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High Efficiency Drug Repurposing for New Antifungal Agents

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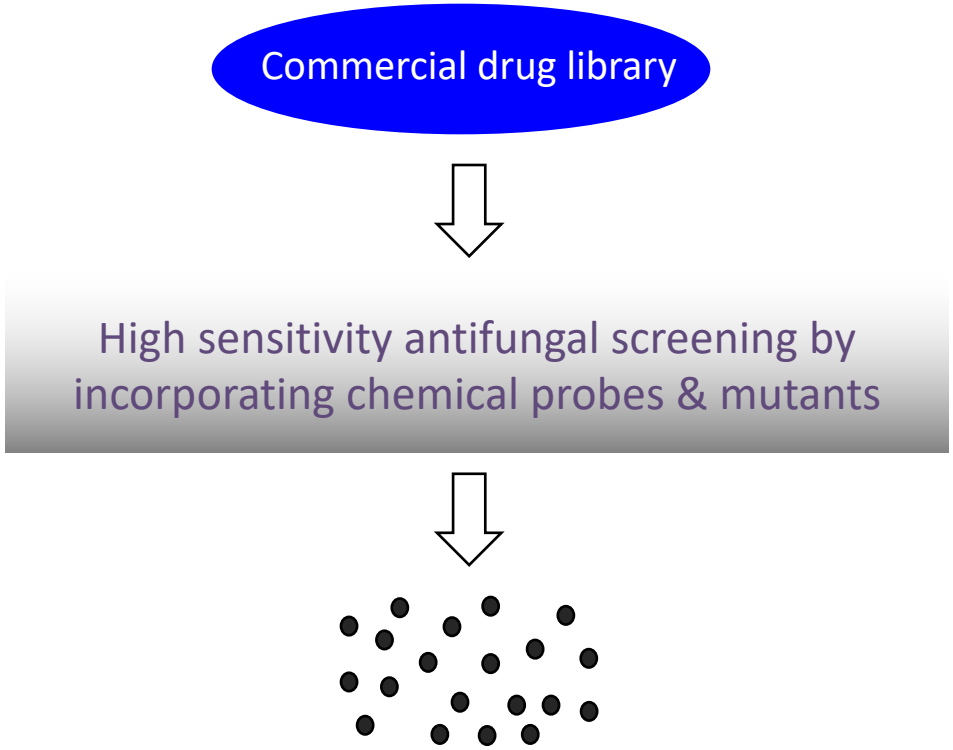
Graphical Abstract

High Efficiency Drug Repurposing for New Antifungal Agents:

Repositioning of marketed/commercial drugs with no known antifungal activities as new antifungal drugs or fungicides



Abstract: There has been a persistent effort to improve efficacy of conventional antimycotic drugs. However, current antimycotic interventions have often limited efficiency in treating fungal pathogens, especially those resistant to drugs. Considering development of entirely new antimycotic drugs is a capital-intensive and time-consuming process, we investigated an alternative approach termed drug repurposing whereby new utility of various marketed, non-antifungal drugs could be repositioned as novel antimycotic agents. As a proof of concept, we applied chemosensitization as a new screening strategy, where combined application of a second compound, viz., chemosensitizer, with a conventional drug could greatly enhance antifungal efficacy of the drug co-applied. Unlike the conventional combination therapy, a chemosensitizer itself does not necessarily have to possess an antifungal activity, but the chemosensitizer significantly debilitates defense systems of pathogens to drugs, enabling improved identification of antifungal activity of off-patent drugs. Of note, inclusion of fungal mutants, such as antioxidant mutants, could facilitate drug repurposing process by enhancing the sensitivity of antifungal screening. Altogether, our strategy led to the development of high efficiency drug repurposing, which enhances the drug susceptibility of targeted fungal pathogens.

Keywords: Antifungal; Chemosensitization; Drug repurposing; Drug resistance; Signaling pathway

Introduction

- Antifungal drug repurposing is the repositioning process of non-antifungal, marketed drugs (previously approved for treating other diseases) to treat fungal infections, where the modes of action, cellular targets or safety of the drugs are already identified (Stylianou et al. 2014). While drug repurposing has become a viable approach to accelerate new antifungal drug development, this strategy still requires highly sensitive screening systems.
- The antioxidant system of fungi is a potential target of antifungal agents (Smits and Brul 2005, Jager and Flohe 2006). Certain natural compounds, such as derivatives of benzoic acid or sulfur-containing compounds, can be redox-active and thus inhibit fungal growth by interfering with cellular redox homeostasis/antioxidant system (Guillen and Evans 1994, Jacob 2006).
- Antifungal chemosensitization is an intervention strategy, in which co-application of a certain natural or synthetic compound, viz., chemosensitizer, with a commercial drug augments the efficacy of the drug co-applied (Kim et al., 2012). While a chemosensitizer does not necessarily have antifungal potency, chemosensitization can lead to: (a) the augmentation of antifungal efficacy of commercial drugs co-applied; (b) overcoming fungal resistance to commercial antifungal drugs; and also (c) enhanced inhibition of mycotoxin production by fungi, such as aflatoxigenic *Aspergillus parasiticus* (Kim et al., 2014).

- The yeast *Saccharomyces cerevisiae* is a useful model system for the identification of antifungal drugs and their molecular targets in view that: (1) the genome of *S. cerevisiae* has been sequenced and well annotated (*Saccharomyces Genome* Database, www.yeastgenome.org), (2) *S. cerevisiae* gene deletion mutant collections (~6,000 mutants) have proven to be very useful for determining drug mechanism of action (Parsons et al, 2004; Norris et al, 2013; Lee et al, 2014), and (3) many genes in *S. cerevisiae* are orthologs of genes of fungal pathogens including *Aspergillus* sp. (Kim et al, 2005).
- Using the model yeast *S. cerevisiae* bioassay, we previously identified several chemosensitizers, which target cellular antioxidant or cell wall integrity systems (See next slide for examples).
- In this *in vitro* study, we tried to develop a high-efficiency drug repurposing strategy for effective control of fungal pathogens. We selected two drugs (aspirin, bithionol) previously investigated, and concentrated on targeting the oxidative stress response system of fungi with redox-active chemosensitizers, viz., 2-isopropyl-5-methylphenol (Thymol), 4-isopropyl-3-methylphenol (Structural analog of thymol) and 3,5-dimethoxybenzaldehyde.
- The susceptibility of fungi to the candidate drug (Bithionol) could be enhanced by co-applying with redox-active chemosensitizers. Bithonol also mitigated fludioxonil tolerance of *Aspergillus fumigatus* antioxidant signaling mutants.

