Targeting the oxidative stress response system of fungi with redox-potent chemosensitizing agents

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The cellular antioxidant system is a target in the antifungal action of amphotericin B (AMB) and itraconazole (ITZ), in filamentous fungi. The *sakA*Δ mutant of *Aspergillus fumigatus*, a mitogen-activated protein kinase (MAPK) gene deletion mutant in the antioxidant system, was found to be more sensitive to AMB or ITZ than other *A. fumigatus* strains, a wild type and a *mpkC*Δ mutant (a MAPK gene deletion mutant in the polyalcohol sugar utilization system). Complete fungal kill ($\geq$99.9%) by ITZ or AMB was also achieved by much lower dosages for the *sakA*Δ mutant than for the other strains. It appears *msnA*, an *Aspergillus* ortholog to *Saccharomyces cerevisiae MSN2* (encoding a stress-responsive C_2H_2-type zinc-finger regulator) and *sakA* and/or *mpkC* (upstream MAPKs) are in the same stress response network under *tert*-butyl hydroperoxide (*t*-BuOOH)-, hydrogen peroxide (H_2O_2)- or AMB-triggered toxicity. Of note is that ITZ-sensitive yeast pathogens were also sensitive to *t*-BuOOH, showing a connection between ITZ sensitivity and antioxidant capacity of fungi. Enhanced antifungal activity of AMB or ITZ was achieved when these drugs were co-applied with redox-potent natural compounds, 2,3-dihydroxybenzaldehyde, thymol or salicylaldehyde, as chemosensitizing agents. We concluded that redox-potent compounds, which target the antioxidant system in fungi, possess a chemosensitizing capacity to enhance efficacy of conventional drugs.

Keywords: amphotericin B, itraconazole, natural compounds, chemosensitization, *Candida*, *Cryptococcus*, *Aspergillus*, oxidative stress response

INTRODUCTION

Recent studies have shown that one of the antimicrobial modes of action of certain drugs involves cellular oxidative stress response in pathogens, which further contributes to the death of microorganisms. Thus, these types of drugs could be defined as oxidative stress drugs.

Examples include amphotericin B (AMB). Although AMB is known as a fungicidal drug by causing ion leakage, studies have shown that forming channels in the cellular membrane was not the sole mechanism of AMB activity (Palacios et al., 2007). Instead, oxidative stress triggered by AMB could be one of the contributing mechanisms for AMB fungicidality. For instance, addition of antioxidants, such as reduced glutathione (GSH), cysteine, etc., could revive endospores of *Coccidioides immitis* treated with AMB (Graybill et al., 1997 and references therein), indicating the involvement of cellular oxidative stress in AMB activity. Results showed that superoxide radical-mediated oxidative damage was involved in AMB activity (Okamoto et al., 2004). Other studies further support involvement of cellular oxidative stress as a component of the antifungal mode of action of AMB (Sokol-Anderson et al., 1986, 1988; Blum et al., 2008; An et al., 2009; González-Párraga et al., 2011).

Itraconazole (ITZ) is another example of an oxidative stress drug. The main mechanism of action of ITZ is similar to other azole agents by inhibiting fungal cytochrome P450 oxidase-mediated biosynthesis of ergosterol, ultimately inhibiting fungal growth. However, a recent study with the Ddr48 protein of *Candida albicans* indicated the oxidative stress response of this pathogen was also triggered by ITZ treatment (Dib et al., 2008). The *C. albicans* Ddr48 protein is essential for fungal filamentation, stress response, and also confers partial resistance to antifungal drug(s). The *DDR48/ddr48* heterozygote mutant strain was susceptible to ITZ in a concentration-dependent manner (Dib et al., 2008). Noteworthy is that this mutant also showed hypersensitivity to hydrogen peroxide (H_2O_2), a strong oxidant, which indicated there was a relationship between ITZ susceptibility and H_2O_2 hypersensitivity (Dib et al., 2008). Thus, it appears that the cellular antioxidant system in yeasts is involved in tolerance to AMB or ITZ.

Stress-signaling/response genes of fungal pathogens are known to play roles in virulence, pathogenesis and defense against oxidative burst (rapid production of reactive oxygen species, ROS) from the host (Washburn et al., 1987; Hamilton and Holdom, 1999; Clemons et al., 2002; de Dios et al., 2010). In fungi, stress signals resulting from oxidative stress are integrated into the upstream mitogen-activated protein kinase (MAPK) pathways, which ultimately regulate the downstream response genes detoxifying the stress (Miskei et al., 2009). In yeasts, such as *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*, the HOG MAPK system plays a key role in countering oxidative stress (Toone and Jones, 1998; Lee et al., 2002; Miskei et al., 2009). SakA and MpkC in *Aspergillus fumigatus* are orthologous proteins to Hog1p of *S. cerevisiae*

(Xue et al., 2004; Reyes et al., 2006). The $sakA\Delta$ ($sakA$ gene deletion) is an osmotic/oxidative stress sensitive mutant, while the $mpkC\Delta$ ($mpkC$ gene deletion) is a mutant of the polyalcohol sugar utilization system (Xue et al., 2004; Reyes et al., 2006).

The $A.$ $flavus$ $msnA$ is an orthologous gene of $S.$ $cerevisiae$ $MSN2$ that encodes a C_2H_2-type zinc-finger regulator, Msn2p. Msn2p is required for yeast cells to cope with a broad range of environmental and physiological stresses (Ruis and Schuller, 1995). Maximum induction of Msn2p-dependent genes, such as $CTT1$ (encoding a catalase), under osmotic/oxidative stress required Hog1p (O'Rourke et al., 2002; see Miskei et al., 2009 for review). We surmised MsnA in $Aspergillus$ would also functionally interact with MAPKs such as SakA and/or MpkC. Recently, an $A.$ $flavus$ $CA14msnA\Delta$ mutant was generated (and also examined in this study). Deletion of the $A.$ $flavus$ $msnA$ gene adversely affected the fungus, as manifested by (1) increased expression of oxidative stress defense genes in $Aspergillus$, and (2) increased levels of ROS in $msnA\Delta$ mutant comparing to the parental strain (Chang et al., 2011).

Thus, it is quite evident that the fungal antioxidant system could serve as an effective antifungal target of redox-potent agents. Such agents could disrupt cellular redox homeostasis in fungi and serve as a means for controlling fungal pathogens (see also Smits and Brul, 2005; Jaeger and Flohe, 2006).

Redox-potent natural phenolics, such as benzaldehyde analogs, or sulfur-containing compounds can be potent redox-cyclers in microorganisms and inhibit microbial growth by interfering with cellular redox homeostasis and/or the function of redox-sensitive components (Guillen and Evans, 1994; Jacob, 2006). We reasoned that redox-potent natural compounds, which destabilize the fungal antioxidant system, could act as potent chemosensitizing agents when co-applied with oxidative stress drugs, such as AMB or ITZ. Redox-potent chemosensitizers and drugs can affect common cellular targets, i.e., the antioxidant system of fungi, which results in synergistic inhibition of fungal growth. Thus, chemosensitization could make the use of toxic antifungal drugs or fungicides more attractive as an antifungal therapeutic strategy (see also Ogita et al., 2006).

