Ctt1 catalase activity potentiates antifungal azoles in the emerging opportunistic pathogen *Saccharomyces cerevisiae*

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Fungi respond to antifungal drugs by increasing their antioxidant stress response. How this impacts antifungal efficacy remains controversial and not well understood. Here we examine the role of catalase activity in the resistance of *Saccharomyces cerevisiae* to the common antifungals, fluconazole and miconazole, for which we report minimum inhibitory concentrations (MICs) of 104 and 19 μM, respectively. At sub-MIC concentrations, fluconazole and miconazole stimulate catalase activity 2-3-fold but, unexpectedly, deletion of cytosolic catalase (*ctt1*) makes cells more resistant to these azoles and to clotrimazole, itraconazole and posaconazole. On the other hand, upregulating Ctt1 activity by preconditioning with 0.2 mM H₂O₂ potentiates miconazole 32-fold and fluconazole 4-fold. Since H₂O₂ preconditioning does not alter the resistance of *ctt1Δ* cells, which possess negligible catalase activity, we link azole potentiation with Ctt1 upregulation. In contrast, *sod2Δ* cells deleted for mitochondrial superoxide dismutase are 4–8-fold more azole sensitive than wild-type cells, revealing that Sod2 activity protects cells against azole toxicity. In fact, the *ctt1Δ* mutant has double the Sod2 activity of wild-type cells so *ctt1* deletion increases azole resistance in part by Sod2 upregulation. Notably, deletion of peroxisomal/mitochondrial *cta1* or cytosolic *sod1* does not alter fluconazole or miconazole potency.

Antimicrobial challenge appears to induce the rewiring of microbial metabolic networks and stress-response pathways regardless of the primary drug-target interaction. Drug lethality increases when major stress responses are disrupted whereas resistance increases in fungi when antioxidant defenses are boosted. Susceptibility to antimicrobial killing decreases when cells are treated with antioxidants. Moreover, deletion of respiratory enzymes or inhibition of cellular respiration, a major source of reactive oxygen species (ROS), decreases antimicrobial lethality. Combined, these observations are consistent with the belief that cidal antibiotics and antifungals increase ROS levels. Hence, understanding the roles of ROS-metabolizing enzymes in antimicrobial efficacy is of critical importance in treating infection.

Several studies have examined the contribution of key ROS-metabolizing enzymes such as catalases and superoxide dismutases (Sods) to bacterial survival following challenge with antibiotics. For example, the ΔrelA ΔspoT mutant of *Pseudomonas aeruginosa*, which is deficient in the (p)ppGpp alarmone, exhibits depressed catalase and superoxide dismutase (Sod) activities and is hypersensitive to antibiotics. This can be reversed by overexpression in the mutant of KatA, the dominant *P. aeruginosa* catalase, or by restoration of Sod activity. Many other bacteria become more susceptible to antibiotics on Sod deletion, including *Enterococcus faecalis* and *Campylobacter jejuni*, *Acinetobacter baumannii*, *Staphylococcus aureus* and *E. coli* in stationary-phase but maybe not in exponentially growing cultures. Deletion of the catalase-peroxidase *katG* or the alkyl hydroperoxide reductase *ahpC* also potentiates some antibiotics in *E. coli*. Antioxidant enzymes also are associated with antifungal potency. For example, sirtuin Hst1 deletion increases catalase activity and lowers multidrug sensitivity in *Candida glabrata*. Deletion of membrane-associated CuSod4 and CuSod5 (Fig. 1) or inhibition of Cu-dependent Sod activity in *Candida albicans* increases the antifungal activity of miconazole and amphotericin B. Fluconazole induces a number of genes responsive to...

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oxidative- and nitrosative-stress in C. albicans and both fluconazole- and amphotericin B-resistant C. albicans and Candida dubliniensis exhibit increased catalase and Sod activities. Previously, it was shown that exposure to a fungistatic dose of miconazole induces catalase activity in both C. albicans and Saccharomyces cerevisiae. Notably, the catalase and superoxide dismutase isoforms present in these yeasts differ considerably (Fig. 1).

