

Overcoming Antifungal Drug Resistance Using Chemosensitization: Targeting Stress Response Pathways of Fungi with Benzo Analogs

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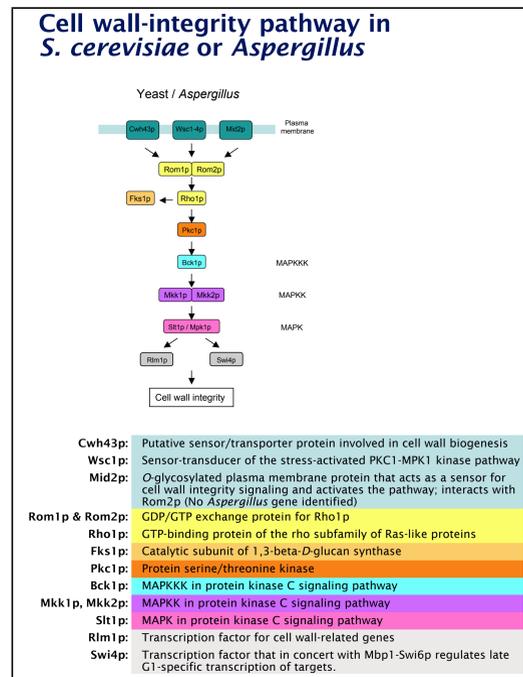
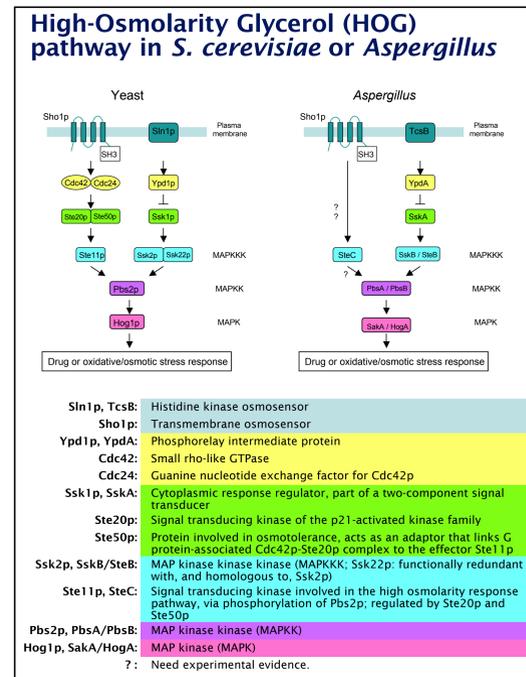
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Purpose of the study

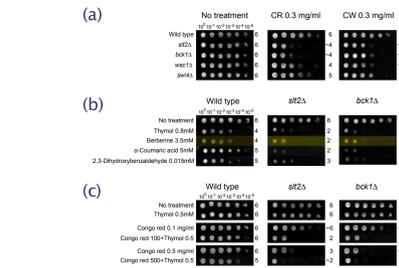
The clinical treatment of infectious fungi is rapidly becoming a major problem as a consequence of the continuous emergence of fungal strains resistant to antifungal drugs. Counteracting this problem with higher dosage levels or combination therapy presents potential risks involving adverse drug sequelae. Historically, the development of drug resistance in fungi has required an unremitting search for new drugs. Here, we present a promising approach wherein a resistant fungal pathogen can be rendered susceptible to antifungal agents through chemosensitization.

Description of the project

Fungal oxidative stress response pathways protect fungal cells from reactive oxygen species (ROS) generated by the host defense-reaction to infection. We chose to target genes in this pathway using safe, natural benzo analogs. For example, fungal tolerance to 2,3-dihydroxybenzaldehyde or its acid analog may rely upon genes encoding mitochondrial superoxide dismutase (Sod2p; Mn-SOD) or glutathione reductase (Glr1p). These genes are regulated by *HOG1*, a mitogen-activated protein kinase signaling pathway gene (MAPK) in *Saccharomyces cerevisiae*. These phenolic compounds could, thus, serve as chemosensitizing agents that weaken the ability of fungi to launch protective responses to conventional antifungal agents and could significantly elevate their sensitivity to commercial antifungal agents.

