International Journal of Antimicrobial Agents ■■ (2016) ■■–■■

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

# Novel cell-based in vitro screen to identify small-molecule inhibitors against intracellular replication of *Cryptococcus neoformans* in macrophages

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ARTICLE INFO

*Article history:*
Received 18 January 2016
Accepted 16 April 2016

*Keywords:*
*Cryptococcus neoformans*
Macrophages
Intracellular proliferation
Drug screening
Anticryptococcal therapy
Fendiline

ABSTRACT

The fungal pathogen *Cryptococcus neoformans* poses a major threat to immunocompromised patients and is a leading killer of human immunodeficiency virus (HIV)-infected patients worldwide. Cryptococci are known to manipulate host macrophages and can either remain latent or proliferate intracellularly within the host phagocyte, a favourable niche that also renders them relatively insensitive to antifungal agents. Here we report an attempt to address this limitation by using a fluorescence-based drug screening method to identify potential inhibitors of intracellular proliferation of *C. neoformans*. The Prestwick Chemical Library® of FDA-approved small molecules was screened for compounds that limit the intracellular replication of a fluorescently-tagged *C. neoformans* reference strain (H99-GFP) in macrophages. Preliminary screening revealed 19 of 1200 compounds that could significantly reduce intracellular growth of the pathogen. Secondary screening and host cell cytotoxicity assays highlighted fendiline hydrochloride as a potential drug candidate for the development of future anticryptococcal therapies. Live cell imaging demonstrated that this $Ca^{2+}$ channel blocker strongly enhanced phagosome maturation in macrophages leading to improved fungal killing and reduced intracellular replication. Whilst the relatively high dose of fendiline hydrochloride required renders it unfit for clinical deployment against cryptococcosis, this study highlights a novel approach for identifying new lead compounds and unravels a pharmacologically promising scaffold towards the development of novel antifungal therapies for this neglected disease.





## 1. Introduction

*Cryptococcus neoformans* is an opportunistic fungal pathogen that can cause fatal infections in immunocompromised individuals. The infection process begins with inhalation of infectious agents (spores or desiccated yeasts) resulting in a primary pulmonary infection, which, in response to immunosuppression, can further disseminate to the central nervous system causing meningitis [1]. Human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) patients are particularly prone to cryptococcal infections, with an estimated overwhelming disease burden of approximately one million cases of cryptococcal meningitis (CM) per year [2,3]. The highest incidence of CM-related deaths in HIV-positive patients occurs in sub-Saharan Africa, with an associated mortality of 70% [2]. Despite the advent of highly active antiretroviral therapy, CM-related mortality remains prevalent among HIV/AIDS patients in developing regions [4,5].

Alveolar macrophages form the first line of defence against *Cryptococcus*, however the fungus is able to survive and replicate within the macrophage phagosome following internalisation [6–8]. The exact mechanisms facilitating intracellular survival of the pathogen within phagosomes are not fully understood, but recent data suggest that the process of phagosomal maturation is subtly perturbed leading to reduced antimicrobial activity in this compartment [9] and phagolysosomal damage [10]. Continued replication of the fungus eventually leads to lysis of the host macrophage or non-lytic release of the pathogen by a process termed vomocytosis [11,12].

Current World Health Organization (WHO) guidelines for CM management in AIDS patients recommend 'gold-standard' combination therapy with amphotericin B (AmB) and 5-flucytosine (5-FC) followed by lifelong maintenance with fluconazole (FLU) [13,14]. However, the need for intravenous dosing together with

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http://dx.doi.org/10.1016/j.ijantimicag.2016.04.018
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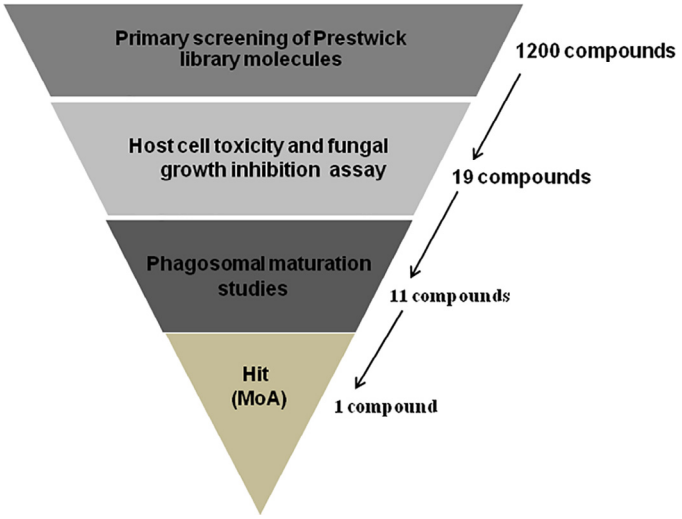


**Fig. 1.** Screening strategy for library molecules. A total of 1200 US Food and Drug administration (FDA)-approved small molecules were screened for intracellular growth inhibition of *Cryptococcus neoformans* in macrophages. The primary screen yielded 19 active compounds that were further probed for host cell cytotoxicity and fungal growth inhibition. Eleven compounds were shortlisted for phagosomal maturation screening, which identified only one promising drug hit candidate, D9 (fendiline hydrochloride). Finally, the mechanism of action of fendiline was investigated. MoA, mode of action.

significant clinical toxicity and thus a requirement for therapeutic monitoring, particularly for optimising 5-FC dosing in patients with renal impairment, has limited deployment of this approach in resource-limited settings [15–17]. Therefore, novel and effective alternatives to these mainstay anticryptococcal drugs are needed. Compounds that drive clearance of cryptococci from the intracellular niche offer a powerful alternative approach to treating cryptococcosis and may hold promise as adjunct therapy to use alongside existing antifungals.

