Antifungal activity of EIPE-1, an eumelanin-inspired compound, against *Cryptococcus neoformans* and *Candida albicans*

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Abstract

In the past century, anti-fungal drugs have had much success in the treatment of fungal pathogens. However, due to similarities between fungal and mammalian cells, many of these treatments are toxic. In addition, fungi have developed resistance against existing antifungal drugs. Currently, there are only four separate classes of antifungal agents that target plasma membrane sterols (ergosterol), nucleic acid synthesis and cell wall constituents, and some of these existing drugs are not effective against all fungal pathogens. To combat this issue along with antibiotic resistance in bacteria, a eumelanin-inspired indoylenepheyleneethynylene, EIPE-1, derived from vanillin was synthesized. The synthetic compound has demonstrated antimicrobial effects on the methicillin resistant *S. aureus* (MSRA), but not on the gram-negative organisms. We hypothesized that EIPE-1 could also be used to kill fungal pathogens. For these studies, we tested EIPE-1 against the fungal pathogens *Cryptococcus neoformans* and *Candida albicans*, which are responsible for over 200,000 yearly deaths. Our results showed that EIPE-1 has considerable anti-fungal effects on *C. neoformans* with a MIC of 1.749 µg/ml and a MIC of 2.705 µg/ml for *C. albicans*. In addition, we conducted scanning electron microscopy (SEM) and transmission EM (TEM) on the exposed cells at varying time points. Cells exposed for four or more hours displayed structural changes to their cell wall. Overall, we conclude that EIPE-1 does display potent anti-fungal activity against *C. neoformans* and *C. albicans*. Future studies will examine the mechanism of the activity of EIPE-1 against these fungal pathogens.

Introduction

Cryptococcus neoformans and *Candida albicans* are opportunistic fungal pathogens that cause infections in immune compromised individuals. *C. neoformans* infects patients with T cell immune deficiencies, such as AIDS patients, transplant patients on immune suppressive drugs, and chemotherapy patients. *C. neoformans* infection can lead to meningitis, which has a 40% fatality rate, even with antifungal treatment. Current estimates in AIDS patients show that 220,000 cases of cryptococcal meningitis occur world-wide each year, with 180,000 yearly deaths. In immune compromised individuals such as hospitalized patients with central venous catheters, *Candida* species can lead to bloodstream infections, which have a 30% fatality rate, even with antifungal treatment. *Candida* is now the 4th leading cause of bloodstream infections in the US, with 46,000 cases per year in the US alone. Despite many advances in antifungal therapies, the currently available antifungal drugs have high toxicity, and fungal organisms have started to acquire resistance to the current antifungal drugs. As a result, there is a limited ability for the management of life-threatening infections, especially in immunocompromised individuals. Therefore, discovery of novel antifungal therapies is critical for fighting these deadly fungal infections. To combat this issue, a eumelanin-inspired compound, EIPE-1, derived from vanillin was synthesized. The synthetic compound was tested against drug-resistant bacteria, and it has demonstrated antimicrobial effects on methicillin resistant S. aureus (MSRA), but not on the gram-negative organisms. In this study, we determined the activity of EIPE-1 against the fungal pathogens *Cryptococcus neoformans* and *Candida albicans*.

Methods

Reagents. YPD media, used in the culturing of *C. neoformans* and *C. albicans* cells, was obtained from Fisher Scientific. Phosphate buffered saline (PBS) was purchased at a 10X concentration from Fisher Scientific and was used for washing cryptococcal cells and was diluted 1:10 with deionized water. The solution was then autoclaved to ensure proper sterilization. The plastic ware was purchased from Fisher Scientific. RPMI with mops media, used in the MIC Assay was purchased from Fisher scientific, diluted to the concentration of 0.165 M. Amphotericin B was purchased and reconstituted to a 5 mg/µL concentration.

***Cryptococcus* and *Candida* Cultures.** *Cryptococcus neoformans* serotype A strain H99 as well as *Candida ablicans* strain SC5314, was readily available in the lab. The stock of cells is stored at -80°C to preserve and prevent contamination. The cells were grown and maintained in yeast-extract-peptone-dextrose (YPD) media (Difco). *Cryptococcus* or *Candida* cells were incubated in YPD broth for 18 hrs at 30°C, in a shaking incubator. After the incubation period, they were washed three times in sterile phosphate-buffered saline (PBS) to remove all remaining residue of YPD. The cells were then quantified by using trypan blue dye exclusion in a hemocytometer.