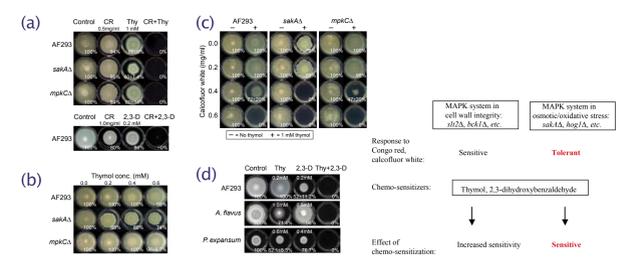


Targeting the cell wall/membrane integrity pathway



(a) Mutant strains of *S. cerevisiae*, i.e., *slt2Δ*, *bck1Δ*, *wsc1Δ* (in cell wall construction/integrity pathway) and *swi4Δ* (PKC-signaling pathway), were sensitive (~10 to 100 times) to Congo red (0.3 mg/ml) and calcofluor white (0.3 mg/ml) compared to the wild type. (b) *S. cerevisiae* *slt2Δ* and *bck1Δ* mutants showed the highest sensitivity (10³ to 10⁴ times) to thymol, 2,3-dihydroxybenzaldehyde, *o*-cumaric acid and berberine compared to the wild type or *wsc1Δ/swi4Δ* strains (*wsc1Δ/swi4Δ* data not shown). (c) Thymol showed a chemosensitizing effect to Congo red, where co-application of thymol (0.5 mM) and Congo red (0.1 and 0.5 mg/ml) resulted in ~10 to 10⁴ times higher sensitivity of wild type, *slt2Δ* and *bck1Δ* mutants compared to the independent treatment of each compound.

Chemosensitization to cell wall-interfering agents by thymol



(a) Co-application of Congo red (0.4 to 1.0 mg/ml) with thymol (1.0 mM) or 2,3-dihydroxybenzaldehyde (0.2 mM) enhanced its antifungal activity against *A. fumigatus* (i.e., ~95% to 100% growth inhibition). (b) *A. fumigatus* *sakAΔ* mutant showed higher (12 to 24%) sensitivity to thymol compared to the wild type or *mpkCΔ* mutant. (c) Co-application of calcofluor white (0.2 to 0.6 mg/ml) with thymol (1.0 mM) achieved 100% growth inhibition of *A. fumigatus* AF293, *sakAΔ* or *mpkCΔ* strains. (d) Co-application of thymol (0.2 to 1.0 mM) with 2,3-dihydroxybenzaldehyde (0.2 to 0.5 mM) completely inhibited the growth of *A. fumigatus* AF293. *A. flavus* 3357 and *Penicillium expansum* 974, indicating these two compounds affect common cellular targets in fungi.

