1st International Electronic Conference on Medicinal Chemistry

2-27 November 2015

chaired by Dr. Jean Jacques Vanden Eynde

sponsored by pharmaceuticals

Augmenting the Efficacy of Antifungal Intervention *via* Chemo-biological Approaches

Jong H. Kim *, Kathleen L. Chan and Luisa W. Cheng

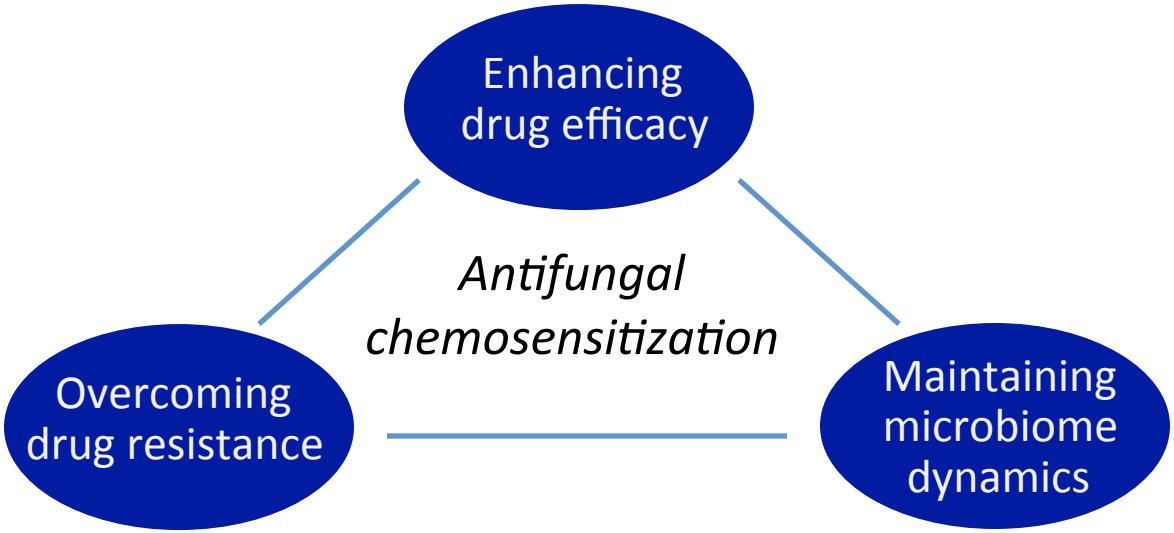
Foodborne Toxin Detection and Prevention Research Unit, Western Regional Research Center, USDA-ARS, 800 Buchanan St., Albany, CA 94710, USA

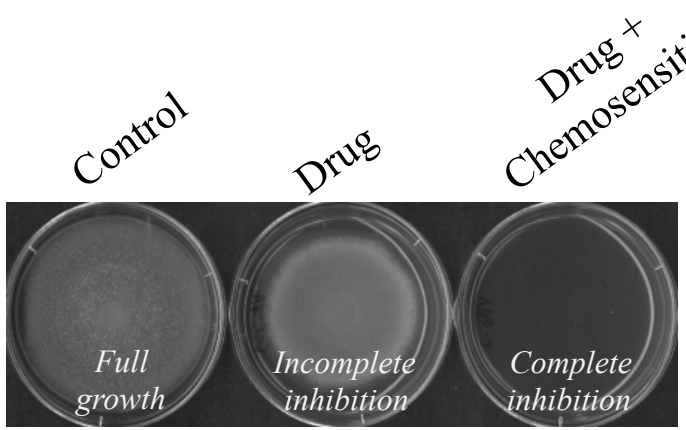
***** Corresponding author: jongheon.kim@ars.usda.gov

USDA United States Department of Agriculture

Agricultural Research Service

Augmenting the Efficacy of Antifungal Intervention *via* Chemo-biological Approaches: *Graphical Abstract*





Abstract: Mycotic infection is becoming a serious health problem since effective antifungal agents for control of pathogenic fungi, especially drug-resistant pathogens, are often very limited. Fungal resistance to antimycotic agents frequently involves mutations caused by environmental stressors. In fungal pathogens, stress signals resulting from oxidative, cell wall stress, etc., are integrated into the upstream mitogen-activated protein kinase (MAPK) pathways that regulate genes countering the stress. Noteworthy is that mutations in MAPK signaling system result in fungal tolerance to cell wall disrupting agents or phenylpyrrole. In a chemo-biological platform to achieve targeted antifungal intervention, the model yeast *Saccharomyces cerevisiae* served as a tool for identifying mechanisms of action of redox-active or cell wall disrupting agents. This also enabled the identification of new utility of known compounds or the utilization of natural products/derivatives as chemosensitizing agents to intensify the efficacy of conventional antimycotic agents. Compounds targeting cellular antioxidant, mitochondrial or cell wall integrity systems effectively inhibited the growth of pathogens and/or overcame fungal tolerance to antimycotic agents. Therefore, chemo-biological approaches lead to the development of novel intervention strategies, such as antifungal chemosensitization, which enhance the drug susceptibility of targeted fungi, and ensure the maintenance of healthy microbiome dynamics.

Keywords: Antifungal; Cell wall integrity; Chemosensitization; Drug resistance; Signaling pathway

Introduction

Chemical biology is powerful approach for the discovery of novel antifungal agents and/or their mechanisms of action. With recent advances in biological and chemical tools, such as relevant phenotypic systems for drug/compound screening, bio- and chemoinformatics, etc., rapid identification of antifungal agents or novel targets is possible. For instance, the yeast *Saccharomyces cerevisiae* is a useful model system for identifying antifungal agents and their gene targets in view that: (1) the genome of *S. cerevisiae* has been sequenced and well annotated (www.yeastgenome.org), (2) *S. cerevisiae* gene deletion mutant collections (~6,000 mutants) have proven to be very useful for genome-wide drug-induced haploinsufficiency screen to determine drug mode of action, and (3) many genes in *S. cerevisiae* are orthologs of genes of fungal pathogens.

Natural compounds that pose no significant medical or environmental side effects are potential sources of antimicrobial agents, either in their nascent structure or as leads for more effective derivatives. For example, natural benzo analogs (e.g., vanillic or caffeic acid) not only inhibited the growth of fungal pathogens (*Aspergillus* sp., *Fusarium* sp., *Penicillium* sp.), but also disrupted the synthesis of toxic secondary metabolites. These fungi are causative agents of human invasive aspergillosis or are producers of mycotoxins, including gliotoxin, aflatoxin, patulin, etc. The redox-active natural compounds, such as phenolic agents, can be potent redox cyclers that prevent fungal growth by interfering cellular redox homeostasis (resultantly, triggering fungal oxidative stress) or by disrupting the integrity of cellular components.

Introduction (continued)

For defense, fungal antioxidant system, cell wall/membrane integrity pathway, etc., play important roles for fungal survival against redox-active compounds administered.

