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Synergy and antagonism between iron chelators and antifungal drugs in *Cryptococcus*

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ABSTRACT

Fungal infections remain very difficult to treat, and developing new antifungal drugs is difficult and expensive. Recent approaches therefore seek to augment existing antifungals with synergistic agents that can lower the therapeutic dose, increase efficacy and prevent resistance from developing. Iron limitation can inhibit microbial growth, and iron chelators have been employed to treat fungal infections. In this study, chequerboard testing was used to explore combinations of iron chelators with antifungal agents against pathogenic *Cryptococcus* spp. with the aim of determining how disruption to iron homeostasis affects antifungal susceptibility. The iron chelators ethylenediaminetetraacetic acid (EDTA), deferoxamine (DFO), deferiprone (DFP), deferasirox (DSX), ciclopirox olamine and lactoferrin (LF) were paired with the antifungal agents amphotericin B (AmB), fluconazole, itraconazole, voriconazole and caspofungin. All chelators except for DFO increased the efficacy of AmB, and significant synergy was seen between AmB and LF for all *Cryptococcus* strains. Addition of exogenous iron rescued cells from the antifungal effect of LF alone but could not prevent inhibition by AmB + LF, indicating that synergy was not due primarily to iron chelation but to other properties of LF that were potentiated in the presence of AmB. Significant synergy was not seen consistently for other antifungal–chelator combinations, and EDTA, DSX and DFP antagonised the activity of azole drugs in strains of *Cryptococcus neoformans* var. *grubii*. This study highlights the range of interactions that can be induced by chelators and indicates that most antifungal drugs are not enhanced by iron limitation in *Cryptococcus*.

1. Introduction

Cryptococcosis is an important fungal infection. *Cryptococcus neoformans* causes infections in human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) and other immunocompromised patients such as cancer and transplant populations [1], whilst its sibling species *Cryptococcus gattii* is a primary fungal pathogen affecting apparently healthy hosts and has caused outbreaks in the Pacific Northwest of North America [2]. In its most severe form, cryptococcosis manifests as cryptococcal meningitis, which is fatal if not treated, and globally it has been estimated that *C. neoformans* kills more than 500,000 people annually worldwide [1]. The preferred induction antifungal treatment for cryptococcal meningitis is an amphotericin B (AmB) formulation combined with 5-flucytosine (5FC) [3]. However, AmB formulations are toxic and must be administered by intravenous infusion, and 5FC is expensive and is not available in many countries, making this combination difficult to administer, particularly in resource-poor settings. Fluconazole (FLC) is safe, cheap and easy to administer and is the drug of choice in consolidation and maintenance therapy, typically after cerebrospinal fluid (CSF) cultures are negative. FLC monotherapy is not recommended, however, due to common failure to sterilise the CSF, and drug resistance is emerging [4]. The triazoles posaconazole and voriconazole (VRC), whilst highly active in vitro, have unpredictable bioavailability, and robust experience with their use is still lacking. Echinocandins, the most recent class of antifungal drugs to come onto the market, have no useful activity against *Cryptococcus*. Taken together, even with current best treatment practices, mortality is high and there are substantial rates of permanent neurological sequelae including blindness, seizures, headache, memory loss and personality disorders [5].

Given the challenges of developing new antifungal drugs, enhancing existing drugs with synergistic agents is a promising alternative approach. Synergy can increase efficacy and lower the

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therapeutic dose, and by working via more than one target, it can slow down the development of resistance. The current use of AmB + 5FC for cryptococcosis is an example of a synergistic drug combination, and synergy has also been seen between calcineurin inhibitors and various antifungal agents against *Cryptococcus*, *Candida* and *Aspergillus* [6–8]. Iron chelation has been explored as an adjunct in the treatment of fungal infections, particularly in salvage therapy [9].

Clinically approved iron-chelating drugs are available and some have been shown to directly inhibit fungal pathogens, including *Cryptococcus*, *Rhizopus*, *Candida* and *Aspergillus* [10–12], illustrating the need for iron in the growth and survival of pathogenic fungi. The combined effects of various iron-chelating and antifungal agents have been tested in vitro against *Aspergillus fumigatus* and *Candida albicans*, and some synergistic combinations have been found [12,13]. However, synergy appears to be species-specific and chelators can sometimes promote growth or act antagonistically with drugs; for example, *Rhizopus* uses the chelator deferoxamine (DFO) as a xenosiderophore to promote iron uptake and this enhances invasive infection [14].

Cryptococcus mutants defective in iron homeostasis have increased susceptibility to antifungal drugs [15,16]; however, an analysis of chelator–antifungal interactions has not been undertaken. The current study sets out to determine how different antifungals interact with a range of chelating agents, including lactoferrin (LF), a globular chelating protein found in milk, tears and saliva; the US Food and Drug Administration (FDA)-approved chelators deferoxamine (DFO), deferiprone (DFP), deferasirox (DSX) and ciclopirox olamine (CPO); and the potent laboratory chelator ethylenediaminetetraacetic acid (EDTA). The aim was to explore a diversity of antifungal–chelator interactions to determine how disruption to iron homeostasis affects antifungal susceptibility, which could then be used to develop novel adjunct therapies.