In this in $vitro$ study, we attempted to develop a chemosensitization strategy for control of fungal pathogens. We focused on targeting the oxidative stress response system of fungi with redox-potent chemosensitizing agents. Research emphasis was on: (1) identification of the level of sensitivities of $Aspergillus$ MAPK or $msnA$ gene deletion mutants to oxidizing agents, conventional oxidative stress drugs, i.e., AMB and ITZ, or redox-potent phenolic compounds, (2) chemosensitization of antifungal drugs with redox-potent phenolic compounds in $Aspergillus$ and yeast pathogens ($Candida$, $Cryptococcus$), and (3) identification of complex III of mitochondrial respiratory chain (MRC) as an alternative oxidative stress target for control of yeast pathogens.

MATERIALS AND METHODS

FUNGAL STRAINS AND CULTURE CONDITIONS

$Aspergillus$ $fumigatus$ AF293, wild type, and $A.$ $fumigatus$ MAPK gene deletion mutants ($sakA\Delta$ and $mpkC\Delta$) were grown at 35°C on potato dextrose agar (PDA) or Sabouraud dextrose agar (SDA; Sigma, St. Louis, MO, USA). $A.$ $terreus$ UAB673, UAB680, and UAB698 (clinical isolates) were procured from Centers for Disease Control and Prevention, Atlanta, GA, USA, and were grown at 35°C on PDA or SDA. $A.$ $flavus$ NRRL3357, procured from the National Center for Agricultural Utilization Research, USDA-ARS, Peoria, IL, USA, was grown at 35°C on PDA or SDA. Also, $A.$ $flavus$ CA14 (parental strain) and CA14$msnA\Delta$ (knockout mutant for $msnA$ gene; Chang et al., 2011) strains were grown at 28°C on PDA.

$C.$ $albicans$ 90028 and $C.$ $krusei$ 6258 (reference strains) were procured from American Type Culture Collection (Manassas, VA, USA). $C.$ $albicans$ CAN276, $C.$ $krusei$ CAN75, $C.$ $tropicalis$ CAN286 and $Cryptococcus$ $neoformans$ CN24 (clinical isolates) were procured from $Instituto$ de $Higiene$ e $Medicina$ $Tropical/CREM,$ $Universidade$ $Nova$ de $Lisboa$, Portugal. $S.$ $cerevisiae$ wild type BY4741 (Mat a $his3\Delta1$ $leu2\Delta0$ $met15\Delta0$ $ura3\Delta0$) and selected single gene deletion mutants, i.e., cytosolic superoxide dismutase (SOD; Cu, Zn-SOD; $sod1\Delta$) mutant, mitochondrial SOD (Mn-SOD; $sod2\Delta$) mutant, antioxidative transcription factor mutant ($yap1\Delta$), glutathione reductase mutant ($glr1\Delta$), vacuolar H^+-ATPase (V-ATPase) assembly mutant ($vph2\Delta$) and V-ATPase subunit A mutant ($vma1\Delta$), were procured from Open Biosystems [Huntsville, AL, USA; see $Saccharomyces$ Genome Database (www.yeastgenome.org; accessed February 2, 2012)]. Yeast strains were cultured on synthetic glucose (SG; Yeast nitrogen base without amino acids 0.67%, glucose 2% with appropriate supplements: uracil 0.02 mg mL^{-1}, amino acids 0.03 mg mL^{-1}) agar, yeast peptone dextrose (YPD; Bacto yeast extract 1%, Bacto peptone 2%, glucose 2%) agar or SDA at 30°C for $S.$ $cerevisiae$ or 35°C for yeast pathogens ($Candida$, $Cryptococcus$), respectively.

CHEMICALS

Antifungal chemosensitizing agents [2,3-dihydroxybenzaldehyde (2,3-DHBA), salicylaldehyde (SA), thymol (THY)], antifungal drugs [antimycin A (AntA), amphotericin B (AMB), itraconazole (ITZ)], strobilurins [pyraclostrobin (PCS), kresoxim methyl (Kre-Me)], and oxidizing agents [$tert$-butyl hydroperoxide (t-BuOOH), hydrogen peroxide (H_2O_2; Sigma product No. H1009, contained stabilizer)] were procured from Sigma Co. Hydrogen peroxide stock was prepared based on molar concentration provided by the manufacturer. Each compound was dissolved in dimethyl sulfoxide (DMSO; absolute DMSO amount: <1% in media), except oxidizing agents, which were dissolved in water, before incorporation into culture media. In all tests, control plates (i.e., "No treatment") contained DMSO at levels equivalent to that of cohorts receiving antifungal agents, within the same set of experiments.

ANTIFUNGAL BIOASSAY

Agar plate bioassay: filamentous fungi

In the plate bioassay, measurement of sensitivities of filamentous fungi to the antifungal agents was based on percent (%) radial growth of treated compared to control ("No treatment") fungal colonies (see text for test concentrations; Vincent, 1947). Minimum inhibitory concentration (MIC) values on agar plates were determined based on triplicate (except duplicate for $A.$ $flavus$ CA14 and CA14$msnA\Delta$ mutant) assays, and defined as the lowest concentration of agents where no fungal growth was visible on the plate. For the above assays, fungal conidia (5×10^4 CFU mL^{-1}) were diluted in phosphate-buffered saline (PBS) and applied as

a drop onto the center of PDA or SDA plates with or without antifungal compounds. Growth was observed for 3–7 days, except *A. flavus* CA14*msnA*Δ mutant (and *A. flavus* CA14 as a control), which needed around 3 weeks for optimal growth to determine cellular responses to drugs/compounds.

Agar plate bioassay: yeasts

Petri plate-based yeast dilution bioassays were performed with the *S. cerevisiae* wild type and mutant [antioxidant (*sod1*Δ, *sod2*Δ, *glr1*Δ, *yap1*Δ), vacuolar (*vph2*Δ, *vma1*Δ)] strains to assess the effects of THY on the antioxidant or vacuolar system of fungi. Yeast strains were exposed to 0.2–1.4 mM of THY. This assay was performed in duplicate on SG agar following previously described protocols (Kim et al., 2008a). *S. cerevisiae* strains were grown at 30°C for 3–7 days.

Petri plate-based yeast dilution bioassays were also performed with the yeast pathogens (*Candida* and *Cryptococcus*) to investigate (1) the level of sensitivity of pathogens to ITZ or *t*-BuOOH (see text for concentrations), (2) sensitivities of pathogens to the inhibitors of complex III of MRC (100 μg mL^{-1}), i.e., AntA, Kre-Me, and PCS (see Chemicals above), and (3) the chemosensitizing activity of 2,3-DHBA to PCS in yeast pathogens. For the chemosensitization test, yeast pathogens were exposed to 100 μg mL^{-1} of MRC inhibitors without or with 0.1–0.4 mM of 2,3-DHBA. Yeast pathogens were grown on SG agar (in duplicate as described above for *S. cerevisiae*) at 35°C for 3–7 days.

