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Chemosensitization prevents tolerance of *Aspergillus fumigatus* to antimycotic drugs

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ABSTRACT

Tolerance of human pathogenic fungi to antifungal drugs is an emerging medical problem. We show how strains of the causative agent of human aspergillosis, *Aspergillus fumigatus,* tolerant to cell wall-interfering antimycotic drugs become susceptible through chemosensitization by natural compounds. Tolerance of the *A. fumigatus* mitogen-activated protein kinase (MAPK) mutant, *sakA*Δ, to these drugs indicates the osmotic/oxidative stress MAPK pathway is involved in maintaining cell wall integrity. Using deletion mutants of the yeast, *Saccharomyces cerevisiae*, we first identified thymol and 2,3-dihydroxybenzaldehyde (2,3-D) as potent chemosensitizing agents that target the cell wall. We then used these chemosensitizing agents to act as synergists to commercial antifungal drugs against tolerant strains of *A. fumigatus*. Thymol was an especially potent chemosensitizing agent for amphotericin B, fluconazole or ketoconazole. The potential use of natural, safe chemosensitizing agents in antifungal chemotherapy of human mycoses as an alternative to combination therapy is discussed.

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Signal transduction pathways of fungi play an important role in susceptibility to antifungal agents. Functionally intact signaling pathways, such as mitogen-activated protein kinase (MAPK) pathways, are required to achieve biocidal activity for certain antifungal agents. For example, *HOG1* or *PBS2* are MAPK and MAPK kinase (MAPKK) genes in the *HOG* (high osmolarity glycerol) pathway of baker's yeast *Saccharomyces cerevisiae*, an osmotic/oxidative stress-responsive MAPK system. Strains of *S. cerevisiae* having mutations in these genes are tolerant to cell wall-interfering agents, such as calcofluor white (CW) [1–3]. Interference of the upstream osmosensors *SHO1* (transmembrane osmosensor) or *SLN1* (histidine kinase osmosensor), of the same pathway, also result in partial tolerance to CW, indicating fungal susceptibility to CW is dependent on the uninterrupted *HOG* signaling system [1–3]. These types of tolerances also apply to the human pathogenic yeast, *Candida albicans* [4]. Also, MAPK mutants of a variety of fungal species have higher tolerance to fludioxonil, a phenylpyrrole fungicide that disrupts cellular glycerol biosynthesis [5,6]. Collectively, these studies indicate gene mutations in MAPK signal transduction pathways can result in tolerance to antifungal agents.

Disruption of cell wall or membrane integrity facilitates control of pathogens by antifungal drugs [7]. The cell wall integrity pathway is well characterized in *S. cerevisiae*. It is controlled by protein kinase C (PKC) and the downstream cell wall integrity-specific MAPK pathway [8]. *In silico* reconstruction studies in three species of *Aspergillus* (*i.e.*, *A. nidulans*, *A. oryzae*, and *A. fumigatus*) reveal that genes in the cell wall integrity pathway in *Aspergillus* and *S. cerevisiae* are well conserved [9]. Also, like *S. cerevisiae slt2*Δ (a cell wall integrity MAPK mutant), *A. nidulans mpkA*Δ (equivalent to *S. cerevisiae slt2*Δ) is hypersensitive to CW [9].

Here, we use *S. cerevisiae* mutants in which genes in MAPK or PKC signaling systems were systematically deleted. This screening process allowed us to identify several natural compounds as chemosensitizing agents when combined with commercial antimycotic agents that disrupt cell wall or membrane integrity. Co-application of thymol completely reversed tolerance of *A. fumigatus* to such agents.

Materials and methods

Microorganisms. Saccharomyces cerevisiae wild type BY4741 (*Mat* a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and selected deletion mutants lacking genes in five different MAPK and PKC signaling systems were obtained from Invitrogen (Carlsbad, CA) and Open Biosystems (Huntsville, AL), as follows: (1) osmoregulation: *hog1*Δ (MAPK), *hog4*Δ (MAPKK; Scaffold activity), *ssk22*Δ (MAPKK kinase; MAPKKK), *ssk2*Δ (MAPKKK), *ste11*Δ (MAPKKK), (2) Cell wall construction/integrity: *slt2*Δ (MAPK), *mkk1*Δ (MAPKK), *mkk2*Δ (MAPKK), *bck1*Δ (MAPKKK), *wsc1*Δ (Sensor-transducer), *mid2*Δ (Sensor), *kss1*Δ (MAPK), *ste7*Δ (MAPKK), *ste11*Δ (MAPKKK), (3) Morphological switch: *kss1*Δ (MAPK), *ste7*Δ (MAPKK), *ste11*Δ (MAPKKK), (4) Mat-