Examples of chemosensitizers targeting antioxidant or cell wall systems in fungi (From the model yeast *S. cerevisiae* bioassay):

Compounds	Antioxidant targets	Cell wall targets	References
2,3-Dihydroxybenzaldehyde	*sod1*Δ, *sod2*Δ, *glr1*Δ		Kim et al. (2008)
trans-Cinnamaldehyde	*sod1*Δ, *sod2*Δ		Kim et al. (2011)
2-Hydroxy-4-methoxy-benzaldehyde		*slt2*Δ, *bck1*Δ	Kim et al. (2015)
2-Hydroxy-5-methoxy-benzaldehyde	*sod1*Δ, *sod2*Δ		Kim et al. (2011)
4-Methoxybenzoic acid		*slt2*Δ, *bck1*Δ	Kim et al. (2015)
3,5-Dimethoxybenzaldehyde	*sod1*Δ, *sod2*Δ, *glr1*Δ	*slt2*Δ, *bck1*Δ	Kim et al. (2011,2015)
2,5-Dimethoxybenzaldehyde	*sod1*Δ, *sod2*Δ	*slt2*Δ, *bck1*Δ	Kim et al. (2011,2015)

Functions of gene products (See also slide #10)**:**
Sod1, Cytosolic superoxide dismutase; Sod2, Mitochondrial superoxide dismutase;
Glr1, Glutathione reductase; Slt2, MAPK of cell wall integrity system;
Bck1, MAPKKK of cell wall integrity system.

Results and discussion

Repurposed drug examples: PubMed search in the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) by using the key words "Drug Antifungal Repositioning" (Search date: May 31, 2018) retrieved 70 articles. We re-evaluated the content of the retrieved articles for their relevance to drug screening, and examples are as shown below.

Examples of repositioned drugs possessing antifungal activities.

Compounds	Functions	Repositioning methods	Target fungi, outcome	References
Aliskiren	Anti-hypertensive drug	CLSI[1] M27-A2 protocol	*C. albicans*	Kathwate and Karuppayil (2013)
Amiodarone	Antiarrhythmic drug	High-throughput adenylate kinase assay.	*C. neoformans*	Butts et al. (2013)
Aspirin	Anti-pain, fever, or inflammation drug	EUCAST[2] protocol	*C. neoformans*, *C. gatti*	Ogundeji et al. (2016)
Auranofin	Rheumatoid arthritis drug	CLSI M27-A3 protocol	*Candida*, *Cryptococcus*	Thangamani et al. (2017)
Bithionol	Antiparasitic drug	High-throughput ATP content assays	*Exserohilum rostratum*	Sun et al. (2013)
Human glycogen synthase kinase 3 (GSK-3) inhibitors	Neurological disorder drug	24-well plate assay using five GSK-3 inhibitors	*A. fumigatus*	Sebastián-Pérez et al. (2016)
Tosedostat	Anti-cancer (Aminopeptidase inhibitor) drug	EUCAST protocol	*C. albicans*, *C. glabrata*	Stylianou et al. (2014)

[1]Clinical & Laboratory Standard Insitute

[2]European Committee on Antimicrobial Susceptibility Testing

We chose **aspirin** and **bithionol** as representative drugs for further investigation.

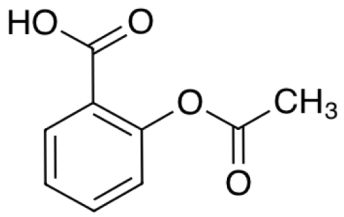
- **Aspirin (Acetyl salicylic acid)** is a non-steroidal anti-inflammatory agent and binds to/acetylates serine residues in cyclooxygenases. This drug decreases synthesis of prostaglandin, platelet aggregation, and inflammation (https://pubchem.ncbi.nlm.nih.gov/compound/2244).

- **Bithionol** is a halogenated anti-infective agent that is used against trematode and cestode infestations. This drug inhibits human soluble adenylyl cyclase (Kleinboelting et al. 2016).

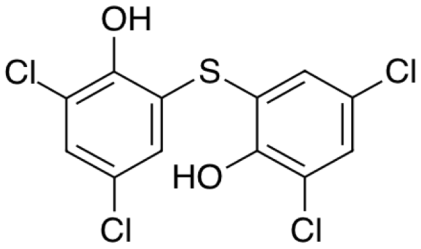
- **Octyl gallate (OG)** was used as a positive control for antifungal bioassay. The mechanism of antifungal action of OG was previously determined as: (a) interrupting the lipid bilayer-protein interface in fungal cells, and (b) functioning as a pro-oxidant (redox-active oxidative stressor), thus triggering cytotoxicity in fungi (Kim et al. 2018).

