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# Augmenting the activity of antifungal agents against aspergilli using structural analogues of benzoic acid as chemosensitizing agents

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ABSTRACT

A number of benzoic acid analogues showed antifungal activity against strains of *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus terreus*, causative agents of human aspergillosis, in *in vitro* bioassays. Structure–activity analysis revealed that antifungal activities of benzoic and gallic acids were increased by addition of a methyl, methoxyl or chloro group at position 4 of the aromatic ring, or by esterification of the carboxylic acid with an alkyl group, respectively. Thymol, a natural phenolic compound, was a potent chemosensitizing agent when co-applied with the antifungal azole drugs fluconazole and ketoconazole. The thymol-azole drug combination demonstrated complete inhibition of fungal growth at dosages far lower than the drugs alone. Co-application of thymol with amphotericin B had an additive effect on all strains of aspergilli tested with the exception of two of three strains of *A. terreus*, where there was an antagonistic effect. Use of two mitogen-activated protein kinase (MAPK) mutants of *A. fumigatus*, *sak*AΔ and *mpk*CΔ, having gene deletions in the oxidative stress response pathway, indicated antifungal and/or chemosensitization activity of the benzo analogues was by disruption of the oxidative stress response system. Results showed that both these genes play overlapping roles in the MAPK system in this fungus. The potential of safe, natural compounds or analogues to serve as chemosensitizing agents to enhance efficacy of commercial antifungal agents is discussed.

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## Introduction

Aspergilli remain the most common causative agents of invasive mould infections among patients with hematologic malignancies, and recipients of solid-organ and hematopoietic stem-cell transplants. There are continuous efforts to improve efficacy of commercial drugs for the treatment of these human mycoses, mainly invasive aspergillosis. Currently, voriconazole, posaconazole, liposomal amphotericin B, amphotericin B lipid complex and caspofungin are some of the antifungals that are used for antifungal therapy; combination therapy, that is, use of two antifungal compounds for treatment, is considered a promising future strategy for first-line treatment (Maschmeyer *et al.* 2007).

We report here another potential approach to improve efficacy of antifungal drugs. This approach is through use of safe,

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natural compounds that act as chemosensitizing agents. Such agents can render the fungus more vulnerable to certain types of drugs by targeting fungal stress response. A recently reported example of such natural product-targeting includes sulfated sterols from the marine sponge, *Topsentia* sp. These compounds were found to be efflux pump inhibitors that reversed efflux pump-based resistance in *Candida albicans* to the antifungal drug, fluconazole (DiGirolamo *et al.* 2009).

The oxidative stress response system of fungi is another particularly good potential target of antimicrobial agents (Smits & Brul 2005; Jaeger & Flohe 2006). It has already been shown that some natural compounds, such as derivatives of benzoic or cinnamic acids, can serve as potential alternatives to conventional antimicrobial agents (Tawata *et al.* 1996; Florianowicz 1998; Beekrum *et al.* 2003). Phenolic or sulfur-containing natural compounds can be redox-active and inhibit microbial growth by interfering with cellular redox homeostasis (Guillen & Evans 1994; Shvedova *et al.* 2000; Jacob 2006). Examples extend to diallyl disulfide (DADS), a constituent of garlic (*Allium sativum*). DADS treatment of the human pathogen *C. albicans* disrupts the yeast's cellular glutathione homeostasis. This disruption is caused by the depletion of thiol resulting in oxidative stress and impairment of normal mitochondrial activity (Lemar *et al.* 2007). In the model yeast *Saccharomyces cerevisiae*, genetic variation in glutathione biosynthesis was associated with sensitivity to pharmacologically active compounds (Kim & Fay 2007). We also observed that an aspirin metabolite, 2,5-dihydroxybenzoic acid, exerted its antifungal activity by disrupting fungal glutathione homeostasis (Kim *et al.* 2007).

Amphotericin B (AmB) is a clinical antifungal drug that disrupts ergosterol biosynthesis and, thus, the fungal membrane structure. In *C. albicans*, AmB not only disrupts the cell membrane but also induces toxicity through oxidative damage (Sokol-Anderson *et al.* 1986; Okamoto *et al.* 2004). *Aspergillus terreus*, a causative agent of human aspergillosis, has high *in vitro* Minimum Inhibitory Concentration (MIC) values and demonstrated poor *in vivo* response to AmB (Walsh *et al.* 2003). Interestingly, *A. terreus* has a higher catalase activity (Blum *et al.* 2008); catalase is an antioxidant enzyme decomposing hydrogen peroxide ($H_2O_2$) to water ($H_2O$) and $O_2$. *A. terreus* AmB resistance may result from its relatively higher catalase activity that effectively reduces oxidative stress.