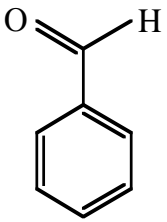
Chemosensitization, a combined application of a certain natural or synthetic compound, i.e., a chemosensitizer, with a conventional antimycotic agent significantly enhances the effectiveness of the antimycotic agent co-applied, and mitigates pathogen resistance to the conventional antimycotic drug. The key value/characteristic of antimycotic chemosensitization is that, in contrast to combination therapy (administration of two or more antimycotic drugs), a chemosensitizer, itself, does not necessarily possess a high level of antifungal potency. However, chemosensitization in pathogen control renders the target microorganism more susceptible to the conventional drug/agent in use, since the chemosensitizer mainly debilitates a defense response of a target pathogen to the conventional drug/agent. Accordingly, chemosensitization strategy further helps the maintenance of healthy mycobiome dynamics.

In this *in vitro* chemosensitization study, strategies for control of fungal pathogens by targeting: (1) the antioxidant, (2) mitochondrial respiration, or (3) cell wall integrity system of fungi are presented.

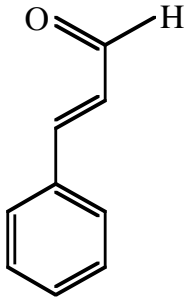
Results and discussion:

I. Antifungal activity of redox-potent benzaldehyde analogs that target cellular antioxidant system

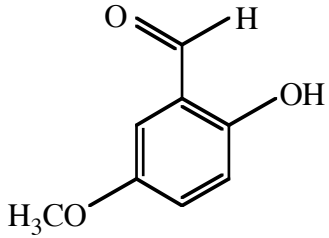
Benzaldehyde analogs were examined as natural antifungal agents against *S. cerevisiae*, a model system for identifying molecular targets of benzaldehydes, and strains of *Aspergillus fumigatus*, a causative agent of human invasive aspergillosis. Seven benzaldehydes exhibited potent antifungal activity.



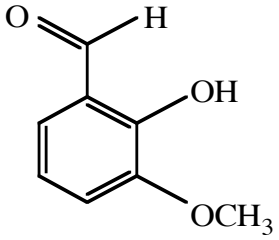
Benzaldehyde
(Basal structure)



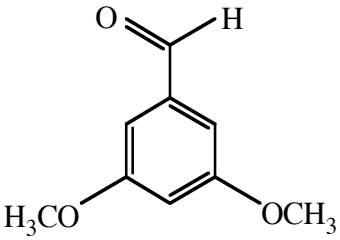
Cinnamaldehyde



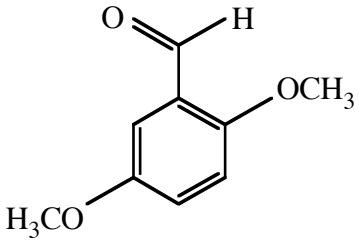
2-Hydroxy-5-methoxy
benzaldehyde



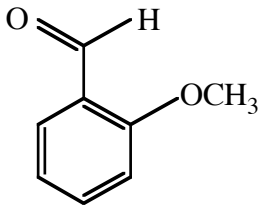
o-Vanillin



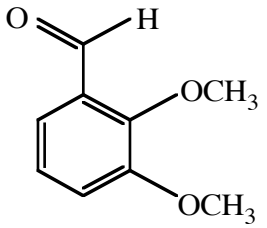
3,5-Dimethoxy
benzaldehyde



2,5-Dimethoxy
benzaldehyde



2-Methoxy
benzaldehyde



2,3-Dimethoxy
benzaldehyde

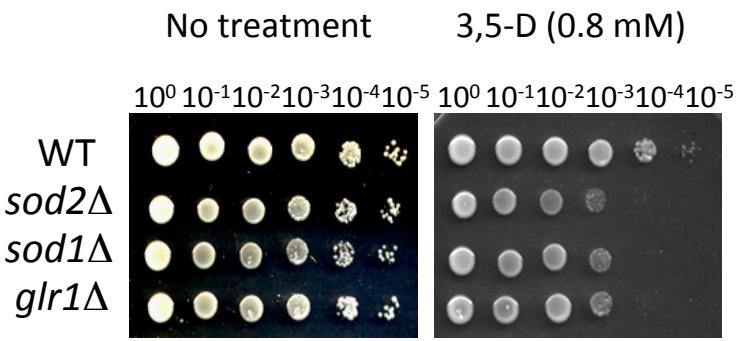
Structure-activity relationship of benzaldehydes in targeting the antioxidant system of fungi

Structures	*sod1*Δ	*sod2*Δ	*glr1*Δ
Cinnamaldehyde	X	X	
2-Hydroxy-5-methoxy-benzaldehyde	X	X	
o-Vanillin	X		
3,5-Dimethoxy-benzaldehyde	X	X	X
2,5-Dimethoxy-benzaldehyde	X	X	
2-Methoxy-benzaldehyde		X	
2,3-Dimethoxy-benzaldehyde	X		

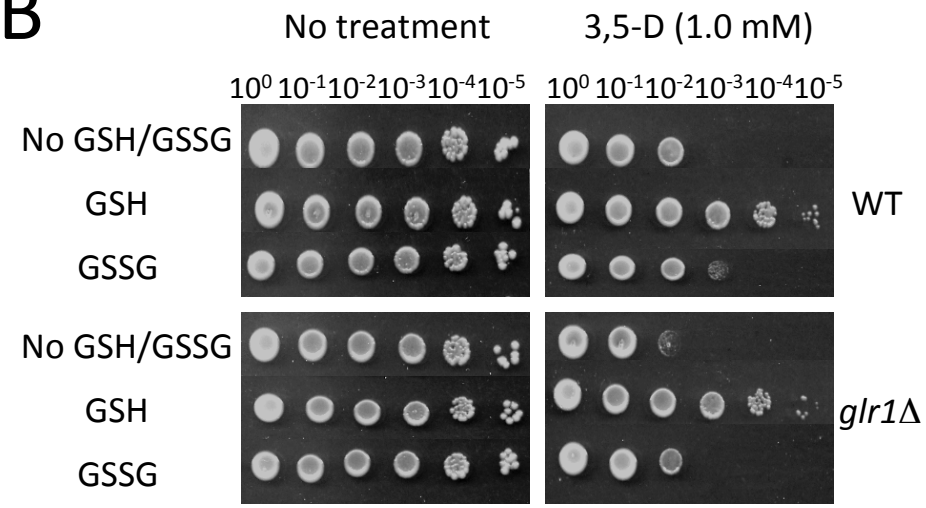
Yeast dilution bioassays showed *S. cerevisiae sod1*△ (cytosolic superoxide dismutase, Cu,Zn-SOD), *sod2*△ (mitochondrial superoxide dismutase, Mn-SOD), or *glr1*△ (glutathione reductase) were sensitive to the seven most active benzaldehyde analogs with structure-activity relationship (X = Sensitive response).