Microtiter plate (microdilution) bioassay: filamentous fungi

To determine antifungal MICs and/or chemosensitizing activities of natural compounds (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 mM) to antifungal drugs (AMB or ITZ; 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 μg mL^{-1}) in filamentous fungi, triplicate checkerboard bioassays (0.4 × 10^4 to 5 × 10^4 CFU mL^{-1}) were performed in microtiter wells using a broth microdilution, according to modified methods outlined by the Clinical Laboratory Standards Institute (CLSI) M38-A (CLSI, 2008). MICs for chemosensitization were defined as the concentrations where no fungal growth was visible at 48 and 72 h.

Microtiter plate (microdilution) bioassay: yeasts

Chemosensitizing activities of natural compounds (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 mM) to antifungal drugs (AMB or ITZ; 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 μg mL^{-1}) were determined by using checkerboard bioassays in microtiter plates (with RPMI 1640 medium; Sigma Co.). To determine changes in MICs of antifungal agents (i.e., drugs and chemosensitizers) in microtiter wells, triplicate assays (0.5 × 10^5 to 2.5 × 10^5 CFU mL^{-1}) were performed using broth microdilution protocols according to modified methods outlined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; Cuenca-Estrella et al., 2003). MICs for chemosensitization were defined as the concentrations where no fungal growth was visible at 24 and 48 h.

DETERMINATION OF CHEMOSENSITIZING ACTIVITY OF SA TO ITZ

Determination of chemosensitizing activity of SA, a volatile benzaldehyde analog, to ITZ was performed in segmented Petri dishes (150 mm × 10 mm; VWR International Co., Radnor, PA, USA).

These dishes are divided into four, isolated sections, and each of two sections was supplied with 6 mL of SDA for fungal growth (without or with 1–7 μg mL^{-1} of ITZ in SDA). Then, conidial suspensions of *A. fumigatus* AF293 (5 × 10^4 CFU mL^{-1}) were diluted in PBS and applied as a drop on the center of each section, providing two fungal inocula within each plate; see also **Figure 3**.).

SA was dissolved in DMSO, and was applied onto a round Whatman™paper (2.5 cm in diameter; GE Healthcare Bio-science Co., Piscataway, NJ, USA; Final volume: Total 150 μL of SA plus DMSO on a Whatman™paper per each plate based on SA concentration tested). Each SA preparation was then placed onto an empty section located between the fungal inocula. The fungal spores inside the Petri plate would be exposed to the volatilized SA. The SA-wet (or DMSO-wet control) Whatman™paper was placed onto a piece of Parafilm® (American National Can Co., Chicago, IL, USA) to avoid the direct contact of SA plus DMSO with the surface of a Petri dish. The plates containing SA (or DMSO control) and fungal inocula were then sealed with two layers of Parafilm® to prevent escape of SA. The plates were incubated at 35°C (5–7 days). The antifungal treatments, therefore, consist of (1) control plates: *A. fumigatus* AF293 inocula without SA vapor (DMSO only) and (2) treated plates: *A. fumigatus* AF293 inocula with SA vapor (9.5–95 mM on a Whatman™paper). Results were based on two replicated plates.
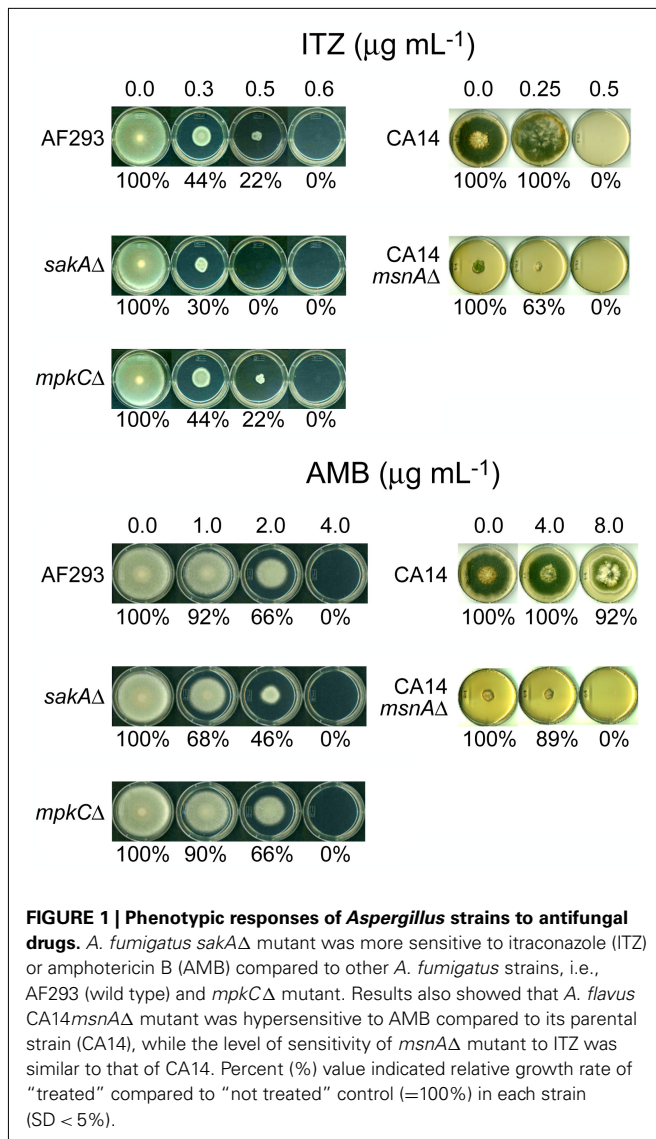
RESULTS

ASPERGILLUS MAPK AND *msnA* MUTANTS WERE SENSITIVE TO OXIDATIVE STRESS

We initially investigated the phenotypic responses (i.e., level of sensitivity) of three *Aspergillus* mutant strains, i.e., deletion mutants for *A. fumigatus* MAPKs, *sakA* and *mpkC*, and *A. flavus msnA* genes, to *t*-BuOOH (an organic peroxide) and H$_2$O$_2$ (hydrogen peroxide). Sensitivities of fungi to the oxidizing agents were determined based on relative fungal radial growth on agar plates. The *sakA*Δ, *mpkC*Δ and *msnA*Δ mutants currently available were derived from two different species of infectious *Aspergillus*. However, we reasoned that results presented in this study would provide the basis for detecting signaling network(s) in *Aspergillus*, in general, under the stress/toxicity triggered by oxidizing agents and/or antifungal drugs. To our knowledge, the *A. flavus msnA* is the first *MSN2* gene ortholog functionally characterized (with gene knockout) in *Aspergillus*.

In our tests, all three *Aspergillus* mutants, i.e., *A. fumigatus sakA*Δ and *mpkC*Δ, *A. flavus* CA14*msnA*Δ, showed marginally higher sensitivity to *t*-BuOOH and H$_2$O$_2$ compared to the wild type strains (Data not shown). Based on these results, we postulated that *msnA* (downstream regulator) and *sakA*/*mpkC* (upstream MAPKs) are located in the same stress response network in *Aspergillus* under the peroxide-mediated oxidative stress.