In the study reported here, we identify a set of benzoic acid analogues as natural antifungal compounds. Some of these benzoic analogues possess enhanced antifungal activity when co-applied with thymol, a redox-active phenolic compound. Moreover, thymol itself is also an effective chemosensitizing agent in combination with the commercial antifungal drugs, fluconazole and ketoconazole; drugs that are normally ineffective against the aspergilli. The efficacy of using natural chemosensitizing agents to enhance control of fungal pathogens by conventional antifungal drugs is further discussed.

## Materials and methods

### Microorganisms

*Aspergillus fumigatus* AF293, wild type, and *A. fumigatus* mitogen-activated protein kinase (MAPK) deletion mutants *sak*AΔ and *mpk*CΔ (Xue *et al.* 2004; Reyes *et al.* 2006) were grown at 37 °C on potato dextrose agar (PDA). *Aspergillus terreus* UAB673, UAB680 and UAB698 were obtained from Centers for Disease Control and Prevention, Atlanta, GA, and were grown at 35 °C on PDA. Also, *Aspergillus flavus* NRRL3357, obtained from the National Center for Agricultural Utilization and Research, USDA-ARS, Peoria, IL, was grown at 28 °C on PDA. The differing incubation temperatures represent the optimum growth temperature for each fungal strain. *Saccharomyces cerevisiae* wild type BY4741 (*Mat* a *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) and selected deletion mutant (*glr1*Δ) were obtained from Invitrogen (Carlsbad, CA) and Open Biosystems (Huntsville, AL; www.yeastgenome.org).

### Chemicals

Thymol [5-methyl-2-(isopropyl)phenol], benzoic acid and 12 synthetic benzo analogues (*i.e.*, 2-, 3-, or 4-methyl, -methoxy, -chloro, -hydroxy benzoic acids), gallic acid and its esters (methyl, ethyl, propyl, octyl and decyl gallates), and antifungal drugs (fluconazole, ketoconazole and amphotericin B) were obtained from Sigma Co. (St. Louis, MO) or Pfaltz & Bauer Co. (for decyl gallate; Waterbury, CT). Each compound was dissolved in dimethylsulfoxide (DMSO; absolute DMSO amount: <2 % in media) before incorporation into media.

### Antifungal bioassays

#### Plate bioassay

Sensitivities of filamentous fungi to the analogues of benzoic acids were based on percent radial growth of treated compared to control colonies. The percent inhibition of growth was calculated using the Vincent equation (Vincent 1947), % inhibition $= 100(C - T)/C$; where $C =$ diameter of fungal colony on control plate (receiving only DMSO), and $T =$ diameter of fungal colony on the treated plate. MIC values were based on triplicate assays and defined as the lowest concentration of agent where no fungal growth was visible on the plate (see Table 1). For the above assays, *Aspergillus* conidia ($5 \times 10^3$) were diluted in phosphate buffered saline and spotted on the center of PDA plates with or without antifungal compounds. Growth was observed for 3–7 d.

To determine the combined effects of salt stress and 2,3-dihydroxybenzoic acid (2,3-DHBA), *Aspergillus flavus* was treated with 0, 0.3, 0.6 or 0.9 M sodium chloride (NaCl; Sigma Co.) and/or 0, 9, 12 or 15 mM 2,3-DHBA. Radial growth of fungal colonies was measured on PDA plates in triplicate and % inhibition of growth was determined using the Vincent equation.

Yeast dilution bioassays on plate were performed on the wild type and a glutathione reductase mutant (*glr1*Δ) to assess the effects of treatments on the oxidative stress response system. Yeast strains were exposed to 1 mM *tert*-butyl hydroperoxide (t-BuOOH; an organic peroxide) with or without gallate (20 mM). These assays were performed in triplicate on SG agar following previously described protocols (Kim *et al.* 2007).

#### Liquid bioassay

Levels and types of compound interactions (chemosensitization) between test benzoic analogues and drugs were based

**Table 1 – MICs (mM) of analogues of benzoic acid against various strains of species of *Aspergillus* that are etiologic agents of human aspergillosis. MICs based on radial growth assays on solid media.**