Structures of repurposed drugs and OG, (+) Control, tested in this study

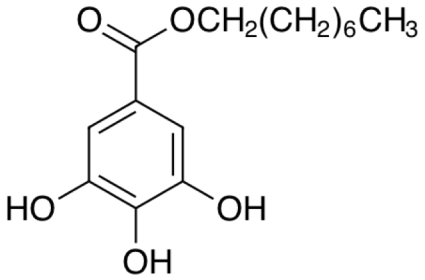
Aspirin



Bithionol



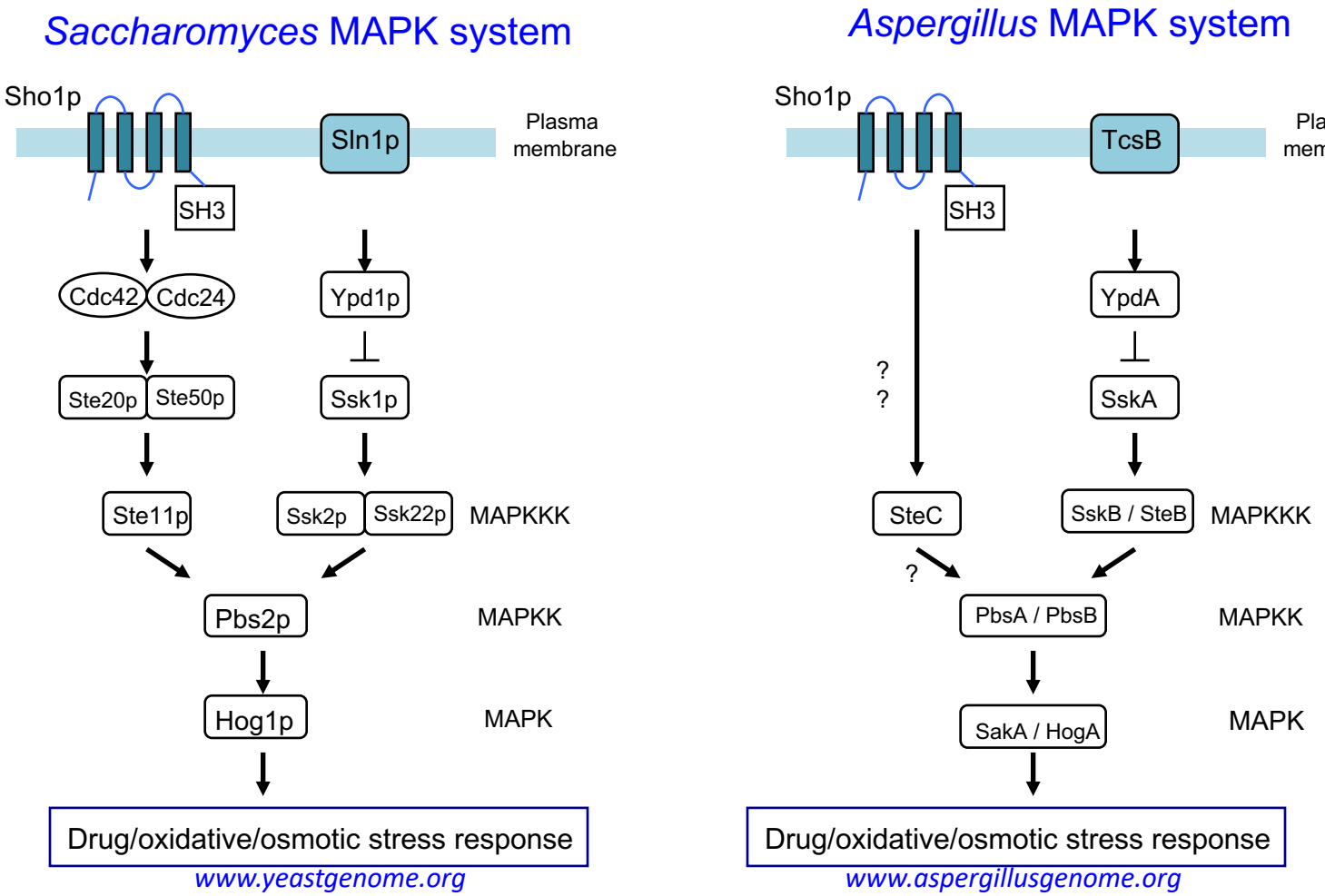
Octyl gallate (OG)



Fungal signaling system as a target:

Meanwhile, oxidative signaling systems, such as **mitogen-activated protein kinase (MAPK)** signaling pathway, have been served as effective antifungal targets for redox-active drugs or compounds (Kim et al. 2012) (Next page).

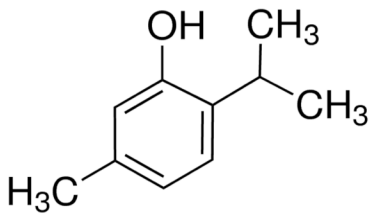
MAPK signaling systems of *Aspergillus* and the model fungus *Saccharomyces cerevisiae* (MAPKK, MAPK kinase; MAPKKK, MAPKK kinase)



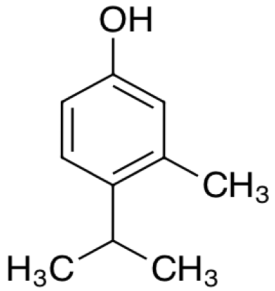
Aspergillus fumigatus is a causative agent of the highly debilitating human invasive aspergillosis, where the *sakA* and *mpkC* genes encode MPAKs in *A. fumigatus*. *A. fumigatus sakA*Δ is an osmotic/oxidative stress sensitive MAPK mutant, while the *mpkC*Δ is a MAPK mutant of the polyalcohol sugar utilization system (Xue et al. 2004; Reyes et al. 2006). We previously determined that both mutants were highly susceptible to redox-active reagents such as amphotericin B, itraconazole or natural phenolics compared to the wild type strain (Kim et al. 2011, 2012).

Use of chemosensitizers for targeting fungal antioxidant systems: Thymol (2-isopropyl-5-methylphenol) is a natural compound, which can disrupt cellular redox homeostasis, and 4-isopropyl-3-methylphenol (4I3M) is a synthetic analog of thymol.