ANTIOXIDANT MUTANTS WERE SENSITIVE TO TOXICITIES OF AMB AND ITZ

Next, we examined the level of sensitivity of *Aspergillus* mutants to ITZ or AMB in plate bioassays. We tried to determine if the function of the fungal antioxidant system is responsive to toxicities triggered by ITZ or AMB. As shown in **Figure 1**, the *A. fumigatus*

FIGURE 1 | Phenotypic responses of *Aspergillus* strains to antifungal drugs. *A. fumigatus sakA*Δ mutant was more sensitive to itraconazole (ITZ) or amphotericin B (AMB) compared to other *A. fumigatus* strains, i.e., AF293 (wild type) and *mpkC*Δ mutant. Results also showed that *A. flavus* CA14*msnA*Δ mutant was hypersensitive to AMB compared to its parental strain (CA14), while the level of sensitivity of *msnA*Δ mutant to ITZ was similar to that of CA14. Percent (%) value indicated relative growth rate of "treated" compared to "not treated" control (=100%) in each strain (SD < 5%).

Table 1 | Levels of fungicidality of itraconazole (ITZ) or amphotericin B (AMB) at various concentrations against *Aspergillus fumigatus* strains (AF293, *sakA*Δ, *mpkC*Δ).

	Strains		
	AF293	*sakA*Δ	*mpkC*Δ
ITZ Conc. (μg mL^{-1})			
1	ND[1]	99.45%	ND
2	ND	99.85%	99.65%
4	ND	99.89%	99.72%
8	ND	**99.92%**	99.76%
16	99.39%	**99.94%**	99.83%
32	99.42%	**99.95%**	99.83%
64	**99.93%**	99.99%	**99.99%**
AMB Conc. (μg mL^{-1})			
1	ND	ND	ND
2	ND	ND	ND
4	ND	**99.95%**	ND
8	ND	**100.00%**	ND
16	99.31%	**100.00%**	99.45%
32	99.43%	**100.00%**	99.68%
64	99.88%	**100.00%**	**99.94%**
128	**99.95%**	**100.00%**	**99.98%**

ND, not determined (<99.00%).
Columns showing over 99.90% of fungicidality are indicated in bold.

*sakA*Δ mutant showed higher sensitivity to ITZ or AMB compared to AF293 or the *mpkC*Δ mutant. The *A. fumigatus sakA*Δ mutant did not grow at 0.5 μg mL^{-1} of ITZ (i.e., sensitive), while AF293 and the *mpkC*Δ mutant did grow (**Figure 1**). Also, the *A. fumigatus sakA*Δ mutant was more sensitive to AMB compared to the AF293 or *mpkC*Δ strains. For example, when treated with AMB, the inhibition rate of fungal growth for the *sakA*Δ mutant was 32–54% (i.e., more sensitive; SD < 5%) at 1.0–2.0 μg mL^{-1} of AMB, while inhibition of AF293 or the *mpkC*Δ mutant was around 8–34% (SD < 5%; **Figure 1**).

To evaluate the negative impact of the deletion of MAPK genes on cell survivability after AMB or ITZ treatment, we also monitored the minimum fungicidal concentrations (MFCs) of AMB or ITZ for *A. fumigatus* AF293, *sakA*Δ and *mpkC*Δ strains in a separate microdilution (microtiter plate) bioassay. Fungi were treated with 1–128 or 1–64 μg mL^{-1} of AMB or ITZ, respectively. In this test, over 99.9% of fungicidality was achieved in the *A. fumigatus sakA*Δ mutant at dosage levels of ITZ or AMB lower than

that for AF293 or the *mpkC*Δ mutant (**Table 1**). Altogether, we concluded that the antifungal activity of AMB or ITZ evoked a cellular oxidative stress response requiring participation of MAPK pathway genes in *A. fumigatus*. However, SakA must play a more significant role in response to AMB or ITZ than MpkC, as represented by the higher sensitivity of *sakA*Δ than *mpkC*Δ to either drug. A similar type of hypersensitivity of the *sakA*Δ mutant was previously observed when it was treated with redox-potent phenolic reagents, where the *mpkC*Δ mutant showed less sensitivity to the same treatment (Kim et al., 2010).

Sensitivity of the *A. flavus* CA14*msnA*Δ mutant to AMB was also examined using plate bioassays. As shown in **Figure 1**, the MIC of the *A. flavus* CA14*msnA*Δ mutant for AMB was between 4.0 and 8.0 μg mL^{-1}, while that of the parental strain, *A. flavus* CA14, was above 8.0 μg mL^{-1} (the highest concentration of AMB tested). However, unlike its response to AMB, there was no difference in level of sensitivity of the *A. flavus* CA14*msnA*Δ mutant and the parental strain to ITZ (i.e., MICs for both strains were between 0.25 and 0.5 μg mL^{-1}; although, some discoloration of the parental strain occurred with ITZ treatment; **Figure 1**). One plausible explanation for this differential response to two different drugs would be that a regulator(s) other than MsnA is involved in fungal response/tolerance to ITZ.

Collectively, the experimental results from the *A. fumigatus* and *A. flavus* strains studied indicate that antifungal activity of ITZ or AMB involves the functioning/response of fungal antioxidant system. Among the molecular genetic components examined in these *Aspergillus* strains, it appears that SakA plays a more significant role for fungal tolerance/response to both ITZ- and AMB-induced toxicity than do either MpkC or MsnA.

A. FLAVUS msnA MUTANT WAS SENSITIVE TO REDOX-POTENT PHENOLIC COMPOUNDS

In prior studies, the *A. fumigatus sakA*Δ mutant showed hypersensitivity to 2,3-DHBA or THY (Kim et al., 2008a,b). These compounds were effective as chemosensitizing agents to conventional antifungal drugs/fungicides (Kim et al., 2008a,b).

In the present study, the *A. flavus* CA14*msnA*Δ mutant was also more sensitive to 2,3-DHBA or THY compared to the parental strain in plate bioassays (Figure data not shown). For example, the MICs for 2,3-DHBA and THY were between 0.8 and 1.6 mM or 3.2 and 6.4 mM, respectively, in the parental strain, while those of the *msnA*Δ mutant were between 0.0 and 0.1 mM or 0.2 and 0.4 mM, respectively. Therefore, including results with the *t*-BuOOH and H_2O_2 experiments (see above), these findings with 2,3-DHBA and THY further provide evidence of antioxidative cellular components (i.e., MAPKs and MsnA) playing a role in fungal response to drugs (ITZ, AMB) or redox-potent compounds (2,3-DHBA, THY) [see **Table 2** for the summary of responses of *Aspergillus* MAPKs (*sakA*Δ, *mpkC*Δ) and *msnA*Δ mutants to the test compounds].

REDOX-POTENT PHENOLIC COMPOUNDS ACT AS CHEMOSENSITIZERS TO ANTIFUNGAL DRUGS IN FILAMENTOUS FUNGI

We next investigated the potential role of redox-potent compounds (2,3-DHBA, THY) as chemosensitizing agents to oxidative stress drugs (AMB, ITZ) in different fungal pathogens. SA was also included in these tests. We reasoned there should be either an additive or synergistic antifungal interaction if the drug and compounds were co-applied, lowering dosages for control of fungi than sole treatment of each drug. At this stage of study, we tried to identify the most effective chemosensitization strategy with various combinations of redox-potent compounds and antifungal drugs.