| Strains | Compd.[a] | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BA | 2-Me | 3-Me | 4-Me | 2-OMe | 3-OMe | 4-OMe | 2-Cl | 3-Cl | 4-Cl | 2-OH | 3-OH | 4-OH |
| *A. flavus* | | | | | | | | | | | | | |
| NRRL3357 | 5.0 | 4.5 | 3.0 | 2.0 | >10.0 | 6.0 | 3.5 | 10.0 | 3.0 | 2.0 | 7.0 | >10.0 | >10.0 |
| *A. fumigatus* | | | | | | | | | | | | | |
| AF293 | 3.5 | 3.0 | 2.0 | 1.5 | 8.0 | 4.5 | 2.5 | 8.0 | 2.5 | 2.0 | 6.0 | >10.0 | >10.0 |
| *sak*AΔ | 2.5 | 3.0[b] | 1.5 | 1.5 | 7.0 | 3.5 | 1.5 | 8.0 | 2.5[b] | 1.5 | 6.0 | >10.0 | >10.0 |
| *mpk*CΔ | 3.0 | 3.0 | 1.5 | 1.5 | 8.0 | 4.0 | 2.0 | 8.0 | 2.5 | 2.0 | 6.0 | >10.0 | >10.0 |
| *A. terreus* | | | | | | | | | | | | | |
| UAB673 | 6.0 | –[c] | – | 2.5 | – | – | 3.5 | – | – | 2.0 | 7.0 | – | – |
| UAB680 | 6.0 | – | – | 2.5 | – | – | 4.0 | – | – | 2.0 | 7.0 | – | – |
| UAB698 | 6.0 | – | – | 2.5 | – | – | 3.5 | – | – | 2.5 | 7.0 | – | – |
| Average MIC[d] (four fungi) | 3.50 | 3.38 | 2.00 | 1.63 | >8.25 | 4.5 | 2.38 | 8.5 | 2.63 | 1.88 | 6.25 | >10.0 | >10.0 |
| Average MIC[e] (seven fungi) | 4.57 | – | – | 2.00 | – | – | 2.93 | – | – | 2.00 | 6.57 | – | – |

a BA, benzoic acid; Me, methylbenzoic acid; OMe, methoxybenzoic acid; Cl, chlorobenzoic acid; OH, hydroxybenzoic acid.
b Smaller colony growth compared to AF293 (parental strain), indicating test compound is redox-active.
c –, not determined.
d Calculated from *A. flavus*, *A. fumigatus* AF293, *sak*AΔ and *mpk*CΔ strains.
e Calculated from *A. flavus*, *A. fumigatus* AF293, *sak*AΔ, *mpk*CΔ, *A. terreus* UAB673, UAB680 and UAB698 strains.

on Fractional Inhibitory Concentration Indices (FICIs) (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) (see Table 2). To determine antifungal MICs for use in calculating FICIs, triplicate assays were performed using a broth microdilution method published by the Clinical Laboratory Standards Institute (CLSI) M38-A (NCCLS 2002), as follows: RPMI 1640 medium (Sigma Co.) was supplemented with 2 % glucose and 0.03 % L-glutamine and buffered with 0.165 M 3-[N-morpholino] propanesulfonic acid ($5 \times 10^2$ conidia well$^{-1}$). Concentrations of test compounds used for chemosensitization assays were: fluconazole, ketoconazole – 0, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 (μg ml$^{-1}$), and thymol/benzo analogues – 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 (mM; millimolar).

## Results

Antifungal structure–activity relationships of benzoic acid analogues were investigated in two respects: changing types of substituents and their positions on the aromatic ring; and, changing lengths of alkyl groups esterified with the carboxyl group. In each modification, we focused on the effectiveness of structural analogues in targeting the cellular oxidative stress response system of fungi.

### *Changing types and positions of substituents on the aromatic ring*

The antifungal activities of 12 benzoic acid analogues (*i.e.*, positions 2-, 3-, or 4- of methyl, methoxyl, chloro, or hydroxyl substituents; see Fig 1) against *Aspergillus fumigatus* and *Aspergillus flavus* are shown in Table 1. These compounds exhibited differential levels of activity depending on structural variation. Antifungal activity increased when the position of either methyl, methoxyl, or chloro groups was moved from position 2 to 4 on the aromatic ring (*i.e.*, 4- > 3- > 2-; higher to lower activity). Alternatively, the trend in antifungal activity of hydroxybenzoic acid analogues was opposite to that of methyl, methoxyl, or chloro benzoic acids where antifungal activity of hydroxybenzoic acids increased as the hydroxyl group was moved from position 4 to 2 (*i.e.*, 2- > 3- > 4-; higher to lower activity). However, adding a hydroxyl group to the aromatic ring actually lowered antifungal activity relative to the unsubstituted benzoic acid (control). In contrast, antifungal activities of 4-methyl, 4-methoxy, or 4-chloro benzoic acids were higher than that of benzoic acid. In tests of the most potent benzo analogues in each structural category (*i.e.*, 4-methyl, 4-methoxy, 4-chloro, and 2-hydroxy benzoic acids) against strains of *Aspergillus terreus*, MIC values were similar to or slightly higher than those for *A. fumigatus* and *A. flavus*. These higher MIC values reflected a general relatively higher resistance level to these compounds of the *A. terreus* strains, compared to other aspergilli (see Table 1).

### *Benzo analogues target MAPK systems of fungi: overlapping roles of SakA and MpkC pathways*

We wanted to determine if the antifungal activity of these compounds resulted from negatively affecting fungal stress response systems, such as the antioxidation system (see Introduction). In order to test this as a potential mode of action for the benzo analogues, we examined the responses of two *Aspergillus fumigatus* MAPK mutants, *sak*AΔ and *mpk*CΔ. *A. fumigatus sak*AΔ is an osmotic/oxidative stress sensitive mutant, while *mpk*CΔ is a mutant of the polyalcohol sugar utilization system (Xue *et al.* 2004; Reyes *et al.* 2006).