C. albicans possesses a single peroxisomal/mitochondrial catalase (Cta1) together with six Sods while S. cerevisiae produces cytosolic Ctt1 in addition to Cta1 but just two Sods, cytosolic CuZnSod1, which also localizes to the mitochondrial intermembrane space, and mitochondrial MnSod2 (for clarity, we indicate Sod metal dependence throughout the text). Thus, a comparison of how deletion of specific antioxidant enzymes alters antifungal potency in these well-characterized yeasts provides an excellent opportunity to gain new insights into pathogen survival strategies and the evolution of antifungal resilience.

In this work, we focus on the role of catalase activity in the response of S. cerevisiae (strain BY4741; Table 1) to challenge with common antifungal azoles. The primary target of these drugs is ergosterol biosynthesis, a sterol found in the cell membrane of fungi. Specifically, we report on the azole resistance of single ctt1 and cta1 knockouts (Table 1) as well as on wild-type cells preconditioned with a low dose of H₂O₂ to stimulate catalase activity. Furthermore, since MnSod2 is induced by the H₂O₂ stress response, we also monitored the Sod activity and azole sensitivity of sod1 and sod2 mutants (Table 1) with and without catalase inhibition. Combined, our unprecedented results shed new light on antioxidant defense and azole resistance in S. cerevisiae, which itself is an emerging opportunistic pathogen.

### Results

#### MICs of azoles for S. cerevisiae and their classification as fungicidal vs. fungistatic.

Starting at an initial cell density of 10⁶ cfu/ml and based on cell growth at different drug concentrations (Fig. 2), we determined the minimum inhibitory concentration (MIC µg/mL; µM) for our S. cerevisiae strain (BY4741) of six medically relevant azoles: itraconazole (32; 45), fluconazole (32; 105), posaconazole (32; 46), voriconazole (>256; >730), miconazole (8; 19) and clotrimazole (4; 12) (Table S1). The structures of the azoles, shown as a footnote to Table S1, reveal that the drugs examined can be classified as triazoles (itraconazole, fluconazole, posaconazole and voriconazole) and imidazoles (miconazole and clotrimazole). The imidazoles are more potent antifungals than the triazoles and, in fact, cells are refractory to voriconazole (Table S1). An azole is classified as fungicidal if 1xMIC or 2xMIC promotes a ≥10³-fold reduction in the viable cfu/mL and Table S2 shows that the imidazoles are fungicidal under the present experimental conditions, whereas the triazoles are fungistatic with the exception of voriconazole.

Cultures of C. albicans (strain SC5314) at the same initial cell density (10⁶ cfu/mL) exhibit MICs of >1 mM for fluconazole and 60 µM for miconazole. Thus, under our culture conditions, S. cerevisiae strain BY4741 is more...
increase in probe fluorescence, respectively, compared to untreated control cells (Fig. 3C,F). Hence, a high level of H$_2$O$_2$ accumulates over time in miconazole-treated wild-type cells, affirming that the fungicidal azole increases levels deplete catalase activity in *S. cerevisiae* and *C. albicans*.

Catalase activity weakly combats miconazole-induced H$_2$O$_2$ accumulation in *S. cerevisiae* cells. Miconazole induces a rise in intracellular H$_2$O$_2$ (Fig. 3C,F) despite also inducing catalase activity in wild-type cells. This led us to examine catalase activity and H$_2$O$_2$ levels in the *cta1Δ* and *ctt1Δ* strains, which lack peroxisomal/mitochondrial and cytosolic catalase, respectively (Fig. 1). Catalase activity (Fig. 3A,B) and H$_2$O$_2$ levels (Fig. 3C) are the same in wild-type and *cta1Δ* cells, which reflects the strong repression of Cta1 by glucose.

Deletion of *ctt1* or inhibition of catalase activity increasesazole resistance in *S. cerevisiae*. Peroxide-metabolizing enzymes have been associated with protection against cidal antimicrobials. However, our observation that Ctt1 does not inhibit miconazole-induced H$_2$O$_2$ accumulation (Fig. 3C,F) led us to ask whether Ctt1 actually protects cells against azole toxicity. As shown in Table 2 and Fig. S1, *ctt1Δ* cells display 4- and 8-fold higher MICs for fluconazole and miconazole, respectively, than the two strains with *ctt1* activity (Tables 2, S1). Given this surprising observation, we additionally determined the fold-change in MIC when *ctt1* was deleted for the four other azoles. Both wild-type and *ctt1Δ* cells are refractory to voriconazole (Table S1) but the *ctt1Δ* strain is 8-fold less sensitive to posaconazole and 2-fold less sensitive to clotrimazole and itraconazole than wild-type cells (Table S1). Thus, Ctt1 appears to potentiate both fungistatic and fungicidal azoles in *S. cerevisiae*.

