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Inhibition of electron transport chain assembly and function promotes photodynamic killing of *Candida*

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Abstract

Respiratory deficiency increases the sensitivity of the pathogenic fungi *Candida albicans* and *C. glabrata* to oxidative stress induced by photodynamic therapy (PDT) sensitized by the cationic porphyrin meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate (TMP-1363). Since disruption of electron transport chain (ETC) function increases intracellular levels of reactive oxygen species in yeast, we determined whether interference with ETC assembly or function increased sensitivity to TMP-1363-PDT in *C. albicans*, *C. glabrata* and the non-pathogenic yeast *Saccharomyces cerevisiae*. Metabolic inhibitor antimycin A and defined genetic mutants were used to identify ETC components that contribute to the sensitivity to PDT. Inhibition of cytochrome *bc₁* (Complex III) with antimycin A increases mitochondrial levels of reactive oxygen species. PDT performed following pretreatment with antimycin A reduced colony forming units (CFU) of *C. albicans* and *C. glabrata* by approximately two orders of magnitude relative to PDT alone. A *S. cerevisiae* mitochondrial glutaredoxin *grx5* mutant, defective in assembly of Fe-S clusters critical for Complex III function, displayed increased sensitivity to PDT. Furthermore, *C. glabrata* and *S. cerevisiae* mutants in cytochrome *c* oxidase (Complex IV) synthesis and assembly were also significantly more sensitive to PDT. These included *suv3*, encoding an ATP-dependent RNA helicase critical for maturation of cytochrome *c* oxidase subunit transcripts, and *pet117*, encoding an essential cytochrome *c* oxidase assembly factor. Following PDT, the reduction in CFU of these mutants was one to two orders of magnitude greater than in their respective parental strains. The data demonstrate that selective inhibition of ETC Complexes III and IV significantly increases the sensitivity of *C. albicans*, *C. glabrata* and *S. cerevisiae* to PDT sensitized with TMP-1363.

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Conflicts of interest
None to declare.

Keywords

photodynamic therapy; *Candida*; oxidative stress; respiration

1. Introduction

Candida species commonly colonize the epithelial surfaces of the body in the majority of the human population. However, few healthy carriers develop clinical signs of candidiasis until the body's physical and immunological defenses are compromised in some way. Patients with diseases such as cancer, HIV/AIDS, or diabetes, as well as premature infants and patients requiring intensive care, are among the groups at risk of developing infection from *Candida*. As a result of its widespread colonization of mucosal and cutaneous surfaces, the bulk of *Candida* infections are located at these sites [1].

While *C. albicans* accounts for nearly half of *Candida* infections, *C. glabrata* is the second most prevalent pathogenic *Candida* species [2]. Infections with non-*albicans* species such as *C. glabrata* often emerge after treatment for an initial *C. albicans* infection, or during prophylaxis for *C. albicans* infection, by virtue of their inherent resistance to commonly used azole antifungals [3]. For example, fluconazole-resistant *Candida* species colonize approximately 81% of AIDS patients receiving therapy for oral candidiasis [4]. In oral candidiasis, *C. glabrata* is seen as either a co-infecting agent with *C. albicans* or as the sole etiologic agent [5]. *C. glabrata* is also emerging as a prominent cause of vaginal infections [2]. Finally, catheters and prosthetic devices serve as substrates for *Candida* biofilms [6], providing a reservoir of drug-resistant cells that can initiate life-threatening disseminated infection [7]. These trends underscore the importance of developing novel strategies for treatment of *Candida* infection, as the microbiology and resistance patterns of clinical isolates evolve in response to selective pressures of current antifungal therapy.

The application of photodynamic therapy (PDT) to microbial infections, also referred to as photodynamic antimicrobial chemotherapy or PACT [8,9], is gaining attention as an alternative treatment against organisms resistant to conventional chemotherapy [10]. However, the mechanisms by which PDT exerts antimicrobial activity are just beginning to be examined. Mammalian cells require mitochondrial respiration for survival and normal growth. However, certain fungi, such the model eukaryotic yeast *Saccharomyces cerevisiae* [11] and its pathogenic relatives *C. albicans* [12] and *C. glabrata* [13], can grow without functional mitochondria if provided with a fermentable carbon source such as glucose. Hence, respiratory-deficient mutant yeasts are useful tools for the study of mitochondrial function. Respiratory-deficient *Candida* display pleiotropic resistance to a number of toxic stresses, including antifungal drugs [13-16].

In contrast, we recently made the striking observation that respiratory-deficient mutants of *Candida* [17], characterized by the deletion of major segments or total absence of mitochondrial DNA [18], are significantly more sensitive than their parental counterparts to oxidative stress induced by PDT mediated by the cationic photosensitizer meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate (TMP-1363). This indicates that there are inherent mechanisms in *Candida* linked to respiratory function that help the pathogen directly counter the oxidative stress and/or repair the damage induced by PDT. Exploiting our understanding of these mechanisms may lead to therapeutic strategies to increase the sensitivity of *Candida* to PDT.