Results

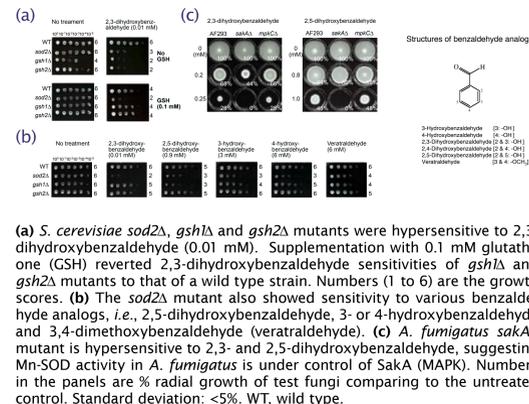
We examined the responses of MAPK deletion mutants of *sakA* and *mpkC*, derived from *Aspergillus fumigatus* AF293 (wild type), to benzo analogs. *SakA* and *MpkC* are orthologs of *Hog1p* of *S. cerevisiae*. Growth of AF293 and the *mpkC* deletion mutant was inhibited 32% and 72% with 0.20 to 0.25 mM 2,3-dihydroxybenzaldehyde, respectively. The *sakA* deletion mutant was more sensitive, showing 56 to 100% growth reduction, respectively, to the same treatments. Like *Hog1p* in *S. cerevisiae*, it appears *SakA* plays a role in regulating Mn-SOD activity and, thus, tolerance to 2,3-dihydroxybenzaldehyde. Minimum Inhibitory Concentrations (MICs) of Congo red, calcofluor white, amphotericin B, fluconazole and ketoconazole, antifungal compounds interfering with cell wall and/or membrane integrity, were significantly lowered against *A. fumigatus* when co-applied with thymol or 2,3-dihydroxybenzaldehyde. Similar co-applications of small amounts of these compounds with amphotericin B, fluconazole and itraconazole resulted in almost complete mortality of clinical strains of *Candida* and *Cryptococcus neoformans* resistant to these drugs.

Conclusion

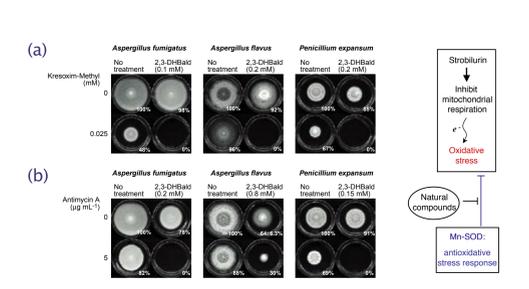
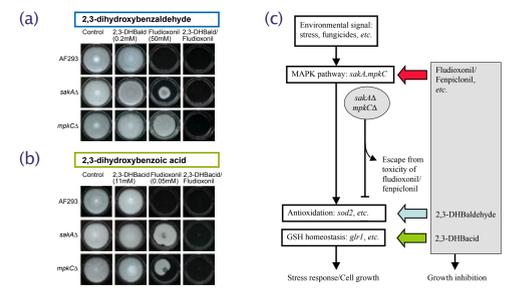
Certain, safe, natural compounds can serve as effective chemosensitizers to commercial antifungal agents, especially when the molecular target is identified. To achieve this chemosensitization, we showed how safe natural products can be used to disrupt the MAPK signaling pathways (the "command and control centers") of fungi so that they are unable to respond to stress. This inability to respond renders them more susceptible to antifungal drugs. Moreover, we show how this approach lowers the MICs against *A. fumigatus* and overcomes resistance in *Candida* and *Cryptococcus* to one or more of the following drugs: amphotericin B, fluconazole, itraconazole and ketoconazole. Thus, chemosensitization using safe, natural compounds has great potential for lowering required dosages, treatment costs and development of resistance to antifungal drugs.

Antifungal activities of various benzaldehyde derivatives

Analog	2,3-	2,4-	2,5-	3-	4-	Benzaldehyde	Veratraldehyde	Vanillin
Fungus								
<i>S. cerevisiae</i> (yeast)	0.08	2.4	1.8	9.0	13.0	>35.0	19.0	10.0
Filamentous fungi:								
<i>Aspergillus fumigatus</i> (wild type)	0.3	2.4	1.2	3.0	9.0	>35.0	7.0	6.0
<i>A. flavus</i>	1.0	3.1	2.8	9.0	12.0	>35.0	7.0	6.0
<i>A. parasiticus</i>	1.0	2.6	2.6	8.0	11.0	>35.0	8.0	10.0
<i>A. oryzae</i>	1.0	3.0	3.0	8.0	9.0	>35.0	8.0	8.0
<i>A. niger</i>	0.9	3.3	3.6	7.0	12.0	>35.0	5.0	7.0
<i>A. nidulans</i>	0.5	2.2	1.7	5.0	8.0	>35.0	5.0	6.0
<i>A. ochraceus</i>	0.7	2.5	2.0	5.0	11.0	>35.0	8.0	7.0
<i>P. expansum</i>	0.5	1.8	1.5	4.0	8.0	>35.0	8.0	8.0
Mean MIC (Filamentous fungi)	0.74	2.61	2.30	6.13	10.0	>35.0	7.0	7.25



