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DOI: 10.1016/j.ijantimicag.2016.04.018
10.1016/j.ijantimicag.2016.04.018

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Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):

Link to publication on Research at Birmingham portal
Novel cell-based in vitro screen to identify small-molecule inhibitors against intracellular replication of *Cryptococcus neoformans* in macrophages

Sweta Samantaray, Joao N. Correia, Mariam Garelnabi, Kerstin Voelz, Robin C. May *, Rebecca A. Hall **

Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, Birmingham, UK

** Corresponding author. Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, Birmingham, UK. Tel.: +44 121 414 5418; fax: +44 (0)121 41 45925.
E-mail address: r.c.may@bham.ac.uk (R.C. May).

** Corresponding author. Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, Birmingham, UK. Tel.: +44 121 414 5581; fax: +44 (0)121 414 3071.
E-mail address: r.a.hall@bham.ac.uk (R.A. Hall).

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 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 antifungal 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 

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% CO₂.

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 × 10⁵ 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, washed three times with phosphate-buffered saline (PBS) and 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 (t₀) 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 (t₅₀) and medium containing drugs was added to the cells and was incubated for a further 18 h at 37 °C in 5% CO₂. After 18 h, the GFP fluorescence was measured (t₁₈) 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 (t₁₈). The total (intracellular plus extracellular) proliferation rate (PR) was quantified as the relative fluorescence ratio of t₁₈/t₅₀, whilst the intracellular proliferation rate (IPR) was quantified as the ratio t₁₈/t₀. The statistical parameters signal-to-basal (S/B) ratio, Z’ factor and percent coefficient of variation (%CV) were calculated as follows [19]:

\[
Z’ = 1 - \frac{3(\sigma_n + 3\sigma_p)}{\mu_n - \mu_p}
\]

\[
S/B = \frac{\mu_n}{\mu_p}
\]

\[
\%CV = \frac{\sigma_n}{\mu_n} \times 100
\]

where \(\mu_n, \sigma_n, \mu_p\) and \(\sigma_p\) are the means (\(\mu\)) and standard deviations (\(\sigma\)) of the negative and positive controls, respectively.
2.5. Cytotoxicity assay

Macrophage cell cytotoxicity of the compounds was tested using the LDH Cytotoxicity Detection Kit (Takara Bio Inc., Kusatsu, Japan) according to the manufacturer’s protocol. Macrophages were seeded into 96-well plates at a density of either 0.25 × 10^5 cells/well or 0.5 × 10^5 cells/well and were cultured overnight. Medium was replaced with serum-free DMEM containing either 5 μM or 10 μM of drug and cells were incubated at 37 °C in 5% CO₂ for a further 18 h. Lactate dehydrogenase (LDH) enzyme released into the cell culture supernatant was measured as a colorimetric change after addition of reaction mixture. The plate was read at 490 nm using a FLUOstar Omega Microplate Reader (BMG Labtech) after 10 min incubation at room temperature. 1% Triton X-100 was used as a positive control. Treatments were done in triplicate and the mean absorbance was calculated. The percent cytotoxicity for samples was expressed as a percentage of the positive control (100% for positive control).

2.6. Live imaging

Time-lapse imaging was performed at 37 °C using a Nikon TE2000 microscope (Nikon, Tokyo, Japan) in a humidified chamber with 5% CO₂ with a 20× or 60× objective (Nikon) as indicated. Images were captured every 5 min for 18 h and were analysed using NIS-Elements Advanced Research software (Nikon). The IPR was determined at 12 h (t12) due to excessive extracellular cryptococcal growth at 18 h precluding measurement at this time point.

2.7. Fungal growth inhibition assay

For the fungal growth inhibition assay, 10⁴ yeast cells were added per well in VPD broth supplemented with 5 μM of drug or the respective control (1.25 μg/mL Amb or 0.1% DMSO) in a 48-well plate (Greiner Bio One). Fungal growth kinetics was measured over a 24-h time period by reading the plate at 600 nm every 30 min using a FLUOstar Omega Microplate Reader. Plates were incubated throughout at 25 °C in the plate reader.