Disruption of the respiratory pathway at selected points can increase intracellular levels of reactive oxygen species in yeast [19,20]. Using a metabolic inhibitor of respiration and defined genetic mutants deficient in respiratory pathway function, we determined whether interference

with electron transport chain assembly or function increased sensitivity to PDT in *C. albicans*, *C. glabrata* and the non-pathogenic yeast *S. cerevisiae*.

2. Materials and methods

2.1 Organisms and culture conditions

C. albicans, *C. glabrata* and *S. cerevisiae* strains used in this study are described in Table 1. Organisms were grown overnight at 30°C in yeast extract-peptone-dextrose (YPDextrose) broth (Difco, Detroit, MI). Organisms were washed twice with sterile dH₂O and adjusted to 10⁷ cells/ml in sterile dH₂O prior to PDT. We have used conventional yeast genetics nomenclature throughout the paper. For example, *PET117* refers to the wild type gene, *pet117* refers to a mutant in that allele and Pet117 refers to the protein encoded by *PET117*.

2.2 Recombinant DNA manipulations

Plasmid DNA was isolated using the QIAprep spin kit (QIAGEN, Valencia, CA). Restriction endonucleases, calf intestinal phosphatase, and T4 DNA ligase were used as specified by the supplier (New England Biolabs, Beverly, MA). DNA fragments were isolated from 0.7% Tris-borate-EDTA agarose gels using the Freeze and Squeeze Kit (Bio-Rad, Hercules, CA). PCR was performed using the PuReTaq Ready-To-Go PCR beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) in a MJ Mini Gradient Thermal Cycler (Bio-Rad). Oligonucleotide primers were obtained from Sigma Genosys (The Woodlands, TX). *Candida* transformation was performed using the Frozen EZ Yeast Transformation II kit (Zymo Research, Orange, CA).

2.3 Photodynamic treatment

For studies on the metabolic inhibition of the electron transport chain, *Candida* species were grown overnight in YPDextrose at 30°C with vigorous aeration, in either the presence or absence of the inhibitor of respiratory Complex III inhibitor antimycin A (10 µM AA) (Sigma-Aldrich, St. Louis, MO) [21]. Following this treatment, organisms were treated with 10 µg/ml of the cationic photosensitizer meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate (TMP-1363; Frontier Scientific, Logan, UT) for 10 min. The organism suspension (2 ml) was added to six well plates for the irradiation step and irradiated at 2.4 J/cm² with visible light from a 48 cm × 48 cm light box equipped with a bank of fluorescent lamps (Sylvania GRO-LUX, 15 W, part no. F15T8/GRO). The irradiance at the surface of the light box was 4.0 mW/cm², and the spectrum of the light, shown in Figure 1, was such that approximately 67% of the power was emitted within the range of 575–700 nm. The fluence of 2.4 J/cm² corresponded to a 10 min. irradiation time with this light source. Previous work [17] has shown that these parameters give a significant PDT response against parental strains but with enough range in the assay to observe enhanced sensitivity of the respiratory-deficient mutants. Untreated organisms and organisms treated with TMP-1363 but shielded from light were used as controls. For subsequent studies, organisms were incubated for 10 min. with different concentrations of TMP-1363 as indicated in the figures, and PDT was carried out as described.

2.4 Colony forming unit (CFU) phototoxicity assay

Following PDT, organism suspensions were diluted in dH₂O. For screening a range of TMP-1363 concentrations, dilutions of the different experimental groups were spotted (2 µl/spot) on YPDextrose plates and grown overnight at 30°C to assay phototoxicity. To assay phototoxicity quantitatively following PDT, organisms were diluted in water, 200 µl was plated on YPDextrose agar and plates were incubated 24–48 h at 30°C to allow colony formation. Data were expressed as CFU/ml and represented as a log₁₀ reduction compared to untreated organisms.

2.5 *C. glabrata* genomic DNA preparation

Organisms were grown overnight at 30°C in YPDextrose broth, cells from 3 ml of culture were washed twice with dH₂O and resuspended in 200 µl of breaking buffer (2% Triton X-100, 1% SDS, 0.1 M NaCl, 1 mM EDTA) containing 0.5 mm Zirconia/Silica beads (BioSpec Products, Bartlesville, OK), followed by the addition of 200 µL of phenol:chloroform:isopropanol (25:24:1) in 10 mM Tris (pH 7.6):1 mM EDTA. Organisms were vortexed for 1.5 min, and placed on ice for the same amount of time. This cycle was repeated 4 times. Following these steps, 200 µL of TE was added the suspension and the contents were centrifuged for 3 min in a microfuge. The DNA was then concentrated from the supernatant by precipitation, and resuspended in 100 µl water.

