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A High Throughput Screening Assay for Assessing Viability of *Cryptococcus neoformans* under Nutrient Starvation Condition

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Abstract

Cryptococcus neoformans causes an estimated 600,000 AIDS-related deaths annually that occur primarily in resource-limited countries. Fluconazole and amphotericin B are currently available for the treatment of cryptococcal-related infections. However, fluconazole has limited clinical efficacy and amphotericin B requires intravenous infusion and is associated with high renal toxicity. Therefore, there is an unmet need for a new orally administrable anti-cryptococcal drug. We have developed a high throughput screening assay for the measurement of *C. neoformans* viability in 1536-well plate format. The signal-to-basal ratio of the ATP content assay was 21.9 fold with a coefficient of variation and Z' factor of 7.1% and 0.76, respectively. A pilot screen of 1,280 known compounds against the wild type *C. neoformans* (strain H99) led to the identification of four active compounds including niclosamide, malonoben, 6-bromoindirubin-3'-oxime, and 5-[(4-Ethylphenyl)methylene]-2-thioxo-4-thiazolidinone. These compounds were further tested against 9 clinical isolates of *C. neoformans*, and their fungicidal activities were confirmed. The results demonstrate that this miniaturized *C. neoformans* assay is advantageous for the high-throughput screening of large compound collections to identify lead compounds for new anti-cryptococcal drug development.

Keywords

Cryptococcus neoformans; cryptococcal infections; cryptococcal meningitis; high throughput screening; ATP content assay

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Introduction

Cryptococcus neoformans (*C. neoformans*) is currently the fourth major cause of mortality in infectious diseases, annually causing an estimated 600,000 AIDS-related deaths globally [1]. Currently, amphotericin B and fluconazole are two available drugs for the treatment of disseminated cryptococcal diseases; mainly cryptococcal meningitis. Amphotericin B is a polyene antibiotic developed in the 1950's that requires intravenous administration. The severe renal toxicity and electrolyte disturbances associated with amphotericin B treatment require close monitoring that is a challenging task in resource limited countries. Fluconazole, a more recent addition, can be orally administered without significant toxicity. However, it has poor therapeutic efficacy against cryptococcal meningitis, resulting in an almost 90% mortality in resource-limited settings [2]. Therefore, there is an urgent need for the identification of a safe and orally administrable new drug with an efficacy exceeding fluconazole for the treatment of cryptococcal meningitis in resource limited countries.

Recently, a cell viability assay utilizing the AlamarBlue dye was reported capable of measuring fungicidal activity against *C. neoformans* [3]. An advantage of the AlamarBlue assay is that it allows screening of fungicidal compounds under conditions of nutrient starvation that may simulate host environments such as cerebrospinal fluid and joint cavities. Cell viability is measured by the oxidation levels of AlamarBlue dye in the mitochondria in the assay. However, the signal-to-basal ratio of that assay is typically low due to a high fluorescence background associated with the dye. The ATP content assay is a newer alternative for the measurement of cell viability that detects cellular ATP levels utilizing a luminescence readout. It possesses high assay sensitivity, and has been applied in high throughput screening for a variety of targets [4-8]. Additionally, the ATP content assay is in a homogenous assay format, therefore eliminating the need for a tedious cell wash. A luminescence signal is generated after a incubation with the reagent mixture consisting of detergents, luciferase, and luciferin substrate. The signal-to-basal ratio is characteristically higher in comparison to the AlamarBlue assay, because there is little innate luminescence background in comparison to the fluorescence background in cells and plates. We report here the development and optimization of an ATP content assay for the measurement of *C. neoformans* cell viability in a miniaturized 1536-well plate format. This assay has been tested in a pilot screen using the Library of Pharmacological Active Compounds (LOPAC) that led to the identification of four fungicidal compounds against *C. neoformans*. The results demonstrate that this miniaturized ATP content assay is highly robust, and well suited for the high-throughput screening of large compound collections to identify novel anti-cryptococcal drug development leads.

