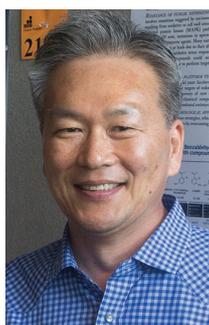


AUGMENTING THE EFFICACY OF FUNGAL AND MYCOTOXIN CONTROL VIA CHEMOSENSITIZATION

Jong H. Kim^{1,*}, Kathleen L. Chan¹, Laura L. McConnell^{2,1} Foodborne Toxin Detection and Prevention Research Unit, Western Regional Research Center, USDA-ARS, 800 Buchanan St., Albany, CA 94710, USA, ²Bayer CropScience, 2 T.W. Alexander Dr., Research Triangle Park, NC 27709, USA describe a new approach to disease control and mycotoxin production. *Correspondence. E-mail: jongheon.kim@ars.usda.gov, Phone: +1 510 559-5841

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Jong Kim



Kathleen Chan



Laura McConnell

Mycotoxins, fungicides and crop production

Infection of crops by fungal pathogens, especially those that produce mycotoxins, *e.g.*, aflatoxins, fumonisins, zearalenone, patulin, ochratoxins, *etc.*, is problematic because effective fungicides for eliminating mycotoxigenic fungi, especially fungicide-resistant pathogens, are sometimes very limited. Recent data showed that mycotoxin contamination damages around 25% of global food and feed crop production (Moretti *et al.* 2013 and references therein). Mycotoxins, such as aflatoxins, can cause serious human or animal health risks as they act as liver-damaging carcinogens. In the European Union, stringent mycotoxin regulations are currently

applied to imported crops, including almonds/pistachios (aflatoxin thresholds 8 to 10 ppm), walnuts (aflatoxin thresholds 2 to 4 ppm), among others (USDA-FAS, 2010). In recent years, the expansion of fungal resistance to conventional fungicides has triggered global agricultural and food safety issues. For example, agricultural fields receiving continuous applications of the most widely used fungicides, such as strobilurins, resulted in the development of insensitivity of some of these fungi to the fungicides. In particular, if strobilurins are applied at suboptimal time-points of fungal growth, the fungicides actually potentiate mycotoxin production by fungi (Ellner, 2005; www.cropscience.bayer.ca/en/Stories/2013/Fungicide-Application-Timing-is-Everything.aspx, Accessed on 18 May, 2015). Fungicide-potential of mycotoxin production in resistant strains has been documented further in a number of aflatoxin-, trichothecene-, citrinin-, and patulin-producing fungal pathogens (Table 1). Therefore, there is a continuous need to enhance the effectiveness of conventional antimycotic agents or discover/develop new intervention strategies, which can ensure safe food and support crop production.

Combined application of antimycotic agents to eliminate fungal infections

In medicine, combination therapy, an administration of two or more antimycotic agents, has been a vital strategy to treat invasive fungal infections. Antifungal drugs with different cellular targets have been administered in combination to eliminate fungal pathogens, especially drug-resistant strains. However, the efficacy of combination therapies often varies depending on antifungal studies investigated. For example, several studies

Table 1. Examples of fungicide-potential of mycotoxin production in resistant pathogens.

Fungi	Fungicides	Mycotoxins	Key literature
<i>Aspergillus parasiticus</i>	Anilinopyrimidine	Aflatoxin	D'Mello <i>et al.</i> , 2000;
<i>Fusarium graminearum</i>	Benzimidazole	Citrinin	Doukas <i>et al.</i> , 2012;
<i>Fusarium culmorum</i> <i>Fusarium sporotrichioides</i>	Carbendazim	Patulin	Malandrakis <i>et al.</i> , 2013.
<i>Penicillium expansum</i>	Fludioxonil	Trichothecene	
<i>Penicillium verrucosum</i>	Flusilazole		
	Iprodione		
	Phenylpyrrole		
	Strobilurins		
	Tebuconazole		

determined a better outcome for fungal control with combination therapy, while others resulted in no added advantage of combination therapy over independent application of each drug alone (Carrillo-Muñoz *et al.*, 2014). Moreover, there are also high risk factors related to combination therapy, such as multi-drug interactions, drug antagonism, delicate dosage dependency and cytotoxicity. Accordingly, application of combination therapy in medicine is generally limited only to severe cases of mycotic diseases (Carrillo-Muñoz *et al.*, 2014).