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ing response: *fus3*Δ (MAPK), **ste7*Δ (MAPKK), **ste11*Δ (MAPKKK), *ste5*Δ (Scaffold protein), *ste2*Δ ("alpha" factor receptor), *ste3*Δ ("a" factor receptor), (5) Sporulation: *smk1*Δ (MAPK), (6) PKC signaling pathway: *rom2*Δ (GDP/GTP exchange protein), *fks1*Δ (β-1,3-*D*-glucan synthase), *fks2*Δ (β-1,3-*D*-glucan synthase), *rlm1*Δ (Transcription factor), *swi4*Δ (Transcription factor), *pkc1*Δ (Protein serine/threonine kinase; diploid), where the asterisk (*) indicates overlapping biological roles of the marked gene in more than one pathway (Reference: www.yeastgenome.org, Accessed on March 27, 2008). 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) at 30 °C. *A. fumigatus* AF293, wild type, and *A. fumigatus* MAPK deletion mutants *sakA*Δ and *mpkC*Δ [10,11] were grown at 37 °C on potato dextrose agar (PDA) medium.

Chemicals. The cell wall-interfering agents [Congo red (CR) and CW] and chemosensitizers (2,3- and 2,5-dihydroxybenzaldehyde; benzoic, salicylic, acetyl salicylic, 2,5-dihydroxybenzoic, cinnamic, *o*-coumaric acids; thymol; berberine hemisulfate) were purchased from Sigma Co. (St. Louis, MO). Each compound was dissolved in dimethylsulfoxide (DMSO; absolute DMSO amount: <2% in media) except CR, CW, and berberine, which were dissolved in water.

Antifungal bioassays. Yeast cell-dilution bioassays were performed on SG agar to examine activity of antifungal compounds following protocols outlined [6]. Sensitivities of filamentous fungi were based on percent radial growth of treated compared to control colonies, receiving only DMSO [Vincent equation: % inhibition = 100 (C − T)/C, C, diameter of fungi on control plate; T, diameter of fungi on the test plate] [12]. Fungi (5×10^3 spores) 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 days. For testing the chemosensitizing effects of thymol or 2,3-dihydroxybenzaldehyde (2,3-D), each compound was incorporated into the growth medium together with cell wall-interfering agents, and radial growth was recorded as described above. Antifungal MICs (minimum inhibitory concentrations; defined as concentrations with no visible fungal growth) and chemosensitization resulting from interactions between drug and natural compounds were also determined by the broth microdilution method in RPMI 1640 medium (Sigma Co.), supplemented with 2% glucose and 0.03% L-glutamine and buffered with 0.165 M 3-[*N*-morpholino] propanesulfonic acid (5×10^3 conidia/ml inoculum) following the methodology published by the Clinical Laboratory Standards Institute M38-A [13].

Growth recovery bioassay. To test the effect of antifungal agents on cell membrane integrity, sorbitol recovery bioassays were conducted (See [14] for method). Tenfold diluted (See "Antifungal bioassays" above) strains of *S. cerevisiae* BY4741, *bck1*Δ, and *slt2*Δ were spotted on (1) SG only, (2) SG + caffeine (5 mM), thymol (0.4 mM) or 2,3-D (0.02 mM) (Testing sensitivity of *bck1*Δ and *slt2*Δ mutants to the compounds), and (3) SG + sorbitol (0.5 M) + caffeine, thymol or 2,3-D (Testing recovery of *bck1*Δ and *slt2*Δ mutants, by sorbitol treatment, from sensitivity to antifungals). Cell growth was monitored for 5–7 days. If the growth score on the sorbitol-containing medium was higher than that on the "no sorbitol" medium, the test compounds were considered to affect cell membrane integrity.