2.8. Statistical analysis

All statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) with all data represented as the mean ± standard deviation (S.D.) from at least three independent experiments unless otherwise stated. Data analyses were done using the Mann–Whitney U-test, one-way analysis of variance (ANOVA) or Fisher’s exact test (two-tailed) as indicated in the respective figure legends.

3. Results

3.1. Assay development to monitor intracellular proliferation of C. neoformans in macrophages and primary screen design

To identify small molecules that inhibit the intracellular growth of C. neoformans in macrophages, a high-throughput fluorescence-based automated screen was designed to score replication of a GFP-expressing C. neoformans in macrophages (Fig. 1). Macrophages were infected with strain H99-GFP in 96-well plates and the IPR of the yeast was quantified by GFP fluorescence after 18 h following phagocytosis and drug addition. As a proof of concept and in order to establish the accuracy of the assay principle in evaluating intracellular proliferation of H99-GFP under different drug treatments, the IPR and total PR in response to DMSO, Amb and FLU were first assessed manually (Fig. 2A). Whilst the IPR values remained mostly consistent for DMSO (the carrier control) and FLU (which has poor penetration into the phagosome), it decreased significantly for Amb (Fig. 2A), demonstrating that the screening set-up is able to discriminate agents that are active intracellularly from those with only extracellular activity. Therefore, we proceeded with the high-throughput screen using DMSO and Amb as negative and positive controls, respectively, which yielded significantly different IPR values for DMSO and Amb treatments (Fig. 2B). High-throughput screens are typically characterised by a Z’ factor ≥0.5 and high S/B values [19,20]. According to Zhang et al, an assay with a Z’ value >0 and <0.5 is considered to be marginal but feasible for screening [19]. Both the Z’ factor (0.37) and S/B (4.3) values for our screen fell within the range that is deemed suitable for high-throughput screening [19,20], and the %CV value of 12% was well below the recommended 20% limit [20] (Table 1; Fig. 2B). Thus, the screening procedure, whilst inherently noisy, appears sufficiently sensitive to identify promising lead compounds.

To exclude the possibility of molecules inhibiting phagocytic uptake of yeasts, the compound library was added after phagocytosis. From the preliminary screening, only those compounds that exhibited a statistically significant decrease in IPR (i.e. 2 S.D. lower than the mean IPR of the total 1200 compounds) were considered as ‘primary hits’ (Fig. 2C). This preliminary screening yielded a total of 19 small molecules that significantly inhibited intracellular growth relative to the DMSO control (data not shown). Compounds that virtually exhibited significant macrophage toxicity or with previously reported antimicrobial (including antifungal) activity were further discarded. Compounds that significantly lowered PR values (i.e. showed potent extracellular fungicidal effects) were also excluded (data not shown). After applying these filters, 13 unique molecules that limit C. neoformans survival in macrophages were identified. These selected compounds were repurchased from commercial sources for further tests and are denoted as D2–D14 (data not shown).

3.2. Validating host cell cytotoxicity and direct antifungal activity of D2–D14 compounds

To confirm the primary screen-specific effects, the 13 selected repurchased compounds were tested under different conditions using a reduced compound concentration (5 μM), lower DMSO content (0.1%) and higher macrophage density. As observed in Fig. 3A, treatment of a macrophage cell density of 0.25 × 10⁵ cells/well with 10 μM compounds (1.25 μg/mL Amb) and a corresponding 2.5% DMSO control yielded a high level of macrophage cytotoxicity. In contrast, treatment with a 5 μM drug dose corresponding to a low DMSO content of 0.1% and higher cell density (0.5 × 10⁵ cells/well) enhanced macrophage survival (Fig. 3B), thereby prompting us to use the latter setting for further investigation.