In particular, one of them, 3,5-dimethoxybenzaldehyde (3,5-D) further targeted glutathione reductase (*GLR1*)

A

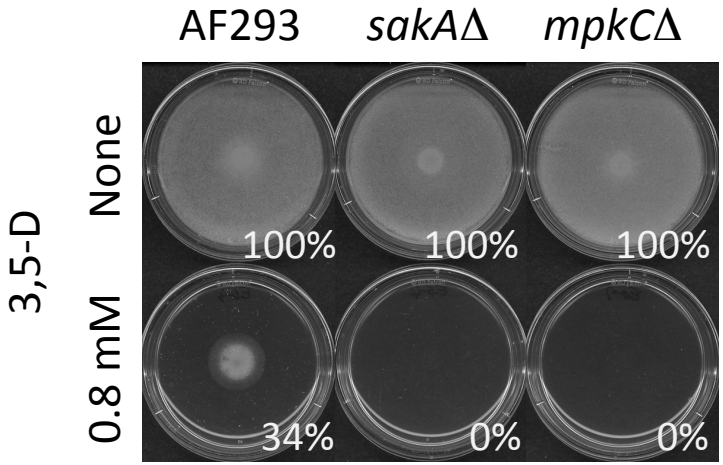


B



(A) Responses of *S. cerevisiae* antioxidant mutants to 3,5-D. (B) Recovery of the growth of yeast cells treated with 3,5-D by reduced glutathione (GSH, antioxidant), but not by oxidized glutathione (GSSG). Result indicated that cellular target of 3,5-D is also fungal GSH homeostasis.

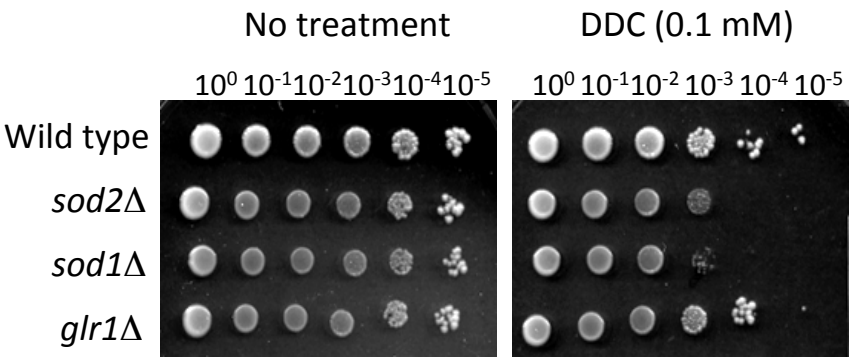
Enhanced susceptibility of *A. fumigatus* oxidative MAPK signaling mutants (sakAΔ, *mpkCΔ)* to 3,5-dimethoxybenzaldehyde (3,5-D), demonstrating 3,5-D targets pathogen's antioxidant system.



In yeasts, i.e., *S. cerevisiae* and *Schizosaccharomyces pombe*, *SOD1*, *SOD2* and *GLR1* are under MAPK control. These systems in fungi play an important role in responding to and/or detoxifying the benzaldehyde administered.

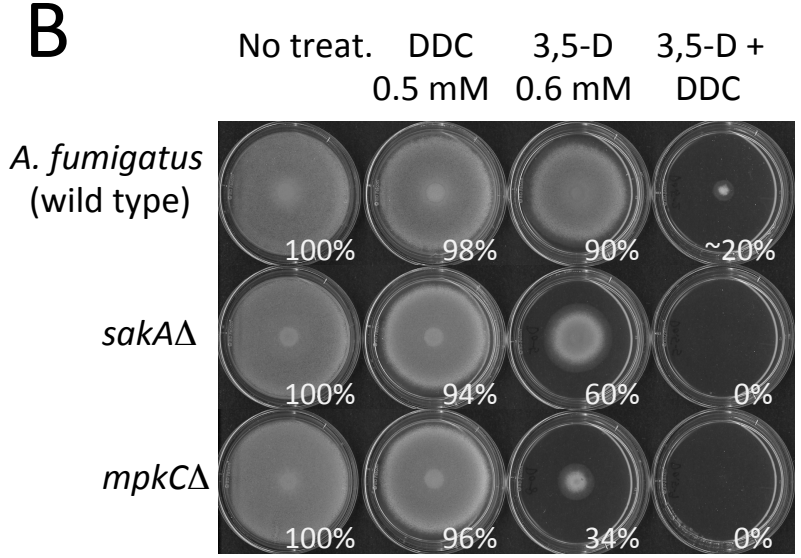
Co-application of benzaldehyde derivatives, e.g., 3,5-D, with dimethyldithiocarbamate (DDC; Cu,Zn-SOD inhibitor) increased the inhibition of fungal growth by the compounds

A



DDC = Dimethyldithiocarbamate

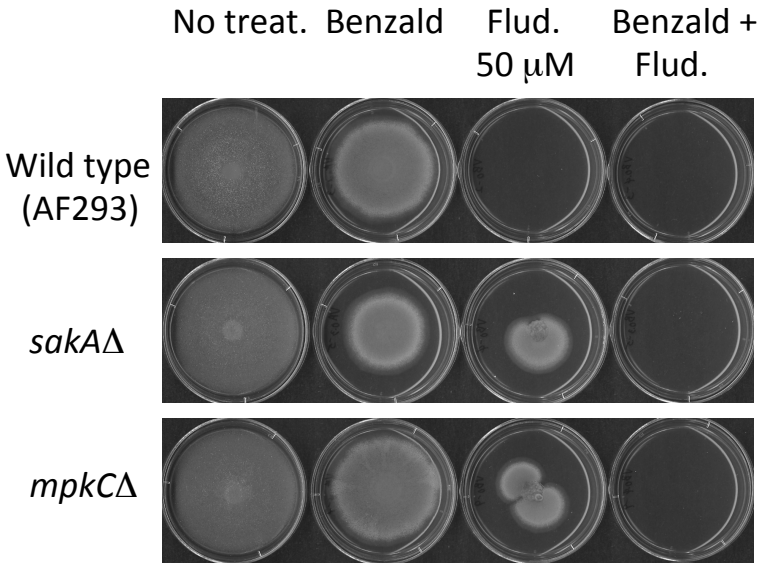
B



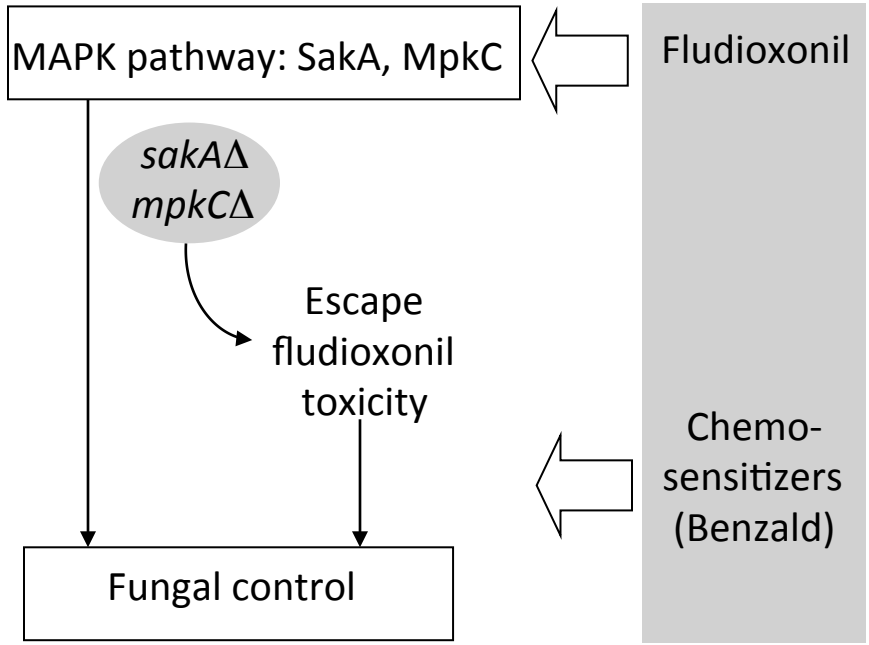
(A) Sensitive responses of *S. cerevisiae* antioxidant mutants to DDC. (B) Co-application of 3,5-D and DDC resulted in enhanced growth inhibition in *A. fumigatus*.