Aminotriazole is a well-documented inhibitor of catalase activity in *S. cerevisiae*. Thus, to directly probe the effect of inhibition of catalase activity on miconazole resistance we added aminotriazole to the cells. This compound did not inhibit the growth of any strain at concentrations as high as 100 mM (data not shown) but
treatment with 25 mM lowers catalase activity to undetectable levels in wild-type cells (Fig. S5). In the presence of 25 mM aminotriazole, both wild-type and ctt1Δ cells have the same miconazole MIC (32 μg/mL; Table 2), which links miconazole potentiation in wild-type cells with Ctt1 catalase activity.

H2O2 preconditioning stimulates Ctt1 catalase activity and lowers the fluconazole and miconazole resistance of S. cerevisiae cells. Prompted by the link between miconazole potentiation and catalase activity, we questioned whether stimulating this activity before azole addition would further sensitize cells to the drug. As we previously reported34, preconditioning wild-type or cta1Δ cells with a low dose of H2O2 (e.g., 0.2 mM) in YPD medium doubles their Ctt1 activity (Fig. 3D,E vs. 3A,B; no azole). The combination of H2O2 preconditioning and 25 mM aminotriazole did not further sensitize cells to miconazole (32 μg/mL; Table 2). Table 2. Fluconazole and miconazole MICs for wild-type and mutant S. cerevisiae cells ± H2O2 preconditioning and ± aminotriazole. *Minimum inhibitory concentrations (MICs) for cultures diluted to an initial cell density of 10⁶ cfu/mL before challenge. Growth conditions are given in the legend to Fig. 2. Cultures were preconditioned with saline (0.85% wt/v aqueous NaCl) or 0.2 mM H2O2 for 30 min under the same conditions. Note that 1.0 μg/mL corresponds to 3.3 μM fluconazole (MW 306 Da) and 2.4 μM miconazole (MW 416 Da). The strains are described in Table 1. MICs were determined from plots of OD600 vs. [azole] shown in the figures listed in the parentheses. ND = not determined. 1Aminotriazole (ATZ) was present at 25 mM during the incubation with miconazole.
and azole challenge (0.5xMIC fluconazole or 0.05xMIC miconazole) increases Ctt1 activity by 4–6-fold above basal levels in wild-type and cta1Δ cells (Fig. 3D,E) and increases their azole sensitivity 4–32-fold (Table 2). In contrast, H2O2 preconditioning has little or no effect on the catalase activity (which remains barely detectable; Fig. 3) or azole sensitivity of the ctt1Δ strain (Table 2), affirming that azole potentiation is linked to increased Ctt1 activity and is not augmented by H2O2 exposure. Moreover, H2O2 preconditioning potentiates miconazole significantly more than fluconazole (Table 2) presumably because the fungicide is a more potent stimulator of Ctt1 activity (Fig. 3B,E vs. 3A,D).

Deletion of ctt1 elevates MnSod2 activity in early log phase and increases miconazole resistance. Although fungicide-dependent ROS production reportedly leads to fungal cell death55,56, we find no link here between elevated H2O2 levels and miconazole sensitivity. In fact, ctt1Δ cells, which are the most miconazole resistant (Table 2), accumulate more H2O2 on challenge with this azole (Fig. 3C,F). However, the miconazole resistance of C. albicans biofilms is dependent on the ROS-detoxifying activity of SodS55, and we57 and others58 have shown previously that suppressing or deleting catalase activity in S. cerevisiae upregulates mitochondrial MnSod2. Thus, we hypothesized that increased MnSod2 activity contributes to the enhanced azole resistance of ctt1Δ cells (Table 2). There are two Sod isoforms in S. cerevisiae (Fig. 1), and we find that the three strains exhibit similar total Sod activity, which doubles between 8 and 24 h but does not increase upon miconazole challenge (Fig. 4A,D). Since MnSod2 accounts for only 10–20% of the total Sod activity in cells growing on glucose52, to unmask any variation in this activity, we selectively inhibited CuZnSod1 with KCN53. This revealed 1.7-fold higher MnSod2 activity in untreated cta1Δ cells (Fig. 4B,E).

