Chemosensitization of fungal pathogens to antimicrobial agents using benzo analogs

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Abstract

Activities of conventional antifungal agents, fludioxonil, strobilurin and antimycin A, which target the oxidative and osmotic stress response systems, were elevated by coapplication of certain benzo analogs (aldehydes and acids). Fungal tolerance to 2,3-dihydroxybenzaldehyde or 2,3-dihydroxybenzoic acid was found to rely upon mitochondrial superoxide dismutase (SOD2) or glutathione reductase (GLR1), genes regulated by the HOG1 signaling pathway, respectively. Thus, certain benzo analogs can be effective at targeting cellular oxidative stress response systems. The ability of these compounds to chemosensitize fungi for improved control with conventional antifungal agents is discussed.

Introduction

Oxidative stress response systems are known to play a role in the virulence of fungal pathogens (Hamilton & Holdom, 1999). Fungal oxidative stress response pathways protect fungal cells from reactive oxygen species (ROS), which are generated as part of the host defense-reaction to infection. Antioxidative responses can also protect fungi from ROS that are induced by exposure to certain environmental stress factors (e.g. UV radiation, drought, etc). As such, oxidative stress response systems of fungi appear to be promising targets of antimicrobial compounds (Smits & Brul, 2005; Jaeger & Flohe, 2006).

Many natural compounds (e.g. phenolics) are promising antifungal agents, or lead compounds, for disrupting fungal oxidative stress response systems. When applied above antioxidant levels, phenolics or sulfur-containing natural compounds can be potent redox-cyclers (i.e. redox-active). This cycling interferes with cellular redox homeostasis and/or the function of redox-sensitive components resulting in poor cell viability and growth (Guillen & Evans, 1994; Shvedova et al., 2000; Jacob, 2006). In order to counter this interference, the oxidative stress response systems are induced in the cell.

Fungal resistance to conventional drugs is an emerging problem (Moore et al., 2000; Cowen et al., 2001; Schoustra et al., 2006). To counteract this problem, efforts have been underway to use natural compounds, such as derivatives of benzoic or cinnamic acid, as antifungal or antimycotoxicogenic agents or lead compounds to new agents (Tawata et al., 1996; Florianowicz, 1998; Beekrum et al., 2003). It is anticipated that such compounds can curtail development of tolerance or resistance to antifungal agents.

Analogs of benzaldehyde, such as anisaldehyde, vanillin (4-hydroxy-3-methoxybenzaldehyde), etc., are frequently found as natural plant constituents. Many possess potent antimicrobial activities (Harborne, 1989; Fitzgerald et al., 2005). Structural–functional relationships, involving the position of the aldehyde group in relation to the position of side-groups on the aromatic ring, can greatly affect the antifungal activity of benzaldehyde compounds (Fitzgerald et al., 2005). In the present study, we characterize antifungal
activities of analogs of 2,3-dihydroxybenzaldehyde. Identification of genes targeted by these compounds shows that chemosensitization is most likely associated with disruption of cellular oxidative stress response systems in the fungus, thus, enhancing conventional antifungal agents. Coapplication of these chemosensitizing compounds with commercial antifungal agents may serve as a promising approach to alleviating health and environmental risks. This alleviation would result by reducing amounts of commercial antifungal agents required to achieve effective control.