2-isopropyl-5-methylphenol (Thymol)



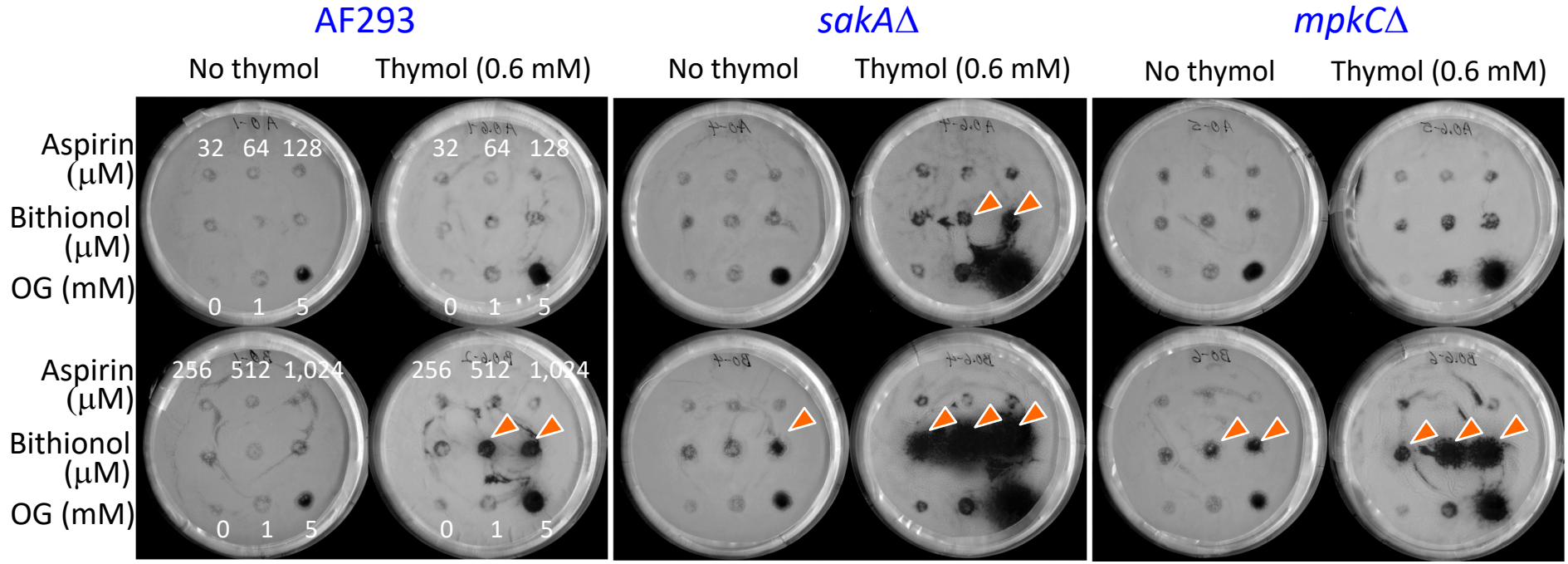
4-isopropyl-3-methylphenol



In the **zone of inhibition bioassays** using *A. fumigatus*, we compared antifungal efficacy of repurposed drugs between (a) wild type (*A. fumigatus* AF293), (b) antioxidant mutants (*A. fumigatus sakA*Δ, *mpkC*Δ), (c) wild type + chemosensitizer (thymol, 4I3M), and (d) mutants + chemosensitizer (thymol, 4I3M).

Bithionol + 2-Isopropyl-5-methylphenol (Thymol):

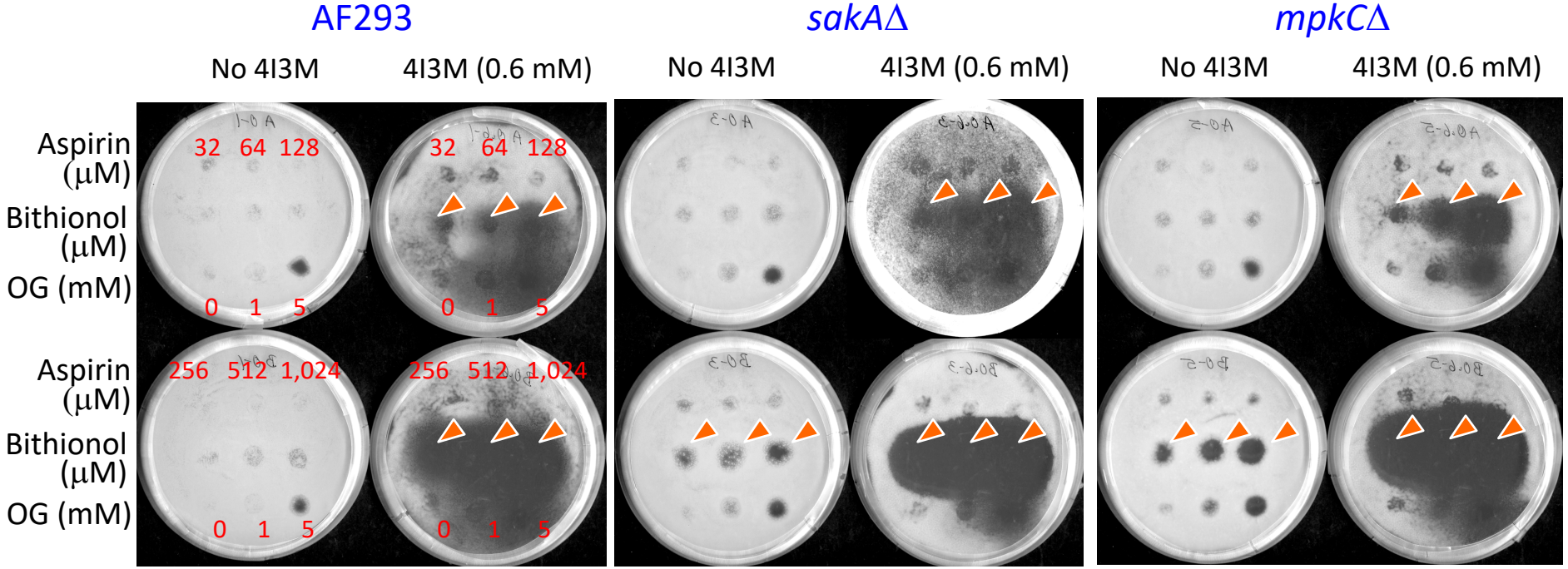
Results showed that antifungal activity of **bithionol** was greatly enhanced in the presence of thymol (chemosensitizer; a chemical probe targeting fungal antioxidant system), while that of **aspirin** was almost not affected, indicating "drug-chemosensitizer specificity" exists for the enhancement of antifungal activity. Results also showed that *A. fumigatus* MAPK mutants were more susceptible to the treatment compared to the wild type, indicating increased susceptibility of antioxidant mutant to the application of redox-active agents, such as thymol (Test concentrations- Aspirin & Bithionol: 32 to 1,024 μM; OG: 1 & 5 mM).