2.6 Construction of the *C. glabrata* PET117 disruption mutant

Genetic recombination with a homologous linear DNA fragment was used to generate an insertion mutant in *C. glabrata* PET117 [22]. Genomic DNA from *C. glabrata* BG2 [23] was used as a template for PCR to amplify fragments from the 5' and 3' ends of PET117 and its flanking regions of 399 bp and 424 bp, respectively. PCR primers with unique restriction sites were used to facilitate cloning into pTEF1/Zeo (Invitrogen Corp., Carlsbad CA) containing a Zeocin resistance (Zeo^R) cassette. The restriction sites are underlined in the primer sequences, and the respective restriction enzyme is listed after the primer sequence. The 5' fragment amplified using primers PET117 5'F (5'

ATGACAGATCTAACTGCAAGGACGTGAGCTTC 3'; *Bgl*III) and PET117 5'R (5' ATGACGCGGCCGCTCTTACTGGCTCGGCTCATTG 3'; *Not*I) was cloned into the 5' polylinker upstream of the Zeo^R cassette, and the 3' fragment amplified using primers PET117 3'F (5' ATGACGAATTCCTACTATGTGCAGGAGCTGGAGC 3'; *Eco*RI) and PET117 3'R (5' TACGGAAGCTTACGCCAATGATTCCATTCTC 3'; *Hind*III) was cloned into the 3' polylinker downstream of the Zeo^R cassette, resulting in the Zeo^R cassette flanked by the 5' and 3' PET117 fragments.

The entire insert was released by restriction digestion, and 100 µg was used to transform both *C. glabrata* BG2 and *C. glabrata* 66032 using the Frozen EZ Yeast Transformation II kit (Zymo Research, Orange, CA). Disruption of native PET117 was achieved by homologous recombination into the chromosome by the fragment containing the Zeo^R cassette. To allow expression of Zeocin resistance, organisms were incubated in YPDextrose broth for 24 h prior to plating in selective media containing Zeocin concentrations ranging from 200-400 µg/mL. Recombinant clones were further screened for inability to grow on YP + 2% glycerol (YPGlycerol) to confirm their respiratory-deficient status.

Disruption of PET117 by the Zeo^R cassette was confirmed by PCR using *C. glabrata*-specific primers flanking those employed to generate the recombinogenic fragment and primers internal to the Zeocin cassette. Primers PET117 A-F (5' AACTGCAAGGACGTGAGCTTC 3') and ZeocinR (5' TCAACACCGCCCCTTAGATT 3') generated an amplicon of approximately 500 bp from the region upstream of the Zeocin cassette; primers ZeocinF (5' GGTCGTGTCCACGAAGCTTCC 3') and PET117 B-R (5' ACGCCAATGATTCCATTCTC 3') generated an amplicon of approximately 900 bp from the region downstream of the Zeocin cassette. The amplicons were characterized by nucleotide sequencing, and specific disruption of PET117 was confirmed by comparison to sequences in the *C. glabrata* genome database (<http://cbi.labri.fr/Genolevures/cagl.php#>).

2.7 Spectrophotometric analysis of photosensitizer association with yeast

C. glabrata parental strain BG2 and ATP-dependent RNA helicase mutant *suvs3*, *C. glabrata* BG2 and cytochrome *c* oxidase assembly mutant *pet117*, and *S. cerevisiae* parental strain S288C and glutaredoxin mutant *grx5* were incubated with the same concentration of TMP-1363

(10 µg/mL) for approximately 30 minutes, washed with dH₂O to remove unbound photosensitizer and resuspended in dH₂O. Steady state fluorescence spectra from these samples were obtained using optimal TMP-1363 excitation wavelengths (near 425 nm) determined experimentally from excitation spectra. In a separate series of experiments, we performed fluorescence excitation and emission spectroscopy on these organisms in the absence of TMP-1363 incubation in order to evaluate a possible role for endogenous porphyrins in conferring enhanced sensitivity to PDT. Excitation and emission spectra were acquired using a Varian Cary Eclipse fluorometer (Varian Instruments, Walnut Creek, CA). Sample volumes were 3 mL and contained approximately $2-4 \times 10^8$ organisms/mL.

2.8 Statistical analysis

To obtain an adequate sample number for statistical analysis, each experimental condition was assayed in triplicate, and each experiment was performed at least three times. Data points for a given condition from each experiment were combined for subsequent data analysis and analyzed as the mean $\pm$ standard deviation. Comparisons of samples in the same group, and between-group comparisons, were made using the Student's *t* test. Comparisons among strains or treatment groups were made by one-way analysis of variance (ANOVA) to permit comparison of multiple groups or treatment conditions in the same analysis. In each case, *p* values ≤ 0.05 were considered significant.