## Materials and methods

### Microorganisms and culture condition

*Saccharomyces cerevisiae* wild-type BY4741 (*Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and selected deletion mutants lacking genes in antioxidative stress response/multidrug resistance systems were obtained from Invitrogen (Carlsbad, CA) and Open Biosystems (Huntsville, AL), as follows (see also Kim et al., 2005, 2007): Gene regulation mutants: yap1Δ, msn2Δ, msn4Δ, hot1Δ, sko1Δ, rim101Δ; Transporter/assembly protein mutants: fla1Δ, yer1Δ, pdr5Δ, vph2Δ, tfp1Δ/vma1Δ; Signal transduction mutants: sho1Δ, sli1Δ, ste50A, ste20A, ypd1Δ, ssh1Δ, ptp2Δ, ptp3Δ, hog1Δ, hog4Δ, ssk22Δ, ssk2Δ, ste11Δ; Antioxidation mutants: ctt1Δ, cta1Δ, osr1Δ, trr1Δ, trr2Δ, rsa1Δ, grx1Δ, grx2Δ, trx1Δ, trx2Δ, glr1Δ, gsh1Δ, gsh2Δ, sod1Δ, sod2A, aph1Δ; DNA damage control/energy metabolism mutants: rad54Δ, gsh1Δ, acc1Δ, gpd1Δ, hor2Δ (Reference for the description of each deletion mutant: www.yeastgenome.org, accessed on August 17, 2007). Yeast strains were grown on YPD (complete medium; 1% Bacto yeast extract, 2% Bacto peptone, 2% glucose) or SG (minimal medium; 0.67% Yeast nitrogen base w/o amino acids, 2% glucose with appropriate supplements: 0.18 mM uracil, 0.2 mM amino acids) medium at 30 °C (5 to 7 days). *Aspergillus fumigatus* AF293, wild type, and *A. fumigatus* mitogen-activated protein kinase (MAPK) deletion mutants sakAΔ and mpkCΔ (Xue et al., 2004; Reyes et al., 2006) were grown at 37 °C on potato dextrose agar (PDA) medium (5–7 days). *Aspergillus flavus* NRRL3537, *Aspergillus parasiticus* NRRL5862, *Aspergillus niger* NRRL326, *Aspergillus ochraceus* NRRL5175, *Penicillium expansum* NRRL974 (obtained from National Center for Agricultural Utilization and Research, USDA, Peoria, IL; http://nrl.ncaur.usda.gov/index.html, accessed on 17 August 2007), *Aspergillus nidulans* A4 and *Aspergillus oryzae* FGSC A815 (obtained from Fungal Genetics Stock Center, Kansas City, MO; www.fgsc.net/aliast.html, accessed on 17 August 2007; McCluskey, 2003), were cultured at 28 °C on PDA (5–7 days). Antifungal compounds fludioxonil, kresoxim-methyl, antimycin A, and benaldeehyde analogs, 2,3-, 2,4-, 2,5-dihydroxybenzaldehyde; 3-, 4-hydroxybenzaldehyde; benzaldehyde, veratraldehyde (3,4-dimethoxybenzaldehyde), vanillin (4-hydroxy-3-methoxybenzaldehyde) and 2,3-dihydroxybenzoic acid were purchased from Sigma Co. (St Louis, MO). Each compound was dissolved in dimethylsulfoxide (absolute amount <20 μL mL⁻¹ media) before use.

### Antifungal bioassays

Yeast cell dilution bioassays were performed on SG agar media to monitor activity of and gene deletion mutant hypersensitivity to antifungal compounds, as follows (see Kim et al., 2005, 2007): 1 × 10⁶ cells of the wild type or respective deletion mutants of *S. cerevisiae*, cultured on YPD medium, were serially diluted 10-fold in SG liquid medium supplemented with amino acids and uracil five times to yield cell dilution cohorts of 10⁶, 10⁵, 10⁴, 10³, 10² and 10 cells. Cells from each dilution of respective yeast strains were spotted adjacently on SG agar medium incorporated with individual benzo analogs to be tested or antifungal reagents and incubated at 30 °C. Results were recorded based on a designated value of the highest dilution where a colony became visible after 5–7 days of incubation, as follows: Score ‘0’ - no colonies were visible from any of the dilutions, Score ‘6’ - colonies were visible from all dilutions, Score ‘1’ - only a colony from the undiluted cells (10⁶ cells), ‘2’ only colonies from the undiluted and 10⁵ cells were visible, etc. Thus each unit of numerical difference was equivalent to a 10-fold difference in the sensitivity of the yeast strain to the treatment.

Sensitivities of filamentous fungi were measured based on percent radial growth of treated fungal colonies compared to control colonies, receiving only dimethylsulfoxide [Vincent equation: % inhibition = 100 (C – T)/C, C is diameter of fungi on control plate; T is diameter of fungi on the test plate; Vincent, 1947]. Fungi (~200 spores) were diluted in phosphate-buffered saline (PBS) and spotted on the center of PDA plates with or without antifungal compounds. Growth was observed for 5–7 days. To measure the potential for chemosensitization by test benzo analogs, compounds were added to the growth media together with either fludioxonil, strobilurin (kresoxim-methyl) or antimycin A. Radial growth was recorded as described above.