Overcoming fludioxonil (Flud; phenylpyrrole) resistance of MAPK mutants, e.g., *A. fumigatus sakA*Δ and *mpkC*Δ, *via* chemosensitization.

A

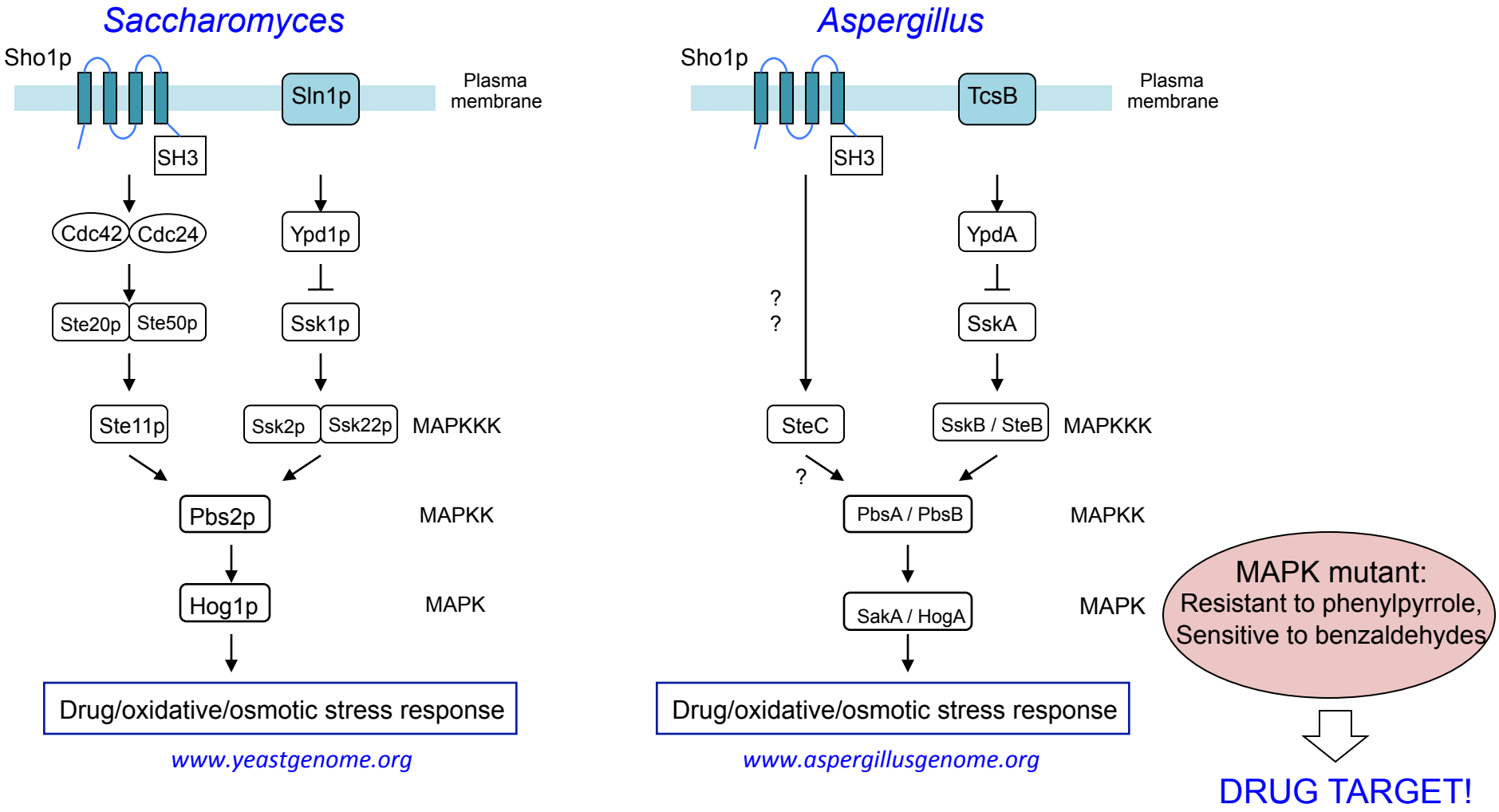


B



(A) Exemplary chemosensitization bioassay to overcome Flud resistance of MAPK mutants by using benzaldehyde analog. (B) Diagram showing the strategy for overcoming Flud resistance of fungal pathogens by using chemosensitizers.

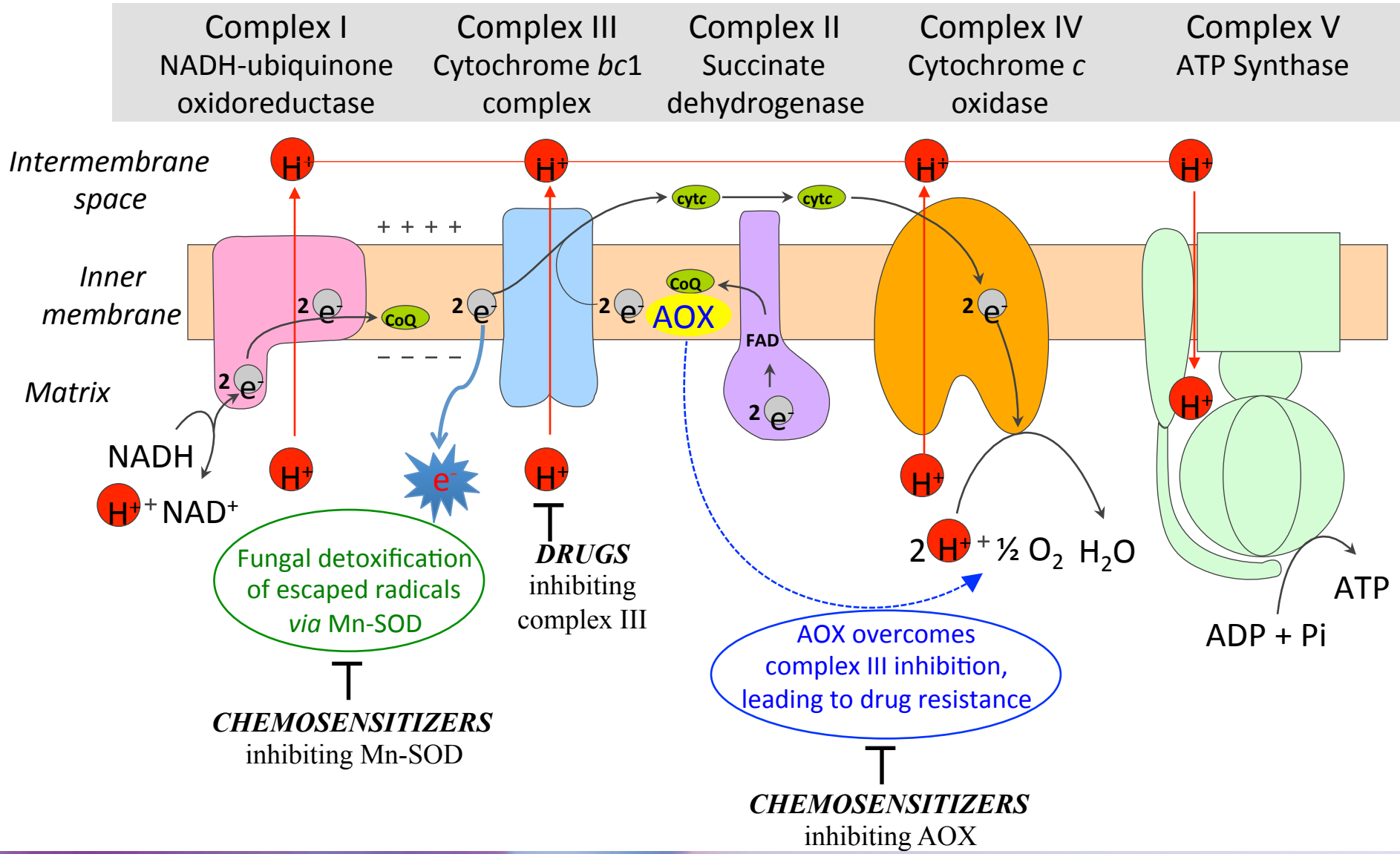
Therefore, oxidative MAPK signaling systems can serve as an effective antifungal drug target