3. Results

3.1 Metabolic inhibition of respiration enhances sensitivity of *Candida* to oxidative stress induced by PDT

C. glabrata possesses only the conventional, cyanide-sensitive cytochrome *c* respiratory pathway [13]. *C. albicans* also possesses the cyanide-insensitive alternative oxidase (AOX) respiratory pathway [24]. Antimycin A (AA) specifically inhibits the Rieske iron-sulfur (Fe-S) protein in the *bc₁* complex (Complex III) of the conventional respiratory pathway [25] by a mechanism that increases oxidative stress in yeast [26]. However, AA does not inhibit the AOX pathway of *C. albicans* [21]. To determine whether AA pre-treatment would increase the sensitivity of *C. albicans* and *C. glabrata* to PDT, organisms were grown overnight in either the presence or absence of AA, and subjected to PDT [17,27]. Pre-treatment with AA significantly enhanced ($p < 0.001$) the sensitivity of both *C. albicans* and *C. glabrata* to PDT compared to untreated organisms (Figure 2), resulting in an additional 2-log₁₀ reduction in CFU compared to cells grown in the absence of AA. AA treatment did not appear to exert toxicity to either species in organisms shielded from irradiation.

3.2 Specific respiratory-deficient mutants display increased sensitivity to PDT

The observations in Figure 2 and our previous studies [17] led us to investigate the role of specific gene products in conferring sensitivity to singlet oxygen-mediated stress induced by PDT. Using *S. cerevisiae* as a model, previous studies by Herrero and colleagues [28] compared gene expression in respiratory-deficient mutants *pet117* and *grx5* following oxidative stress, and observed *grx5* to be hypersensitive to oxidative stress. We used a *S. cerevisiae* non-essential gene, haploid deletion library [29] available to us (courtesy of Dr. Damian J. Krysan, University of Rochester, Rochester, NY) to compare *pet117* and *grx5* to parental strain S288C for their sensitivity to PDT (Figure 3). *Pet117* participates in the assembly of cytochrome *c* oxidase [30]. *Grx5* is a glutaredoxin found in the mitochondrial matrix, where it participates in Fe-S cluster formation in proteins, including cytochromes [31]. Both *S. cerevisiae* respiratory-deficient mutants *pet117* and *grx5* displayed significantly increased sensitivity to oxidative stress mediated by PDT ($p < 0.001$) compared to parental strain S288C. The *pet117* mutant was also significantly more sensitive to PDT ($p = 0.003$) compared to *grx5*. These data indicate that cytochrome assembly and maturation are likely important in determining sensitivity to PDT.

3.3 *C. glabrata* *pet117* insertion mutants are respiratory deficient and display increased sensitivity to PDT

To compare mutants in *S. cerevisiae* demonstrating increased sensitivity to PDT with mutants in orthologous genes in *C. glabrata*, we utilized the method of Alderton, *et al.* [22] for generating targeted mutants in *C. glabrata* by homologous recombination. Briefly, linear DNA is recombinogenic in yeasts such as *S. cerevisiae* and *C. glabrata*. Transformation of contiguous genomic DNA separated by a selectable marker will undergo homologous recombination at high frequency, and selection is achieved by plating on the appropriate medium. We initiated our studies by targeting *C. glabrata* *PET117*. To corroborate phenotypes observed in *C. glabrata* parental strain BG2 [23], we also transformed a second strain, *C. glabrata* 66032 [32] (American Type Culture Collection, Manassas, VA). Since we predicted *C. glabrata* *pet117* would be respiratory-deficient, we screened transformants by colony transfer to YPDextrose and YPGlycerol agar plates to identify clones that grew on YPDextrose but not YPGlycerol, since glycerol is a non-fermentable carbon source. We identified numerous Zeocin-resistant transformants in each strain background that were respiratory-deficient based on their inability to grow on YPGlycerol. Representative clones are shown in Figure 4A. In addition, we confirmed the genomic location of the Zeocin resistance cassette in the *PET117* locus by PCR amplification, nucleotide sequencing and BLAST analysis of the amplicons (data not shown). As seen in Figure 4B, *C. glabrata* *pet117* exhibited significantly enhanced sensitivity to PDT compared to parental strain BG2 ($p < 0.001$), with a reduction of more than 4-log_{10} CFU in the irradiated samples. In contrast, wild type BG2 had approximately 1.5- $\log_{10}$ reduction of CFU. There was no dark toxicity observed in the shielded samples of either the parental strain or *pet117*.

3.4 Selected pathways leading to respiratory deficiency in *C. glabrata* contribute to increased susceptibility to PDT

We proceeded to examine *Tn7* insertion mutants of *C. glabrata* with disruptions in genes known to contribute to mitochondrial function for increased sensitivity to PDT. The mutants have been described in detail [23] and were generously provided by Dr. Brendan P. Cormack (Johns Hopkins University, Baltimore, MD). We addressed the question of whether disruption of all pathways that led to respiratory deficiency resulted in increased sensitivity to PDT. Examination of a limited set of mitochondrial *Tn7* insertion mutant strains [23] showed that this was not the case, and illustrates that selected pathways contribute more significantly to sensitivity to PDT.