We next compared the relative levels of O2•− in the three strains. Staining cells with the fluorescent dye, DHE, which is preferentially oxidized by O2•−,54 we uncovered 2-fold less O2•− in the ctt1Δ strain relative to wild-type or cta1Δ cells (Fig. 4C). O2•− levels were a factor of ~1.3 higher in the cultures challenged with miconazole but remained significantly lower in ctt1Δ cells (Fig. 4C). The O2•− levels tripled between 8 and 24 h such that the 24-h miconazole-challenged cells contained >10-fold more O2•− than the untreated 8-h cells (Fig. 4F vs. 4C). Also, the 24-h cultures have comparable O2•− levels and MnSod2 activity (Fig. 4E,F) so azole resistance must be associated with the O2•− detoxifying activity of MnSod2 during exponential growth. Thus, we conclude that ctt1Δ cells are more azole resistant (Table 2) because they possess the higher MnSod2 activity in early log phase (Fig. 4B,C).
S. cerevisiae cells deleted for sod2Δ exhibit decreased fluconazole and miconazole resistance. To further explore the importance of Sod activity in azole resistance, we measured the fluconazole and miconazole MICs for sod1Δ and sod2Δ cells. MICs are the same for sod1Δ and wild-type cells, revealing that CuZnSod1 deletion does not impact miconazole resistance (Table 2), which is consistent with 1 mM TEMPO having no protective effect (Table 3). However, the sod2Δ strain possesses fluconazole and miconazole MICs that are 4- and 8-fold lower, respectively (Table 2). These results confirm that MnSod2 activity protects cells from azole toxicity and upregulation of MnSod2 activity in the ctt1Δ strain (Fig. 4B,E) increases its azole resistance (Table 2).

Inhibiting catalase activity in the sod2Δ strain enhances miconazole resistance less than in wild-type cells. If Ctt1 activity potentiates the azoles by suppressing MnSod2, then inhibiting catalase activity in the sod2Δ strain should not enhance resistance. Treatment of sod2Δ cells with 25 mM aminotriazole resulted in undetectable catalase activity as seen for wild-type cells (Fig. S5). The MIC for miconazole increased from 1 to 4 µg/mL vs. the increase to 32 µg/mL seen on aminotriazole treatment of wild-type cells (Table 2). Hence, Ctt1 activity potentiates miconazole in large part by depressing MnSod2 activity or in other words, the O2•− detoxifying activity of MnSod2 combatsazole toxicity and its deletion by Ctt1 activity lowers azole resistance in S. cerevisiae.

Discussion

Cytosolic Ctt1 catalase activity, not elevated intracellular H2O2, potentiates azole toxicity. De Nollin et al. found that fungistatic doses of miconazole stimulate catalase activity in S. cerevisiae48 and proposed that this rescues cells from H2O2 intoxication. We report here that sub–MIC concentrations of miconazole induce Ctt1 catalase activity up to 3-fold in our wild-type S. cerevisiae strain (BY4741) (Fig. 3) but this does prevent cells from accumulating ~4-fold more H2O2 over 24 h than untreated cells (Fig. 3F). Furthermore, ctt1Δ cells with negligible catalase activity, accumulate more miconazole-induced H2O2 than wild-type or cta1Δ cells (Fig. 3) but are more resistant to theazole (Table 2). Therefore, contrary to expectation14, our results reveal that azole-induced H2O2 production does not alter cell viability. In fact, cytosolic Ctt1 ineffectively combats H2O2 accumulation in wild-type and cta1Δ cells, and the increased azole sensitivity of H2O2-preconditioned wild-type cells results from Ctt1 upregulation and not exposure to exogenous H2O2. In sum, the azoles potentiate their own toxicity by induction of Ctt1 and not H2O2.