In agriculture, application of fungicide mixtures (or formulated mixtures), namely inclusion of two or more fungicides with different mechanisms of action, is often recommended for the improvement of fungal disease control and/or management of fungicide resistance during crop production (www.frac.info, accessed 18 May, 2015). In principle, the fungicide mixtures can broaden the spectrum of target fungal pathogens infecting crops, and hence, can increase the effectiveness of fungicides co-applied through synergistic interaction. However, as with the combination therapy in medicine, there are certain restrictions for the use of fungicide mixtures in agricultural fields, as follows: (a) each component included in the fungicide mixture must not show cross-resistance; (b) the dosage levels of individual components incorporated into the mixture should be sufficient to control sensitive fungal isolates when applied alone; and (c) highly effective single-site fungicides, rather than multi-site fungicides, should be used in the mixture considering that more environmental regulatory restrictions are currently applied to the multi-site fungicides (www.frac.info, accessed 18 May, 2015). Therefore, care should be taken when combined application of two or more fungicides is considered/ designed for control of phyto- and/or mycotoxigenic fungal pathogens in agricultural production.

Antifungal chemosensitization as the means to control fungal pathogens

Recently, a mechanism termed antifungal “chemosensitization” has been investigated for the effective control of fungal pathogens. Chemosensitization is an intervention strategy where combined application of a certain compound, namely, a chemosensitizer (natural or synthetic), with a conventional drug/fungicide can enhance the effectiveness of the antimycotic agent co-applied (Campbell *et al.*, 2012 and references therein). Chemosensitization in pathogen control renders fungi highly susceptible to the conventional antimycotic agent in use, where the chemosensitizer significantly debilitates a defense response of a pathogen to the conventional antimycotic agent. The key value or characteristic of antifungal chemosensitization is that, in contrast to the combination therapy or fungicide mixtures described previously, a chemosensitizer does not necessarily possess a high level of antifungal potency. However this intervention strategy can lead to: (a) the enhancement of antifungal efficacy of conventional drugs or fungicides co-applied; (b) mitigation of pathogen resistance to conventional antimycotic agents; and (c) enhanced disruption of mycotoxin production by fungi (Campbell *et al.*, 2012 and references therein). Consequently, chemosensitization-based intervention could complement combination therapy or usage of fungicide mixtures in agriculture.

Examples of chemosensitization-based intervention for pathogen control

For instance, in medicine, the efficacy of the azole drug fluconazole (FLC), an inhibitor of sterol biosynthesis, and consequently, a disruptor of cell membrane synthesis, is being compromised due to the emergence of resistant strains of pathogenic yeasts, such as *Candida albicans* (Youngsaye *et al.*, 2013). Although inhibitors of fungal defense signaling system, including calcineurin or heat shock protein 90, could reverse the FLC resistance, those inhibitors also negatively affect the functions of human homologues. To identify fungal-selective chemosensitizers for effective pathogen control, ~300,000 candidate molecules (from the National Institutes of Health Molecular Libraries Probe Production Centers Network compound collection, USA) have been screened in the presence of a sub-lethal concentration of FLC, which resulted in the identification of the chemical piperazinyl quinolone (Youngsaye *et al.*, 2013). This molecule selectively reversed FLC resistance of *C. albicans* when co-applied with the drug, while the compound showed no antifungal activity when administered alone.

Targeting the functions of pathogens’ mitochondria is another example. In the fungal pathogen *Pneumocystis jirovecii*, the drug atovaquone (ATQ; hydroxy-1,4-naphthoquinone) interferes with the mitochondrial respiratory chain (MRC). ATQ also disrupts the production of cellular energy (ATP) and/or the inner mitochondrial membrane potential ($\Delta\Psi_m$) in the malarial parasites *Plasmodium* (Kim *et al.*, 2013 and references therein). It is noteworthy that, in malarial parasites, co-application of the chemosensitizer proguanil (a modulator of mitochondrial function) further enhanced the anti-malarial activity of ATQ. Interestingly, proguanil-mediated chemosensitization efficacy was specific for the drug ATQ. Proguanil did not improve the activity of other MRC-inhibitory drugs, such as myxothiazole or antimycin A (Kim *et al.*, 2013 and references therein). Therefore, studies indicate drug-chemosensitizer specificity also exists during antifungal chemosensitization.

As mentioned above, strobilurins are one of the most widely used fungicide classes in orchards and other crops. As with ATQ, the cellular target of strobilurins is the MRC (complex III; ubiquinol-cytochrome c oxidoreductase, EC 1.10.2.2). Strobilurins specifically bind to the Q_p (Q_o) center of cytochrome b, and thus inhibit the energy production and trigger cellular oxidative stress caused by an abnormal release of electrons from the MRC (www.frac.info, accessed 18 May, 2015). However, in addition to the high potential for fungal development of strobilurin resistance, the activity of cellular alternative oxidase (AOX) can also overcome the antifungal activity of strobilurins, rendering the completion of electron transfer *via* MRC. Therefore, molecules, such as benzhydroxamic or salicylhydroxamic acid, have been co-applied with strobilurins as chemosensitizers to interfere with the function of fungal AOX (Kim *et al.*, 2014a and references therein).