Materials and Methods

Reagents and Fungal Strains

Amphotericin B (catalog number A9528) was purchased from Sigma-Aldrich. The ATP content kit (either ATPlite, catalog number 6016941 or CellTiter-Glo, catalog number G7572) was obtained from PerkinElmer or Promega. AlamarBlue (catalog number DAL1100) and Opti-MEM reduced serum medium without phenol red (catalog number

11058-021) were purchased from Life technologies. The 1536-well white and black sterile tissue culture treated polystyrene plates (catalog number 789092-F and 789173-F) were purchased from Greiner Bio-One (Monroe, NC). *C. neoformans* strain H99 (wild type, WT) was the kind gift of J. Perfect. The nine clinical isolates of *C. neoformans* were provided by KJ Kwon-Chung.

Fungal Culture

Wild type strain H99 was cultured at 30°C for 24h to mid-log phase in YPD growth media that consists of 2% glucose, 2% bactopectone, and 1% yeast extract. Subsequently, the cells were harvested after centrifugation, rinsed twice with sterile ASN media that is made up of 1g/L asparagine and 10 mM sodium phosphate, pH 6.5, and finally re-suspended in the ASN media for dispensing [3].

ATP Content Assay

The ATP assay kit, consisting of a cell lysis buffer and luminescence detection reagents, was used to quantitate cellular ATP levels for all *C. neoformans* strains described in this manuscript. All experiments for assay optimization and miniaturization were preformed in white 1536-well plates. Briefly, the *C. neoformans* suspension was plated at a seeding density of 2200, 4500 and 9100 cells/well with a final volume of 5 µl/well in white 1536-well plates using the Multidrop-Combi dispenser (Thermo Scientific). The resulting assay plates were incubated for 24, 48, and 72 hours at 37°C supplied with 5 % CO₂. The ATP assay kit reagent mixture was prepared according to the manufacture's protocol, and added at 5 µl/well followed by a 10 minute incubation at room temperature. The assay plates were read in luminescence mode on a ViewLux plate reader (PerkinElmer).

AlamarBlue Assay

The AlamarBlue assay, previously developed in a 96-well plate format [3], was miniaturized to a black 1536-well plate format. Briefly, the *C. neoformans* suspension was plated at a seeding density of 2200, 4500 and 9100 cells/well with a final volume of 5 µl/well using the Multidrop-Combi dispenser in black 1536-well plates. Cells were incubated for 24, 48, and 72 hours at 37°C supplied with 5 % CO₂. The AlamarBlue dye (10× stock supplied by the manufacture) was prepared as a 2× working solution using Opti-MEM medium and added at 5 µl/well. Plates were incubated at 37°C supplied with 5 % CO₂ for 2 hrs, and subsequently measured in a fluorescence intensity mode (Ex= 525 nm, Em= 598 nm) on the ViewLux plate reader.

Compound Screen

To avoid contamination of our compound library with *C. neoformans*, the compound solution was first added to the ASN medium in the assay plates prior to the addition of *C. neoformans* (Table 1). Briefly, 2.5 µl/well ASN growth media was dispensed into 1536-well white assay plates using a Multidrop-Combi dispenser. Compound in DMSO solution was transferred in a volume of 23 nl/well using the NX-TR Pintool station (WAKO Scientific Solutions, San Diego, CA). The *C. neoformans* suspension, prepared in ASN growth medium, was added at 2.5 µl/well for a final seeding density of 5,000 cells/well using the

Multidrop-Combi dispenser. The assay plates were incubated for 24 hours at 37°C supplied with 5 % CO₂. The ATP assay reagent (prepared according to manufactures protocol) for measurement of cell viability was added at 5 µl/well to the assay plates that were incubated at room temperature for an additional 10 minutes followed by a detection in a luminescence mode of the ViewLux plate reader.

Compound Library and Instrumentation

The compound library was prepared and handled as previously described [9]. Briefly, the powder samples of Library of Pharmacologically Active Compounds (LOPAC) containing 1,280 known compounds were purchased from Sigma-Aldrich. Compounds were weighed individually and dissolved in DMSO as 10 mM stock solutions that were further serially diluted at a 1:5 ratio in 384 well plates to 7 concentrations and then reformatted into 1536-well compound plates. A CyBi®-Well dispensing station with a 384-well head (Cybio Inc., Woburn, MA) was used to reformat compounds from 384-well plate to 1536-well plate. A volume of 23 nl of compound in DMSO solution was transferred to 1536-well assay plates using the NX-TR pintool workstation. The cells and detection reagent were dispensed into 1536-well assay plates at 1 to 5 µl/well using a Multidrop Combi or a BioRAPTR FRD™ workstation (Beckman Coulter). The quality of compounds in DMSO solution was examined after compound dilution to ensure greater than 90% in purity. The quality of cherry-picked hit compounds was also examined on a routine base (before the confirmation experiments).