Minimum inhibitory concentrations (MICs) of antifungal compounds in *S. cerevisiae* or filamentous fungi were based on the lowest concentrations of compounds where no visible growth of cells were observed either on SG or PDA plates, respectively.

### Results

**Fungal tolerance to benzo analogs depends on cellular mitochondrial superoxide dismutase (Mn-SOD)**

Based on yeast cell dilution bioassays, 2,3-dihydroxybenzaldehyde had the highest antifungal activity, i.e. no visible
growth of wild-type *S. cerevisiae* at ≥0.08 mM, among eight benzo analogs tested (Table 1). Highest to lowest antimicrobial activity was, as follows: 2,3-dihydroxybenzaldehyde >2,5-dihydroxybenzaldehyde >2,4-dihydroxybenzaldehyde >3-hydroxybenzaldehyde >vanillin, 4-hydroxybenzaldehyde, veratraldehyde > benzaldehyde. An almost identical relationship in the relative antifungal activities of the analogs was observed among the various filamentous fungi, i.e. aspergilli and *Penicillium*, tested (Table 1).

Among 45 mutants of *S. cerevisiae* examined, where genes in oxidative stress response/multidrug resistance systems were individually deleted, the sod2Δ (Mn-SOD deletion), gsh1Δ (γ-glutamylcysteine synthetase deletion) and gsh2Δ (glutathione synthetase deletion) mutants showed 10³–10⁴-fold hypersensitivity to 2,3-dihydroxybenzaldehyde (at 0.01 mM) compared with the wild-type strain (Fig. 1). Sensitivity of gsh1Δ and gsh2Δ mutants to 2,3-dihydroxybenzaldehyde was mainly a result of glutathione (GSH)

Table 1. MICs (mM) of benzaldehyde analogs in *S. cerevisiae* and filamentous fungi

<table>
<thead>
<tr>
<th>Analog</th>
<th>2,3-</th>
<th>2,4-</th>
<th>2,5-</th>
<th>3-</th>
<th>4-</th>
<th>Benzaldehyde</th>
<th>Veratraldehyde</th>
<th>Vanillin</th>
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<tr>
<td><em>S. cerevisiae</em></td>
<td></td>
<td></td>
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<tr>
<td>Wild type</td>
<td>0.08</td>
<td>2.4</td>
<td>1.8</td>
<td>9.0</td>
<td>13.0</td>
<td>&gt;35.0</td>
<td>19.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Filamentous fungi</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>A. fumigatus AF293 wild type</td>
<td>0.3</td>
<td>2.4</td>
<td>1.2</td>
<td>3.0</td>
<td>9.0</td>
<td>&gt;35.0</td>
<td>7.0</td>
<td>6.0</td>
</tr>
<tr>
<td>A. flavus NRRL 3357</td>
<td>1.0</td>
<td>3.1</td>
<td>2.8</td>
<td>9.0</td>
<td>12.0</td>
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<td>7.0</td>
<td>6.0</td>
</tr>
<tr>
<td>A. parasiticus NRRL 5862</td>
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<td>2.6</td>
<td>2.6</td>
<td>8.0</td>
<td>11.0</td>
<td>&gt;35.0</td>
<td>8.0</td>
<td>7.0</td>
</tr>
<tr>
<td>A. oryzae FGSC A815</td>
<td>1.0</td>
<td>3.0</td>
<td>3.0</td>
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<td>8.0</td>
<td>8.0</td>
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<tr>
<td>A. niger NRRL 326</td>
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<td>3.6</td>
<td>7.0</td>
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<td>5.0</td>
<td>7.0</td>
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<tr>
<td>A. nidulans FGSC A4</td>
<td>0.5</td>
<td>2.2</td>
<td>1.7</td>
<td>5.0</td>
<td>8.0</td>
<td>&gt;35.0</td>
<td>5.0</td>
<td>6.0</td>
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<tr>
<td>A. ochraceous NRRL 1757</td>
<td>0.7</td>
<td>2.5</td>
<td>2.0</td>
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<td>&gt;35.0</td>
<td>8.0</td>
<td>7.0</td>
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<tr>
<td>P. expansum NRRL 974</td>
<td>0.5</td>
<td>1.8</td>
<td>1.5</td>
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<td>8.0</td>
<td>&gt;35.0</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Mean MIC (Filamentous fungi)</td>
<td>0.74</td>
<td>2.61</td>
<td>2.30</td>
<td>6.13</td>
<td>10.0</td>
<td>&gt;35.0</td>
<td>7.0</td>
<td>7.25</td>
</tr>
</tbody>
</table>