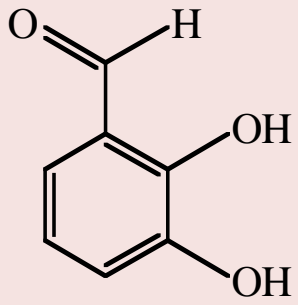
II. Chemosensitization to disrupt mitochondrial respiration

The mitochondrial respiratory chain (MRC) can serve as a molecular target for effective control of fungal pathogens. Conventional inhibitors of MRC, such as antimycin A, strobilurins, mucidin, etc., interfere with the fungal energy (ATP) production, thus debilitate fungal viability. However, coinciding with this interference is an abnormal escape of electrons from MRC. The escaped electrons trigger oxidative damage/stress to essential components in fungal cells, such as DNAs, cell membranes and proteins, resulting in fungal necrosis. Consequently, the antioxidant system in fungi plays a crucial role in such cases, maintaining cellular redox-homeostasis or integrity from reactive oxygen species. Of note, fungi can also overcome the toxic effect of MRC inhibitors *via* the function of alternative oxidase (AOX), enabling the completion of electron transfer *via* MRC (AOX is insensitive to MRC inhibitors). Therefore, the antioxidant system and AOX can be antifungal targets of chemosensitizing agents.

Scheme of mitochondrial respiratory chain in fungi

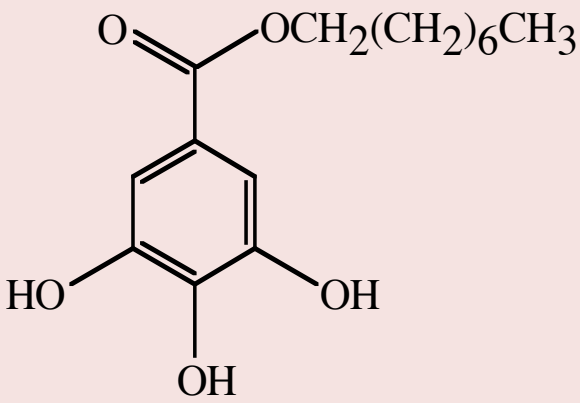


Mitochondrial target of chemosensitizing agents tested, i.e., 2,3-dihydroxybenzaldehyde (2,3-DHBA) and octyl gallate (OG)



2,3-DHBA

Target: Mitochondrial superoxide dismutase (Mn-SOD)



OG

Target: Mitochondrial alternative oxidase (AOX)

Antifungal chemosensitization of OG or 2,3-DHBA (mM) to complex III inhibitor, i.e., pyraclostrobin (PCS; μg/mL), tested against *Cryptococcus* or *Candida**

	Compd	MIC alone	MIC combined	FICI		MFC alone	MFC combined	FFCI
Cryptococcus	OG	0.04	0.01	0.3		0.04	0.01	0.3
	PCS	18.2	1.3			32.0	2.6	
Candida	OG	0.07	0.05	1.2		0.1	0.08	1.5
	PCS	32.0	14.8			32.0	21.9	1.5
Cryptococcus	2,3-DHBA	0.2	0.05	0.4		1.5	0.8	0.9
	PCS	18.2	1.9			32.0	11.6	
Candida	2,3-DHBA	0.3	0.2	1.1		6.8	6.8	2.0
	PCS	32.0	15.3			32.0	32.0	

*Determined by EUCAST-based bioassays. Arendrup et al. *Clin. Microbiol. Infect.* **2012**, *18*, E246–E247.

Cryptococcus (Non-fermenting pathogens) are more susceptible to the chemosensitization than *Candida* (Fermenting pathogens) (Kim et al., 2013).

Non-fermenting pathogens are more susceptible to the chemosensitization than the fermenting pathogens.