Data Analysis

The primary screen data and curve fitting were analyzed using software developed internally at the NIH Chemical Genomics Center (NCGC) [10]. IC₅₀ values were calculated using the Prism software (Graphpad Software, Inc. San Diego, CA). The signal-to-basal ratio (S/B) was calculated by dividing the signal generated from the *C. neoformans* only wells by the basal signal derived from *C. neoformans* in the presence of 50 µM amphotericin B, our positive control. All values were expressed as the mean ± SD (n=3).

Results

ATP Content Assay Development

An ATP content assay utilizing a luminescent readout was applied to measure *C. neoformans* cell viability. The assay procedure is sensitive and amiable to 1536-well plate format for the screening of large compound libraries. The assay procedure involves a single reagent addition followed by a 10 minute incubation prior to detection in luminescence mode. The reagent mixture of this assay kit is comprised of detergents, luciferase enzyme, and luciferin substrate that interact with the ATP released from cells to generate a luminescence signal. Because of the high sensitivity and robustness of the ATP content assay, assay development was carried out directly in 1536-well plates for all *C. neoformans* strains. Amphotericin B was used as a positive control compound in the assay development.

The number of *C. neoformans* cells per well was first optimized by testing seeding densities of 2200, 4500 and 9100 cells/well in 1536-well plates. A peak luminescence signal was

reached with the cell density of 4500 cells/well. The signal-to-basal ratios (S/B) for 2200, 4500 and 9100 cells/well were 15.5, 14.5 and 9.5 fold, respectively (Fig. 1A and B). The IC₅₀ value of amphotericin B (~250 nM) was similar for all cell densities tested (Fig. 1C). Therefore, a seeding density of 4500 cells/well was selected for all subsequent experiments as an optimal condition. We then determined the optimal incubation time with the positive control compound, amphotericin B. The signal-to-basal ratio determined after 24, 48, and 72 hours incubation ranged from 12 to 15 fold, and the IC₅₀ value of amphotericin B was consistent for all three incubation times (Fig. 2A and B). Thus, an incubation time of 24 hours was selected as an optimal condition for subsequent experiments. Additionally, the IC₅₀ value of amphotericin B obtained from the ATP content assay was similar to that determined in the AlamarBlue assay (Fig. 3). Together, these results demonstrate that the ATP content assay has similar compound sensitivity, but has significantly higher signal-to-basal ratio in comparison to the AlamarBlue assay.

Primary Compound Screen

To assess the property of this assay for compound screening, a DMSO plate was initially tested with a final concentration of 0.46% DMSO that was the equivalent concentration of DMSO used in compound screen experiments. The signal-to-basal ratio was 21.9 fold, CV was 7.1% and Z' factor of 0.76 in this experiment (Fig. 4), indicating a robust assay that is suitable for high-throughput screening.

The LOPAC library of 1,280 compounds was then screened at compound concentrations ranging from 0.074 to 46 μ M in the ATP content assay to identify fungicidal compounds against *C. neoformans* (strain H99). A concentration response of amphotericin B was used as an internal positive control in every assay plate. The average signal-to-basal ratio was 22 fold and Z' factor was 0.75 in this screen. Six compounds were identified as primary hits with a selection criteria of IC₅₀ <5 μ M and greater than 95% cell killing activity and the hit rate for this LOPAC library screen was 0.46 %.

Hit Confirmation and Profiling

Four of the six compounds identified from the primary LOPAC screen were selected and cherry-picked for confirmation. They were retested in the same ATP content assay against the *C. neoformans* H99 strain. The IC₅₀ value for amphotericin B in the confirmation assay was 0.32 μ M, consistent with previous experiments. The activities of all four compounds; NCGC00015735, NCGC00015992, NCGC00094112, and NCGC00185994, were confirmed and their IC₅₀ values were 0.17, 0.38, 0.29 and 3.37 μ M, respectively (Table 2).