Previous studies showed that SakA and MpkC MAPK signaling pathways are differentially utilized. For example,

**Table 2 – Ranges of MICs (based on cell growth in liquid culture) of three antifungal drugs (μg ml$^{-1}$), fluconazole, ketoconazole and amphotericin B, antifungal benzoic derivatives (mM), 4-methyl (4-MeBA), 4-chloro (4-ClBA) benzoic acids and octyl gallate (OcGal), tested alone or in combination with thymol (displayed below respective antifungal agent) against strains of *Aspergillus terreus*, *A. flavus* and *A. fumigatus*. Compound interactions are based upon FICI[a].**

| Compounds | MIC alone | MIC combined | FICI[a] | MIC: alone | MIC: combined | FICI | MIC: alone | MIC: combined | FICI |
|---|---|---|---|---|---|---|---|---|---|
| | *A. terreus* UAB698 | | | *A. terreus* UAB680 | | | *A. terreus* UAB673 | | |
| Amphotericin B | 2–4 | 0.25–0.5 | 0.63 A[b] | – | – | Antagonistic | – | – | Antagonistic |
| Thymol | 3.2–6.4 | 1.6–3.2 | | | | | | | |
| Fluconazole | >128[c] | 0–0.125 | 0.50 S | >128[c] | 1–2 | 0.26 S | >128[c] | 0.125–0.25 | 0.50 S |
| Thymol | 1.6–3.2 | 0.8–1.6 | | 3.2–6.4 | 0.8–1.6 | | 3.2–6.4 | 1.6–3.2 | |
| Ketoconazole | 2–4 | 0.125–0.25 | 0.56 A | 2–4 | 0.125–0.25 | 0.56 A | 2–4 | 0.125–0.25 | 0.56 A |
| Thymol | 1.6–3.2 | 0.8–1.6 | | 1.6–3.2 | 0.8–1.6 | | 1.6–3.2 | 0.8–1.6 | |
| 4-MeBA | 3.2–6.4 | 1.6–3.2 | 0.53 A | 3.2–6.4 | 1.6–3.2 | 0.56 A | 3.2–6.4 | 1.6–3.2 | 0.75 A |
| Thymol | 3.2–6.4 | 0.1–0.2 | | 6.4[d] | 0.4–0.8 | | 1.6–3.2 | 0.4–0.8 | |
| 4-ClBA | 3.2–6.4 | 1.6–3.2 | 0.63 A | 3.2–6.4 | 1.6–3.2 | 0.75 A | 3.2–6.4 | 0.8–1.6 | 0.50 S |
| Thymol | 0.8–1.6 | 0.1–0.2 | | 0.8–1.6 | 0.2–0.4 | | 1.6–3.2 | 0.4–0.8 | |
| OcGal | 0.1–0.2 | 0.05–0.1 | 0.63 A | 0.1–0.2 | 0.0–0.05 | 0.50 S | 0.05–0.1 | 0.0–0.05 | 0.75 A |
| Thymol | 0.8–1.6 | 0.1–0.2 | | 1.6–3.2 | 0.4–0.8 | | 1.6–3.2 | 0.4–0.8 | |
| | *A. flavus* 3357 | | | *A. fumigatus* AF293 | | | | | |
| Amphotericin B | 2–4 | 0.125–0.25 | 0.56 A | 2–4 | 0.125–0.25 | 0.56 A | | | |
| Thymol | 1.6–3.2 | 0.8–1.6 | | 3.2–6.4 | 1.6–3.2 | | | | |
| Fluconazole | >128[c] | >128[c] | 2.00 N | >128[c] | 0.125–0.25 | 0.50 S | | | |
| Thymol | 0.8–1.6 | 0.8–1.6 | | 0.8–1.6 | 0.4–0.8 | | | | |
| Ketoconazole | 4–8 | 4–8 | 2.00 N | 16–32 | 0.125–0.25 | 0.51 A | | | |
| Thymol | 0.8–1.6 | 0.8–1.6 | | 0.8–1.6 | 0.4–0.8 | | | | |
| 4-MeBA | 3.2–6.4 | 0.8–1.6 | 0.75 A | 3.2–6.4 | 0.1–0.2 | 0.53 A | | | |
| Thymol | 0.8–1.6 | 0.4–0.8 | | 0.8–1.6 | 0.4–0.8 | | | | |
| 4-ClBA | 3.2–6.4 | 0.8–1.6 | 0.75 A | >6.4[d] | 1.6–3.2 | 0.75 A | | | |
| Thymol | 0.8–1.6 | 0.4–0.8 | | 0.4–0.8 | 0.2–0.4 | | | | |
| OcGal | 0.1–0.2 | 0.05–0.1 | 0.63 A | 0.05–0.1 | 0.0–0.05 | 0.75 A | | | |
| Thymol | 0.8–1.6 | 0.1–0.2 | | 0.4–0.8 | 0.1–0.2 | | | | |

a Compound interactions were determined as described by Isenberg (1992) using the higher concentrations of the MIC ranges (mM for benzoic analogues; μg ml$^{-1}$ for antifungal drugs), as follows: synergistic – FICI ≤ 0.5; additive – 0.5 < FICI ≤ 1; neutral – 1 < FICI ≤ 2; antagonistic – 2 < FICI.