Non-fermenting yeasts:
Cryptococcus

⇩

HIGH
sensitivity to
MRC inhibitors

⇩

Increased
sensitivity w/
chemosensitization in
"most" strains

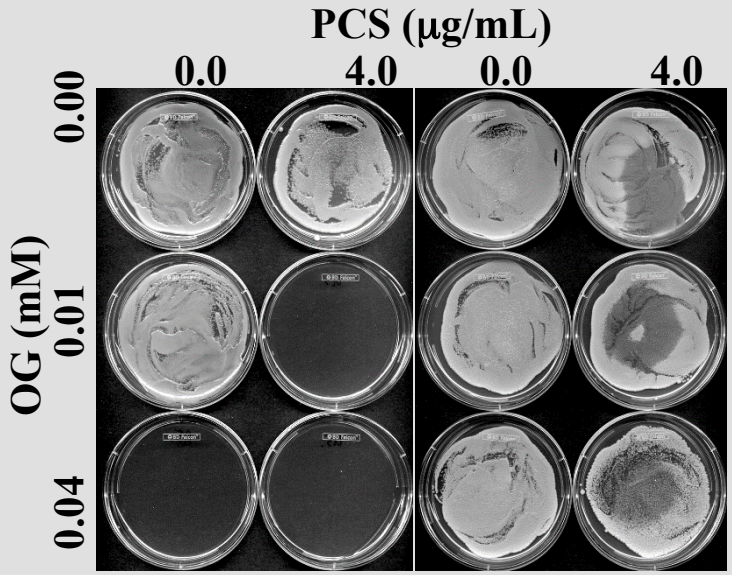
Fermenting yeasts:
Candida

⇩

LOW
sensitivity to
MRC inhibitors

⇩

Increased
sensitivity w/
chemosensitization in
"selected" strains

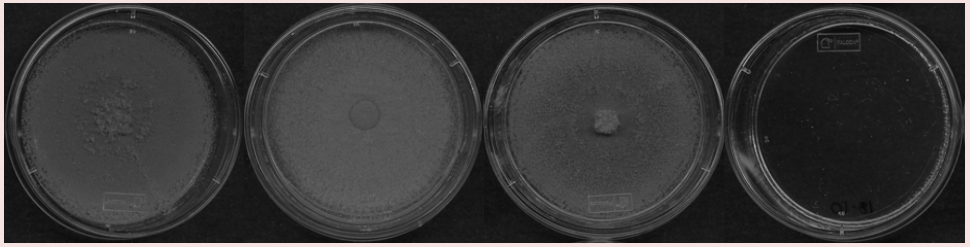


***C. neoformans* 208821** ***C. albicans* 90028**

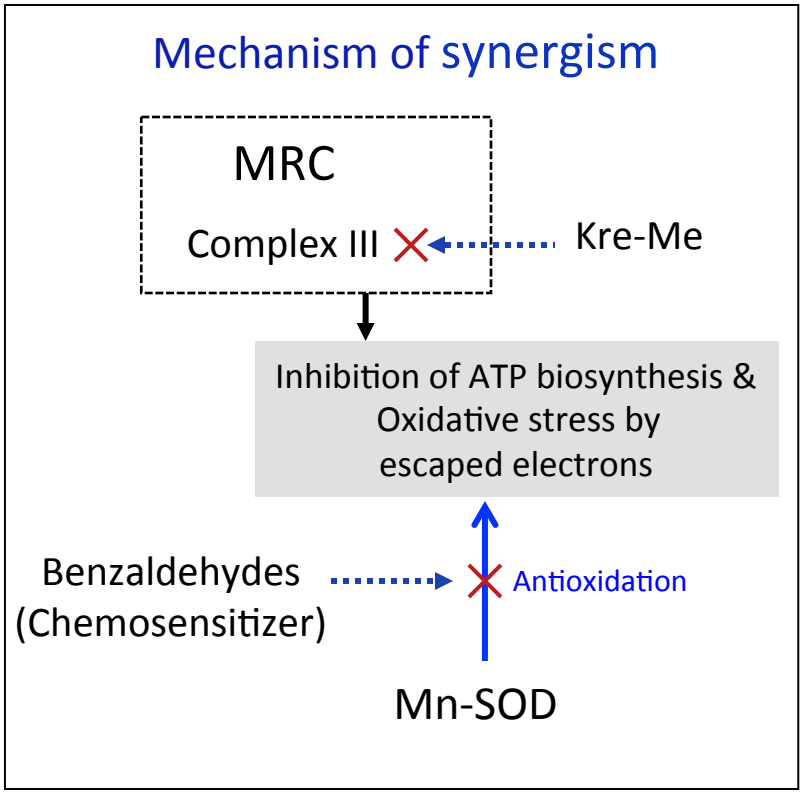
Octyl gallate (OG)-mediated chemosensitization to pyraclostrobin (PCS; complex III inhibitor) in *Cryptococcus* (non-fermenter) or *Candida* (fermenter) showed that *C. neoformans* was much more susceptible to the chemosensitization than *C. albicans.*

Chemosensitization to disrupt mitochondrial respiration in the filamentous fungal pathogen, *Aspergillus* sp.

No treatment | Kre-Me 25μM | Benzald. 4mM | Stro + Benzald.



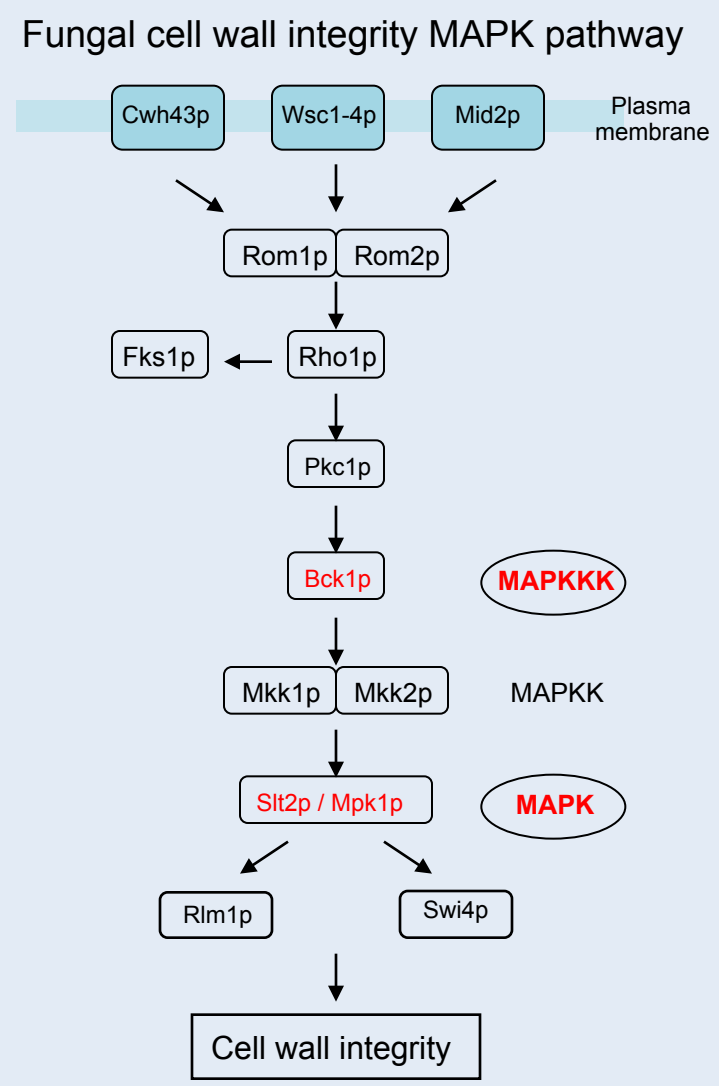
Kre-Me (Kresoxim-methyl): Complex III inhibitor



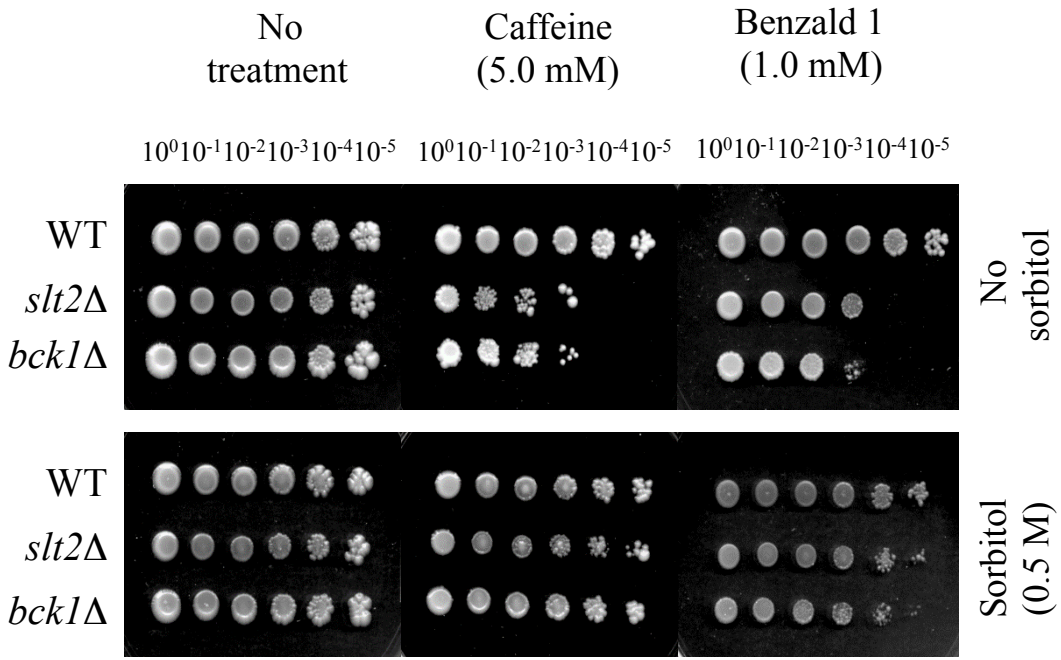
III. Chemosensitization to disrupt fungal cell wall integrity