To identify such compounds, we have employed a high-throughput fluorescence-based screening approach to probe 1200 US Food and Drug Administration (FDA)-approved small molecules for their ability to inhibit intracellular proliferation of *C. neoformans* in murine macrophages (Fig. 1). Shortlisted compounds were further probed for host cell cytotoxicity and antifungal activity, and lead molecules were validated in vitro by intracellular killing assays, leading to the identification of fendiline hydrochloride as a potential candidate compound. Finally, we demonstrated that fendiline hydrochloride improves the phagosomal maturation rate and thus facilitates killing of *C. neoformans* within the host cell.

## 2. Materials and methods

### 2.1. Yeast cells and growth conditions

All reagents were purchased from Sigma unless otherwise stated. Green fluorescence protein (GFP)-expressing *C. neoformans* serotype A strain (H99-GFP) and *Cryptococcus gattii* serotype B strain (R265-GFP) were used for this study [18] and were grown overnight in YPD medium (2% glucose, 1% peptone and 1% yeast extract) on a rotator revolving at 240 rpm at 25 °C prior to all experiments.

### 2.2. Macrophage cell line culture

Cells from the murine macrophage-like cell line J774A.1 were used between passages 4 and 14 after thawing and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin and 10% foetal bovine serum (FBS) at 37 °C and 5% $CO_2$.

### 2.3. Assay development and primary screening assay

The Prestwick Chemical Library® containing approximately 1200 FDA-approved small molecules was accessed via the Birmingham Drug Discovery Facility (University of Birmingham, Birmingham, UK). Shortlisted compounds were repurchased from Sigma-Aldrich unless otherwise mentioned. The drugs were dissolved in dimethyl sulphoxide (DMSO), which served as a negative control, and were used at a final assay concentration of 10 μM, and AmB (final assay concentration 1.25 μg/mL) was used as a positive control. Primary screening of compounds was performed at the Drug Discovery Unit (University of Birmingham) using a Hamilton STAR liquid handling robot (Hamilton Robotics, Bonaduz, Switzerland) integrated with a microplate reader (BMG LABTECH, Ortenberg, Germany) enabling GFP fluorescence measurement. Macrophages ($0.25 \times 10^5$ cells/well) were seeded in a glass-bottom 96-well plate (Greiner Bio One Ltd., Stonehouse, UK) 18 h before infection. An overnight culture of strain H99-GFP was harvested by centrifugation at 6500 rpm for 2.5 min, was washed three times with phosphate-buffered saline (PBS) and was opsonised with 5% pooled human serum (not heat-inactivated) for 1 h at room temperature prior to infection. Macrophages were activated with 150 ng/mL phorbol myristate acetate (PMA) for 1 h in DMEM without FBS and were infected with yeast cells [multiplicity of infection (MOI) 10:1] for 2 h at 37 °C. At this point, total GFP fluorescence was measured ($t_0'$) to control for well-to-well variation in cryptococcal exposure. Then, the medium was aspirated and cells were washed with PBS to remove extracellular yeasts. The GFP fluorescence corresponding to only intracellular yeast was measured ($t_0$) and medium containing drugs was added to the cells and was incubated for a further 18 h at 37 °C in 5% $CO_2$. After 18 h, the GFP fluorescence was measured ($t_{18}'$) to determine the total yeast burden. Extracellular yeast was then removed by washing with PBS and a final GFP fluorescence reading (representing intracellular yeast only) was taken ($t_{18}$). The total (intracellular plus extracellular) proliferation rate (PR) was quantified as the relative fluorescence ratio of $t_{18}'/t_0'$, whilst the intracellular proliferation rate (IPR) was quantified as the ratio $t_{18}/t_0$. The statistical parameters signal-to-basal (S/B) ratio, Z′ factor and percent coefficient of variation (%CV) were calculated as follows [19]:

$$Z' = 1 - (3\sigma_n + 3\sigma_p)/|\mu_n - \mu_p|$$

$$S/B = \mu_n/\mu_p$$

$$\%\,CV = \sigma_n/\mu_n \times 100$$

where $\mu_n$, $\sigma_n$, $\mu_p$ and $\sigma_p$ are the means (μ) and standard deviations (σ) of the negative and positive controls, respectively.

### 2.4. Secondary screening (phagosomal maturation studies)

Macrophages ($0.5 \times 10^5$ cells/well) were seeded into a glass-bottom 96-well plate and were infected with serum-opsonised H99-GFP cells (MOI 10:1) following activation with PMA as described above. Where necessary, yeasts were heat-killed at 55–60 °C for 30 min before serum opsonisation and infection as described previously [9]. At 2 h post-infection, medium was replaced with serum-free DMEM supplemented with 5 μM of drugs (equivalent to 0.1% DMSO) or 1.25 μg/mL AmB containing the acidotropic dye 50 nM LysoTracker® Red DND-99 (Invitrogen, Molecular Probes, Waltham, MA). Cells were then taken for live imaging for 18 h.



Please cite this article in press as: Sweta Samantaray, Joao N. Correia, Mariam Garelnabi, Kerstin Voelz, Robin C. May, Rebecca A. Hall, Novel cell-based in vitro screen to identify small-molecule inhibitors against intracellular replication of *Cryptococcus neoformans* in macrophages, International Journal of Antimicrobial Agents (2016), doi: 10.1016/j.ijantimicag.2016.04.018