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Functional disruption of yeast metacaspase, Mca1, leads to miltefosine resistance and inability to mediate miltefosine-induced apoptotic effects

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ABSTRACT

Miltefosine (MI) is a novel, potential antifungal agent with activity against some yeast and filamentous fungal pathogens. We previously demonstrated in the model yeast, *Saccharomyces cerevisiae*, that MI causes disruption of mitochondrial membrane potential and apoptosis-like cell death via interaction with the Cox9p sub-unit of cytochrome c oxidase (COX). To identify additional mechanisms of antifungal action, MI resistance was induced in *S. cerevisiae* by exposure to the mutagen, ethyl methanesulfonate, and gene mutation(s) responsible for resistance were investigated. An MI-resistant haploid strain (H-C101) was created. Resistance was retained in the diploid strain (D-C101) following mating, confirming dominant inheritance. Phenotypic assessment of individual D-C101 tetrads revealed that only one mutant gene contributed to the MI-resistance phenotype. To identify this gene, the genome of H-C101 was sequenced and 17 mutated genes, including metacaspase-encoding *MCA1*, were identified. The *MCA1* mutation resulted in substitution of asparagine (N) with aspartic acid (D) at position 164 ($MCA1^{N164D}$). MI resistance was found to be primarily due to $MCA1^{N164D}$, as single-copy episomal expression of $MCA1^{N164D}$, but not two other mutated genes ($FAS1^{T1417I}$ and $BCK2^{T104A}$), resulted in MI resistance in the wild-type strain. Furthermore, an *MCA1* deletion mutant (*mca1Δ*) was MI-resistant. MI treatment led to accumulation of reactive oxygen species (ROS) in MI-resistant ($MCA1^{N164D}$-expressing and *mca1Δ*) strains and MI-susceptible (*MCA1*-expressing) strains, but failed to activate Mca1 in the MI-resistant strains, demonstrating that ROS accumulation does not contribute to the fungicidal effect of MI. In conclusion, functional disruption of Mca1, leads to MI resistance and inability to mediate MI-induced apoptotic effects. Mca1-mediated apoptosis is therefore a major mechanism of MI-induced antifungal action.

1. Introduction

Invasive fungal infections (IFIs) cause substantial morbidity and mortality, especially in critically ill and immunocompromised patients, and are costly to treat. Although most IFIs are caused by *Candida* and *Aspergillus* spp. (Richardson, 2005; Pfaller and Diekema, 2007, 2010), IFIs caused by uncommon pathogens including *Fusarium* and *Scedosporium* species, the dematiaceous moulds and the mucormycetes (previously zygomycetes) are becoming more prevalent (Husain et al., 2003; Pfaller and Diekema, 2004, 2010; Richardson, 2005). As most of these fungi are resistant or less susceptible to standard antifungal agents, treatment is problematic. The identification of new antifungal agents directed toward novel molecular targets is essential (Husain et al., 2003; Pfaller and Diekema, 2004, 2010; Richardson, 2005; Fluckiger et al., 2006).

Recently, miltefosine (MI), a drug licensed to treat leishmanial infections, was shown to exhibit *in vitro* antifungal activity against *Candida* and *Aspergillus* spp., *Scedosporium prolificans*, *Fusarium solani* and the dermatophytes (Widmer et al., 2006; Tong et al., 2007). Furthermore, the combination of MI with either posaconazole or voriconazole demonstrated synergy against *Fusarium* spp. and the mucormycetes (Biswas et al., 2013b).

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MI is an analogue of phosphatidylcholine (PC) and inserts into cell membranes, potentially disrupting function of membrane-associated proteins (Wieder et al., 1999; Zuo et al., 2011; Dorlo et al., 2012). Despite identification of numerous molecular targets of MI in neoplastic mammalian cells, *Leishmania* and *Trypanosoma*, the same is not true of fungi. In *Cryptococcus neoformans*, MI inhibits, although not as a primary target, the membrane-associated and secreted virulence determinant phospholipase B (PLB1) (Cox et al., 2001; Djordjevic et al., 2005; Widmer et al., 2006; Siafakas et al., 2007), which hydrolyses PC as its major substrate (Chen et al., 2000). In the model yeast, *Saccharomyces cerevisiae*, MI was rapidly incorporated into yeast cells (Zuo et al., 2011) and was effluxed from them via the hexose transporter *HXT13* (Biswas et al., 2013a). Cell-associated MI concentrates in the mitochondrial inner membrane, where it disrupts membrane potential via an interaction with the *COX9*-encoded subunit VIIa (Cox9p) of the COX complex of the electron transport chain. This MI-induced disruption of mitochondrial membrane potential leads to an apoptosis-like cell death as defined by DNA degradation, detected using the TUNEL assay (Zuo et al., 2011).

In mammalian cells, apotosis can be triggered by a series of caspases (Earnshaw et al., 1999) and many studies have now concluded that functional caspase homologues present in plants, protists and fungi, known as a metacaspases, can also trigger an apoptosis-like cell death (Madeo et al., 2002; Mazzoni and Falcone, 2008; Ramsdale, 2008). *S. cerevisiae*, *Candida albicans* and *Schizosaccharomyces pombe* contain a single metacaspase-encoding gene: *YCA1/MCA1* in *S. cerevisiae* and *C. albicans* and *PCA1* in *S. pombe* (Ramsdale, 2008), while *Aspergillus fumigatus*, *Aspergillus nidulans*, *Neurospora crassa* and *C. neoformans* possess two metacaspase-encoding genes (Ramsdale, 2008). Previous studies in *S. cerevisiae* have demonstrated that Mca1 triggers an apoptosis-like cell death in response to hydrogen peroxide and acetic acid stress (Madeo et al., 2002; Mazzoni and Falcone, 2008). However, as metacaspase-independent apoptotic mechanisms also function in yeast (Wissing et al., 2004; Liang et al., 2008), drug/MI-induced apoptosis may not necessarily be Mca1-dependent and thus requires further investigation.