Disruption of cell wall integrity system should be an effective strategy for control of fungal pathogens. The cell wall integrity pathway is well characterized in the model fungus *S. cerevisiae*, where the upstream MAPK signaling system, required for cell wall maintenance, is controlled by protein kinase C. Genome and functional analyses indicated that genes in the cell wall integrity system in fungi are well conserved. To augment the cell wall disruption efficacy of conventional drugs, such as caspofungin (CAS), chemosensitization capacities of benzaldehydes were evaluated against strains of *S. cerevisiae* wild type (WT), *slt2*Δ and *bck1*Δ, i.e., mutants of the MAPK and MAPK kinase kinase, respectively, in the cell wall integrity pathway. OG also targeted fungal cell wall integrity, where OG-mediated chemosensitization to CAS lead to targeted intervention of pathogens, such as *Penicillium* (i.e., *Penicillium* strains are more susceptible to OG + CAS than *Aspergillus*).

Cell wall MAPK system as the target of benzaldehydes



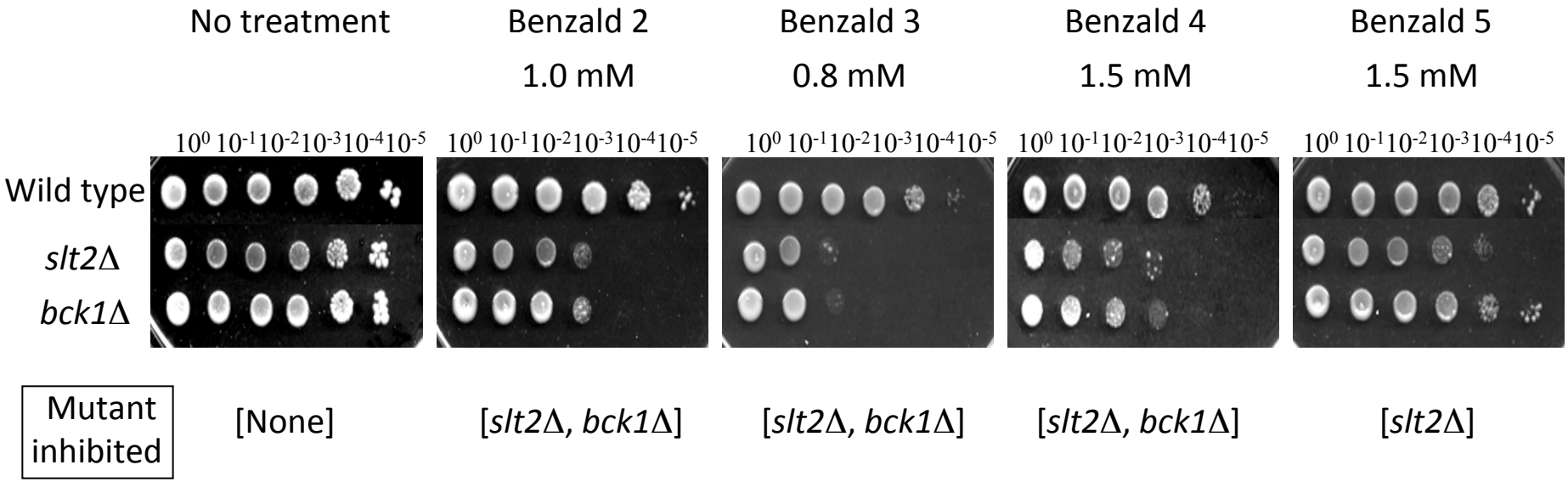
www.yeastgenome.org & www.aspergillusgenome.org



Yeast dilution bioassay showing sensitivity of *S. cerevisiae slt2*Δ (MAPK) and *bck1*Δ (MAPK kinase kinase) mutants to caffeine (5 mM; control) and benzaldehyde 1 (1.0 mM) was remediated by sorbitol (osmotic protectant). Results indicate benzaldehyde 1 negatively affected cell wall integrity system of fungi.

Cell wall MAPK system as the target of benzaldehydes:

Other examples of benzaldehydes targeting cell wall integrity MAPK system



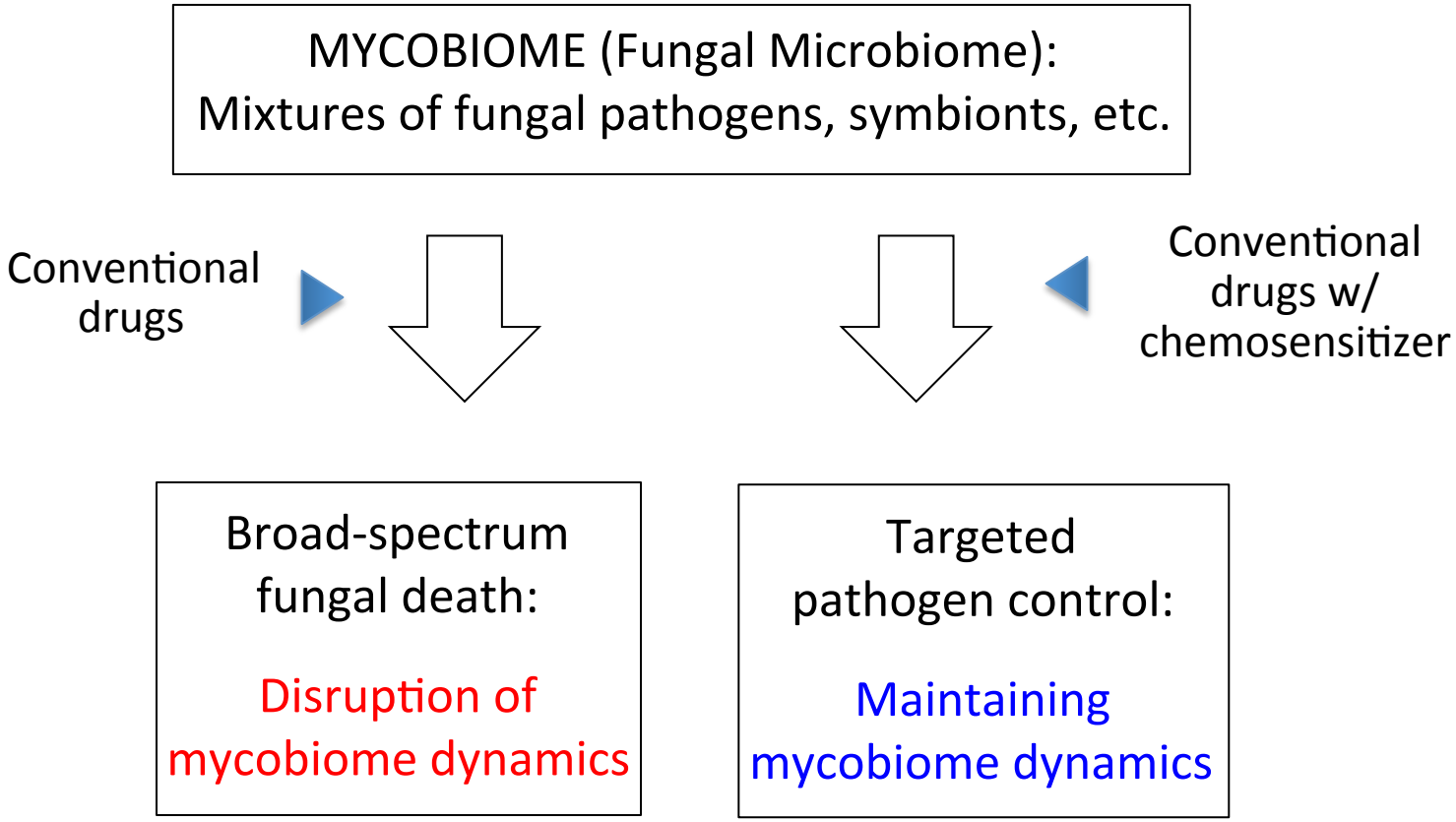
Higher sensitivity of *Penicillium* sp. to OG +CAS chemosensitization compared to *Aspergillus* sp.*