Cytosolic Ctt1 activity potentiates the azoles partly by depressing MnSod2 activity. Since ctt1Δ cells with the highest MnSod2 activity of the strains examined here (Fig. 4) are 4–8-fold more azole resistant than wild-type cells, we conclude that depression of MnSod2 activity on Ctt1 stimulation potentiates the azoles. MnSod2 is not essential for fermenting S. cerevisiae33 but sod2Δ cells exhibit 4–8-fold lower azole resistance than wild-type cells (Table 2). However, we note that H2O2, preconditioned wild-type cells are 4-fold more miconazole sensitive than sod2Δ cells (Table 2). Thus, strong Ctt1 induction may potentiate miconazole by additional mechanisms. For example, miconazole may bind to the heme of Ctt1 as reported for CYP5158, the 14α-demethylase in the ergosterol biosynthetic pathway38. This could promote heme-catalyzed azole autoxidation with the formation of reactive, cytotoxic species via mechanisms analogous to those we reported for hydrazides59.

Elevated MnSod2 activity in early log phase increases azole resistance. The ctt1Δ cells from 8-h cultures possess higher MnSod2 activity and less O2•− than wild-type and cta1Δ cells (Fig. 4). Respiration is a major source of O2•−, and induction of respiration by miconazole reportedly increases its toxicity in S. cerevisiae whereas genetic blockage of respiration (by deleting TCA-cycle and ETC components) has the opposite effect2. Respiration-derived O2•− inactivates aconitase60,61 with the release of free iron, which catalyzes the production of highly toxic hydroxyl radicals via Fenton chemistry62,63.

We have previously reported on the positive biochemical and physiological effects of elevated MnSod2 activity in young cells deleted for cytochrome c peroxidase (ccp1Δ)64. Like ctt1Δ cells, the ccp1Δ mutant exhibits low O2•− and high H2O2 levels plus it possesses stable aconitase activity, accumulates low amounts of free iron and hydroxyl radicals, amasses mitochondrial damage more slowly and lives longer than wild-type cells46. These traits arise from the beneficial mitochondrial H2O2 stress response known as mitohormesis, which requires MnSod2 upregulation17,36,37,64. Presumably, the advantages of elevated MnSod2 activity in early log phase contribute to the increased miconazole resistance of ctt1Δ cells. At 24 h after miconazole treatment, the three strains possess comparable MnSod2 activity and O2•− levels (Fig. 4). Nonetheless, based on Rhod123 staining65, miconazole does not increase respiration in ctt1Δ cells (Fig. S6), suggesting that mitohormesis protects mitochondrial function65.

Catalase and azole resistance in S. cerevisiae vs. C. albicans and C. glabrata. Given their different catalase and Sod isozymes (Fig. 1), it is informative to compare azole sensitivity in S. cerevisiae and C. albicans. It was reported in the 1970s that fungistatic doses of miconazole induce catalase activity in S. cerevisiae and C. albicans whereas fungicidal doses inhibit this activity47. We confirm these results for S. cerevisiae but show that only Ctt1 activity is induced (Fig. 3B) since peroxisomal/mitochondrial Cta1 is repressed by glucose66. Cta1 is the

| Miconazole | Miconazole + 1 mM TEMPO | Miconazole + 1 mM mito-TEMPO |
|------------|------------------------|-------------------------------|
| 8 (6)      | 8 (6)                  | 16 (4)                       |

Table 3. Miconazole MIC for fermenting wild-type S. cerevisiae cells ± O2•− scavengers. aSee Footnotes a-c to Table 2. The number of independent observations of a given MIC is in brackets in red font.
only catalase isoform in C. albicans29 (Fig. 1), and synergistic killing of C. albicans biofilms by fluconazole and H$_2$O$_2$ has been reported but no molecular mechanism was suggested29.

Like S. cerevisiae, the opportunistic yeast C. glabrata possesses Cta1, cytosolic CuZnSod1 and mitochondrial MnSod2. Antifungals also induce ROS production and stimulate catalase, Sod and glutathione peroxidase activities in C. glabrata34,45. Azole resistance is associated with increased catalase activity2 and increased protein levels of thiol peroxidases44, but whether deletion of these antioxidant enzymes alters azole resistance in C. glabrata remains to be seen.