b A = additive; S = synergistic, N = neutral.

c MIC tests were performed up to 128 μg ml$^{-1}$ of fluconazole, 256 μg ml$^{-1}$ (doubling of 128 μg ml$^{-1}$) was used for FICI calculations.

d MIC tests were performed up to 6.4 mM of benzoic analogue, 12.8 mM (doubling of 6.4 mM) was used for FICI calculations.

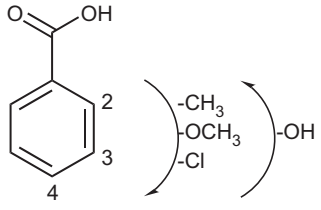


**Fig 1 – Benzoic acid and its structural analogues, *i.e.*, 2-methyl (2-$CH_3$), 3-methyl (3-$CH_3$), 4-methyl (4-$CH_3$), 2-methoxy (2-$OCH_3$), 3-methoxy (3-$OCH_3$), 4-methoxy (4-$OCH_3$), 2-chloro (2-Cl), 3-chloro (3-Cl), 4-chloro (4-Cl), 2-hydroxy (2-OH), 3-hydroxy (3-OH) or 4-hydroxy (4-OH) benzoic acid, used in this study. The direction of each arrow indicates the incremental increase of antifungal activity of benzo analogues as the positions of each residue change either from 2 to 4 (for methyl, methoxyl or chloro group) or 4 to 2 (for hydroxyl group) on the aromatic ring (see text).**

SakA-responsive conditions (oxidative stress, *etc.*) did not result in an observable phenotype for the *mpk*CΔ mutant (Xue *et al.* 2004; Reyes *et al.* 2006). Based on these observations, it was concluded that SakA and MpkC MAPK signaling pathways did not have overlapping roles.

However, in our present study, both *sak*AΔ and *mpk*CΔ mutants were more sensitive to certain benzo analogues compared to the wild type (Fig 2; Table 1). The heightened sensitivity of both of these mutants, relative to the wild type, indicated that the compounds may target the fungal MAPK system (*e.g.*, oxidative stress response). Thus, it appears SakA and MpkC pathways do have overlapping roles in responding to antifungal benzo analogues. Moreover, in addition to polyalcohol sugar utilization, MpkC may play a role in tolerance to and/or detoxification of redox-active compounds in *A. fumigatus*.

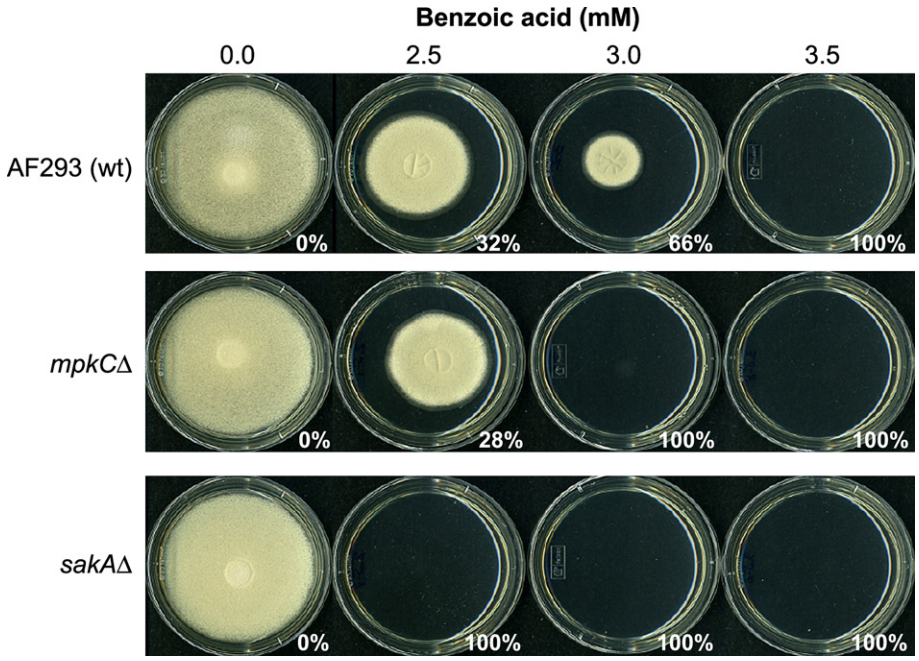


**Fig 2 – Exemplary plate bioassay showing MAPK mutants (*i.e.*, *sakA*Δ and *mpkC*Δ) of *A. fumigatus* were more sensitive to benzoic acid or its structural analogues (see text for detail), indicating SakA and MpkC MAPK pathways play overlapping roles in response to antifungal benzo analogues. Numbers in percent (%) indicate the rate of growth inhibition for each treatment based on the Vincent equation (see Materials and methods). SD < 5 % (triplicate for each treatment).**