▲, Enhanced susceptibility; OG, Octyl gallate (+ control)

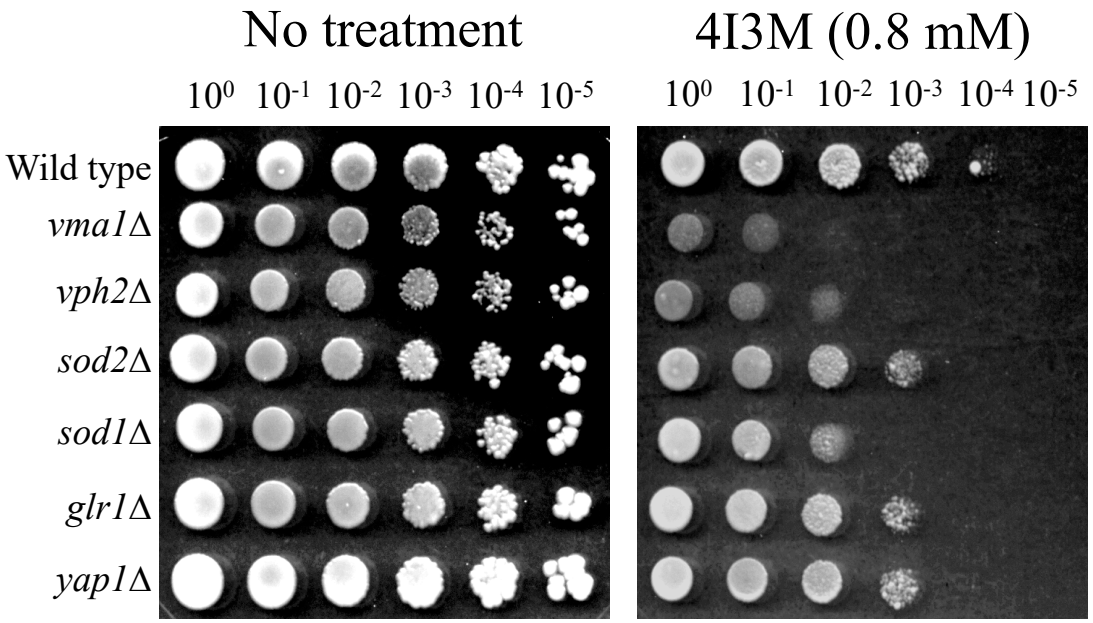
Bithionol + 4-Isopropyl-3-methylphenol (4I3M; Thymol analog):

We found the level of bithionol activity was enhanced further when the structural analog of thymol, viz., 4I3M, was co-applied as a chemosensitizer. For example, antifungal activity of bithionol was enhanced at much lower concentration of bithionol (32 to 128 μM), and the sizes of zone of inhibition were also larger than that with thymol. Therefore, results indicated that 4I3M could be more effective chemosensitizer to bithionol. As observed in thymol, the activity of **aspirin** was almost not affected (Test concentrations- Aspirin & Bithionol: 32 to 1,024 μM; OG: 1 & 5 mM).



▲, Enhanced susceptibility; OG, Octyl gallate (+ control)

Yeast dilution bioassay showing the “sensitive” response of the model fungus *Saccharomyces cerevisiae* gene deletion mutants, i.e., vacuolar (*vph2*Δ, *vma1*Δ) and antioxidant (*sod2*Δ, *sod1*Δ, *glr1*Δ, *yap1*Δ), to 4-isopropyl-3-methylphenol (4I3M). Results shown are representative data from treatment with 0.8 mM of 4I3M.