The four confirmed compounds were further tested against nine clinical isolates of *C. neoformans*; Et27a, Et1a, Et90a, Et125a, NIH7, NIH9, NIH38, NIH157, Bt68a, and MRL#862. The antifungal profiles against these nine isolates of *C. neoformans* are shown in Table 2. The activities of amphotericin B (a positive control compound) against the Bt27a and NIH7 clinical isolates were 10 and 7.6 fold weaker in comparison to the wild type *C. neoformans* H99 strain, respectively. Interestingly, the variations of fungicidal activity of the three newly identified compounds (NCGC00015735, NCGC00094112, and NCGC00185994) on the nine clinical isolates were smaller in comparison to amphotericin

B. For example, NCGC00015735 (Fig. 5A) resulted in less than a 3.5 fold reduction in IC₅₀ values for all the nine isolates compared to the wild type strain (Fig 5E). However, the mechanism of action against *C. neoformans* is still to be elucidated for these four compounds.

Discussion

Although the first line of defense against *C. neoformans* infection in immunocompromised patients is typically amphotericin B, this drug is accompanied by significant renal toxicity and inconvenience of intravenous administration that makes it a poor option in many resource-limited countries. Fluconazole, a second-line agent against *C. neoformans* infections, is widely available through a compassionate use program but has limited efficacy against cryptococcal meningitis [11]. This may be due to its lack of fungicidal activity in comparison to amphotericin B under nutrient deficient condition [12,13], although growth inhibition of the fungus by fluconazole has been well established under nutrient rich conditions [14]. Therefore, the nutrient conditions used in the screening of *C. neoformans* may play a critical role in identifying lead compounds for the treatment of cryptococcal meningitis, because nutrients such as glucose are limited in the cerebrospinal fluid and this could potentially lead to a decrease in compound efficacy in an *in vivo* environment[15]. Consequently, a new screening methodology is needed for the identification of more effective drugs to treat *C. neoformans* related infections.

The ATP content assay has been used in mammalian cell lines and other live pathogens for high-throughput screening [4,16]. This luminescence based assay has high sensitivity and high signal-to-basal ratio that is particularly important for the assay using *C. neoformans* cells under starvation conditions where metabolic activity is reduced. We have developed a sensitive ATP content assay that measures the viability of *C. neoformans* for the high-throughput screening of large libraries to identify new lead compounds for drug development. Because the luciferase enzyme is an exogenous protein, the background generated in this assay is negligible, resulting in a high signal-to-basal ratio. Our data has demonstrated that this *C. neoformans* viability assay is sensitive, robust, and well suited for the high-throughput screening of large molecular libraries.

Using the ATP content assay, four new active compounds from the LOPAC library screen have been identified against the *C. neoformans* (strain H99). Briefly, Niclosamide (NCGC00015735) is an approved drug for the treatment of tapeworms with a mechanism of action related to uncoupling oxidative phosphorylation in the tapeworm[17]. Malonoben (NCGC00015992) is a pesticide, 6-bromoindirubin-3'-oxime (NCGC00094112) has been reported as a GSK-3 inhibitor [18,17], and 5-[(4-Ethylphenyl)methylene]-2-thioxo-4-thiazolidinone (NCGC00185994,) has been reported as a c-Myc inhibitor [19]. It's interesting to note that the activities for three of these four compounds were similar against the nine clinical isolates in comparison to the wild type *C. neoformans* H99 strain. These results indicate that the new lead compounds may have better efficacy against the amphotericin B resistant strains of *C. neoformans*. Interestingly, none of these four compounds identified here were found in a recent repurposing screen of *C. neoformans* conducted under nutrient rich conditions[20]. This may be due to the different cellular

signaling pathways and growth environment involved and/or induced by the opposing nutrient conditions in *C. neoformans*. However, additional testing will be required to confirm the *in vivo* efficacy of these four lead compounds.