Chemosensitization of ITZ with THY

First, we performed a yeast dilution bioassay on SG agar containing THY (0.2–1.4 mM), using a number of different *S. cerevisiae* gene deletion mutants (see Fungal Strains and Culture Conditions). As shown in **Figure 2**, vacuolar [*vph2*Δ, *vma1*Δ; "growth" at no dilution (10^0) and 10^1 dilution spots only] and antioxidant (*sod2*Δ, *sod1*Δ, *glr1*Δ; "growth" at no dilution, 10^1 and/or 10^2 dilution spots only) mutants exhibited higher sensitivity to THY (0.8 mM) compared to the wild type ("growth" from no dilution to 10^4 dilution). These results indicated that, like carvacrol (a structural isomer of THY; Rao et al., 2010), THY disrupted cellular

ion and also redox homeostasis in fungi. The vacuolar mutants appeared to be more sensitive to THY compared to the antioxidant mutants. The *yap1*Δ, an antioxidative transcription factor mutant, was sensitive to THY but less so than the other antioxidant mutants examined.

Next, we examined the chemosensitizing efficacy of THY to ITZ in five different *Aspergillus* strains using microdilution bioassays. As shown in **Table 3**, co-application of THY and ITZ enhanced the antifungal efficacy of either compounds. Namely, MICs of THY and ITZ were lowered under co-application compared to independent treatment of each compound, alone, in most fungi. Fungal response to chemosensitization (i.e., THY plus ITZ) was strain dependent, where *A. fumigatus* AF293 was the most sensitive/responsive strain (i.e., both MIC and MFC were lowered), while *A. flavus* 3357 was the least affected by chemosensitization [i.e., no effect on MIC or MFC (data not shown)].

MsnA as an effective target of chemosensitization: AMB with 2,3-DHBA

First, chemosensitization of fungi by 2,3-DHBA to AMB (see Microtiter Plate (Microdilution) Bioassay: Filamentous Fungi for concentrations) was investigated in five *Aspergillus* strains using microdilution bioassays. All the compound interactions between 2,3-DHBA and AMB were additive ($0.6 \leq FICI \leq 1.0$), depending on *Aspergillus* strain (Data not shown). However, no
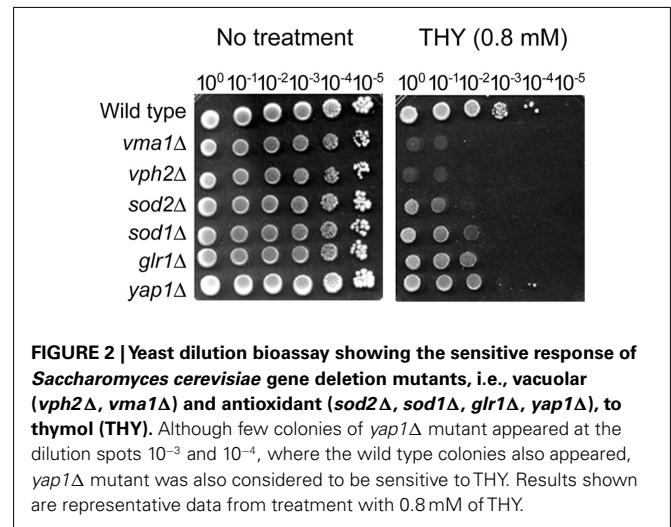

FIGURE 2 | Yeast dilution bioassay showing the sensitive response of *Saccharomyces cerevisiae* gene deletion mutants, i.e., vacuolar (*vph2*Δ, *vma1*Δ) and antioxidant (*sod2*Δ, *sod1*Δ, *glr1*Δ, *yap1*Δ), to thymol (THY). Although few colonies of *yap1*Δ mutant appeared at the dilution spots 10^{-3} and 10^{-4}, where the wild type colonies also appeared, *yap1*Δ mutant was also considered to be sensitive to THY. Results shown are representative data from treatment with 0.8 mM of THY.

Table 2 | Summary of sensitivities of *Aspergillus* MAPKs (*sakA*Δ, *mpkC*Δ) and *msnA*Δ mutants to the test compounds.[1]

Strains	Compounds					
	t-BuOOH	H_2O_2	AMB	ITZ	2,3-DHBA	THY
*A. flavus msnA*Δ	S	S	S	N	S	S
*A. fumigatus sakA*Δ	S	S	S	S	S[2]	S[2]
*A. fumigatus mpkC*Δ	S	S	N	N	N[2]	N[2]

[1] S, sensitive; N, not sensitive (compared to the parental strains).

[2] Determined in previous studies (see Kim et al., 2008a,b).

Table 3 | Chemosensitization of itraconazole (ITZ; μg mL^{-1}) with thymol (THY; mM) tested against pathogenic *Aspergillus*.

Strains	Compounds	MIC alone	MIC combined	FICI[1]
A. fumigatus AF293	THY, ITZ	0.4–0.8, 2–4	0.2–0.4, 1–2	1.0
A. flavus NRRL3357	THY, ITZ	0.8–1.6, 0.5–1	0.8–1.6, 0.5–1	2.0
A. terreus UAB673	THY, ITZ	1.6–3.2, 0.5–1	0.8–1.6, 0.25–0.5	1.0
A. terreus UAB680	THY, ITZ	1.6–3.2, 0.5–1	0.8–1.6, 0.25–0.5	1.0
A. terreus UAB698	THY, ITZ	0.8–1.6, 0.5–1	0.4–0.8, 0.25–0.5	1.0
Strain	**Compounds**	**MFC alone**	**MFC combined**	**FFCI[2]**
A. fumigatus AF293	THY, ITZ	1.6–3.2, >16[3]	0.8–1.6, 0.5–1	0.5

[1]*Levels of compound interactions (i.e., changes in MICs) between antifungal drug and chemosensitizer were based on Fractional Inhibitory Concentration Indices (FICI; Isenberg, 1992), where FICI = (MIC of compound A in combination with compound B/MIC of compound A, alone) + (MIC of compound B in combination with compound A/MIC of compound B, alone). Compound interactions were: synergistic (FICI ≤ 0.5), additive (0.5 < FICI ≤ 1), neutral (1 < FICI ≤ 2), or antagonistic (FICI > 2). If preferred, the Odds' (2003) methodology can be substituted in parallel calculations of "synergism," where FICI values ≤0.5 indicate "synergy" and values >0.5–4 indicate "indifference."*

[2]*To calculate Fractional Fungicidal Concentration Indices (FFCI), minimum fungicidal concentrations (MFCs) were used. To obtain MFCs, the entire volume of each microtiter well (200 μL), after determination of MICs, was spread onto individual YPD or SDA plates, and cultured for an additional 48 and 72 h. MFC was defined as the lowest concentration of agent where ≥99.9% fungal death was achieved. Compound interactions were: synergistic (FFCI ≤ 0.5), additive (0.5 < FFCI ≤ 1), neutral (1 < FFCI ≤ 2) or antagonistic (FFCI > 2; Isenberg, 1992). If preferred, the Odds' (2003) methodology can be substituted in parallel calculations of "synergism," where FFCI values ≤0.5 indicate "synergy" and values >0.5–4 indicate "indifference."*

[3]*For calculation (FFCI) purpose, 32 μg mL^{-1} (doubling of 16 μg mL^{-1}) was used.*

chemosensitization was detected for lowering MFCs in all fungi tested (data not shown), indicating the efficacy of chemosensitization by using 2,3-DHBA plus AMB was at the level of lowering MICs of each agent, only.