Chemosensitization to conventional fungicides by benzo analogs



Enhanced activity of fludioxonil (0.05 mM) by co-application with (a) 2,3-dihydroxybenzaldehyde (0.2 mM) or (b) 2,3-dihydroxybenzoic acid (11 mM). These combined treatments prevented escape of *sakAΔ* 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 GSH homeostasis by 2,3-dihydroxybenzoic acid), which enhances toxicity in wild type cells or prevents escape of MAPK mutants from antifungal effects. Standard deviation: <5%.

Targeting the mitochondrial antioxidative stress system with 2,3-dihydroxybenzaldehyde in combination with (a) strobulin (kresoxim-methyl) or (b) antimycin A enhanced the antifungal effect against the filamentous fungi, *A. fumigatus* AF293, *A. flavus* NRRL 3357 or *P. expansum* NRRL 974. Standard deviation: <5%, except where noted.

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Chemosensitization to conventional antifungal drugs by thymol and benzo analogs

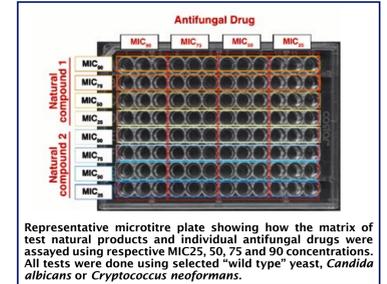
Compounds	MIC ₅₀ Agent alone	MIC ₅₀ Agent combined	FIC index ¹
Ketoconazole	2 < n ₁ < 4 ² 0.8 < n ₂ < 1.6 ³	0.125 < n ₁ < 0.25 ⁴ 0.4 < n ₂ < 0.8 ⁵	0.5625 additive
Fluconazole	32 < n ₁ < 64 0.8 < n ₂ < 1.6	2 < n ₁ < 4 0.4 < n ₂ < 0.8	0.5625 additive
Amphotericin B	1 < n ₁ < 2 0.8 < n ₂ < 1.6	0.125 < n ₁ < 0.25 0.4 < n ₂ < 0.8	0.625 additive

¹For calculation purposes, the higher concentration of n₁ to n₂ in each column was used. MIC₅₀ is where no visible fungal growth was detected. Fractional inhibitory concentrations (FICs) were based on the formula FIC = (MIC₅₀ of antifungal compound A in combination with antifungal compound B / MIC₅₀ of antifungal compound A alone) + (MIC₅₀ of antifungal compound B in combination with antifungal compound A / MIC₅₀ of antifungal compound B alone). Compound interactions were designated according to the following FIC calculations: synergistic (FIC index ≤ 0.5), additive (0.5 < FIC index ≤ 1), neutral (1 < FIC index ≤ 2) or antagonistic (2 < FIC index).

²n₁ = MIC₅₀ (μg/ml) of drug alone.
³n₂ = MIC₅₀ (μM) of thymol alone.
⁴n₁ = MIC₅₀ (μg/ml) of drug in combination with thymol.
⁵n₂ = MIC₅₀ (μM) of thymol in combination with drug.