We validated that the selected doses of the compounds (5 μM and corresponding 0.1% DMSO) were not directly toxic to the fungi. For this, the growth inhibitory activity of the selected compounds was tested against an H99-GFP axenic culture over a period of 24 h (Fig. 3C). Compounds D5 and D6 significantly inhibited fungal growth to the same extent as the positive control Amb and were therefore excluded from further screening. The remainder of the compounds exhibited comparable growth kinetics to the untreated control, suggesting that these compounds do not exhibit

| Table 1: Summary of quality control parameters for screening assay (n = 100). |
|-----------------|-----------------|
| Statistical parameter | Value |
| Z’ factor | 0.37 |
| S/B ratio | 4.3 |
| %CV | 12.01 |
direct antifungal activity but rather suppress intracellular growth of the fungus (Fig. 3C).

3.3. Secondary screening reveals D9-triggered phagosomal acidification in macrophages

To probe how the remaining 11 compounds reduced the intracellular proliferation of *C. neoformans*, their effect on phagosomal maturation was assessed. Phagosome maturation is a multistep process eventually resulting in phagolysosome fusion, acidification and enhanced antimicrobial properties [21]. Several intracellular pathogens such as *Mycobacterium tuberculosis*, *Legionella pneumophilia*, *Salmonella Typhimurium* and *Leishmania donovani* evade macrophage killing by perturbing the phagosomal maturation process [22,23]. Recently, we showed that *C. neoformans* is also capable of altering phagosomal acidification in host cells to facilitate intracellular survival [9]. Therefore, we considered the possibility that compounds identified as reducing intracellular growth of crypto-cocci may do so by improving phagosomal maturation. To test this hypothesis, phagosome acidification was monitored using the flu-
orescent acidotropic dye LysoTracker Red in response to drug treatment during infection. Whilst live untreated cryptococci-containing phagosomes hardly acquired a LysoTracker-positive signal, almost 100% of the phagosomes in the heat-killed control became LysoTracker-positive over the 18 h period of infection (Fig. 4A). Most of the drug treatments, including the DMSO control, did not yield any significant phagosomal acidification compared with the untreated control (Fig. 4A). Interestingly, however, treatment with drug D9 significantly enhanced phagosomal acidification and yielded a striking 87.14% LysoTracker-positive phagosomes compared with untreated and DMSO controls (Fig. 4A–D; Supplementary Movies S1–S3). Surprisingly, D9 yielded significantly higher...
LysoTracker-positive signal than AmB, which is a mainstay anticytocol drug with fungicidal activity against cryptococci but generated only 43.53% LysoTracker-positive phagosomes up to 18 h of infection (Fig. 4A; Supplementary Movie S1). Upon careful analysis of time-lapse movies, it was found that the D9-induced acidification events occurred typically within the first 4–5 h of infection (Fig. 4D; Supplementary Movie S3). Conversely, untreated and DMSO-treated phagosomes remained mostly negative at that time point (Fig. 4B, C; Supplementary Movies S2 and S3) and failed to retain a significant amount of LysoTracker-positive signal even 18 h post-infection. Thus, D9 appears to act by enhancing phagosomal maturation and either killing or disabling the intracellular cryptococci.

3.4. Drug D9 is active against intracellular cryptococci at 5 μM but loses efficacy at 1 μM

Next, we were prompted to analyse the efficacy of D9 at a lower dose of 1 μM that would be ideal for clinical applications. However, D9 failed to retain its activity upon lowering the drug dose and the LysoTracker-positive signal significantly dropped to 3.58% compared with that of the 5 μM dose (Fig. 5A). Furthermore, the effect of D9 at both doses on intracellular proliferation of C. neoformans in macrophages was assessed from time-lapse movies until 12 h post infection (Fig. 5B). Congruently with its effect on phagosomal acidification, a 5 μM dose of D9 significantly reduced the IPR of H99-GFP cells in macrophages compared with untreated and DMSO

![Figure 4](image_url)
controls. Conversely, D9 failed to inhibit intracellular growth of Cryptococcus at the lower dose of 1 μM and retained an IPR level comparable with the untreated control, suggesting loss of efficacy at this dose (Fig. 5B). This strong threshold effect itself reflects a requirement for a minimum signalling flux to activate the phagosome maturation pathway in order to successfully kill intracellular cryptococci.