Meanwhile, in separate plate bioassays, we exposed *A. flavus* CA14 (parental strain) and the *A. flavus* CA14*msnA*Δ mutant to 2,3-DHBA plus AMB. Co-application of AMB (2 μg mL^{-1}) and 2,3-DHBA at 20 μM (which is a much lower concentration compared to that used in the microdilution bioassay above) completely inhibited the growth of the *A. flavus* CA14*msnA*Δ mutant, while independent treatment of each compound, at these concentrations alone, allowed full (with 2,3-DHBA) to slightly sensitive (with AMB) growth of this mutant (Data not shown). However, almost no chemosensitization was achieved in the parental strain under the same condition (Data not shown). Thus, it appears that *msnA* could be an effective antifungal target of redox-potent drugs/compounds, where disruption of its function enhanced the antifungal interaction between 2,3-DHBA and AMB.

Chemosensitization of ITZ with SA

We examined the chemosensitizing efficacy of SA, a volatile benzaldehyde analog, to ITZ in *A. fumigatus* AF293 in plate bioassays. We reasoned that the volatile characteristic of SA would facilitate the development of targeted delivery of this compound to the infection site, such as pulmonary aspergillosis. Previously, the growth of *Aspergillus* was inhibited up to 10–75% by co-application of volatilized SA with either AntA or strobilurin, both inhibitors of complex III in the MRC (Kim et al., 2011a). We identified the fungal antioxidant system as one of the molecular targets of SA, where the model yeast *S. cerevisiae sod1*Δ, *sod2*Δ, *glr1*Δ, and *vph2*Δ mutants showed hypersensitivity to SA (Kim et al., 2011a).

As shown in **Figure 3**, combined application of volatile SA (at 37.5–45.0 mM) and ITZ (2–3 μg mL^{-1}, incorporated into SDA media) completely inhibited the growth of *A. fumigatus* AF293, while individual treatment of each compound, alone, allowed fungal growth. Therefore, like the chemosensitization of SA to the MRC inhibitors (Kim et al., 2011a), volatilized SA also enhanced the antifungal activity of ITZ as a chemosensitizing agent of *A. fumigatus*.

CORRELATION BETWEEN ITZ AND *t*-BuOOH SENSITIVITIES IN *CANDIDA* AND *CRYPTOCOCCUS*

Since positive correlation between the level of drug/compound sensitivity and antioxidation capacity was identified in the filamentous fungi (see above), we also investigated if such a relationship occurred in yeast pathogens using ITZ and *t*-BuOOH. As shown in **Figure 4**, *C. albicans* 90028, CAN276, *C. krusei* CAN75, and *C. tropicalis* CAN286 were relatively more tolerant (i.e., growth up to the 10^5 dilution spot) to ITZ compared to *C. krusei* 6258 and *C. neoformans* CN24 (i.e., growth at no cellular dilution only). Noteworthy is that *C. krusei* 6258 and *C. neoformans* CN24, two ITZ-sensitive strains, were also sensitive to *t*-BuOOH (**Figure 4**). This finding indicated there might be a connection between ITZ sensitivity and antioxidation capacity in yeast pathogens [Our recent data with yeast pathogens also indicated the positive correlation between AMB sensitivity (of *C. albicans* CAN276) and thiol-oxidant sensitivity (Manuscript submitted)].

REDOX-POTENT PHENOLIC COMPOUNDS ACT AS CHEMOSENSITIZERS TO ANTIFUNGAL DRUGS IN YEAST PATHOGENS
Chemosensitization of AMB with THY

We examined the chemosensitizing efficacy of THY to AMB in six different yeast pathogens. As shown in **Table 4**, most of the compound interactions (FICI) between THY and AMB were additive,

except for *C. neoformans* CN24, which was a neutral interaction. The level of MFC was also lowered in *C. albicans* CAN276 and *C. krusei* CAN75, where the FFCI was determined as additive and synergistic, respectively. Whereas, no chemosensitization was achieved in other strains for lowering the MFCs. We concluded that *C. albicans* CAN276 and *C. krusei* CAN75 were the most sensitive/responsive strains for this chemosensitization.

Chemosensitization of ITZ with 2,3-DHBA

Next, we investigated the chemosensitizing activity of 2,3-DHBA to ITZ (see Microtiter Plate (Microdilution) Bioassay: Yeasts for concentrations) in yeast pathogens. The compound interactions (FICI) between the two compounds in *C. albicans* 90028, CAN276 and *C. tropicalis* CAN286 were synergistic to additive ($0.5 \leq$ FICI ≤ 0.6), while those of the remaining yeasts were neutral (Data not shown). No chemosensitization achieved in any of the strains for lowering MFCs, indicating that, compared to other chemosensitization tests (see above), co-application of 2,3-DHBA and ITZ resulted in limited chemosensitizing efficacy, i.e., chemosensitization in three yeast pathogens for lowering MICs only (Data not shown).

INHIBITION OF COMPLEX III OF THE MRC: ALTERNATIVE OXIDATIVE STRESS TARGET FOR CONTROL OF YEAST PATHOGENS

We examined the antifungal efficacy of three inhibitors of complex III of the MRC, i.e., AntA and strobilurins (Kre-Me, PCS), in four clinical yeast isolates to investigate whether yeast pathogens could
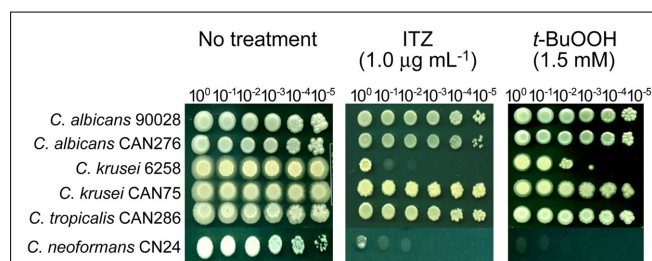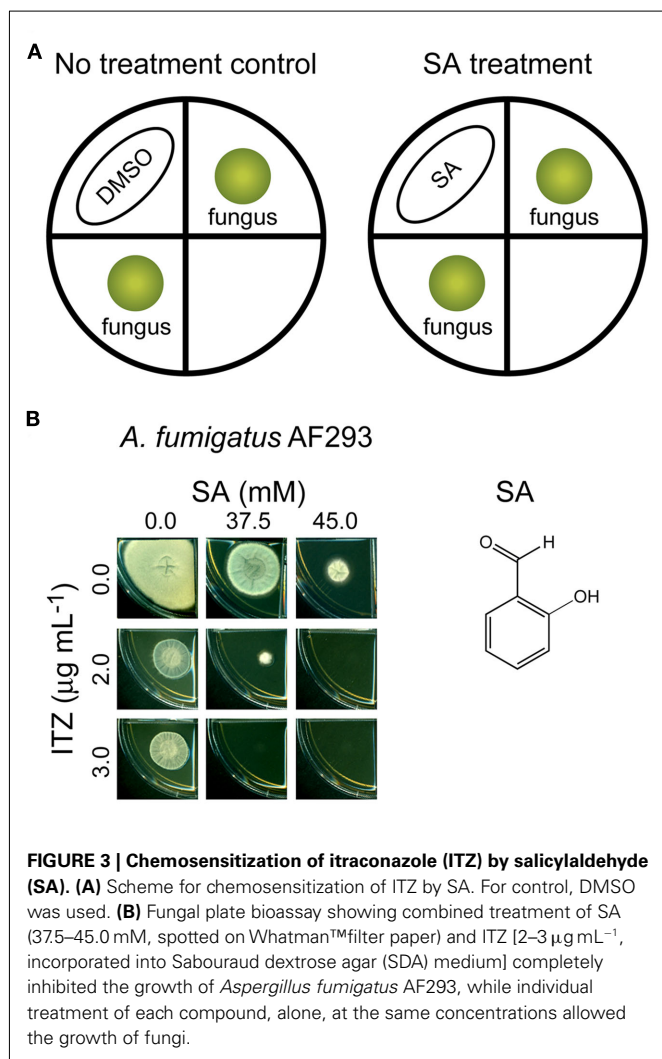