MRPL4 encodes a structural protein in the large subunit of the mitochondrial ribosome. The *mrpl4* mutant is respiratory competent and fluconazole sensitive. However, when *mrpl4* was challenged by plating on YPDextrose agar supplemented with fluconazole [23], clones were selected that were respiratory-deficient, based on an inability to grow using the non-fermentable substrate glycerol as a carbon source (*mrpl4* RD). *SHE9* encodes a mitochondrial inner membrane protein of unknown function. Similar to the *mrpl4* strains, the original *she9* mutant was respiratory-competent, but following challenge with fluconazole, respiratory-deficient clones were again isolated. It is unknown why the survivors of fluconazole treatment became respiratory-deficient. *SUV3* encodes an ATP-dependent mitochondrial RNA helicase [33,34]. In contrast to *mrpl4* and *she9*, the initial *suv3* mutant was respiratory deficient in *C. glabrata*. We compared the sensitivity of the mutant strains to PDT with a URA⁺ transformant that is used as a control strain for the *C. glabrata* mutants. The URA⁺ strain has equivalent sensitivity to PDT to the parental BG2 strain from which the mutants were derived (data not shown). Representative results are shown in Figure 5. PDT using 1.0 µg/ml TMP-1363 showed the greatest contrast among the strains. While the *she9* and *mrpl4* respiratory-deficient strains were somewhat more sensitive to PDT than their respiratory-competent counterparts, *suv3* was at least an order of magnitude more sensitive than the other RD mutants, and over 2 orders of

magnitude more sensitive than the URA⁺ strain. Taken together, the data in Figure 5 suggest not all mitochondrial mutations result in sensitivity to oxidative stress mediated by PDT, and some respiratory deficient mutants are more sensitive to PDT than others.

3.5 Hypersensitivity of respiratory-deficient mutants to PDT is not a result of increased accumulation of TMP-1363

We investigated the possibility that the hypersensitivity to PDT of the respiratory-deficient mutant strains resulted from differences in uptake of the photosensitizer by comparing three parental/respiratory-deficient mutant pairs. *C. glabrata* parental strain BG2 and ATP-dependent RNA helicase mutant *suv3*, *C. glabrata* BG2 and cytochrome *c* oxidase assembly mutant *pet117*, and *S. cerevisiae* parental strain S288C and glutaredoxin mutant *grx5* were incubated with the same concentration of TMP-1363 (10 µg/mL) for approximately 30 minutes, washed with dH₂O to remove unbound photosensitizer and resuspended in dH₂O. Steady state fluorescence spectra from these samples were obtained using optimal TMP-1363 excitation wavelengths (near 420 nm) determined experimentally from excitation spectra. Fluorescence characteristic of TMP-1363 was the overwhelmingly dominant feature in all emission spectra. Peak TMP-1363 emission from wild type strains was consistently higher than that from the mutants, as indicated in the representative pair of spectra for the *C. glabrata* BG2/*suv3* pair shown in Figure 6. In each case, TMP-1363 emission was approximately 2-fold to 8-fold higher in the respective parental strains vs. their mutant counterparts, thus enabling a clear rejection of the hypothesis that increased sensitizer accumulation contributed to the hypersensitivity to PDT of these specific mutant strains.

3.6 Hypersensitivity of respiratory-deficient mutants to PDT is not a result of increased accumulation of endogenous porphyrins

We also considered the possibility that mitochondrial mutants could accumulate abundant endogenous porphyrins as a result of defects in the heme synthesis pathway. Indeed, it has been suggested [35] that endogenous porphyrins contribute to photosensitivity of yeast. We investigated this possible mechanism in two ways. First, we performed steady state excitation and fluorescence emission spectroscopy on the 3 pairs of wild type and corresponding mutant organisms described above. No detectable emission was observed when cells were excited at 425 nm, the optimal wavelength for TMP-1363 emission spectra (Figure 6). Excitation spectroscopy revealed a peak near 500 nm. Using this excitation wavelength, we did not observe any fluorescence spectral features or amplitudes that distinguished the wild type *C. glabrata* cells from their mutant counterpart, as shown in Figure 7. Similar results were observed with the other 2 pairs of wild type and mutant organisms.

Further, the amplitudes of the endogenous fluorescence signatures were 50-100 fold lower than those of TMP-1363, making it extremely unlikely that these fluorophores contribute to the PDT response of wild type or mutant cells. Finally, the fluorescence spectra were not consistent with that of protoporphyrin IX or with any of the fluorescent precursors in the heme synthesis pathway. Second and more directly, we have performed light-only (no TMP-1363) control experiments in all of the PDT-sensitive respiratory-deficient mutant strains and have observed no phototoxicity, even at fluences 3-fold above those used in PDT experiments (data not shown).