Incubation of *Cryptococcus* 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 to every well, with the exception of column 1. An additional 80 µL of RPMI MOPS was added to column 4 for a total of 180 µL/per well. Amphotericin B or EIPE-1 was added to columns 2 and 4 with a volume of 20 µL at a concentration of 1 mg/mL. Contents were mixed, then serially diluted 100 µL down the plate until column 11. The last 100 µL was discarded, leaving column 12 blank. *C. neoformans* was added at a volume of 100 µL per well from column 4 to column 12, so the final volume of the wells was 200 µL. Plates were incubated at 35°C in a humid incubator for 48 hrs.

Incubation of *Candida albicans* with EIPE-1. *C. albicans* 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 to every well, with the exception of column 1. An additional 80 µL of RPMI MOPS was added to column 4 for a total of 180 µL/per well. EIPE-1 was added to columns 2 and 4 with a volume of 20 µL at a concentration of 1 mg/mL. Contents were mixed, then serially diluted 100 µL down the plate until column 11. The last 100 µL was discarded, leaving column 12 blank. *C. neoformans* was added at a volume of 100 µL per well from column 4 to column 12, so the final volume of the wells was 200 µL. Plates were incubated at 35°C in a humid incubator for 48 hrs.

Data Analysis. Data analysis was conducted using GraphPad Prism version 5.00 for Windows. Depending on the data collected and interaction observed between the *Cryptococcus* or *Candida* and the compounds, the one-way ANOVA with the Tukey's multiple comparison test was used to compare the data.

Cell fixation of *Cryptococcus*. *C. neoformans* were suspended in RPMI with MOPs, 0.165M, at a concentration of 2.0×10^6 cells/mL. RPMI Mops media was added to a 2 mL collection tube in a volume of 100 µL. EIPE-1 was added to each tube at the minimum inhibitory concentration 1.749 µg/mL at a volume of 100 µL . The tubes were incubated at 35°C in a humid incubator for set time points of 4 hrs, 8 hrs, 12hrs, or 24hrs. Once removed, the tubes were placed in a centrifuge for 1 min. at 5000 rpm. Media was removed. The pellet was resuspended in 2.0% glutaraldehyde in 0.1M cacodylate buffer at a volume of 1 mL for a minimum of 2 hrs.

SEMs of *Cryptococcus*. Fixed *C. neoformans* cells were placed in a centrifuge for 1 min at 5000 rpm. Media was removed and *C. neoformans* was rinsed 3 times in a buffered wash at fixed intervals of 15 mins. Rinsed cells were resuspended in 1% OsO_4 at room temperature for 1 hr in a 36-well plate with a cover slip. 1% OsO_4 was removed. *C. neoformans* was rinsed 3 times in a buffered wash at fixed intervals of 15 mins. *C. neoformans* were dehydrated in ethanol at 50%, 70%, 90%, 95%, and 100% three times at fixed intervals of 15 mins. *C. neoformans* were wash two times with hexamethyldisilane at a time interval of 5 mins. Cover slips were placed on a clear sheet for 12hrs to dry. Cover slips were mounted on stubs with silver paint. The cover slips were covered in an Au-Pd coat.

SEM Imagery. Imagery was taken with a FEI Quanta 600 field-emission gun Environmental Scanning Electron Microscope with a Bruker EDS X-ray microanalysis system and HKL EBSD system

Results

Figure 1. MIC concentrations for *C. neoformans* and *C. albicans*.

Table 1. Minimum inhibitory concentration (MIC) data for the isolate of *C. neoformans*

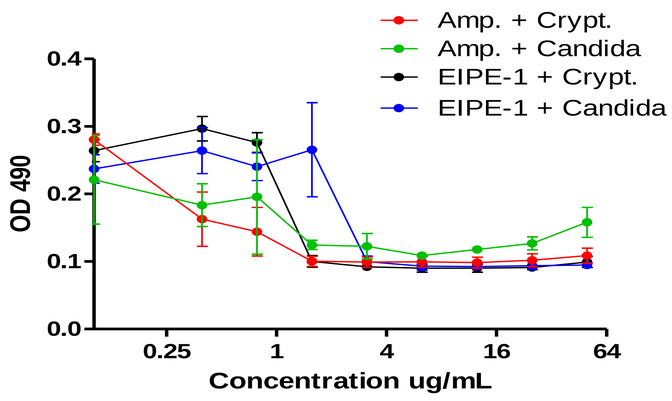
Antifungal	*MIC µg/mL*
Amphotericin B	0.6825 ± 0.2925
EIPE-1	1.749 ± 0.1080

Table 2. Minimum inhibitory concentration (MIC) data for the isolate of *C. albicans*

