Antifungal Activity of Novel Compound EIPE-1 Against *Cryptococcus neoformans*

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Abstract

Cryptococcus neoformans is an opportunistic fungal pathogen of the respiratory tract, which is responsible for over 200,000 deaths annually. Antifungal drugs have been used to treat fungal infections for many decades; however, due to similarities between fungal and mammalian cells, these drugs are often toxic. In these last few decades, the fungi have also become resistant to the antifungal drugs. EIPE-1 was synthesized from vanillin, and was shown to have activity against methicillin resistant *S. aureus* (MRSA), and other gram-positive bacterial pathogens. We hypothesized that EIPE-1 could be used to kill fungal pathogens. For this study, we tested EIPE-1 against *C. neoformans* using a minimum inhibitory concentration (MIC) assay and an *in vitro* model of intracellular macrophage growth using RAW macrophages. EIPE-1 has antifungal activity in our MIC assay, with an MIC value of 1.749 µg/ml. In addition, after incubation of *C. neoformans* with RAW macrophages and EIPE-1, treatment with EIPE-1 had significant antifungal effects on *C. neoformans* compared to *C. neoformans* alone and compared to *C. neoformans* with RAW macrophages. In further studies, we will examine the mechanism of EIPE-1 anti-fungal activity, and we will also test EIPE-1 against other fungal pathogens including *Candida albicans*.

Introduction

Cryptococcus neoformans is an opportunistic fungal pathogen that is acquired through inhalation, but can disseminate to the brain to cause meningitis. *C. neoformans* infects patients with immune deficiencies such as AIDS, chemotherapy patients, and those on immune suppressive drugs. It can cause meningitis, which has a 40% mortality rate, even after treatment with antifungal drugs. Each year, there are 200,000 reported cases of cryptococcal meningitis and nearly 181,000 deaths each year worldwide. In spite of the many advances in antifungal therapies, the antifungal drugs that are available now have high toxicity. In addition, the fungal organisms have begun to acquire resistance to the antifungal drugs that are currently being used today. As a result, there is a limited ability for the regulation of life-threatening infections, especially for individuals that are immunocompromised. To address this issue, a eumelanin-inspired compound, EIPE-1, which is derived from vanillin was synthesized. This synthetic compound was tested against drug-resistant bacteria, and it has demonstrated antimicrobial effects on methicillin-resistant *S. aureus* (MRSA), but not on gram-negative organisms. In this study, we first determined the effects of EIPE-1 against the fungal pathogen *C. neoformans* in a minimum inhibitory concentration assay. Next, because *C. neoformans* is thought to traffic from the lungs to the brain inside of macrophages, we tested the efficacy of EIPE-1 during macrophage phagocytosis of *C. neoformans* to determine whether EIPE-1 could be used therapeutically

Methods

***Cryptococcus* cultures**: *C. neoformans* strain H99 (serotype A) was grown for 18 hours at 30°C with YPD broth in a shaking incubator. The cells were collected by centrifugation and washed three times with sterile phosphate-buffered saline (PBS), to remove any extra residue from the YPD broth. Fungal cells were quantified by trypan dye exclusion using a hemocytometer.

MIC Assays with Amphotericin B or EIPE-1. *C. neoformans* were suspended in RPMI with MOPS, 0.165M, at a concentration of 0.5×10^3 cells/ml. RPMI MOPS media was added to a 96-well plate in a volume of 100µL in each well, with the exception of the column containing the highest concentration of drug Amphotericin B or EIPE-1 was added at a concentration of 100 mg/µl and serially diluted 1:2. Control wells included one row with only RPMI MOPS media. *C. neoformans* was added to all wells at a volume of 100 µl per well, so the final volume of the wells was 200 µL. Plates were incubated at 35°C in a humid incubator for 48 hrs. Data were analyzed visually and by reading ODs at 490nm on a platereader (BioTek).

RAW macrophage cell culture: Immortalized RAW macrophages (ATCC) were grown in RAW cell culture media (DMEM + 10% heat-inactivated fetal bovine serum) per manufacturer's instructions. Cells were incubated at 37°C, 5% CO_2 in T75 tissue culture flasks and passaged twice per week.

Antifungal activity of EIPE-1 with *Cryptococcus* and RAW macrophages: The RAW macrophages were split and counted before the experiment. RAW macrophages and *C. neoformans* were counted and adjusted in RAW media and added to 96-well plates at 2×10^5 per well for macrophages and 1×10^4 per well for *C. neoformans*. Anti-GXM opsonizing antibody was also added to each well. The 96-well plate was incubated at 37°C, 5% CO_2 for 24 hours. A portion of the inoculum was set aside for diluting and plating on YPD agar, followed by incubation at 30°C for 2 days, followed by quantification of the inoculum CFU. Following 24h incubation of the *C. neoformans* with RAW macrophages, plates were centrifuged and the supernatant was discarded, while a pellet was left at the bottom. 100µl of sterile cell culture grade water was added to each well to lyse the macrophages for 15 minutes at room temperature. Contents of the wells were serially diluted and plated on YPD agar. The plates were incubated at 30°C for 2 days and then CFU were counted.