To verify if benzoic acid derivatives markedly sensitize fungi to oxidative stress (and hence the oxidative stress-responsive MAPK system), we examined the effects on a different fungal system, the model yeast *Saccharomyces cerevisiae*, its wild type and *glr1*Δ mutant (glutathione reductase gene deletion). Gallate was used as a representative benzoate compound. As shown in Fig 3, both the wild type and *glr1*Δ strains exposed to the oxidative stress agent, *t*-BuOOH, exhibited increased growth inhibition by the addition of gallate. Of note is the fact that *glr1*Δ mutant showed higher sensitivity (~100 times) to gallate than the wild type. *GLR1* [an antioxidation gene maintaining cellular glutathione (GSH) homeostasis] is one of the genes regulated by the oxidative stress-responsive MAPK pathway in yeast (Rep *et al.* 2001). These results provide evidence that gallate derivatives can target and sensitize fungal antioxidation systems, such as that regulated by MAPK pathway genes *sakA* or *mpkC* in *A. fumigatus*.

We also examined whether benzoate derivatives have an effect consistent with that of osmotic stress. Both of these types of stresses, oxidative and osmotic, are known to dramatically affect MAPK signaling where salt/osmotic stress ultimately triggers oxidative stress in cells (Toone & Jones 1998). For this test, we included 2,3-dihydroxybenzoic acid (2,3-DHBA), a benzo analogue previously shown to affect cellular GSH homeostasis (Kim *et al.* 2008a). As shown in Fig 4, *Aspergillus flavus* stressed by salt (0.9 M of NaCl) showed 8 % growth inhibition. However, addition of 2,3-DHBA (12 mM; an oxidative stress agent) resulted in complete inhibition of fungal germination.

Collectively, these results indicate that benzoate derivatives target the oxidative stress response system in fungi. Moreover, it appears that targeting/sensitizing this cellular oxidative stress response system is applicable for a broad spectrum of benzo analogues.

### *Effects of alkyl chain length*

Changing lengths of the alkyl chain on antifungal efficacy was examined using gallic acid (3,4,5-trihydroxybenzoic acid) as

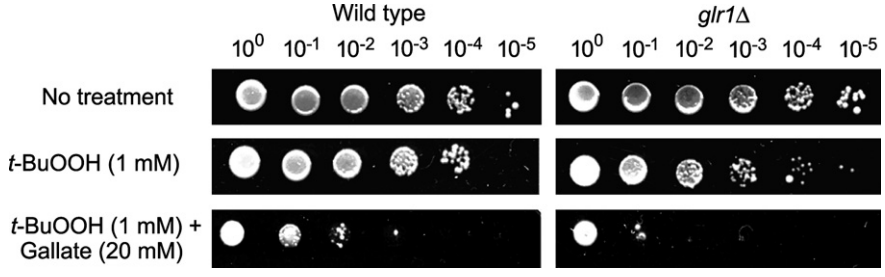


**Fig 3 – Exemplary yeast dilution bioassay showing gallate sensitizes fungi through antioxidation gene(s) regulated by the oxidative stress-responsive MAPK system (see text for detail). *t*-BuOOH: *tert*-butyl hydroperoxide.**

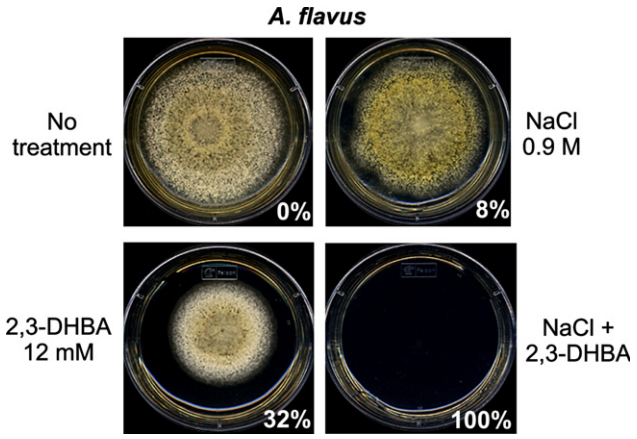


**Fig 4 – Plate bioassay showing 2,3-dihydroxybenzoic acid (2,3-DHBA; 12 mM) sensitizes salt (NaCl; 0.9 M)-stressed *A. flavus*. In cells, salt (osmotic) stress ultimately triggers oxidative stress (see Toone & Jones 1998). These results further indicated benzoate derivatives target cellular oxidative stress response system. Numbers in percent (%) indicate the rate of growth inhibition for each treatment based on the Vincent equation (see Materials and methods). SD < 5 % (triplicate for each treatment).**