*2,3-, 2,3-dihydroxybenzaldehyde; 2,4-, 2,4-dihydroxybenzaldehyde; 2,5-, 2,5-dihydroxybenzaldehyde; 3-, 3-hydroxybenzaldehyde; 4-, 4-hydroxybenzaldehyde; Vanillin, 4-hydroxy-3-methoxybenzaldehyde; Veratraldehyde, 3,4-dimethoxybenzaldehyde.

Fig. 1. Antifungal activities of various benzaldehyde derivatives. (a) The Saccharomyces cerevisiae sod2Δ, gsh1Δ and gsh2Δ mutants were hypersensitive to 2,3-dihydroxybenzaldehyde (0.01 mM). Supplementation with 0.1 mM glutathione reverted 2,3-dihydroxybenzaldehyde sensitivities of gsh1Δ and gsh2Δ mutants to that of a wild-type strain. Numbers (1–6) designated on the right side of each panel are the growth scores of test strains (see ‘Materials and methods’). (b) The sod2Δ mutant also showed sensitivity to various benzaldehyde analogs, i.e., 2,5-dihydroxybenzaldehyde, 3- or 4-hydroxybenzaldehyde and 3,4-dimethoxybenzaldehyde (veratraldehyde). (c) Aspergillus fumigatus sakAΔ mutant is hypersensitive to 2,3- and 2,5-dihydroxybenzaldehyde, suggesting Mn-SOD activity in A. fumigatus is under control of SakA (MAPK). Numbers in the panels are % radial growth of test fungi compared to the untreated control. SD < 5%.
auxotrophy. After supplementation with 0.1 mM glutathione, the gsh1Δ and gsh2Δ mutants treated with 2,3-dihydroxybenzaldehyde showed similar levels of cell growth as the wild type (Fig. 1). Supplementation by glutathione did not result in recovery of either the wild type or the sod2Δ mutant from originally observed levels of sensitivity to 2,3-dihydroxybenzaldehyde (Fig. 1).

Because sensitivity of the gsh mutants resulted from glutathione auxotrophy, attention was focused on the Mn-SOD gene/mutant. The Mn-SOD gene is downstream in the yeast HOG1 (MAPK) oxidative stress signaling-pathway (Boy-Marcotte et al., 1998; Rep et al., 2000, 2001) and appeared to be a promising candidate as a target for fungal control.

Importantly, the sod2Δ mutant showed higher sensitivity to the benzo analogs than two other mutants involved in oxidative stress response, sod1Δ and glr1Δ (Fig. 1) This greater sensitivity strongly indicated Mn-SOD activity is crucial for fungal response/tolerance against toxicity of benzaldehyde derivatives. The sod1Δ mutant, lacking the cytosolic SOD gene, was either less sensitive to 2,3-dihydroxybenzaldehyde, 3- and 4-hydroxybenzaldehyde, or equally sensitive to 2,5-dihydroxybenzaldehyde and veratraldehyde compared with the sod2Δ strain (Fig. 1). Tolerance of glr1Δ, a glutathione reductase mutant, to the same compounds was 10–1000-fold greater than the sod2Δ mutant (Fig. 1).