Antifungal	*MIC µg/mL*
Amphotericin B	0.5606 ± 0.1219
EIPE-1	2.705 ± 0.3962

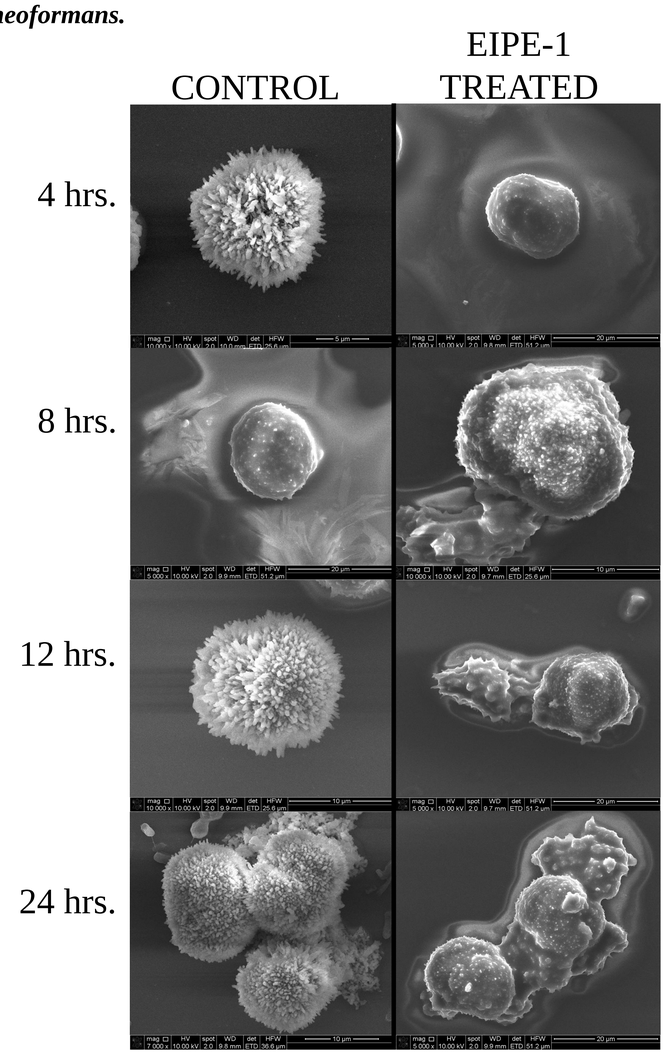
Conclusion: *C. neoformans* minimum inhibitory concentration average is 0.684 µg/mL with Amphotericin B across four experiments. With EIPE-1, the MIC for the organism is 1.67 µg/mL across seven MIC assays, which is more than double of Amp B. *Candida* MIC is 0.5606 µg/ml for Amp B for two experiments and MIC of 2.46 µg/mL for the compound EIPE-1 across seven experiments.

Figure 2. Optical Density for EIPE-1 and Amphotericin B drug inhibition of *C. neoformans* and *C. albicans*.



Conclusion: Each drug displays lower optical densities at 3.625 µg/mL concentrations. However, ODs vary considerably after this concentration in all 18 experiments with fungal organisms exposed to EIPE-1 displaying the most variability in ODs.

Figure 3. Scanning Electron Microscopy (SEMs) Images of *C. neoformans*.



Conclusion: *C. neoformans* was exposed to the EIPE-1 at the minimum inhibitory concentration for various timed intervals. It appeared the compound affected the fungal cells early with changes to the cell wall shown at 4 hrs. However, at prolong exposure the cells begin to display what appears to be a release of its internal organs or potentially a deformed budding cell.

Conclusions/Future Directions

Conclusions:

- EIPE-1 is effective at inhibiting the growth of *Cryptococcus neoformans* and *Candida albicans* as shown by the MIC experiments.
- EIPE-1 is altering the shape and/or structure of *Cryptococcus neoformans*. It begins to affect the fungal cells after 4 hours of exposure.

Future Directions:

- The mechanism behind the synthetic compound's fungal growth inhibition is currently unknown. Further studies will be conducted future to identify the antifungal mechanism of EIPE-1.
  - Electron microscopy will be used to visualize the organisms grown in the presence of the compound to identify any visible changes to the cell wall or membrane.
  - If structural changes are observed, we will perform Western blot assays for proteins from the cell wall integrity pathway of *C. neoformans* and *C. albicans*.
  - In addition, RNA sequencing will be used to determine if there is a change in the expression of certain pathways in C. neoformans after exposure to the drug EIPE-1.

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