Conclusions

Although high catalase activity has been linked to azole resistance in C. albicans, C. glabrata and S. cerevisiae, the present study reveals that azole-induced upregulation of Ctt1 activity potentiates azole toxicity by depressing MnSod2 activity in S. cerevisiae. Hence, MnSod2 is an interesting antifungal target in this yeast but target antioxidant enzymes are likely to be species dependent. Therefore, to expand our knowledge of the role of a given antioxidant activity in fungal survival strategies, we need to establish the potency of antifungal drugs in yeasts singly deleted for the antioxidant enzyme of interest as performed here for S. cerevisiae.

Materials and Methods

Reagents. Suppliers of chemicals/biochemical were as follows: Peptone, yeast extract, microbiological agar, phenylmethylsulfonyl (PMSF), tetramethylethylenediamine (TEMED), tris(hydroxymethyl)aminomethane (Tris, electrophoresis grade 99%), glycine and sodium chloride (Biohop); glucose and 30% H$_2$O$_2$ (v/v) (Fisher Scientific); buffer salts, 3-amino-1,2,4-triazole (aminotriazole or ATZ), 2,2,6,6-tetramethyl-1-piperidinylxoyl (TEMPO*) and 2-(2,2,6,6-tetramethyl-1-piperidinylxoyl-4-ylamino)-2-oxoetyl triphenylphosphonium chloride (Mito-TEMPO*) (Sigma); fluconazole and miconazole nitrate (Santa Cruz Biotechnology); itraconazole, clotrimazole, posaconazole and voriconazole (Cayman Chemicals). The Bradford reagent and other electrophoresis reagents were obtained from Biorad.

Yeast strains. The Saccharomyces cerevisiae wild-type and mutant BY4741 strains used in this work are listed in Table 1. The wild-type strain was purchased from the EUROSCARF. The ctt1Δ, ctt1Δ, sod1Δ and sod2Δ mutant strains are derived from the Yeast Deletion Project46,47 and were kindly provided by Professor Christopher Brett (Department of Biology, Concordia University).

Growth conditions and H$_2$O$_2$ preconditioning. Precultures (10 mL) were obtained by growing single colonies of each strain in YPD (1% yeast extract, 2% peptone and 2% dextrose) for 24 h at 30 °C with high aeration (medium-to-flask ratio of 1:5 and shaking at 225 rpm). These cultures were used to inoculate 25 mL of fresh YPD (OD$_{600}$ 0.50; 12 h) and preconditioned with 0.2 mM H$_2$O$_2$ for 30 min at 30 °C/225 rpm where indicated. Cells (3 mL) were grown under the same conditions to mid-log phase (OD$_{600}$ 0.15; 106 cfu/mL) in fresh YPD before MIC determination. Our initial cell density is higher than suggested by suppliers of chemicals/biochemical.

Determination of azole minimum inhibitory concentration (MIC). The solid azoles were dissolved in 100% ethanol to give stocks of 50 mg/mL fluconazole, 10 mg/mL voriconazole, 1 mg/mL miconazole and clotrimazole; and in 100% dimethyl sulfoxide (DMSO) to give stocks of 1 mg/mL posaconazole and itraconazole. Since H$_2$O$_2$ preconditioning causes a 25–30% reduction in viable ctt1Δ cells34, the liquid cultures were diluted to OD$_{600}$ 0.15 (10$^6$ cfu/mL) in fresh YPD before MIC determination. Our initial cell density is higher than suggested by the Clinical and Laboratory Standards Institute (10$^5$ cfu/mL)48 to provide sufficient cells for the biochemical analyses. Cells were exposed to different azole concentrations in 96-well plates (final volume of 200 μL per well) and MICs were determined as described44. Briefly, cells were mixed with the drug and OD$_{600}$ was measured on a SpectraFluor Plus Tecan plate reader at t$=0$ and t = 24 h after growth at 30 °C without shaking. The MIC for each azole was determined from a plot of OD$_{600}$ at t = 24 h minus that at t = 0 vs. [azole]. The MIC is the lowest antifungal concentration that results in no detectable growth after 24 h incubation44. MICs for cultures simultaneously treated with the azole and 1 mM TEMPO*; 1 mM mito-TEMPO* (Sod mimetics)55,56 or 25 mM aminotriazole (catalase inhibitor)52 were determined in the same way in 96-well plates. To establish if an azole was fungicidal or fungistatic, wells containing 1xMIC and 2xMIC of the drug were serially diluted 10x after 24 h at 30 °C, plated onto YPD agar and grown for 2 days at 30 °C to measure the viable cfu/mL. A drug was considered fungicidal if 1xMIC or 2xMIC promoted a ≥10$^3$-fold reduction in viable cfu44.