To test whether the activity of D9 is species-specific, the effect of D9 on phagosomal acidification against the other main pathogenic cryptococcal species, C. gattii (R265-GFP), was also tested. As with C. neoformans, D9 effectively increased LysoTracker-positive of C. gattii phagosomes, an effect that dropped significantly with reduced drug dosage (Supplementary Fig. S1), suggesting that this drug is effective against both human-infective species of Cryptococcus.

Unblinding the screening compounds revealed D9 to be fendiline hydrochloride, a known inhibitor of L-type Ca²⁺ channels that reportedly increases the intracellular Ca²⁺ concentration [Ca²⁺i], via calcium release from endoplasmic reticulum stores [24, 25]. Therefore, to assess the intracellular calcium flux in macrophages in response to fendiline, drug concentrations ranging from 1 to 20 μM were tested to check the concentration-dependent effects previously reported for other cell types with this drug [24–26] (see Supplementary method). Interestingly, fendiline barely had an effect on [Ca²⁺i], at a concentration of 1 μM compared with the DMSO control (Supplementary Fig. S2A), but the [Ca²⁺i], markedly increased at 5 μM or 20 μM (Supplementary Fig. S2A). Thus, this sharp concentration threshold is likely attributable to the differential efficacy observed between 1 μM and 5 μM. Furthermore, to investigate whether the rise in [Ca²⁺i] upon fendiline treatment is mainly due to discharge of Ca²⁺ from intracellular stores, we tested whether the inhibitor thapsigargin specifically blocking endoplasmic reticulum Ca²⁺ pumps could alleviate the rise in [Ca²⁺i], observed with fendiline [25, 26] (see Supplementary method). Indeed, exposing the cells to 0.5 μM thapsigargin caused a significant drop in [Ca²⁺i] compared with the cells exposed to only 5 μM fendiline (Supplementary Fig. S2B). These results suggest that fendiline induces a Ca²⁺ signalling flux in J774A.1 macrophages that involves Ca²⁺ influx to the cytoplasm from intracellular stores.

4. Discussion

Considering the poor status of current antifungal drugs, new treatment options for cryptococcosis are much needed. Studies from several groups have attempted to address this and have mostly focused on drugs that act on extracellular, rather than intracellular, cryptococci [27, 28]. Recently, Butts et al screened the Prestwick library of FDA-approved molecules to identify compounds with direct fungicidal activity against C. neoformans based on loss of cellular integrity and thereby release of intracellular enzyme adenylate kinase [16]. Although our screening assay utilised the same compound library, it is complementary in approach since it was designed to identify only those molecules that induce intracellular killing, excluding all molecules with direct antifungal activity (including those identified by Butts et al). Importantly, there are no previously reported screens designed to identify adjunct therapies that may augment macrophage-based killing of cryptococci. Therefore, we developed a simple approach to screen the Prestwick compound library by infecting macrophages with the H99-GFP strain and quantifying the GFP fluorescence as an index for intracellular proliferation. Similar approaches to probe intracellular replication using GFP expression have been successfully applied for other intracellular pathogens such as M. tuberculosis [29] and Leishmania [30]. Intracellular screens are technically challenging, as reflected by the relatively poor Z’ score and S/B ratio of our screen, and thus carry a high false-negative rate. However, our screen was supported by a valid %CV value and the fact that it identified the current
frontline anticyptococcal drug (AmB) included in the library. However, we note that there is a possibility that additional anticyptococcal compounds within the collection may have been missed by our screening strategy. Nonetheless, the screen succeeded in identifying a small set of lead compounds, at least one of which appears to be a potential drug candidate.