Results and discussion

Tolerence of A. fumigatus sakA∆ to cell wall-interfering agents

We tested responses of two MAPK mutants, *sakA*Δ and *mpkC*Δ, of *A. fumigatus* to commercial agents that disrupt cell wall integrity. *A. fumigatus sakA*Δ is an osmotic stress-sensitive mutant, while *mpkC*Δ is a mutant of the poly-alcohol sugar utilization system [10,11]. In plate bioassays, *A. fumigatus sakA*Δ was more tolerant to CR (40% growth inhibition at 1.0 mg/ml) or CW (30% growth inhibition at 1.4 mg/ml) compared to the wild type (CR: 60% or CW: 48%) or *mpkC*Δ (CR: 64% or CW: 50%) (Fig. 1). This greater tolerance indicated *A. fumigatus* SakA, like *C. albicans* Hog1, plays a role in maintaining cell wall integrity. These results also indicate a functional and continuous SakA signaling system is necessary for susceptibility to antifungal agents that interfere with cell wall integrity.

Screening natural chemosensitizers using yeast mutants sensitive to cell wall-interfering agents

Use of *S. cerevisiae* deletion mutants can serve as a model fungal system to identify gene-targets of chemicals or drugs [15]. In this study, we used 24 mutants of *S. cerevisiae* lacking key genes in five different MAPK and PKC signaling pathways, and determined their susceptibilities to cell wall-interfering agents. We observed that four mutants, *i.e.*, *slt2*Δ, *bck1*Δ, *wsc1*Δ, and *swi4*Δ, were especially sensitive (∼10–100 times more) to CR/CW compared to the wild type or other mutants (Fig. 2A). Results confirmed that the cell wall integrity MAPK system plays a more critical role for fungal susceptibility to CR/CW than other MAPK signaling pathways. Because of their greater sensitivity to CR/CW, these four mutants were used to screen potential chemosensitizing agents.

Using *slt2*Δ, *bck1*Δ, *wsc1*Δ, and *swi4*Δ mutants of *S. cerevisiae*, identified above, we examined the antibiotic activity of (1) benzaldehyde analogs: 2,3-D (0.015 mM), 2,5-dihydroxybenzaldehyde (1.2 mM), benzoic acid (1.5 mM), salicylic acid (1.6 mM), acetyl salicylic acid (3 mM), 2,5-dihydroxybenzoic acid (10 mM), (2) cinnamic acid analogs: cinnamic acid (0.4 mM), *o*-coumaric acid (5 mM), and (3) other natural compounds: thymol (0.8 mM) and berberine (3.5 mM). All compounds are phenolic derivatives except berberine, an alkaloid. Safe natural compounds have potential to serve as an alternative to conventional antimicrobial agents [16,17]. We found four compounds, thymol, 2,3-D, *o*-coumaric acid, and berberine, were most effective as antifungal agents in yeast. The *slt2*Δ and *bck1*Δ mutants showed highest sensitivity (10^3–10^4 times higher) compared to wild type or *wsc1*Δ/*swi4*Δ strains (Fig. 2B; data not shown for *wsc1*Δ and *swi4*Δ).

Chemosensitization to cell wall- and cell membrane-interfering agents

Combined treatment of thymol (0.5 mM) with CR (0.1 and 0.5 mg/ml) resulted in 10–10^4 times more reduction in yeast cell propagation compared to the compounds, individually (Fig. 2C). We next tested the chemosensitizing capacity of the identified compounds to CR or CW on pathogenic *A. fumigatus*. When thymol (1.0 mM) or 2,3-D (0.2 mM) was co-applied with CR (0.4–1.0 mg/ml), the growth of *A. fumigatus* strains was almost completely inhibited (Fig. 3A and D-a). In contrast, no chemosensitizing effect was observed with co-application of *o*-coumaric acid or berberine with CR or CW (data not shown). Growth of *A. fumigatus* was completely inhibited by co-application of thymol (1 mM) with CW (*sakA*Δ: at 0.4 mg/ml; AF293 and *mpkC*Δ: at 0.6 mg/ml; Fig. 3B). Thus, the chemosensitizing effect of both natural products resulted in complete prevention of *sakA*Δ tolerance to cell wall-interfering agents. In fact, *A. fumigatus sakA*Δ was more sensitive to sole treatment of thymol (*i.e.*, >20% more reduction in radial growth at 0.6 mM; Fig. 3C) or 2,3-D (*i.e.*, >20% more reduction in radial growth at 0.2 mM; Figure data not shown) compared to the wild type or *mpkC*Δ.