FIGURE 3 | Chemosensitization of itraconazole (ITZ) by salicylaldehyde (SA). (A) Scheme for chemosensitization of ITZ by SA. For control, DMSO was used. **(B)** Fungal plate bioassay showing combined treatment of SA (37.5–45.0 mM, spotted on Whatman™ filter paper) and ITZ [2–3 μg mL^{-1}, incorporated into Sabouraud dextrose agar (SDA) medium] completely inhibited the growth of *Aspergillus fumigatus* AF293, while individual treatment of each compound, alone, at the same concentrations allowed the growth of fungi.

FIGURE 4 | Phenotypic responses of yeast pathogens to itraconazole (ITZ) and *tert*-butyl hydroperoxide (*t*-BuOOH). Results showed that *Candida krusei* 6258 and *Cryptococcus neoformans* CN24, two ITZ-sensitive strains, were also sensitive to *t*-BuOOH, indicating the correlation between ITZ toxicity and oxidative stress. Results shown here are representative data from the treatment with 1.0 μg mL^{-1} of ITZ and 1.5 mM of *t*-BuOOH, respectively.

Table 4 | Chemosensitization of amphotericin B (AMB; μg mL^{-1}) with thymol (THY; mM) tested in yeast pathogens.

Strains	Compounds	MIC alone	MIC combined	FICI[1]
C. albicans ATCC 90028	THY, AMB	0.8–1.6, 1–2	0.4–0.8, 0.125–0.25	0.6
C. albicans CAN276	THY, AMB	0.8–1.6, 0.5–1	0.4–0.8, 0.125–0.25	0.8
C. tropicalis CAN286	THY, AMB	0.8–1.6, 1–2	0.4–0.8, 0.25–0.5	0.8
C. krusei ATCC 6258	THY, AMB	0.8–1.6, 1–2	0.4–0.8, 0.5–1	1.0
C. krusei CAN75	THY, AMB	0.8–1.6, 1–2	0.4–0.8, 0.5–1	1.0
Cryptococcus neoformans CN24	THY, AMB	0.4–0.8, 1–2	0.4–0.8, 1–2	2.0

Strains	Compounds	MFC alone	MFC combined	FFCI[2]
C. albicans CAN276	THY, AMB	0.8–1.6, 1–2	0.4–0.8, 0.5–1	1.0
C. krusei CAN75	THY, AMB	1.6–3.2, 2–4	0.8–1.6, 0–0.125	0.5

[1,2] See footnotes of **Table 3** for calculations.

also be chemosensitized by 2,3-DHBA to increase vulnerability to the complex III inhibitors.

As shown in **Figure 5A** (i.e., yeast dilution bioassay on SG agar containing $100 \, \mu g \, mL^{-1}$ of MRC inhibitors), *C. neoformans* CN24 was relatively more innately sensitive to the MRC inhibitors compared to other pathogens. Results indicated disruption of complex III of MRC, alone, could be an effective strategy for control of *C. neoformans* CN24. The remaining three pathogens, relatively tolerant to the complex III inhibitors, were further examined for chemosensitization with 2,3-DHBA in plate bioassays.

Disruption of complex III of the MRC results in an abnormal release of electrons that additionally damage cellular components by oxidative stress (Takimoto et al., 1999). Therefore, antioxidant enzymes, such as Mn-SOD, play important roles in protecting cells from oxidative damage caused by MRC inhibitors. However, when fungal cells are treated with redox-potent chemosensitizers, cellular demand for Mn-SOD will continuously increase as more and more oxidative stress is applied. In this situation, the levels of antioxidant capacity in fungi, such as antioxidant enzymes, would not be sufficient for detoxifying the concerted activities of multiple oxidative stressors (e.g., MRC inhibitors/oxidative stress drug + redox-potent chemosensitizer), resulting in increased inhibition of fungal growth. Hence, we surmised redox-potent benzaldehydes could be useful chemosensitizing agents also in yeast pathogens when co-applied with the complex III inhibitors of the

MRC. We used 2,3-DHBA as a representative molecule for this chemosensitization.

As shown in **Figure 5B**, co-application of 2,3-DHBA (0.1 mM) and PCS ($100 \, \mu g \, mL^{-1}$) enhanced growth inhibition of *C. albicans* CAN276 and *C. tropicalis* CAN286 compared to the control, while *C. krusei* CAN75 maintained robust growth under the same condition. Thus, *C. krusei* CAN75 seemed to be more tolerant to chemosensitization by 2,3-DHBA (A relatively lackluster response of *C. krusei* CAN75 to 2,3-DHBA chemosensitization was also observed above, with ITZ). However, slight enhancement [i.e., a 10-fold increase in number of yeast cells needed to survive (one cellular dilution less)] of growth inhibition of *C. krusei* CAN75 was achieved by increasing the concentration of 2,3-DHBA to 0.4 mM (see **Figure 5B**). In summary, these results indicated that: (1) The MRC (e.g., complex III) could be an alternative oxidative stress target for yeast pathogens, (2) Benzaldehyde analogs, such as 2,3-DHBA, could be developed as potent chemosensitizers in yeasts, especially with MRC inhibitors, and (3) Fungal sensitivity to chemosensitization (i.e., with 2,3-DHBA plus PCS), is strain specific, wherein *C. krusei* CAN75 was least sensitive.

DISCUSSION

Cellular antioxidant systems of fungi appear to be promising targets of redox-potent natural phenolics for control of fungal pathogens. The natural phenolics studied, *in vitro*, targeted MAPK

FIGURE 5 | Chemosensitization of pyraclostrobin (PCS), an inhibitor for complex III in mitochondrial respiratory chain (MRC) with 2,3-dihydroxybenzaldehyde (2,3-DHBA) in yeast pathogens. (A) *Cryptococcus neoformans* CN24 was relatively more sensitive to MRC inhibitors ($100 \, \mu g \, mL^{-1}$) compared to other pathogens (*Candida albicans*

CAN276, *C. krusei* CAN75, *C. tropicalis* CAN286). **(B)** Co-application of 2,3-DHBA (0.1 mM) and PCS ($100 \, \mu g \, mL^{-1}$) enhanced the inhibition of the growth of *C. albicans* CAN276 and *C. tropicalis* CAN286, while similar type of growth inhibition of *C. krusei* CAN75 could be achieved by increasing the concentration of 2,3-DHBA up to 0.4 mM.

signaling and/or the antioxidant enzymes, Cu, Zn-SOD, Mn-SOD, or glutathione reductase. THY further targeted the vacuolar system, such as *VPH2* and *VMA1*, as examined in *S. cerevisiae*. Also, THY or benzaldehyde analogs, such as 2,3-DHBA and SA, can be used as potent chemosensitizing agents to enhance antifungal activity of AMB, ITZ, or PCS.