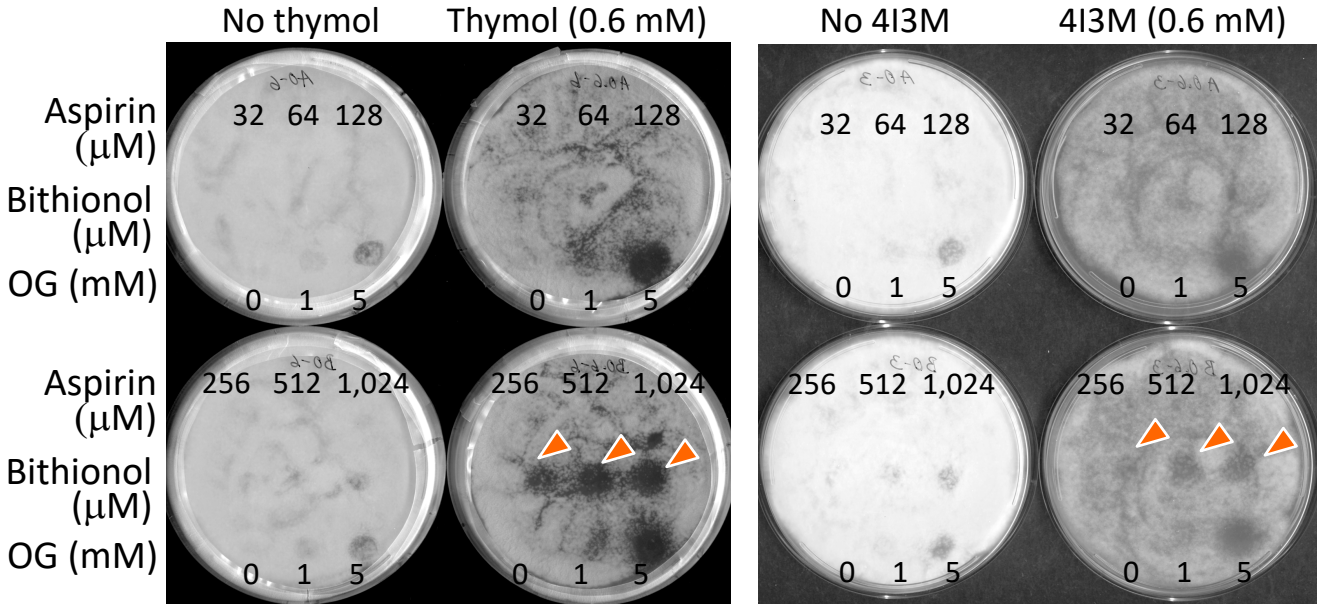


Results indicated 4I3M negatively affects both cellular ion and redox homeostasis in fungi. Similar results were also observed with thymol in a previous study (Kim et al. 2012), indicating 4I3M and thymol share analogous cellular targets in fungi.

Test in *Aspergillus parasiticus*, a mycotoxigenic fungus producing hepato-carcinogenic aflatoxins (Bithionol + Thymol or 4I3M):

Similar results were obtained in *A. parasiticus* 2999 strain, where thymol exhibited higher activity comparing to its analog 4I3M. Also, the sizes of zone of inhibition were generally smaller than that observed in *A. fumigatus*, indicating "strain specificity" also exists for the efficacy of "bithionol + thymol/4I3M" treatment (Test concentrations- Aspirin & Bithionol: 32 to 1,024 μM; OG: 1 & 5 mM).

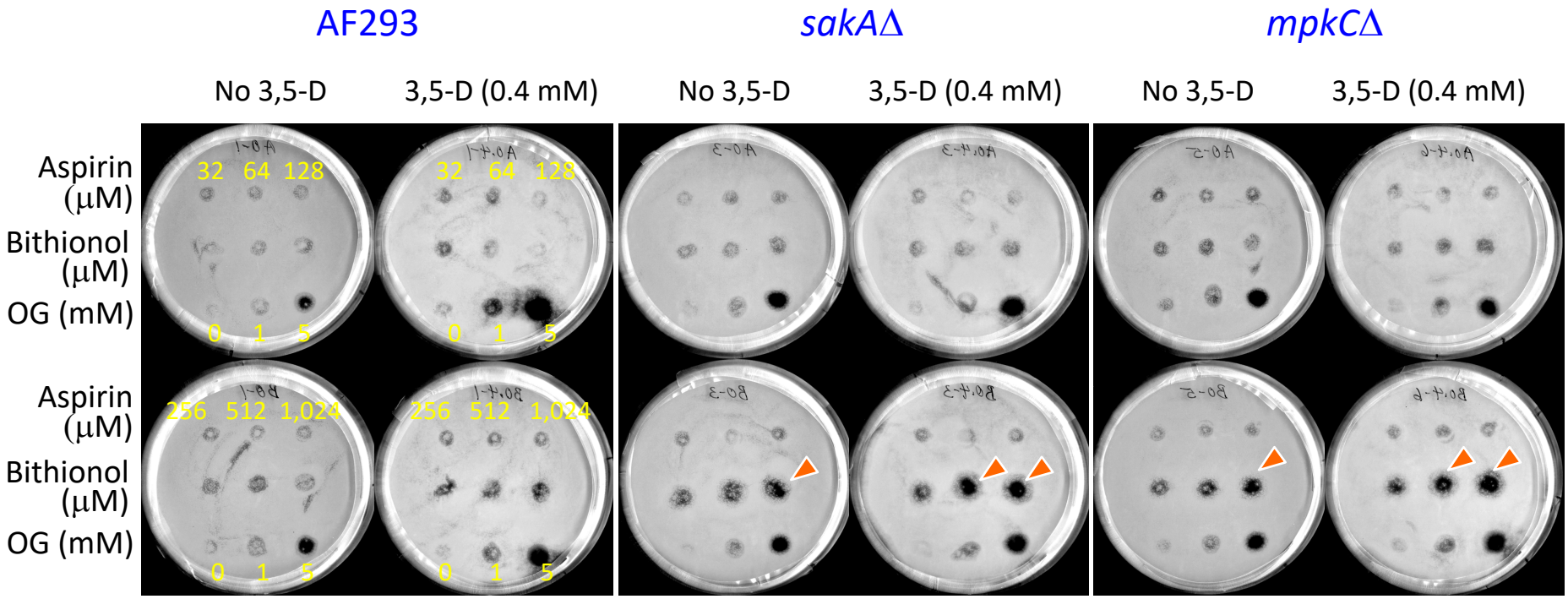
A. parasiticus 2999



▲, Enhanced susceptibility; OG, Octyl gallate (+ control)

Bithionol + 3,5-Dimethoxybenzaldehyde (3,5-D):

We investigated the effect of other types of chemosensitizer for the enhancement of bithionol activity. Results showed that antifungal activity of bithionol was also increased when co-applied with 3,5-D. 3,5-D was also shown to negatively affect cellular antioxidant system, such as superoxide dismutases or glutathione reductase (Kim et al. 2011). As observed in thymol/4I3M, antifungal activity of aspirin was almost not affected. In general, the level of the enhancement of bithionol activity with 3,5-D was lower than that with thymol/4I3M (Test concentrations- Aspirin & Bithionol: 32 to 1,024 μM; OG: 1 & 5 mM).