4. Discussion

In the model yeast *S. cerevisiae*, mitochondrial function is required for innate resistance to oxidative stress caused by reactive oxygen species (ROS) produced during oxidative phosphorylation occurring in the mitochondrial inner membrane, independent of the adaptive response to oxidative stress [36]. Therefore, intact mitochondrial function is likely to provide

a basal level of innate anti-oxidant defense against PDT-induced phototoxicity in both *S. cerevisiae* and *Candida*. In an earlier study [17], we found that respiratory-deficient mutants of *C. albicans* and *C. glabrata* were significantly more susceptible to killing by PDT using TMP-1363 compared to their respective parental strains.

In the present report, we sought to identify specific pathways affecting mitochondrial function that contributed to this increase in sensitivity to PDT. While multiple pathways lead to impairment of respiratory function in fungi [37], our results suggest that selected, not all, pathways leading to respiratory deficiency contribute to increased susceptibility to oxidative stress induced by PDT. The pathways we identified that increase sensitivity to PDT involve the assembly and function of the electron transport chain (ETC). Based on extensive studies in *S. cerevisiae* (reviewed in [25,31,38]) and recent work on *Candida* [20], we propose that impairment of ETC function at selected sites adds to oxidative stress in the cell, thereby increasing its sensitivity to the added oxidative stress induced by PDT.

The increased sensitivity of *Candida* to PDT following pre-treatment with cytochrome *bc₁* (Complex III) inhibitor antimycin A supports this concept. During respiration, Complex III embedded in the mitochondrial inner membrane accepts two electrons from ubiquinol near the outer surface of the inner membrane. Superoxide anion is produced during each of the two electron transfers via the leakage of electrons to molecular oxygen present within the inner membrane. Binding of antimycin A to the cytochrome *b* subunit blocks electron flux by maintaining the *b* cytochromes in a reduced state. As a consequence, electron flow to the Rieske iron-sulfur protein is also blocked [38]. In *S. cerevisiae*, this results in a significant increase in both mitochondrial superoxide anion [26] and hydrogen peroxide production [39].

Antimycin A binds stoichiometrically with comparable affinity to cytochrome *bc₁* complexes from *S. cerevisiae* and bovine heart mitochondria [40]. The Complex III inhibitor ilicicolin H binds to the same (Qi) site on cytochrome *bc₁* as antimycin A, but less tightly to the bovine enzyme by nearly two orders of magnitude compared to the yeast enzyme [40]. This observation would indicate that structural differences between fungal and mammalian cytochrome *b* subunits could be exploited to advantage by increasing fungal sensitivity to PDT while reducing potential toxicity of the Complex III inhibitor to host tissue.

A second potential target within respiratory Complex III is illustrated by the increased sensitivity of *S. cerevisiae grx5* to PDT compared to its parental strain. We initially chose respiratory-deficient *S. cerevisiae grx5* for analysis because of its increased sensitivity to other oxidants [28]. Grx5 is located in the mitochondrial matrix where its primary biological role is participation in the late stages of Fe-S cluster incorporation into proteins, including the Rieske Fe-S protein of Complex III of the respiratory chain [41]. *S. cerevisiae grx5* displays inactive Fe-S enzymes and accumulates intracellular iron [31,41]. The excess iron can replace the manganese cofactor of *S. cerevisiae* mitochondrial superoxide dismutase Sod2, leading to enzyme inactivation [42]. Following PDT, the excess iron may also participate in the formation of additional reactive oxygen radicals via the Fenton reaction, thereby increasing damage to macromolecules [43]. This possibility is supported by the observation that constitutive carbonylation of proteins, an indicator of protein oxidation, is observed in *S. cerevisiae grx5* even without the application of external oxidative stress, suggesting Grx5 protects against oxidative damage of proteins [44]. Together, these events potentially lead to increased oxidative stress in *grx5* [28] that likely increases its sensitivity to PDT compared to its parental strain.

Initial studies in *S. cerevisiae* examining gene expression in *grx5* following oxidative stress [28] utilized respiratory-deficient mutant *pet117* [30] as a comparator to control for the effects of the petite [18] phenotype on the transcriptome. Pet117 is an assembly factor required for

cytochrome *c* oxidase (Complex IV) and, as indicated by the *pet* designation, is essential for respiratory function. Interestingly, when we compared the sensitivity to PDT of *S. cerevisiae* *pet117* and *grx5* (Figure 3), *pet117* was significantly more sensitive to PDT than both parental strain S288C and *grx5*, indicating Complex IV of the respiratory pathway is also important for sensitivity to PDT.

Given the close orthologous relationship of Pet117 in *S. cerevisiae* and *C. glabrata* (*Saccharomyces* Genome Database; www.yeastgenome.org), we generated a *pet117* insertion mutant in two different *C. glabrata* parental strains. In both backgrounds, *C. glabrata* *pet117* was more sensitive to PDT than its corresponding parental strain, suggesting the increased sensitivity of *pet117* was not strain specific. While respiratory chain components are highly conserved in structure, several assembly factors for Complex IV found in yeast are absent in mammals including Pet100, Cox14, Cox17, and Coa1 (*Saccharomyces* Genome Database; www.yeastgenome.org), and thus constitute potential therapeutic targets.