Antifungal activity of EIPE-1 with *Cryptococcus* and RAW macrophages following 2-hour phagocytosis: To determine if EIPE-1 could penetrate the RAW macrophages after phagocytosis of *C. neoformans*, the RAW macrophages, *C. neoformans*, and opsonizing antibody were incubated as described above for 2h. Following this incubation, EIPE-1 was added for an additional 24h, followed by diluting and plating for CFU.

Data analysis: The inoculum plates and experiment plates were counted and CFU were determined. Prism software was used to generate graphs and for statistical analyses. For these studies, ANOVA with a Tukey's post-test (to compare all pairs) was conducted to determine statistically significant differences ($p<0.05$).

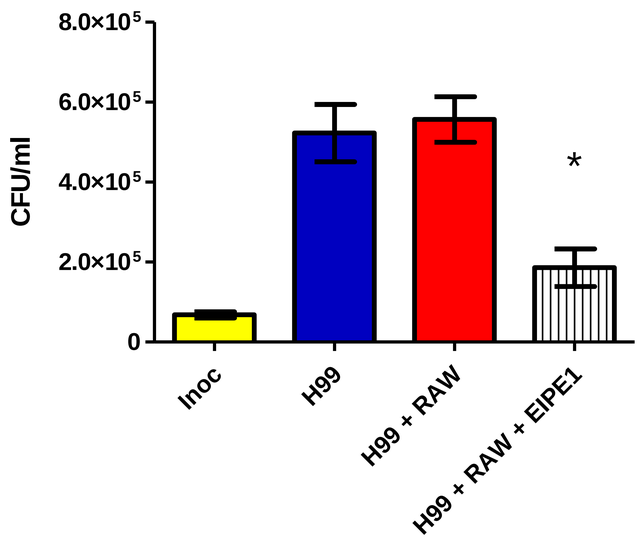
Results

Table 1. EIPE-1 Inhibits *Cryptococcus* Growth

Antifungal	*MIC µg/mL*
Amphotericin B	0.682 ± 0.292
EIPE-1	1.749 ± 0.108

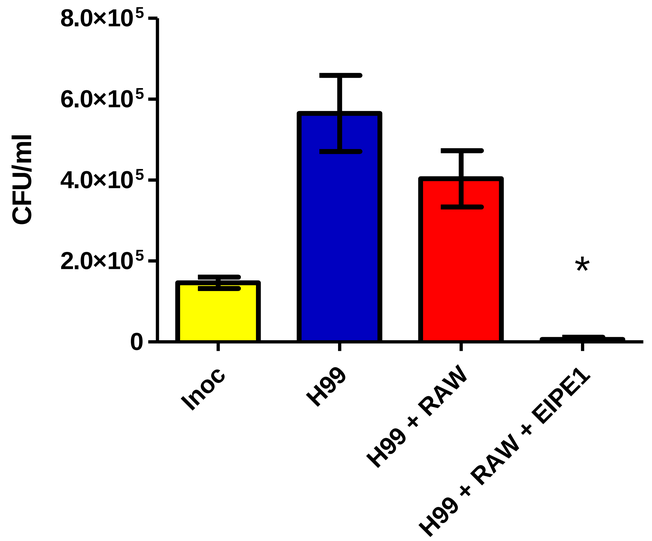
Conclusion: EIPE-1 inhibits the growth of *C. neoformans*, with a minimum inhibitory concentration (MIC) of 1.749 µg/ml compared to an MIC of 0.682 µg/ml with the antifungal drug Amphotericin B. Data shown are from 7 experiments with EIPE-1 and 4 experiments with Amphotericin B.

Figure 1. EIPE-1 has Antifungal Activity Against *C. neoformans* During Macrophage Co-culture



Conclusion: Incubation of *C. neoformans* with RAW macrophages and EIPE-1 for 24h led to significant inhibition of growth compared to *C. neoformans* grown alone ($p<0.05$). Data shown are from three experiments, with each condition conducted in triplicate.

Figure 2. EIPE-1 has Antifungal Activity Against *C. neoformans* After Macrophage Uptake



Conclusion: EIPE-1 has significant antifungal activity against *C. neoformans* after 2 hour phagocytosis by RAW macrophages compared to *C. neoformans* grown in media alone ($p<0.05$). Data are shown from two experiments, with each condition conducted in triplicate.

Conclusions

- EIPE-1 is effective at inhibiting the growth *of C. neoformans* in the MIC assay
- EIPE-1 is effective at inhibiting the growth of *C. neoformans* during incubation with RAW macrophages
- EIPE-1 is effective at inhibiting the growth of *C. neoformans* after phagocytosis by RAW macrophages

Future Directions

- Conduct EIPE-1 antifungal studies with another fungal pathogen, *Candida albicans*
  - MIC assays, RAW macrophage assays
- Identify the antifungal mechanism of action EIPE-1 on fungal pathogens
  - Collect RNA form EIPE-1 treated fungal pathogens
    - Conduct RNA-sequencing experiments to identify fungal genes affected following EIPE-1 treatment

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