In summary, we have developed a *C. neoformans* viability assay utilizing an ATP content assay format. This sensitive and homogenous assay has been miniaturized into 1536-well plate format and is robust for high throughput screening. The assay has been validated by the control compound amphotericin B, and a pilot screen of the LOPAC library. Four active compounds against *C. neoformans* have been identified and confirmed from this screen. Therefore, the ATP content *C. neoformans* viability assay, under nutrient starvation conditions, is a useful tool for high throughput screening to identify new lead compounds for anti-cryptococcal drug development.

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Fig. 1.

Cell density titrations. (A) *C. neoformans* cell viability compared with those after treatment with 50 μ M amphotericin B for 24 hours at seeding densities of 2200, 4500, and 9100 cells/well. (B) The signal-to-basal ratio from different cell densities calculated from Figure 1A in the 1536-well assay. (C) Concentration response curves of amphotericin B determined at different cell densities. The IC_{50} values the seeding densities of 2200, 4500, and 9100 cells/well were 0.267, 0.331 and 0.284 μ M, respectively.

Fig. 2.

Compound incubation time. (A) Signal-to-basal ratio of the *C. neoformans* ATP content assay. The control compound amphotericin B (50 μ M) was used to define the basal signal, where cells were incubated with *C. neoformans* for 24, 48, and 72 hours. (B) Concentration response curves of amphotericin B at different incubation times. The IC₅₀ values of amphotericin B after the treatment for 24, 48, and 72 hours were 0.331, 2.65 and 0.137 μ M, respectively.

Fig. 3.

Comparison of amphotericin B activity against *C. neoformans* in the ATP content and AlamarBlue assays. The IC_{50} values of amphotericin B were similar in both assays (0.344 μ M in ATP content assay and 0.372 μ M for the AlamarBlue assay). However, the AlamarBlue assay exhibited high basal signal due to the fluorescence properties of the AlamarBlue dye.

Fig. 4.

Scatter plot of results from a DMSO plate screen in the *C. neoformans* ATP content assay. The total signal was calculated from the wells without compound treatment (DMSO solvent only, column 1 and 4) and the basal signal was defined by the wells treated with 50 μ M Amphotericin B for 24 hr (column 2). A titration of amphotericin B was used as an internal control with the compound concentrations ranged from 0.1 nM to 10 μ M (column 3). The signal-basal ratio, CV, and Z' were 21.9 fold, 7.1%, and 0.76, respectively.

Fig. 5. Structures and concentration responses of the four confirmed hits identified from the LOPAC library screen. The potencies of these compounds; NCGC00015735 (A), NCGC00015992 (B), NCGC00094112 (C) and NCGC00185994 (D) against the wild type *C. neoformans* H99 strain and the nine clinical isolates were established in the ATP content assay. Amphotericin B (E) was used as a positive control in the experiment. The IC₅₀ values of these compounds are populated in Table 2.

Table 1Assay protocol for the measurement of *C. neoformans* viability in 1536-well plates

Step	Parameter	Value	Description
1	Medium	2.5 µl/well	ASN medium
2	Compound	0.023 µl/well	Compound in DMSO solution
3	<i>C. neoformans</i>	2.5 µl/well	Suspension (2×10^6 /ml) in ASN medium
4	Incubation	24 hr	37°C, 5% CO ₂
5	Detection reagent	5 µl/well	ATP content assay reagent
6	Incubation	10 min	Room temperature
8	Plate reading	Luminescence mode	ViewLux plate reader

Table 2
IC₅₀ values (μM) of four confirmed compounds against 9 clinical isolate strains of *C. neoformans*.

	H99 (WT)	Bt27α	Bt12α	Bt90α	NIH7	Bt125α	NIH38	Bt68α	NIH9	NIH157
Amphotericin B	0.320	3.21	0.521	0.501	2.430	0.336	0.484	0.087	0.250	0.209
NCGC00015735	0.169	0.518	0.208	0.281	0.479	0.592	0.422	0.168	0.284	0.320
NCGC00015992	0.378	3.48	0.428	0.732	1.70	3.32	0.796	0.384	0.815	2.15
NCGC00094112	2.88	16.4	3.93	4.30	15.2	16.5	10.8	4.24	6.01	6.55
NCGC00185994	3.37	11.1	11.6	13.1	15.6	14.5	7.54	8.23	14.1	13.1