the base benzo analogue. The derivatives tested were methyl, ethyl, propyl, octyl and decyl gallate (see Fig 5A). In this study, we observed that esterification with an octyl group greatly enhanced the antifungal activity of gallic acid against the aspergilli (*i.e.*, *Aspergillus fumigatus* AF293, *sakA*Δ, *mpkC*Δ; *Aspergillus flavus* 3357; *Aspergillus terreus* UAB673, UAB680, UAB698). The MIC range for octyl gallate was 0.1–0.2 mM. In comparison, the MIC for all other alkyl gallates (methyl, ethyl, propyl)

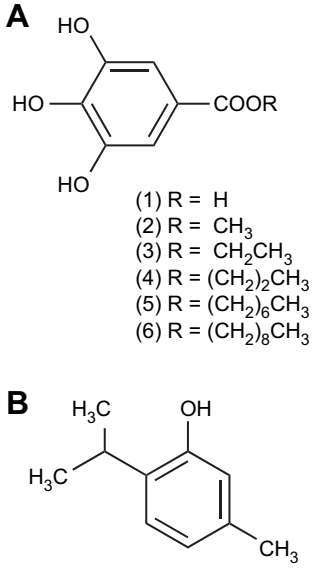


**Fig 5 – (A) Gallic acid and its structural analogues used in this study. (1) gallic acid, (2) methyl gallate, (3) ethyl gallate, (4) propyl gallate, (5) octyl gallate, (6) decyl gallate. (B) Chemical structure of thymol [5-methyl-2-(isopropyl)phenol] used in this study.**

exceeded >10 mM (tested in RPMI 1640 liquid medium; figure data not shown). Our attempts to test decyl gallate were unsuccessful since the compound precipitated in liquid medium at ≥0.3 mM (fungi grew at these concentrations), and thus no MIC could be calculated. After determining that octyl gallate was the most potent of the alkyl gallates, we selected it for antifungal chemosensitization tests (see below).

### *Enhancement of activities of antifungal agents by chemosensitization*

Chemosensitization involves enhancing the effectiveness of antifungal agents by co-applying a second compound. The second compound does not necessarily have much antifungal potency alone, but debilitates the ability of the fungus to launch a protective response to the antifungal agent (Niimi *et al.* 2004). We previously observed that thymol was redox-active chemosensitizing agent. In this case, strains of *Aspergillus fumigatus* having a mutation in their antioxidation system (*e.g.*, MAPK gene deletion) were more sensitive to this compound than the parental non-mutant strain (Kim *et al.* 2008b). Therefore, we reasoned that thymol could synergize redox-active benzo analogues for affecting common cellular targets, such as the antioxidation system of fungi. In the study presented here, we investigated the effect of chemosensitization of *A. fumigatus*, *Aspergillus flavus* and *Aspergillus terreus* by co-applying thymol (Fig 5B) with benzo analogues already observed to have antifungal activity (*viz.*, 4-methyl-, 4-methoxy- or 4-chloro benzoic acids and octyl gallate). We also tested chemosensitization with thymol in concert with conventional antifungal azole drugs, fluconazole, ketoconazole, and the polyene drug, amphotericin B.

Results showed that chemosensitization with thymol against aspergilli resulted in either an additive or synergistic interaction between thymol and most of the antifungal agents tested (see Table 2). This activity varied, depending upon the antifungal agent and the species or strain of *Aspergillus* tested. For example, synergistic interactions were observed when thymol was co-applied with either fluconazole for all strains of *A. terreus* and with *A. fumigatus*, or with octyl gallate against *A. terreus* UAB680, or 4-chloro benzoic acid against *A. terreus* UAB673 (Table 2). Additive effects were observed in most of the other combinations of thymol and antifungal agents among the aspergilli tested, with the exception of amphotericin B, where there was antagonism against *A. terreus* strains UAB680 and UAB673, discussed below (Table 2). Interestingly, the combination of thymol with 4-methylbenzoic acid was additive against all strains tested. However, except for an additive interaction (FICI = 0.625) in *A. fumigatus*, we observed only neutral effects of thymol with 4-methoxybenzoic acid (data not shown). Also, with *A. flavus*, no chemosensitizing effect, only a neutral interaction, was detected when thymol was co-applied with either azole drug (Table 2).