A better understanding of the molecular targets of MI and/or its mechanism of antifungal activity is essential if MI is to be added to the antifungal armamentarium, or be used as a lead compound for the development of more effective antifungal agents. The aim of this study was to induce MI resistance in *S. cerevisiae* by chemical mutagenesis and identify the mutation(s) responsible for such resistance. Mutations would be expected to abolish MI interaction with novel molecular target(s) and/or disrupt pathway(s) downstream of known MI target(s). A mutant strain was created (H-C101) in which dominant resistance to MI was associated with functional disruption of the *MCA1*-encoding metacaspase, Mca1, and inability to undergo MI-induced apoptosis-like cell death. Our results further understanding of the mode of action of MI in yeast and provide insight into potential mechanisms of development of drug resistance in fungi.

2. Materials and methods

2.1. Strains, plasmids, media and growth conditions

Strains used in this study are listed in Table 1. Haploid wild-type (WT) strains of *S. cerevisiae*, M2915-6A (with adenine auxotrophy), BY4741 and BY4742 (Table 1) were used as "standards". The *S. cerevisiae MCA1* deletion mutant (*mca1*Δ) derived from strain BY4741 (YeastMATa Knock Out Strain YOR197W; CloneId:2453) was obtained from the Yeast Knockout Collection (Thermo Scientific, Millennium Science Pty. Ltd., VIC, Australia). Strains were maintained on Yeast Peptone Dextrose (YPD) [USB Affymetrix Inc., Cleveland, OH, USA] medium or Yeast Nitrogen Base (YNB) without amino acids and supplemented with synthetic drop-out medium lacking uracil (BD, Sydney, Australia). Sporulation agar was prepared using the following recipe, 1% potassium acetate (Sigma–Aldrich, MO, USA), 0.1% yeast extract (Sigma–Aldrich, MO, USA), 0.05% glucose, 1% uracil supplement (Sigma–Aldrich, MO, USA), 1% leucine supplement (Sigma–Aldrich, MO, USA) dissolved in 1 l of water.

The single-copy plasmid, p416, containing the constitutive glycerol-3-phosphate dehydrogenase (GPD) promoter and the uracil biosynthetic marker *URA3*, was obtained from American Type Culture Collection (ATCC; Manassas, VA). For transformation in bacteria, One Shot® OmniMAX™ 2 T1 Phage-Resistant (T1R) *Escherichia coli* competent cells (Life Technologies, Mulgrave, VIC, Australia) were used as host cells and were cultured in Luria broth (LB; Sigma–Aldrich, USA) or on agar plates containing 100 μg/ml ampicillin (Sigma–Aldrich).

2.2. Inducing miltefosine resistance with ethyl methanesulphonate

MI (Cayman Chemical Corp, Ann Arbor, MI) was prepared as a 50 mg/ml stock solution in water and stored at −80 °C. *S. cerevisiae* strain M2915-6A was grown overnight in 20 ml YPD broth at 30 °C. Cells were pelleted by centrifugation (13,000*g*), and washed twice with sterile water. The final pellet was resuspended in 4% ethyl methanesulphonate (EMS) (Sigma–Aldrich) in phosphate buffered saline (PBS) and incubated at 30 °C for 1 h. Cells were pelleted by centrifugation, washed twice with PBS, resuspended in YPD broth and incubated for 3 h at 30 °C to allow cell recovery. The cells were again pelleted and resuspended in sterile water, and 100 μl aliquots were spread onto YPD agar plates with and without 10 μg/ml of MI. Colonies that grew at 30 °C on YPD agar plates containing 10 μg/ml MI, a concentration 5–15 times greater than the MI MIC (2 μg/ml) of the WT strain (Zuo et al., 2011; Biswas et al., 2013a), were resistant and isolated for further screening. The selected colonies were named "C-*n*" where C stands for colony and *n* for the colony number.

2.3. Confirming miltefosine resistance using a drop dilution assay

A stable MI resistance phenotype in any haploid and diploid mutants, as well as in the *S. cerevisiae MCA1* deletion mutant (*mca1*Δ) was confirmed using the drop dilution assay. Strains were grown overnight in YPD or YNB (without uracil) broth and the cell density adjusted to 10^6 cells/ml with water. Fivefold serial dilutions (2×10^5 – 320 cells/ml) were prepared and 3 μl of each dilution was dropped onto YPD/YNB agar plates with and without 10 μg/ml of MI. Plates were incubated at 30 °C for 3 days.

2.4. Confirming dominant inheritance of miltefosine resistance

A mating assay was performed using strain M29156A and the MI-resistant mutants derived from it, and strain BY4742, and tetrad analysis was carried out on a resulting diploid strain (D-C101), which demonstrated inherited (dominant) resistance (Fig. 2). Three microlitres of a suspension of each mating partner (diluted to 1×10^6 cells/ml) were combined and dropped onto YNB agar containing leucine and uracil. A similar volume containing each haploid mating partner was dropped separately onto the same plate on either side of the mating drop. Plates were incubated for 3 days at 30 °C. Growth of diploid strains was indicative of successful mating, with the auxotrophic requirements of each mating partner being satisfied by the opposite parental haploid strain, and by supplementation of the media with leucine and uracil.