inoculum and was determined by CFU counts of each organism incubated with lysosomal extract compared to controls.

Incubation of purified lysosomal enzymes with *Cryptococcus*: Purified enzyme killing of *Cryptococcus* was also tested using nostrin, calmodulin, and coronin 1a. These enzymes were tested at 2µg/ml, 1mM, and 100µg/ml, respectively, in 10 mM phosphate buffer with *Cryptococcus*, as described above. Plates were then incubated for 24 hours at 37°C, 5% CO_2. Following incubation, organisms were diluted 1:10 in sterile PBS and plated on YPD agar to determine viable colony-forming units (CFU). Killing of *Cryptococcus* was defined as CFU below the initial inoculum and was determined by CFU counts of each organism incubated with lysosomal extract compared to controls.

Supernatants Analysis: After incubation with lysosomal proteins and *C.neoformans,* supernatants were spun by centrifuge and then collected. Spent media analysis was then preformed by Xell.

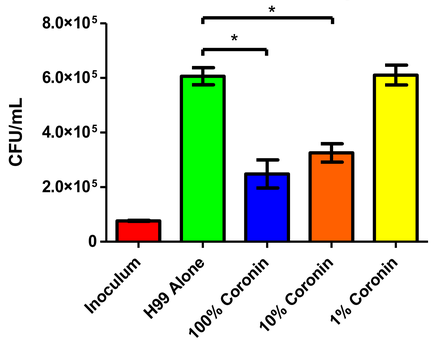
Results

Figure 1. Effect of Calmodulin on *C. neoformans* Growth



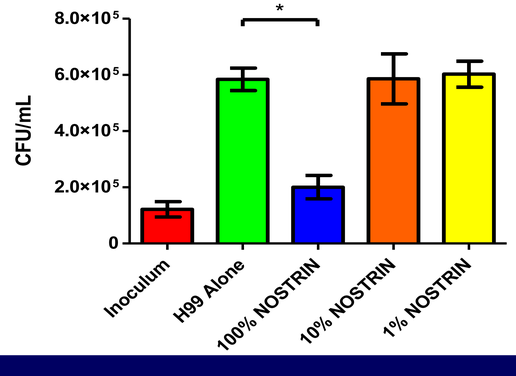
Conclusion: Treatment with calmodulin showed significantly increased growth of *Crypotococcu*s compared to no treatment. This observation supports the idea that calmodulin facilitates growth of *Cryptococcus.*

Figure 2. Effect of Coronin on *C. neoformans* Growth



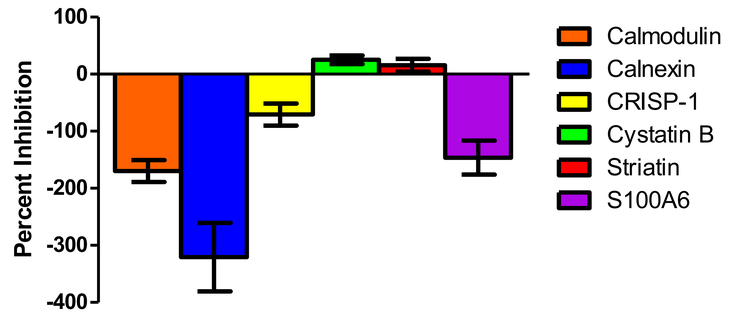
Conclusion: Treatment with coronin showed significant antifungal activity against *C. neoformans* in a dose-dependent manner.

Figure 3. Effect of Nostrin on *C. neoformans* Growth



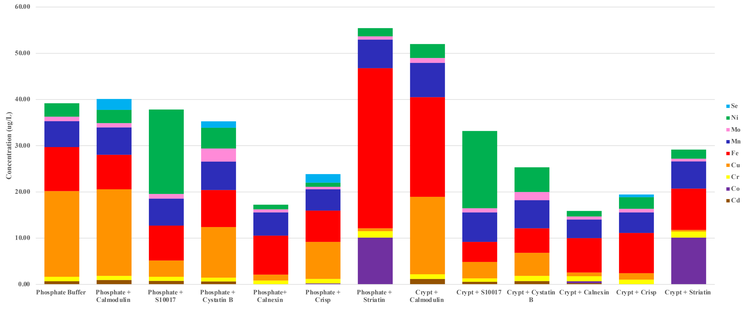
Conclusion: Nostrin is a lysosomal protein that showed antimicrobial activity. Nostrin significantly inhibits the growth of *Cryptococcus*.

Figure 4. *C. neoformans* Enhanced Growth by Lysosomal Proteins



Conclusion: Several of the lysosomal proteins enhanced the growth of *C. neoformans*. This observation demonstrates that some of the proteins may break down the media to make nutrients more available to aid in *C. neoformans* growth.

Figure 5. Trace Metals in Supernatants after Incubation of *C. neoformans and* Lysosomal Proteins



Conclusion: It was found that Cd, Fe, and Mn were increased following incubations with *Cryptococcus* . With CRISP-1, calmodulin, and cystatin B, incubation with *C. neoformans* led to increase biotin in the media.

Conclusions/Future Directions

Conclusions/Future Directions

-DC lysosomal extract is capable of inhibiting growth of *Cryptococcus*

-Nostrin and coronin display evidence of antifungal activity against *Cryptococcus neoformans* strain H99

-Calmodulin, calnexin, CRISP-1, and S100A6 enhance cryptococcal growth

-After incubation, the metals Cd, Fe, Mn are sometimes found in increased in concentrations in *C.neoformans*

-Future studies will examine the mechanism for increased cryptococcal growth by Nostrin, Coronin, HNE, and MMP25, and MPO

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