The sakAΔ mutant of A. fumigatus is hypersensitive to benzaldehyde derivatives

Responses of sakAΔ and mpkCΔ (MAPK deletion) mutants derived from A. fumigatus AF293 (wild type), to the benzo analogs was also examined. The MAPKs, SakA and MpkC are orthologous proteins to Hog1p of S. cerevisiae (Xue et al., 2004; Reyes et al., 2006). Growth of AF293 and mpkCΔ was inhibited by 32–72% with 0.2–0.25 mM 2,3-dihydroxybenzaldehyde, respectively (Fig. 1). The sakAΔ strain was more sensitive to these treatments, showing a 56–100% reduction, respectively, in radial growth. Like Hog1p in S. cerevisiae, SakA may play a role in regulating Mn-SOD activity and, thus, tolerance to 2,3-dihydroxybenzaldehyde. The same trends were observed with 2,5-dihydroxybenzaldehyde, but higher concentrations (0.8–1 mM) were needed to achieve similar levels of growth inhibition (Fig. 1).

2,3-Dihydroxybenzoic acid inhibits fungal growth by disrupting cellular glutathione homeostasis

The acid derivative of 2,3-dihydroxybenzaldehyde, 2,3-dihydroxybenzoic acid, was also examined in order to investigate structure-activity relationships with regard to acid or aldehyde moieties. We previously observed that the glr1Δ mutant of S. cerevisiae was hypersensitive to 12 mM 2,5-dihydroxybenzoic acid, an aspirin metabolite. This hypersensitivity indicated this compound interfered with cellular glutathione homeostasis. Furthermore, supplementation of glutathione (0.1 mM) to 2,5-dihydroxybenzoic acid-treated fungi resulted in recovery of cellular growth of the glr1Δ mutant (Kim et al., 2007).

In this study, 2,3-dihydroxybenzoic acid inhibited growth of S. cerevisiae (MIC in wild type ≥ 7 mM). Also, growth of a number of S. cerevisiae deletion mutants was inhibited by 2,3-dihydroxybenzoic acid at 4 mM, including glr1Δ, gsh1Δ, gsh2Δ, vph2Δ (vacuolar ATPase assembly protein deletion), vma1Δ (vacuolar ATPase deletion). Also, similar to treatments with 2,5-dihydroxybenzoic acid in our prior study, cited above, exogenously supplemented glutathione resulted in a strong recovery of growth of these S. cerevisiae strains (Fig. 2). These findings suggest the mechanism of antifungal activity of 2,3-dihydroxybenzoic acid is, as with the 2,5-analog, disruption of cellular glutathione homeostasis. Thus, the glutathione reductase gene (GLR1), another gene relatively downstream within the HOG1 signaling pathway, may play an important role for fungal tolerance to this, or related, compounds. The sensitivities of the vph2Δ and vma1Δ mutants may result from disruption of the normal ability for transporting, sequestrating and detoxifying compounds in cell vacuoles.

The concordance of these results demonstrates there is a structure-activity relationship between the acid and aldehyde moieties in that they affect different target genes in the

**Fig. 2.** Yeast bioassy showing glutathione at 0.1 mM recovered the growth of Saccharomyces cerevisiae (wild type, glr1Δ, vph2Δ, vma1Δ, gsh1Δ, gsh2Δ) from the toxicity of 2,3-dihydroxybenzoic acid. This recovery indicates this compound interrupts glutathione homeostasis in cells. Numbers (1–6) designated on the right side of each panel are the growth scores of test strains (see ‘Materials and methods’).
HOG1-signaling pathway. The 2,3- and 2,5-dihydroxybenzaldehydes targeted SOD2, whereas, their acids analogs targeted GLR1, disrupting glutathione homeostasis. In addition, the S. cerevisiae sod1Δ or sod2Δ mutants were not hypersensitive to either of these acid derivatives.

Chemosensitization to conventional fungicides by 2,3-dihydroxybenzaldehyde and benzoic acid derivatives: targeting cellular signal transduction/oxidative stress response systems