Thymol (0.2–1.0 mM) was co-applied with 2,3-D (0.2–0.5 mM) to determine synergistic antimycotic effects. This co-application resulted in complete growth inhibition of *A. fumigatus* (Fig. 3D-

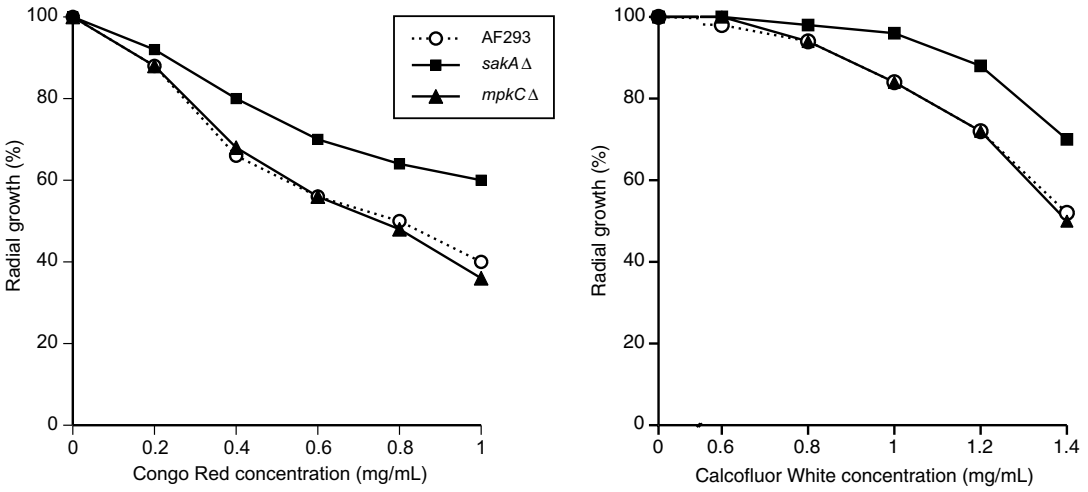

Fig. 1. Graph showing mean radial growth of strains of *A. fumigatus* after treatment with increasing concentrations of Congo red (CR) and calcofluor white (CW). Growth of the *sakA*Δ mutant showed higher tolerance to CR and CW than AF293 (wild type) or the *mpkC*Δ mutant. Values are mean of triplicates. All standard deviations of means <5%.