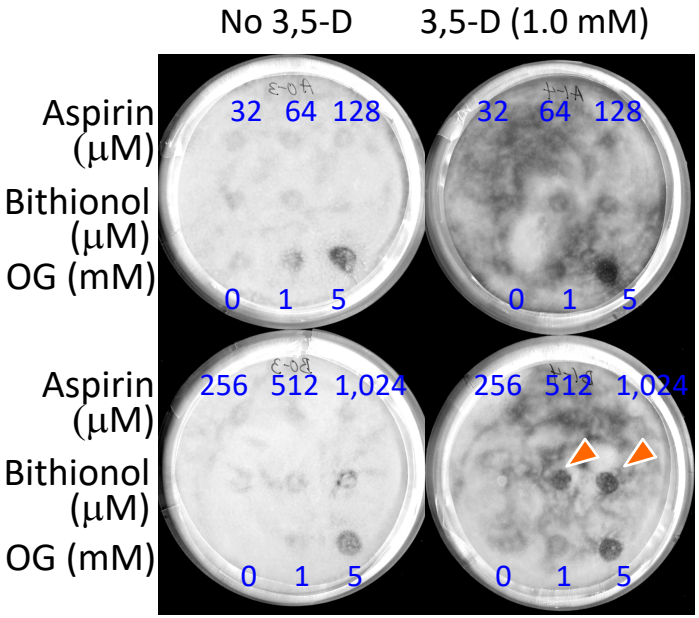


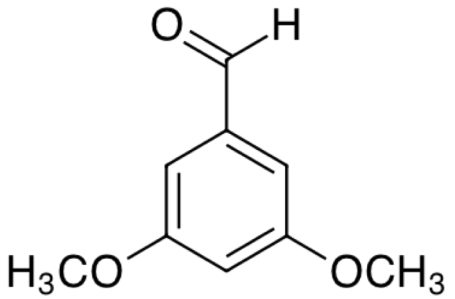
▲, Enhanced susceptibility; OG, Octyl gallate (+ control)

Test in the mycotoxigenic *Aspergillus parasiticus* (Bithionol + 3,5-Dimethoxybenzaldehyde):

Similar results were obtained in *A. parasiticus* 5862 strain, where the sizes of zone of inhibition were generally smaller than that observed in *A. fumigatus*, further indicating "strain specificity" for the efficacy of "bithionol + 3,5-dimethoxybenzaldehyde" treatment (Test concentrations- Aspirin & Bithionol: 32 to 1,024 μM; OG: 1 & 5 mM).

A. parasiticus 5862

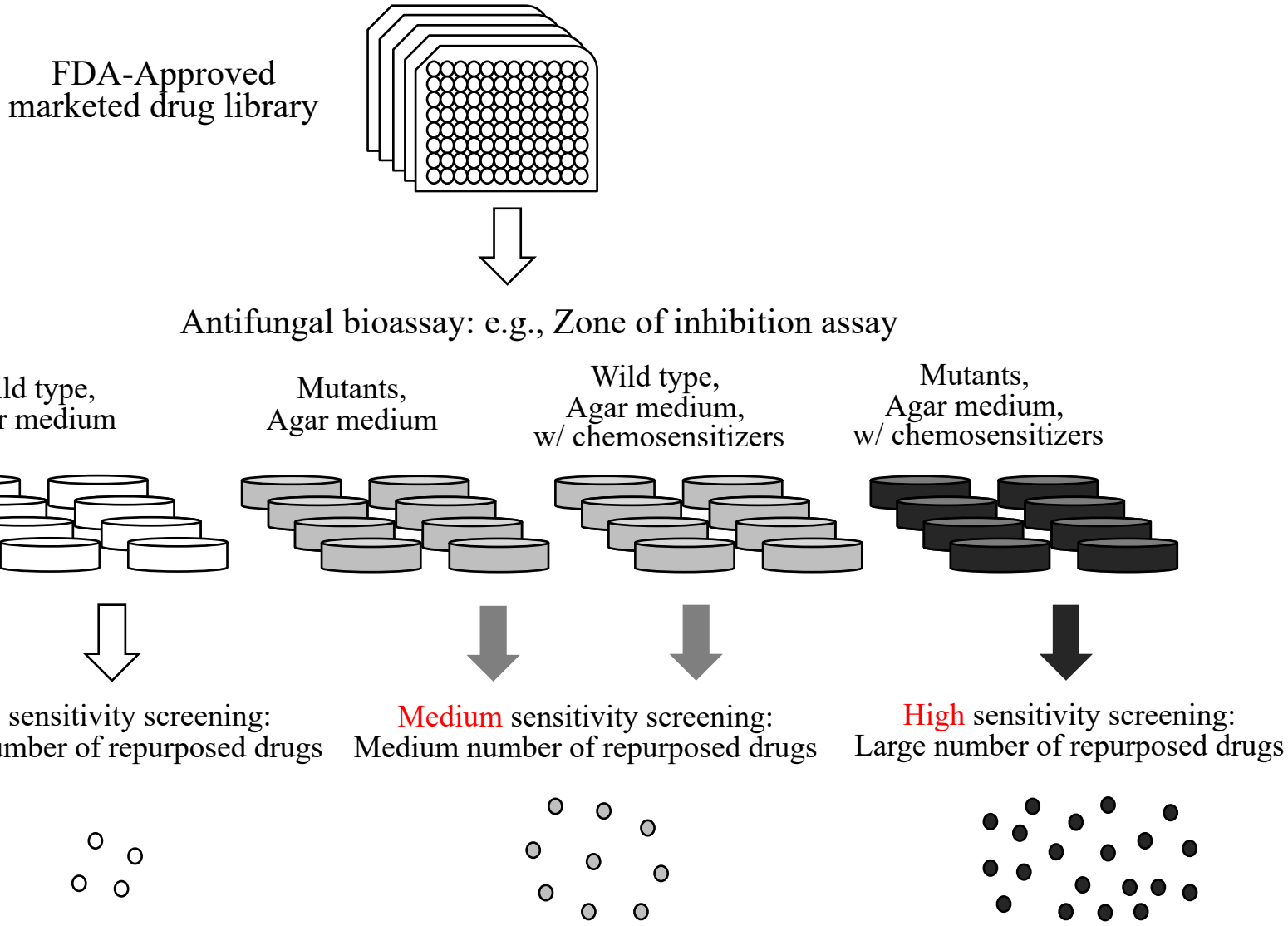




3,5-Dimethoxybenzaldehyde

▲, Enhanced susceptibility; OG, Octyl gallate (+ control)