A. fumigatus MAPK (*sakA*, *mpkC*) and *A. flavus msnA* gene deletion mutants were sensitive to organic and hydrogen peroxides. This common sensitivity indicates that *msnA*, a gene regulator downstream of the MAPKs *sakA* and *mpkC*, and these MAPKs, are all located in the same stress response network under *t*-BuOOH or H_2O_2 stress. Also confirmed was that oxidative stress is one of the contributing mechanisms of toxicities of AMB and ITZ in fungal pathogens. In *A. fumigatus*, SakA appeared to play a more important role for fungal tolerance to ITZ and/or AMB than MpkC, the deletion mutant of which was relatively insensitive to these drugs. Meanwhile, in the yeast pathogens, ITZ-sensitive strains (i.e., *C. krusei* 6258, *C. neoformans* CN24) were also sensitive to *t*-BuOOH. Thus, results indicated that the level of sensitivity (and/or tolerance) to oxidative stress drugs was correlated with the antioxidant capacity of fungal pathogens (both in ascomycetous and basidiomycetous fungi).

Involvement of stress response signaling systems in drug resistance has been previously documented in fungal pathogens. For example, the heat shock protein Hsp90, an essential molecular chaperone regulating cell signaling, was shown to govern azole drug resistance of *C. albicans* either in planktonic or biofilm conditions (LaFayette et al., 2010; Robbins et al., 2011). In the case of Hsp90, its inhibition/depletion resulted in reduction of calcineurin and the terminal cell wall integrity MAPK, Mkc1, in planktonic conditions, whereas marked decrease in matrix glucan levels occurred in biofilms (LaFayette et al., 2010; Robbins et al., 2011). The Mkc1-mediated pathway is also activated in response to oxidative stress (de Dios et al., 2010 and references therein). Like the SakA shown in our study, another Hog1 pathway component, i.e., Hrk1 (Hog1-regulated kinase 1) of *C. neoformans* was recently shown to be involved in the response to azole drug treatment (Kim et al., 2011b). Also, ROS-inducing effect of miconazole, and involvement of SODs of *C. albicans* in biofilm persistence against miconazole (Vandenbosch et al., 2010; Bink et al., 2011) were recently reported, further demonstrating the role of fungal antioxidant system such as SODs in drug resistance.

We also found differences in effects of tested compounds depending upon (1) types of mutation in the antioxidant system (i.e., MsnA or MAPK gene deletions) and (2) types of drugs. For example, *Aspergillus* deletion mutants for *sakA* or *msnA* genes were hypersensitive to AMB (**Figure 1**), while the *A. flavus msnA*Δ mutant was less sensitive to ITZ compared to the *A. fumigatus sakA*Δ mutant. Presumably, regulator(s) other than MsnA might be involved in fungal response/tolerance to ITZ. Regarding the AMB sensitivity of both *sakA*Δ and *msnA*Δ mutants of *Aspergillus*, *S. cerevisiae* could serve as a model for explaining their sensitive phenotype. In *S. cerevisiae*, functional interaction between the *HOG* signaling system and Msn2p (and Msn4p, which is a Msn2p analog) under oxidative stress has been well documented (Görner et al., 1998; O'Rourke et al., 2002 and references therein). Thus, functions of Hog1p and Msn2p/Msn4p are tightly linked

under stress conditions. Considering SakA in *A. fumigatus* is an orthologous protein to Hog1p of *S. cerevisiae*, a similar phenomenon might also occur with the treatment of AMB in filamentous fungal pathogens.

We observed similar levels of sensitivity in both *Aspergillus msnA*Δ and *A. fumigatus sakA*Δ mutants to redox-potent phenolic compounds. These similar responses indicated that the signaling route through "SakA–MsnA" might also be governing fungal response to redox-potent phenolics, such as 2,3-DHBA and THY in *Aspergillus* (**Table 2**). A recent study showed significant changes occurred in transcription levels of environmental stress response genes of *S. cerevisiae* treated with THY (Bi et al., 2010). Moreover, these environmental stress genes are mainly controlled by Msn2p/Msn4p transcription factors. These results from *S. cerevisiae* agree with our findings of the hypersensitivity of the *A. flavus msnA*Δ mutant. Msn2p/Msn4p-regulated genes contain one or more stress response element (STRE) motifs in their promoter regions (Bi et al., 2010), further emphasizing the important roles of MsnA and/or Msn2p/Msn4p in fungal tolerance to THY.

We also demonstrated the chemosensitization of fungal pathogens to conventional drugs by redox-potent phenolic compounds. We found that THY was a better chemosensitizing agent than 2,3-DHBA in combination with ITZ or AMB. All results with THY, in both filamentous fungi and yeasts, had lowered MFCs of drugs/compounds. Whereas, the least effective chemosensitization was found with 2,3-DHBA plus ITZ. Therefore, there were some unique interrelationships between levels of fungal response and types of chemosensitizers applied. *C. neoformans* CN24 was the least sensitive strain to any chemosensitization examined in our test. THY also possessed intrinsic antifungal activity when treated alone (Pinto et al., 2006). However, chemosensitization strategy can lower dosages of THY required for effective control of fungi, as shown in this study.

Fungi could also be sensitized by compounds and antifungal agents that are inhibitor(s) of complex III of the MRC. We were able to demonstrate this with 2,3-DHBA in yeast pathogens. However, there is differential fungal strain sensitivity to chemosensitizers/MRC inhibitors (e.g., the low sensitivity of *C. krusei* CAN75 to 2,3-DHBA plus PCS). Accordingly, doses and/or types of MRC inhibitors or chemosensitizers should be precisely determined for effective control of fungi in the future. Noteworthy is that artemisinin, the wormwood herb used as an antimalarial drug, was recently shown to inhibit the growth of *S. cerevisiae*. In this case, mitochondrial respiration stimulates the effect of this drug, and the mitochondria are subsequently damaged (i.e., depolarization of mitochondrial membrane) by the ROS generated locally (Li et al., 2005).

In conclusion, cellular antioxidant systems can serve as promising molecular targets of redox-potent phenolics for control of fungi. Benzaldehyde analogs, such as 2,3-DHBA, SA, etc., or THY can be developed as chemosensitizing agents to enhance the efficacy of antifungal drugs. Future studies are needed for comprehensive determination of optimum chemosensitization in different fungal pathogens by including additional redox-potent compounds. Further *in vivo* studies are also warranted to determine if the activities shown in this *in vitro* study can translate

into a clinically effective therapeutic strategy for control of fungal pathogens.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.