MFC range CAS (μg/mL)	0 < MFC ≤ 32	32 < MFC ≤ 128
CAS alone		*Aspergillus* sp. *Penicillium* sp.
CAS combined w/ OG	*Penicillium* sp. (more sensitive)	*Aspergillus* sp. (less sensitive)

*Determined by Clinical and Laboratory Standards Institute (CLSI) Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi: Approved Standard. Second Edition. Vol. 22 CLSI; Wayne, PA, USA: 2008. MFC, Minimum Fungicidal Concentration; CAS, Caspofungin (Cell wall disrupting drug); OG, Octyl gallate (chemosensitizer)

The effectiveness of OG-mediated chemosensitization with CAS was fungal strain-specific, where *Penicillium* strains required lower dose of CAS to achieve ≥ 99.9% fungal death. Thus, chemosensitization can lead to targeted intervention of fungal pathogens (such as, intervening *Penicillium* with OG-based chemosensitization to CAS) (Manuscript submitted).

Therefore, targeted pathogen control could be achieved during chemosensitization, enabling the maintenance of mycobiome dynamics



Conclusions

- Cellular antioxidant, mitochondrial respiration, or cell wall integrity system can serve as molecular targets of natural phenolics/derivatives for the effective control of fungal pathogens. Benzaldehyde analogs can be used as potent chemosensitizing agents to enhance antimycotic activity of conventional antifungal drugs.
- The antifungal effects of benzaldehydes are determined in selected filamentous fungi, yeast pathogens and the strains of the *S. cerevisiae* (used to identify mode of action).
- OG and 2,3-DHBA show potent chemosensitizing activity to PCS (complex III inhibitor), resulting in the great enhancement of antifungal activity. This capacity is most effective against *Cryptococcus* (non-fermenting pathogens), compared to *Candida* (fermenting pathogens).
- OG also possesses chemosensitizing capability to the cell wall disrupting drug CAS, thus lowers effective dosages of the drug. Of note, OG-based chemosensitization can lead to targeted intervention of fungal pathogens, where *Penicillium* species are more susceptible to CAS (when co-applied with OG), compared to *Aspergillus*.
- Therefore, methods presented here can reduce costs, abate resistance, alleviate negative side effects associated with current antifungal treatments, while maintaining healthy dynamics in the mycobiome.
- Further *in vivo* studies are required to determine whether the *in vitro* antifungal activities described herein can translate to clinically effective therapeutic resolution of mycoses.

References

- Campbell BC, Chan KL & Kim JH (2012) Chemosensitization as a means to augment commercial antifungal agents. Front. Microbiol. 3:79.
- Costa-de-Oliveira S, Sampaio-Marques B, Barbosa M, Ricardo E, Pina-Vaz C, Ludovico P & Rodrigues AG (2012) An alternative respiratory pathway on *Candida krusei:* Implications on susceptibility profile and oxidative stress. FEMS Yeast Res. 12:423–429.
- Fuchs BB & Mylonakis E (2009) Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. Eukaryot. Cell 8:1616-1625.
- Fujita K, Tani K, Usuki Y, Tanaka T & Taniguchi M (2004) Growth inhibition dependent on reactive oxygen species generated by C9-UK-2A, a derivative of the antifungal antibiotic UK-2A, in *Saccharomyces cerevisiae.* J. Antibiot. (Tokyo) 57:511–517.
- Grant CM (2001) Role of the glutathione/glutaredoxin and thioredoxin systems in yeast growth and response to stress conditions. Mol. Microbiol. 39:533–541.
- Guillen F & Evans CS (1994) Anisaldehyde and veratraldehyde acting as redox cycling agents for H_2O_2 production by *Pleurotus eryngii.* Appl. Environ. Microbiol. 60:2811–2817.
- Inoue K, Tsurumi T, Ishii H, Park P & Ikeda K (2012) Cytological evaluation of the effect of azoxystrobin and alternative oxidase inhibitors in *Botrytis cinerea.* FEMS Microbiol. Lett. 326:83–90.
- Jacob C (2006) A scent of therapy: pharmacological implications of natural products containing redox-active sulfur atoms. Nat. Prod. Rep. 23:851–863.

References (continued)

- Kim JH, Yu J, Mahoney N, Chan KL, Molyneux RJ, Bhatnagar D, Cleveland TE, Nierman WC & Campbell BC (2008) Elucidation of the functional genomics of antioxidant-based inhibition of aflatoxin biosynthesis. Int. J. Food Microbiol. 122:49-60.
- Kim JH, Chan KL, Mahoney N & Campbell BC (2011) Antifungal activity of redox-active benzaldehydes that target cellular antioxidation. Ann. Clin. Microbiol. Antimicrob. 10:23.
- Kim JH, Haff RP, Faria NCG, Martins M de L, Chan KL & Campbell BC (2013) Targeting the mitochondrial respiratory chain of *Cryptococcus* through antifungal chemosensitization: a model for control of non-fermentative pathogens. Molecules 18: 8873- 8894.
- Longo VD, Gralla EB & Valentine JS (1996) Superoxide dismutase activity is essential for stationary phase survival in *Saccharomyces cerevisiae.* Mitochondrial production of toxic oxygen species *in vivo.* J. Biol. Chem. 271:12275–12280.
- Parsons AB, Brost RL, Ding H, Li Z, Zhang C, Sheikh B, Brown GW, Kane PM, Hughes TR & Boone C (2004) Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways. Nat. Biotech. 22:62-69.
- Roemer T & Boone C (2013) Systems-level antimicrobial drug and drug synergy discovery. Nat. Chem. Biol. 9:222-231.
- Ruy F, Vercesi, AE & Kowaltowski AJ (2006) Inhibition of specific electron transport pathways leads to oxidative stress and decreased *Candida albicans* proliferation. J. Bioenerg. Biomembr. 38:129–135.

Acknowledgments

This research was conducted under USDA-ARS CRIS Project 2030-42000-037-00D.