The dihydroxybenzo analogs, 2,3-dihydroxybenzaldehyde and 2,3-dihydroxybenzoic acid, were examined as potential chemosensitizing agents for target-gene based control of fungi. Some fungi having mutations in certain MAPK genes, involved in signal transduction of oxidative stress responses, can escape toxicity of phenylpyrrole fungicides, such as fludioxonil (Kojima et al., 2004). In this regard, we found MAPK mutants; sakAΔ and mpkCΔ, of A. fumigatus were tolerant to fludioxonil toxicity (Fig. 3). However, coapplication of 2,3-dihydroxybenzaldehyde (at 0.2 mM) or 2,3-dihydroxybenzoic acid (at 11 mM) with fludioxonil effectively prevented these mutants from developing this tolerance to fludioxonil (Fig. 3). This prevention of tolerance by coapplication of either of these compounds may result from the disruption of genes downstream in this MAPK pathway. In particular, based on the results with the deletion mutants of S. cerevisiae (Figs 1 and 2) it is likely that these aldehyde and acid analogs target the antioxidative gene sod2 and the glutathione homeostasis genes glr1 and gsh1/2, respectively (Fig. 3).

The potential chemosensitizing effect of 2,3-dihydroxybenzaldehyde was also tested on the activity of kresoxim-methyl, a strobilurin fungicide, and antimycin A. Both of these fungicides disrupt complex III of the mitochondrial respiratory chain. This disruption eventually results in cellular oxidative stress caused by abnormal release of electrons from the respiratory chain and the production of toxic superoxide. Mn-SOD plays an important role in detoxifying this oxidative stressor. Coapplication of 2,3-dihydroxybenzaldehyde enhanced the antifungal activity of both fungicides against the filamentous fungi examined, A. fumigatus, A. flavus and P. expansum. Coapplication of 0.1 or 0.2 mM 2,3-dihydroxybenzaldehyde to kresoxim-methyl (0.025 mM; Fig. 4) or antimycin A (5 μg mL⁻¹; Fig. 4) resulted in complete (100%) inhibition of fungal growth, except A. flavus (70% inhibition). Alternatively, application of these compounds individually at these rates results in only slight inhibition of fungal growth.

Discussion

The results presented in this study indicate certain benzaldehyde derivatives can be used to target genes in the fungal oxidative stress response system regulated by the HOG1 (MAPK) signaling pathway. We identified the Mn-SOD gene as being a crucial genetic component for fungal tolerance to these analogs. In addition a benzoic acid analog, 2,3-dihydroxybenzoic acid, was found to disrupt cellular glutathione homeostasis that is maintained by the GLR1 gene. Our conclusion that these compounds targeted these stress response systems was evident in using bioassays involving strains of the model yeast, S. cerevisiae. These assays clearly showed that the sod2Δ and glr1Δ mutants were hypersensitive to the benzaldehyde analogs and to 2,3-dihydroxybenzoic acid, respectively. The benzaldehyde analogs may also act via the glutathione pathway, but are more effective via Mn-SOD. The effects of the benzoic acids occur at higher concentrations than those of the benzaldehydes. This suggests the glutathione pathway may be a less efficient target compared to the SOD pathway. Further research is necessary to define the precise mechanism(s) of action of benzo analogs as antifungal agents. Various benzaldehyde analogs have previously been shown to inhibit growth of a number of food-borne bacterial pathogens (Ramos-Nino et al., 1999; Friedman et al., 2003).

Other studies have shown that antimicrobial activities of a number of compounds can be linked to disruption of the cellular oxidative stress defense system. For example, the level of ROS was increased when bacterial pathogens were treated with ciprofloxacin, a fluoroquinolone antibiotic inhibiting DNA topoisomerases (Goswami et al., 2006 and references therein). Importantly, application of glutathione or ascorbic acid (an antioxidant) reversed the toxicity of fluoroquinolones. In addition, transfection of SOD genes into E. coli also resulted in greater survival of cells exposed to low concentrations (~10 ng mL⁻¹) of these compounds (Goswami et al., 2006). These observations suggest O₂⁻ and H₂O₂ are probably involved in the antimicrobial activity of ciprofloxacin. A report has also shown that treatment of fungal cells with fungitoxic dimethyldithiocarbamic acid or thiram [bis(dimethylthiocarbamoyl) disulfide] results in a rapid decrease in levels of glutathione (Elskens & Penninckx, 1997). This decrease indicates the mode of action of this fungicide results from disruption of the oxidative stress response system.

Our results show benzaldehyde moieties having ortho and para positions simultaneously occupied by hydroxyl groups possess higher antifungal activity than meta or mono-hydroxy analogs. This higher antifungal activity may be explained by dihydroquinone/semiquinone/quinone redox cycling resulting in higher redox activity than the mono-substituted analogs (Brunmark & Cadenas, 1989).