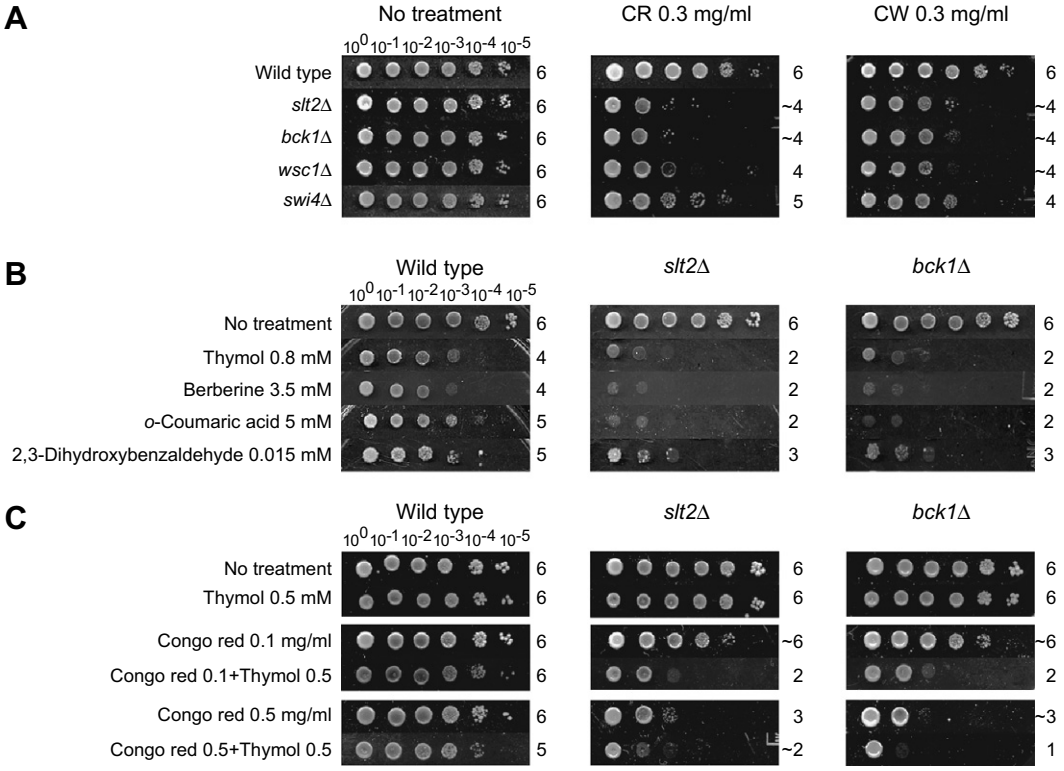

Fig. 2. Yeast cell-dilution bioassays showing levels of sensitivity of strains of *S. cerevisiae*, *slt2*Δ, *bck1*Δ, *wsc1*Δ, and *swi4*Δ to: (A) Congo red (CR) and calcofluor white (CW). Sensitivities of mutants were ~10–100 times higher to CR (0.3 mg/ml) and CW (0.3 mg/ml) compared to the wild type strain; (B) thymol, berberine, *o*-coumaric acid, and 2,3-dihydroxybenzaldehyde. *S. cerevisiae slt2*Δ and *bck1*Δ mutants showed highest sensitivity (10^3–10^4 times) to thymol, 2,3-dihydroxybenzaldehyde, *o*-coumaric acid, and berberine compared to the wild type or *wsc1*Δ/*swi4*Δ strains (*wsc1*Δ/*swi4*Δ data not shown); (C) thymol (0.5 mM) or CR (0.1 or 0.5 mg/ml), alone, or in combination. Thymol produced a chemosensitizing effect (~10–10^4 times higher sensitivity) when co-applied with CR compared to treatment of each compound, alone.

b). A similar result also occurred with other filamentous fungi examined, including *A. flavus* and *Penicillium expansum* (data not shown). These elevations in antimycotic potency resulting from co-application of these two compounds suggest they target common cellular components.

Thymol is known to disrupt cell membrane integrity by forming lesions and by reducing ergosterol content [18]. Therefore, we examined the chemosensitizing effect of thymol to azole, fluconazole and ketoconazole, or a polyene, amphotericin B, drugs affecting cell membrane integrity. As shown in Table 1, application of thymol resulted in chemosensitization for all drugs tested. The FICs (Fractional Inhibitory Concentrations) of these co-applications defined the interactions as "additive", close to "synergistic" (Table 1). This further indicated thymol functions as a chemosensitizing agent to both cell wall- and cell membrane-interfering agents. However, 2,3-D did not have obvious chemosensitizing activity with azole or polyene drugs, as found with thymol (data not shown).