Soluble protein extracts. Cells were grown in YPD at 30 °C/225 rpm for 24 h, OD$_{600}$ values were measured, and soluble proteins were extracted as described previously13,24,25. Briefly, after centrifugation at 2000 × g, cells were washed 2x with 100 mM potassium phosphate buffer at pH 7.0 (KPi) containing 0.1 mM PMSF, the pellets were diluted into KPi/PMSF, and mixed with an equal volume of acid-washed glass beads (400–600μm). Cells were disrupted by vortexing 4 × 15 s, the homogenates were spun at 13000 × g for 10 min at 4°C, and the total protein concentration in the supernatants was determined by the Bradford assay with BSA as a standard71.

Catalase and Sod activity assays. Cells exposed to azole concentrations below the MIC (sub-MIC) were used in the biochemical analyses to avoid the general metabolic collapse and down regulation of multiple enzyme activities seen at lethal drug concentrations13,24. To assay for catalase activity, 25–150 μL aliquots of soluble protein extract were diluted to contain 20–100 μg protein were added to 1.0 mL of 20 mM H$_2$O$_2$ in 50 mM KPi in a cuvette. H$_2$O$_2$ decomposition was monitored at 240 nm (ε$_{240}$ = 43.6 M$^{-1}$ cm$^{-1}$)72. One unit of catalase activity catalyzes the degradation of 1 μmol of H$_2$O$_2$ per min44,72. Sod activity was assayed using the Superoxide Dismutase Detection Kit (Cell Technologies, C80100), where O$_2^-$ is generated by xanthine/xanthine oxidase and oxidized by XTT.
(2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) –. One unit of Sod activity inhibits the rate of XTT reduction by O$_2^·$ by 50% and was assayed according to the manufacturer's instructions using 2–10 µg of total protein in 96-well plates. To determine MnSod2 activity only, lysates at 2 mg/mL total soluble protein were preincubated with 5 mM KCN for 30 min at room temperature to fully inhibit Cu/ZnSod1 prior to assaying 5–50 µg of total protein for sod 2 activity.

**Relative ROS levels.** Relative levels were estimated as we described before using the fluorescent probes, dihydrorhodamine 123 (DHR) for H$_2$O$_2$ and dihydroethidium (DHE) for O$_2^·$. Cultures were diluted to OD$_{600}$ 0.15 in 3 mL of fresh YPD in a 15-mL Falcon tube, vehicle (ethanol) or 0.05xMIC (0.4 µg/mL or ~1 µm) miconazole was added, cells were incubated at 30°C/225 rpm for 8 or 24 h, harvested at 2000 × g for 10 min, washed once and resuspended in PBS (10 mM NaPi and 150 mM NaCl, pH 7.0) to a final density of 10$^7$ cells/mL. One mL of suspension was stained with 5µM DHR or 5µM DHE at 30°C for 120 and 60 min, respectively, the cells were pelleted, diluted to 10$^6$ cells/mL in PBS, fixed with 2% formalin (v/v) and analyzed by flow cytometry (BD Accuri C6, BD Biosciences). The fluorescence from individual cells was measured and expressed as relative fluorescence units (RFU). Relative H$_2$O$_2$ and O$_2^·$ levels are estimated from the median RFU of 10,000 cells for each sample.

**Statistical analyses.** These were performed using the two-tailed Student's t-test calculated using Graph Pad Prism 7 software. The analyses compare each sample with the wild-type untreated control (see figure legends). Probabilities < 5% are considered significant (p < 0.05).

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**Author Contributions**
D.M. and A.M.E. designed research; D.M. performed the experimental work. D.N. contributed new reagents and analytical tools; D.M. and A.M.E. analyzed the data; and D.M., A.M.E., and D.N. wrote the paper.

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