Currently, antifungal drug repurposing is underway using the following scheme:

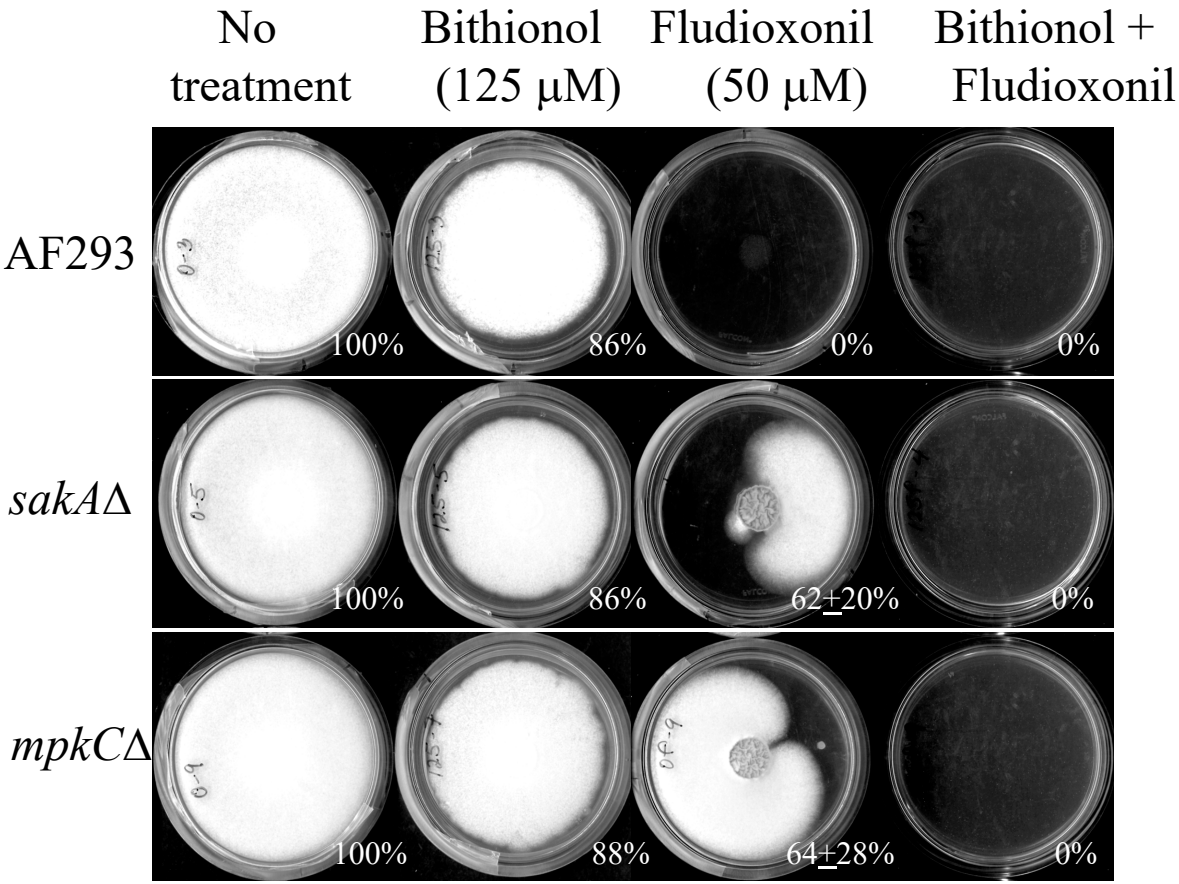


Overcoming Fludioxonil Tolerance of *Aspergillus fumigatus* MAPK Mutants by Bithionol:

Fludioxonil is a commercial phenylpyrrole fungicide, which triggers abnormal and excessive stimulation of the antioxidant MAPK signaling system (Kojima et al. 2004). This abnormal activation of MAPK system triggers cellular energy deprivation via metabolic shifts from normal fungal growth to exhaustive oxidative stress defense. Therefore, application of fludioxonil prevents the growth of fungal pathogens. However, fungi having mutations in components of upstream signaling system, viz., antioxidant MAPK signaling pathway, can escape fludioxonil toxicity (Kojima et al. 2004).

As shown in the figure (next page), *A. fumigatus* MAPK mutants *sakA*Δ and *mpkC*Δ were tolerant to fludioxonil (50 µM), thus developed radial growth on potato dextrose agar (PDA), whereas the growth of wild type was completely inhibited. However, co-application of sub-fungicidal concentration of bithionol (125 µM) with fludioxonil (50 µM) effectively prevented fungal tolerance to fludioxonil, thus achieving complete inhibition of the growth of MAPK mutants. Comprehensive determination of the efficacy of bithionol as an antifungal drug warrants future in-depth study.

Bithionol overcomes fludioxonil resistance of *Aspergillus fumigatus* MAPK mutants (%, Radial growth rate):



Conclusions

- High sensitivity antifungal screening method was investigated by incorporating redox-active chemosensitizers (chemical probes) and antioxidant mutants of *A. fumigatus*.
- Thymol, 4I3M or 3,5-D can be used as potent chemosensitizers to enhance antimycotic activity of the repurposed drug bithionol, while the efficacy of the other drug aspirin was almost not affected, indicating “chemosensitizer – drug specificity" exists.
- While similar enhancement of antifungal efficacy was also observed in the mycotoxigenic *A. parasiticus*, the level of sensitivity of this species was not comparable to that in *A. fumigatus*, thus indicating “strain specificity” also exists during chemosensitization.
- In summary, current data could be used for achieving high-efficiency, large-scale repositioning of marketed drugs with no known antifungal activities as new antifungal drugs, which can reduce costs, abate resistance, alleviate negative side effects associated with current antifungal treatments.

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