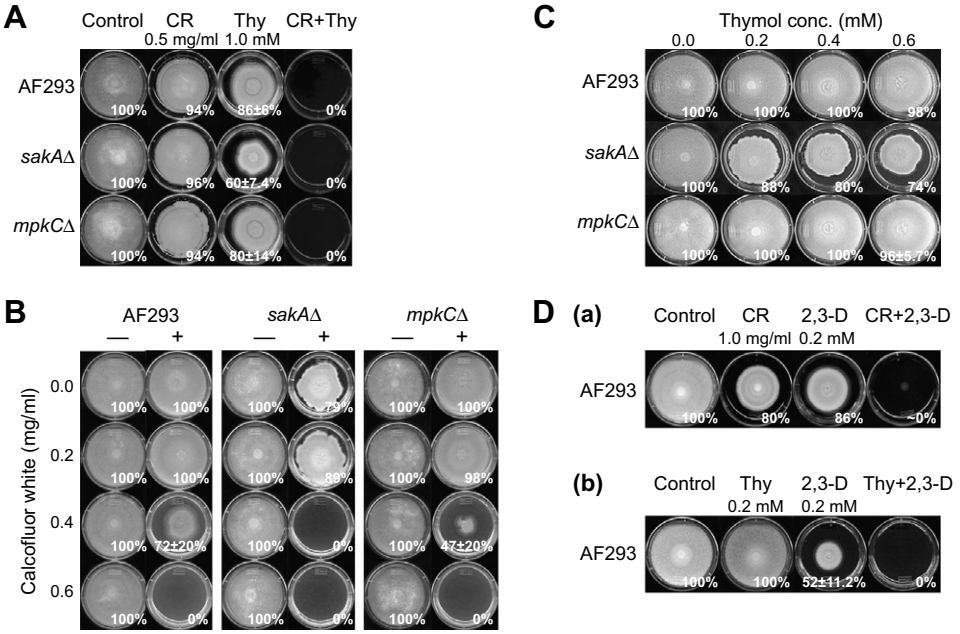

Fig. 3. Growth of AF293, *sakA*Δ, and *mpkC*Δ strains of *A. fumigatus* to treatments with thymol, 2,3-dihydroxybenzaldehyde (2,3-D), Congo red (CR), and/or calcofluor white (CW). (A) CR, thymol and co-application of CR and thymol. Results shown are with 0.5 mg/ml CR with 1.0 mM thymol showing ~95–100% enhancement of growth inhibition than CR, alone. Co-applications with 2,3-D (0.2 mM) and CR (1.0 mg/ml) show similarly enhanced antifungal activity (See D-a); (B) application of CW (0.0–0.6 mg/ml) with (+) or without (−) thymol (1.0 mM), showing 100% growth inhibition of all strains at 0.6 mg/ml CW and thymol; (C) *A. fumigatus sakA*Δ mutant showed higher (12–24%) sensitivity to thymol compared to AF293 or *mpkC*Δ mutant; (D) (a) enhanced antifungal activity of co-application of CR (1.0 mg/ml) and 2,3-D (0.2 mM) against *A. fumigatus* (no germination), (b) co-application of thymol (0.2–1.0 mM) with 2,3-D (0.2–0.5 mM) completely inhibit growth of AF293, indicating these two compounds target common cellular components. Results shown are with 0.2 mM of thymol and 2,3-D.

Table 1
Compound interactions between thymol and antifungal drugs tested on *A. fumigatus* AF293

Compounds	MIC: agent alone	MIC: agent combined	FIC[a]
Ketoconazole	$2 < n_1 < 4$[b]	$0.125 < n_3 < 0.25$[d]	0.563
	$0.8 < n_2 < 1.6$[c]	$0.4 < n_4 < 0.8$[e]	Additive
Fluconazole	$32 < n_1 < 64$	$2 < n_3 < 4$	0.563
	$0.8 < n_2 < 1.6$	$0.4 < n_4 < 0.8$	Additive
Amphotericin B	$1 < n_1 < 2$	$0.125 < n_3 < 0.25$	0.625
	$0.8 < n_2 < 1.6$	$0.4 < n_4 < 0.8$	Additive

[a] For calculation purposes, the higher concentration of n_1–n_4 in each column was used. Compound interactions were determined as described by Isenberg [30] as follows: FICs = (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: synergistic (FIC index $\leqslant 0.5$), additive ($0.5 <$ FIC index $\leqslant 1$), neutral ($1 <$ FIC index $\leqslant 2$) or antagonistic ($2 <$ FIC index).
[b] n_1, MIC (μg/ml) of drug alone.
[c] n_2, MIC (mM) of thymol alone.
[d] n_3, MIC (μg/ml) of drug in combination with thymol.
[e] n_4, MIC (mM) of thymol in combination with drug.

Remediation of slt2Δ and bck1Δ mutants to chemosensitizing agents by sorbitol: evidence for disrupting cell membrane integrity

Caffeine promotes disorganization of cell membranes. Fungal mutants having abnormalities in cell surface integrity are frequently sensitive to caffeine [19]. Caffeine also activates the PKC pathway [20]. Importantly, caffeine sensitivity of *slt2*Δ and *bck1*Δ was remedied by treatment with sorbitol [14,20].

In our test, sensitivity of *slt2*Δ and *bck1*Δ to thymol or 2,3-D was also remedied by sorbitol. The growth rate of *slt2*Δ and *bck1*Δ on sorbitol-containing media was ~10–100 times higher compared to controls without sorbitol (Fig. 4A for thymol; 2,3-D: 10 times, data not shown). This remediation strongly indicates disruption of cell wall/membrane integrity is one mechanism of action of how these compounds generate chemosensitization.

The results presented in this study indicate certain phenolic derivatives can serve as natural chemosensitizers to augment activity of commercial antifungal agents, especially ones disrupting the fungal cell wall or membrane. We identified *A. fumigatus sakA*Δ was tolerant to such cell wall-interfering agents. This tolerance indicates, like in yeasts [3], constitutive functionality of the osmotic/oxidative stress-responsive MAPK (SakA) is necessary for maintaining cell wall integrity. Previous studies showed that fungal mutants having lower levels of chitin in their cell walls were more resistant to CR/CW [21,22]. Thus, increased chitin levels in fungal cell walls may actually promote CR/CW hypersensitivity [22,23]. Levels of cell wall glucans also appear to be an important factor for fungal susceptibility to cell wall-interfering agents. For example, the *S. cerevisiae pbs2*Δ (MAPKK mutant) possessing lower glucan synthase activity is tolerant to CW [2]. The genes for chitin or glucan biosynthesis are identified in *A. fumigatus* [24,25]. Further, biochemical and genetic studies involving these genes could elucidate mechanism(s) of fungal tolerance to cell wall-interfering agents, especially in the *sakA*Δ mutant.