The screening identified a novel compound D9 (fendiline hydrochloride), an L-type calcium–channel blocker widely used in the treatment of angina [25,31]. Notably, D9 does not exhibit a direct antifungal effect against C. neoformans, but instead triggers significant phagosomal acidification in host macrophages. Fendiline is thought to act by transiently elevating [Ca\(^{2+}\)]\(_i\), in certain cell types via release from intracellular endoplasmic reticulum calcium stores [25,26]. In agreement, we observed an increase in [Ca\(^{2+}\)]\(_i\) levels at doses of fendiline above 5 μM, which is inhibited by blocking calcium release from the endoplasmic reticulum with thapsigargin. Interestingly, a previous study by Huang et al showed that a 1 μM dose of fendiline barely triggered any significant increase in [Ca\(^{2+}\)]\(_i\), above baseline [25]. This may explain the strong threshold effect at 5 μM, but almost none at the 1 μM drug dose. Considering that intracellular calcium dynamics are altered during cryptococcal infection [9], it is tempting to speculate that fendiline may re-adjust intracellular calcium signalling to allow full phagosome maturation and thus fungal killing, a hypothesis that would be worthy of future investigation in appropriate animal models.

Pharmacologically, fendiline offers some attractive properties relevant for C. neoformans infection. Its physiochemical structure comprises a highly lipophilic region linked to a weak base, which enables accumulation in intracellular acidic compartments such as the phagolysosome [32,33]. In addition, it is known to be an FDA-approved neuroprotective drug and is included in the Neurodegeneration Drug Screening Consortium of 1040 compounds, most of which have access to the blood–brain barrier [34]. This makes the drug scaffold relevant for the treatment of CM infections [35]. However, fendiline itself appears only to be active in vitro at concentrations significantly higher than the typical serum level of 0.6 μM following oral administration [36]. Although this makes fendiline itself unfit for clinical translation, the drug scaffold still offers scope for potential optimisation in the future to improve either efficacy or bioavailability. Alternatively, existing FDA-approved compounds with structural similarity to fendiline may provide promising lines of investigation that would not necessitate extensive pre-clinical testing prior to clinical trials. Importantly, previous studies have highlighted a role for calcium–channel blockers in inhibition of drug efflux pumps and drug resistance in M. tuberculosis infection, which shares a similar intraphagosomal lifestyle [37,38], and thus it is possible that fendiline-derived compounds may have wide applicability.

In conclusion, this work demonstrates an effective screening platform using a whole-cell-based approach to identify inhibitors against intracellular proliferation of C. neoformans that can be potentially applied to other intracellular pathogens. We identified a novel off-patent drug, fendiline hydrochloride, that itself is not fungicidal to C. neoformans but potently manipulates intraphagosomal replication and viability within the cell. Taken together, these findings propose a role for calcium–channel blockers as potential inhibitors of intracellular survival of C. neoformans in infected macrophages and therefore represent a promising strategy for future anticyptococcal drug design and therapy.

Acknowledgements

The authors gratefully acknowledge Dr. Luke Alderwick and the Birmingham Drug Discovery and Screening Facility (University of Birmingham, Birmingham, UK) for help and guidance in setting up this screen. The authors are also thankful to the School of Medicine, University of Birmingham, for providing the perfusion chamber system for microfluorimetry analysis.

Funding: This work was supported by funding from the Medical Research Council [MR/J008176/1] and the European Research Council under the European Union Seventh Framework Programme [FP/ 2007–2013/ERC Grant Agreement No. 614562 (project MitoFun)]. RCM is supported by a fellowship from the Lister Institute for Preventive Medicine and a Royal Society Wolfson Research Merit Award; RAH by a Career Development Award from the Medical Research Council [MR/L00903X/1]; KJ by funding from the Wellcome Trust ISSF grant; and MG by a fellowship from the Islamic Development Bank.

Competing interests: None declared.

Ethical approval: Not required.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2016.04.018.

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Acknowledgements

The authors gratefully acknowledge Dr. Luke Alderwick and the Birmingham Drug Discovery and Screening Facility (University of Birmingham, Birmingham, UK) for help and guidance in setting up this screen. The authors are also thankful to the School of Medi-