Cryptococcus neoformans strain 24	Amphotericin B				Itraconazole			
	MIC ₅₀	MIC ₇₅	MIC ₉₀	MIC ₂₅	MIC ₅₀	MIC ₇₅	MIC ₉₀	MIC ₂₅
Inhibition by drug alone →	5.99	1.90	1.79	64.89	24.65	3.54		
Thymol	MIC ₅₀ 98.31	98.26	97.88	97.65	98.54	98.62	98.49	98.28
	MIC ₇₅ 82.96	82.19	81.50	73.66	98.26	90.66	84.21	77.62
	MIC ₉₀ 70.46	67.90	65.82	61.91	98.11	78.57	66.51	61.46
	MIC ₂₅ 64.58	57.91	57.66	33.55	97.78	69.61	41.06	12.99
2,5-Dihydroxybenzaldehyde	MIC ₅₀ 96.98	96.52	95.77	95.41	97.46	96.03	94.38	94.81
	MIC ₇₅ 95.09	93.84	93.31	93.02	96.47	93.09	92.85	92.40
	MIC ₉₀ 93.25	92.70	88.34	80.59	92.73	81.63	80.96	79.83
	MIC ₂₅ 81.74	81.25	80.41	79.08	80.83	74.36	65.38	61.96
2,3-Dihydroxybenzaldehyde	MIC ₅₀ 97.16	98.02	98.22	98.08	98.32	98.26	98.12	97.81
	MIC ₇₅ 97.06	97.89	98.01	97.78	98.14	97.73	97.60	92.44
	MIC ₉₀ 96.97	96.82	96.13	91.46	98.06	80.88	57.70	51.56
	MIC ₂₅ 87.84	72.44	68.03	47.70	97.83	32.86	9.13	0.71

Candida krusei strain 75	Amphotericin B				Itraconazole				Fluconazole			
	MIC ₅₀	MIC ₇₅	MIC ₉₀	MIC ₂₅	MIC ₅₀	MIC ₇₅	MIC ₉₀	MIC ₂₅	MIC ₅₀	MIC ₇₅	MIC ₉₀	MIC ₂₅
Inhibition by drug alone →	1.10	0.49	0.05	60.52	14.04	12.76	65.57	7.83	3.21			
Thymol	MIC ₅₀ 99.69	99.54	99.51	99.48	99.93	99.89	99.80	99.52	99.94	99.89	99.88	99.80
	MIC ₇₅ 99.71	99.48	99.47	99.32	99.84	99.63	99.51	99.73	99.87	99.86	99.82	99.67
	MIC ₉₀ 99.41	99.40	99.30	98.87	99.76	99.64	99.49	99.25	99.83	99.74	99.65	99.54
	MIC ₂₅ 59.74	51.08	45.75	38.58	99.71	95.99	76.03	33.57	62.75	42.88	37.51	37.56
2,5-Dihydroxybenzaldehyde	MIC ₅₀ 97.47	97.29	97.06	95.48	96.72	95.94	95.26	93.43	96.92	96.70	96.62	96.19
	MIC ₇₅ 92.61	89.98	89.28	51.62	95.66	79.96	77.95	67.18	90.10	89.24	83.56	87.52
	MIC ₉₀ 91.00	86.26	74.64	50.64	94.62	79.79	61.33	32.54	65.38	64.64	63.53	48.90
	MIC ₂₅ 49.11	38.05	30.90	22.86	85.75	65.95	36.70	20.79	34.48	24.82	21.65	19.76
2,3-Dihydroxybenzaldehyde	MIC ₅₀ 80.36	71.50	66.13	60.75	99.74	79.22	75.07	55.89	49.17	48.07	41.41	40.24
	MIC ₇₅ 61.65	57.17	47.27	47.06	99.46	78.27	57.03	36.94	40.13	36.55	29.35	29.30
	MIC ₉₀ 34.72	34.31	33.47	29.74	98.17	74.26	52.57	36.71	23.87	25.68	15.45	15.37
	MIC ₂₅ 27.53	24.23	20.85	16.54	96.23	72.07	40.96	20.17	18.47	15.14	12.76	6.63



Representative microtiter plate showing how the matrix of test natural products and individual antifungal drugs were assayed using respective MIC₂₅, 50, 75 and 90 concentrations. All tests were done using selected "wild type" yeast, *Candida albicans* or *Cryptococcus neoformans*.

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