Taking advantage of a set of *C. glabrata* transposon insertion mutants related to mitochondrial function [23], we demonstrated that *suv3* displayed the highest degree of sensitivity to PDT. In *S. cerevisiae*, the ATP-dependent RNA helicase Suv3 and exonuclease Dss1 form a tight functional complex termed the “degradosome” [45] that participates in the processing of 3' ends of the eight multi-genic *C. glabrata* mitochondrial transcriptional units [46] to within two bases of a conserved dodecamer protected by a specific, phosphorylated binding protein [33]. Suv3 is also predicted to participate in degradation of three spliced introns [34] from the cytochrome *c* oxidase subunit 1 transcript in *C. glabrata* [46].

In yeast, a notable characteristic of the mitochondrial gene expression machinery is the close physical association of its components [47]. Suv3 and Dss1 demonstrate a tight functional interdependence in *S. cerevisiae*, and co-purify as a complex when expressed in recombinant form. Furthermore, Suv3 is not functional in the absence of Dss1, and Dss1 is only minimally active in the absence of Suv3 [45]. It is reasonable to suggest that the same physical interaction will be true for *C. glabrata*, since no other candidates for the mitochondrial degradosome have been identified in this pathogen. Since neither Suv3 nor Dss1 has a predicted transmembrane domain, it is also likely that these two proteins interact in the mitochondrial matrix at or near the site of translation, the mitochondrial ribosome, in both *S. cerevisiae* [48] and *C. glabrata*. A potential strategy to increase the sensitivity of *C. glabrata* to PDT is to disrupt the interaction between these two components of the mitochondrial “degradosome” to induce respiratory deficiency [48].

The tetra-cationic photosensitizer TMP-1363 has been highly effective in our previous *in vitro* studies of PDT against both *C. albicans* and *C. glabrata* [17,27]. TMP-1363 is also effective in antibacterial PDT *in vitro* in the presence of serum [8]. Preliminary studies indicate that efficacy of PDT against *Candida* is reduced using photosensitizers with fewer cationic substitutions compared to TMP-1363. Similar trends are observed in both parental and respiratory-deficient strains. At present, it is unknown whether respiratory deficiency enhances sensitivity to PDT in other pathogenic microorganisms. Our data suggest a possible two-step therapeutic strategy, where fungal respiration is first selectively inhibited, increasing susceptibility to oxidative stress induced by PDT and leading to organism death. Characterizing specific metabolic pathways and gene products that can be targeted to increase the sensitivity of *Candida* to PDT brings us closer to the identification of specific inhibitors that may be used to initiate such a two-step strategy. This may permit the use of lower PDT doses to eliminate fungal infection, while increasing the therapeutic index.

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Figure 1. Spectrum of visible radiation from the light box used for PDT

The inset is an expanded view of the region of the spectrum in the wavelength range of 575 - 700 nm that accounts for ~ 67% of the total power emitted from the light source.

Figure 2. Metabolic inhibition of respiratory Complex III by antimycin A increases the sensitivity of *C. albicans* and *C. glabrata* to PDT

C. albicans (panel a) and *C. glabrata* (panel b) were grown overnight to early stationary phase in YPDextrose at 37°C with vigorous aeration, in either the presence (closed bars) or absence (open bars) of the inhibitor of respiratory complex III inhibitor antimycin A (10 μM AA). Organisms were adjusted to 10^7 cells/mL, incubated with 10 $\mu\text{g/mL}$ of TMP-1363 for 10 min and irradiated at 2.4 J/cm² with broadband visible light. Untreated organisms and organisms treated with TMP-1363 but shielded from light were used as controls. Organism killing was determined by the colony forming unit (CFU) assay and represented as a log_{10} reduction compared to untreated organisms. Data represent the mean $\pm$ S.D. of three experiments

performed in duplicate. Pre-treatment with AA significantly (*) enhanced ($p<0.001$) the sensitivity of both *C. albicans* and *C. glabrata* to PDT compared to untreated organisms.

Figure 3. Genetic evidence that Pet117 and Grx5 participate in sensitivity to PDT in *S*
 Early stationary phase yeast of *S. cerevisiae* S288C, *pet117* and *grx5* were incubated with 10 μ g/ml TMP-1363 for 10 min and irradiated with a fluence of 2.4 J/cm² broadband visible light (open bars). Untreated organisms and organisms treated with TMP-1363 but shielded from light (closed bars) were used as controls. Organism killing was determined by the CFU assay and represented as a log₁₀ reduction compared to untreated organisms. Data represent the mean $\pm$ S.D. of three experiments performed in triplicate. Both *S. cerevisiae* respiratory-deficient mutants *pet117* and *grx5* displayed significantly (*) increased sensitivity to oxidative stress mediated by PDT ($p < 0.001$) compared to parental strain S288C. The *pet117* mutant was also significantly (#) more sensitive to PDT ($p = 0.003$) compared to *grx5*.