Thymol is known to affect integrity of the cell membrane [18]. Since cell membranes also contain cell wall proteins [26], treatment of fungi with thymol results in disrupting the normal architecture of the cell wall and membrane. As shown in our study, co-application of thymol with CR/CW, azoles or polyene drugs resulted in complete inhibition of fungal growth at much lower doses than the drugs, alone. Many phenolics such as 2,3-D are promising antifungal agents, or lead compounds, for disrupting fungal oxidative stress response systems [27,28]. In our study, 2,3-D served as a chemosensitizing agent to CR/CW. However, it did not provide chemosensitizing activity with either azole or polyene drugs. This result indicates, although 2,3-D and thymol can synergize each other (Fig. 3D-b), 2,3-D may function differently when co-applied with cell membrane-interfering agents. Therefore, specificity regarding compound interactions must be considered to achieve optimum control of fungi.

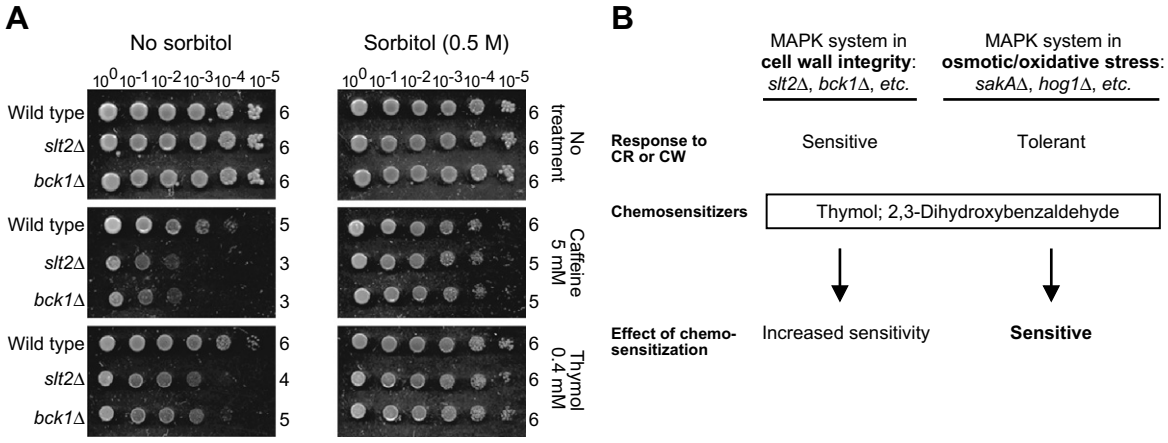

Fig. 4. (A) Yeast cell-dilution bioassay showing sensitivity of *A. fumigatus slt2*Δ and *bck1*Δ mutants to caffeine and thymol was remediated by sorbitol. Similar remediation by sorbitol was found from treatment with 2,3-dihydroxybenzaldehyde (results not shown). (B) Diagram summarizing results of chemosensitization effects of thymol or 2,3-dihydroxybenzaldehyde in enhancing the antifungal activity of cell wall-interfering agents and prevention of fungal tolerance to CR/CW (*e.g.*, *sakA*Δ, *hog1*Δ, *etc.*).

Combination therapy involving cercosporamide, a Pkc1 kinase inhibitor, and an echinocandin analog, a β-1,3-glucan synthase inhibitor, has shown these compounds to have highly synergistic antimycotic activities [29]. Similarly, we presented in our study, how chemosensitization that targets cell wall integrity prevents development of fungal tolerance to conventional drugs.

In summary, cell wall/membrane integrity pathways can be molecular targets for chemosensitization by phenolic agents to enhance antimycotic activity of commercial antifungal agents. Combined application of phenolics with cell wall/membrane-interfering agents can effectively suppress fungal growth or circumvent fungal tolerance to drugs (Fig. 4B).

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