2. Methods

2.1. Strains and growth media

Strains with fully sequenced and annotated genomes were used for initial minimum inhibitory concentration (MIC) and chequerboard studies as these could be used in downstream studies to characterise the cellular response to synergistic combinations. These included *C. neoformans* KN99α (molecular genotype VNI), *C. gattii* strains R265 (VGIIa) and R272 (VGIIb), and *Saccharomyces cerevisiae* reference strain S288c. In addition, *C. gattii* strain 97/170 was included as it has an intrinsically high MIC to azole drugs and it has been sequenced in our laboratory (unpublished). Subsequent testing of drug–chelator pairs of interest was done on strains from the major molecular genotypes in *C. neoformans* and included H99 and WM148 (*C. neoformans* var. *grubii* VNI), WM626 (*C. neoformans* var. *grubii* VNII) and WM629 and JEC21α (*C. neoformans* var. *neoformans* VNIV). *Candida krusei* ATCC 6248 and *Candida parapsilosis* ATCC 22019 were used as quality control strains for in vitro susceptibility assays.

Prior to experiments, cryptococcal strains were grown in yeast nitrogen broth (YNB) (Becton, Dickinson and Company) buffered with 0.165 M MOPS [3-(*N*-morpholino)propanesulfonic acid] (Sigma-Aldrich, Castle, NSW, Australia) and supplemented with 0.5% (w/v) D-glucose. RPMI-1640 medium (In Vitro Technologies, Noble Park North, VIC, Australia) buffered with 0.165 M MOPS supplemented with 0.03% (w/v) L-glutamine and 2% (w/v) D-glucose was used as a growth medium for *Candida* and *Saccharomyces* as specified in Clinical and Laboratory Standards Institute (CLSI) standards [17]. All media were adjusted to pH 7.0 and were filter sterilised.

2.2. Iron-chelating agents and antifungal drugs

Six different iron chelators were studied that encompass a diverse range of structures and iron binding ratios. These included EDTA, a substituted diamine that chelates iron in a 1:1 ratio; deferoxamine (DFO), a siderophore produced by *Streptomyces pilosus* that chelates iron in a 1:1 ratio; deferiprone (DFP), an α-ketohydroxypyridine chelator that chelates intracellular iron and binds in a 3:1 ratio; deferasirox (DSX), a bis(hydroxyphenyl)triazole that chelates intracellular iron and binds in a 2:1 ratio; ciclopirox olamine (CPO), a hydropyridone derivative that chelates iron in a 3:1 ratio, and bovine lactoferrin (LF), an iron-binding glycoprotein that chelates iron in a 1:2 ratio. Antifungal agents included amphotericin B (AmB), fluconazole (FLC), itraconazole (ITC), voriconazole (VRC) and caspofungin (CAS). EDTA, DFO, DFP, CPO, AmB, FLC, ITC and VRC were purchased from Sigma-Aldrich; DSX was from Novartis Pharma AG; LF was from MP Biomedicals (Seven Hills, NSW, Australia); and CAS was purchased from Merck Research Laboratories (Rahway, NJ).

2.3. Minimum inhibitory concentration (MIC) testing

MIC testing was performed according to the CLSI protocol for drug testing in yeasts [17]. Following preliminary analyses, iron chelators were assayed using concentration ranges of 0.5–256 μg/mL for EDTA, DFP, DFO, DSX and LF and 0.03–32 μg/mL for CPO. Antifungals were tested from 0.015 to 16 μg/mL for AmB and CAS, 0.125–256 μg/mL for FLC and 0.007–16 μg/mL for ITC and VRC. Plates were incubated at 37 °C for 48 h for *Saccharomyces* and *Candida* strains and for 72 h for *Cryptococcus*. MICs for the fungicidal drugs AmB and CPO were read as the lowest drug concentration that completely inhibited growth; for the fungistatic azole drugs, these were read at 80% inhibition compared with the positive growth control. Based on preliminary MIC tests with EDTA and the study by Kobayashi et al for LF [18], EDTA and LF were read at 50% inhibition. All assays were performed in duplicate with each drug in a single experiment, and at least two independent biological replicates were performed on different days.

2.4. Synergy testing by chequerboard assay

Concentrations of antifungal drugs and iron-chelating agents were selected to encompass the MICs determined for each strain. Beginning at 4× MIC, serial two-fold dilutions of the drug and chelating agent were prepared in horizontal and vertical directions, respectively, in 96-well microtitre plates, with 50 μL of each drug and chelator aliquoted into the appropriate wells. Inoculations, incubation conditions, assay readings and back plating were performed according to the CLSI guidelines [17]. At least two independent assays were performed for each yeast strain. Inhibition was read visually, and cell density was assessed by a spectrometer for MacSynergy™II analysis [19].

2.5. Assessment of interactions by fractional inhibitory concentration index (FICI) and MacSynergy™II

The FICI was calculated as $FICI = FIC_A + FIC_B$, where FIC (fractional inhibitory concentration) = MIC of drug in combination/MIC drug alone. FICI values of ≤0.5 were defined as synergistic, >0.5–4 as indifferent and >4 as antagonistic [20].

MacSynergy™II uses the Bliss independence algorithm to calculate synergy, which is defined by the equation $Exy = Ex + Ey - (Ex Ey)$, where (Exy) is the additive effect of drugs x and y as predicted by their individual effects (Ex and Ey) [19]. MacSynergy™II is modelled in Microsoft Excel (Microsoft Corp., Redmond, WA) and generates a three-dimensional response curve of the synergy–antagonism landscape by representing the predicted indifferent effect