Many quinone derivatives inhibit a variety of cellular processes, for example, mitochondrial respiration, enzyme activity, etc, based on their redox activity. Adaphostin, a promising dihydroquinone (para-dihydroxy substituted)
derivative for chemotherapy of cancer, targets complex III of the mitochondrial respiratory chain. This generates ROS, rather than direct redox-cycling of the hydroquinone moiety, which are cytotoxic to cancer cells (Le et al., 2007). In this case, Mn-SOD would be essential to detoxification of adaphostin-generated ROS. Like the adaphostin-treated cells, benzaldehyde analogs used in our study may affect fungal mitochondrial respiration in a similar manner. Functional complementation of the Mn-SOD gene in an S. cerevisiae Mn-SOD deletion mutant (sod2Δ) with an orthologous gene (sodA) from A. flavus reversed hypersensitivity of this mutant to antifungal phenolic compounds, such as veratraldehyde, vanillin, and others (Kim et al., 2006). This reversal of hypersensitivity strongly indicates cellular Mn-SOD activity is important for fungal tolerance to such compounds and ROS. Our future efforts will employ a dihydrofluorescein probe to detect any potential ROS build-up in cells treated with our test compounds (Diaz et al., 2003), especially in mitochondria.

In addition to generating ROS, quinone derivatives can also directly disrupt enzyme activity, such as becoming covalently attached through the Michael addition reaction.

Fig. 3. Enhanced activity of fludioxonil (0.05 mM) by coapplication with (a) 2,3-dihydroxybenzaldehyde (0.2 mM) or (b) 2,3-dihydroxybenzoic acid (11 mM). These combined treatments prevented escape of sakΔ or mpkCΔ MAPK mutants from the fungicidal effects of fludioxonil. (c) Scheme showing where phenylpyrrole fungicides (e.g. fludioxonil) target MAPK signaling pathway genes. MAPK mutants escape toxicity to fludioxonil by lack of signaling induced by phenylpyrrole fungicides, thus avoiding over-induced oxidative/osmotic stress responses. Application of natural compounds disrupts cellular oxidative stress defense system (e.g. Sod2 by 2,3-dihydroxybenzaldehyde or glutathione homeostasis by 2,3-dihydroxybenzoic acid), which enhances toxicity in wild-type cells or prevents escape of MAPK mutants from antifungal effects. SD < 5%, except where noted.
Collectively, the above studies indicate cellular oxidative stress response systems, such as SOD and/or glutathione homeostasis, can play important roles in responding to treatment of certain antimicrobial agents. As such, these stress response systems should serve as effective targets for control of fungi.

Our findings showed that certain benzaldehyde analogs also possess chemosensitizing capacity against the ascomycetous fungi *A. fumigatus*, *A. flavus*, *P. expansum*, augmenting activities of conventional antifungal agents. Coapplications of 2,3-dihydroxybenzaldehyde with kresoxim-methyl, antimycin A or fludioxonil, and 2,3-dihydroxybenzoic acid with fludioxonil, resulted in a considerable enhancement of overall fungitoxicity. This enhanced toxicity shows that disruption of the fungal oxidative stress defense system through chemosensitization can be an effective way to control fungal pathogens. Moreover, disruption of this system prevented escape of the *A. fumigatus* MAPK mutants from toxicity of fludioxonil.

In summary, oxidative stress response systems could be efficient molecular targets of phenolic agents for control of fungi. Combined application of redox-active phenolics with other antifungal agents can effectively suppress fungal growth.
growth or circumvent mechanisms of resistance. In this study, we also showed the utility of the model yeast, *S. cerevisiae*, as a tool for screening effectiveness of antifungal compounds, while providing information on gene-targets. Such information can facilitate development of antifungal agents against a broad spectrum of fungal pathogens. Safe, natural products, such as the benzo analogs in this study, can be used as chemosensitizing agents to augment efficacy and lower dosages of antifungal agents. These lower dosages can result in reduced costs and alleviate health and environmental risks often associated with application of such agents.

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**References**