Co-application of thymol with amphotericin B, a drug theorized to act in part through oxidative mechanisms, produced an antagonistic effect when tested against *A. terreus* strains UAB680 and UAB673. Overall, there was a much lower sensitivity of these strains to amphotericin B, alone, possibly the result of increased catalase production. However, additive effects of thymol and amphotercin B were also observed in

*A. fumigatus*, *A. flavus* and *A. terreus* UAB698 strains. We do not know the reason for these differences at this time; more strains per species need to be tested to draw any conclusion from these observations. Also, since the compositions of media for the agar-based and broth assays were different, *i.e.*, PDA for agar assay and RPMI for broth assay, we were not able to directly compare the agar-based MIC (Table 1) and broth-based MIC (Table 2) in this study.

## Discussion

In this study, we show that certain natural compound(s) can be used as potent chemosensitizing agent(s) to enhance *in vitro* antimycotic activity of conventional antifungal agents and/or other antifungal phenolic compounds. As proof of concept, we present evidence that the *in vitro* activity of two azole drugs (fluconazole, ketoconazole that have limited or no activity against aspergilli) increases in combination with thymol against etiologic agents of human aspergillosis. Co-application of thymol with other benzo analogues also resulted in complete inhibition of fungal growth at much lower doses than the analogues, alone.

In the present study, we focused on the effectiveness of structural analogues in targeting the cellular antioxidation system of fungi. We also present some evidence for the basis of antifungal and/or chemosensitizing activity using various fungal deletion mutant bioassays. Modes of action for therapeutic compounds have been discovered previously by screening of yeast heterozygous deletion strains (Baetz *et al.* 2004; Lum *et al.* 2004; Parsons *et al.* 2004). Use of such deletion mutant screening can also assist in determining structure−activity relationships of antifungal compounds. For example, it was previously shown that gallic acid alkyl esters (*e.g.*, octyl gallate) reduce fluidity and/or inhibit H(+)-ATPase of the fungal plasma membrane, resulting in inhibition of fungal growth (Fujita & Kubo 2002). However, in our examination of *Saccharomyces cerevisiae* mutant strains, we showed that gallate derivatives target and sensitize fungal antioxidation systems (see Fig 3). We found that the deletion mutants of *S. cerevisiae*, *sod2*Δ [mitochondrial superoxide dismutase (Mn-SOD) gene deletion], and *Aspergillus fumigatus*, *sak*AΔ (osmotic/oxidative stress sensitive), are more sensitive to alkyl gallates (*e.g.*, methyl, ethyl or octyl gallate) than respective wild types (unpublished results). Like the *GLR1* (see Fig 3), the Mn-SOD gene is also downstream in the yeast *HOG1* (MAPK) oxidative stress signaling pathway (Boy-Marcotte *et al.* 1998; Rep *et al.* 2000, 2001). Collectively, these results indicate that gallate derivatives can target fungal antioxidation systems, such as MAPK pathway-regulated genes in *A. fumigatus*. Thus, the model yeast has served as a useful tool for screening therapeutic compounds by determining mode of action.

Our results showed that addition of a methyl, methoxy, or chloro group at position 4 ( *para*) of the aromatic ring increased the antifungal activity of a benzoic acid parent structure (Table 1). This did not occur if this position was substituted with a hydroxyl group. Moreover, both *sak*AΔ and *mpk*CΔ mutants of *A. fumigatus* were more sensitive to these compounds, compared to the wild type (Fig 2; Table 1). The greater sensitivity of these mutants suggests these compounds target the fungal MAPK system involved in cellular oxidative stress response. Future study is warranted, however, to further investigate the specific mode of antifungal action of benzoic acid analogues. Our study only monitored interactions after 48 h of incubation. Interaction levels, especially for those regarding fungicidal effects, require further investigation.

Of note is the fact that phenolic analogues can also be modified to target MAPK pathways involved in other cellular system(s). In a prior study, esterification of alkyl side chains to another phenolic compound, dihydroferulic acid (DFA), resulted in enhancement of antifungal activity of DFA (Beck *et al.* 2007). In *S. cerevisiae*, the *SLT2* and *BCK1* genes are involved in the signal transduction pathway for cell wall construction/integrity. The *slt2*Δ strain, which lacks the MAPK gene, and *bck1*Δ strain, lacking the MAPK kinase kinase (MAPKKK) gene in cell wall construction, were sensitive to the DFA propyl ester compared to the wild type strain. This study is another example where use of deletion mutants provides evidence of mode of action, in this case that the phenolic ester of DFA targets other genes in the MAPK pathway than those shown in our current study. Therefore, the comparative genomic approach we present here is applicable for the future identification of antimicrobial natural compounds, chemosensitizers as well as their cellular targets, among related fungi.

In summary, we presented in this study the potential for using natural compounds and their analogues as chemosensitizing agents. Such agents can enhance activity, lower resistance, and alleviate health and environmental risks by reducing amounts of commercial antifungal agents required to achieve effective therapy. Further *in vivo* studies are necessary to determine if the *in vitro* activities demonstrated herein can translate to *in vivo* treatment efficacy and safety.

## Disclaimer

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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