Figure 4. *C. glabrata* *pet117* insertion mutants are respiratory deficient and display increased sensitivity to PDT

(a) Representative clones in both the *C. glabrata* BG2 and *C. glabrata* 66032 background incubated at 30°C for 48 h are shown. The diagram on the right shows the organization of the isolates on the YPDextrose and YPGlycerol agar plates. Both transformants show the “petite” phenotype of smaller colony size and reduced growth on YPDextrose, and the putative *pet117* insertion mutants are unable to grow on YPGlycerol, confirming their respiratory deficiency (lower quadrants). The parental strains BG2 and 66032 grew well on both media (upper quadrants).

(b) Early stationary phase yeast of *C. glabrata* BG2 and *pet117* were treated with 0.5 µg/ml TMP-1363 for 10 min and irradiated at a fluence of 2.4 J/cm² with broadband visible light (open bars). Untreated organisms and organisms treated with TMP-1363 but shielded from light (closed bars) were used as controls. Sensitivity to PDT was assessed by the CFU assay. Data are expressed as a log₁₀ reduction in CFU compared to untreated organisms, and represent the mean + S.D. of three experiments performed in triplicate. *C. glabrata pet117* exhibited significantly (*) enhanced sensitivity to PDT compared to parental strain BG2 ($p<0.001$).

Figure 5. Selected pathways leading to respiratory deficiency in *C. glabrata* contribute to increased susceptibility to PDT

To better reveal differences in sensitivity to PDT among the strains, organisms were adjusted to 10⁷ cells/ml, incubated with a range of concentrations (0.5 – 2.0 µg/ml) of TMP-1363 for 10 min and irradiated with a fluence of 2.4 J/cm² broadband visible light (Irradiated). Organisms treated with TMP-1363 but shielded from light were used as controls (Shielded). Organism killing was determined by spotting 2 µl of ten-fold serial dilutions of treated cell suspensions on YPDextrose plates, followed by incubation at 37°C for 24 h. Representative results are shown. Abbreviations: RC (respiratory-competent); RD (respiratory-deficient).

Figure 6. Comparison of cell-associated photosensitizer levels in *C. glabrata* parental and respiratory-deficient strains

TMP-1363 steady state fluorescence from wild type *C. glabrata* BG2 (solid line) and respiratory-deficient mutant *suv3* (dashed line) strains. The excitation wavelength was 425 nm. The shapes of the TMP-1363 excitation spectra (not shown) from these two strains were indistinguishable, thus the differences in amplitude reflect significant differences in sensitizer concentration. In this case, the level of TMP-1363 associated with respiratory-deficient mutant *C. glabrata suv3* was less than its parent BG2.

Figure 7. Comparison of endogenous fluorescence levels in *C. glabrata* parental and respiratory-deficient mutant strains

Endogenous fluorescence from wild type *C. glabrata* BG2 (solid line) and respiratory-deficient mutant *suv3* (dashed line) was excited at 500 nm. The spectral shape and amplitude of the emission from these two strains were indistinguishable. The displayed amplitudes were scaled to illustrate the 50-100-fold lower emission intensities of the endogenous fluorescence relative to that of TMP-1363 (Fig. 6).

Table 1

Strains used in this study.

Name	Description	Reference
<i>C. albicans</i>		
SC5314	Parental	17
<i>C. glabrata</i>		
MRO-084-R	Parental	17
66032	Parental	32
BG2	Parental (RC ^a)	23
<i>Apet117</i>	Zeo ^R insertion mutant in BG2; cytochrome <i>c</i> oxidase assembly (RD ^b)	This study
BG462	URA+ transformant of <i>ura3</i> BG2 (RC)	23
BG663	<i>suv3::Tn7</i> ; ATP-dependent RNA helicase (RD)	23
BG769	<i>she9::Tn7</i> ; mt inner memb. protein (RC)	23
BG824	FLC ^{RC} derivative of <i>she9</i> (RD)	23
BG643	<i>mrpl4::Tn7</i> ; mt large ribosomal subunit protein (RC)	23
BG820	FLC ^R derivative of <i>mrpl4</i> (RD)	23
<i>S. cerevisiae</i>		
S288C	Parental (RC)	29
<i>Apet117</i>	S288C deletion mutant; cytochrome <i>c</i> oxidase assembly (RD)	29
<i>Agx5</i>	Deletion mutant; mt glutaredoxin (RD)	29

^aRC Respiratory competent.^bRD Respiratory deficient.^cFLC^R Selected on YPD plates supplemented with 256 µg/mL fluconazole. ²³