Table 2. Examples of antimycotic chemosensitization by using natural chemical compounds or analogues.

Chemosensitizer	Target fungi	Mechanism of action	Key literature
Octyl gallate (OG)	<i>A. flavus</i> , <i>A. parasiticus</i> , <i>A. fumigatus</i>	Disruption of fungal cell wall integrity	Kim <i>et al.</i> , 2011, 2013, 2014a, 2014c
Kojic acid	<i>Penicillium</i>	Disruption of fungal antioxidant system	
OG, 2,3-Dihydroxybenzaldehyde	<i>Candida</i> , <i>Cryptococcus</i>	Inhibition of mitochondrial respiration	
Salicylaldehyde (SA)	<i>A. flavus</i> , <i>A. parasiticus</i>	Inhibition of mitochondrial respiration/antioxidant system, vacuolar function	

Identification of new antifungal chemosensitizers for agriculture: use of model fungal systems

Certain natural chemical compounds or their structural derivatives that pose no significant environmental side effects are potential sources of antimycotoxigenic and/or antifungal chemosensitizers. For example, vanillic or caffeic acid or compounds screened from natural product libraries not only inhibited the growth of fungi, but also reduced the mycotoxin production by *Aspergillus* (Kim *et al.*, 2014b and references therein), *Fusarium* (Beekrum *et al.*, 2003; Pani *et al.*, 2014), or *Penicillium* (Neri *et al.*, 2006). These compounds are redox-active, and thus can function as potent redox cyclers that inhibit fungal growth as well as mycotoxin production by disrupting cellular redox homeostasis (Kim *et al.*, 2014a and references therein). However, cellular/molecular targets of the majority of natural chemical compounds are still largely unknown, while antimycotic agents need to be developed with an understanding of their modes of action.

In this respect, a chemo-biological approach using model fungal systems could serve as a powerful engine for discovering novel antifungal/antimycotoxigenic chemosensitizers and their mechanisms of action (Roemer & Boone, 2013). With recent advances in biological and chemical tools, such as interpretable phenotypic systems for compound screening, genomics and informatics, *etc.*, rapid identification of new antifungal/antimycotoxigenic reagents, their mechanisms of action, or novel targets are achievable. Baker's yeast *Saccharomyces cerevisiae* is a good example. *S. cerevisiae* has recently been shown to be a useful model system for identifying antifungal reagents and their gene targets in view that: (1) the genome of *S. cerevisiae* has been fully sequenced and well characterized (www.yeastgenome.org, accessed 18 May, 2015); (2) *S. cerevisiae* mutant collections (~6,000 gene deletion mutants) have proven to be very useful for determining antimycotic mode of action (namely, genome-wide drug-induced haploinsufficiency screen) (Parsons *et al.*, 2004); and (3) many genes in *S. cerevisiae* are structurally closely related to the genes of other fungal pathogens, including mycotoxin-producing fungi.

Compared to *S. cerevisiae*, progress in the development of genetic or genomic tools in mycotoxigenic fungi, such as aflatoxin-producing *Aspergillus flavus* and *A. parasiticus*, is very slow. Although several genomic studies have been pursued in *A. flavus*, many characterized genes in these

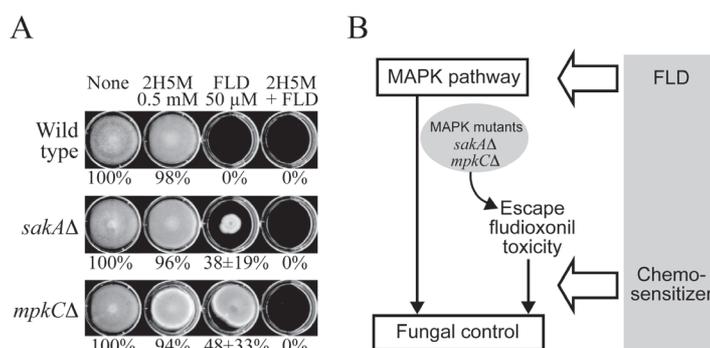


Figure 1. Overcoming fludioxonil (FLD; 50 μM) resistance of *Aspergillus* MAPK mutants (*A. fumigatus sakAΔ* and *mpkCΔ*) via chemosensitization. (A) Example chemosensitization bioassay using 2-hydroxy-5-methoxybenzaldehyde (2H5M; 0.5 mM) (%: Growth inhibition rate, SD < 5% except where noted). (B) Diagram showing the strategy for overcoming FLD resistance of fungal pathogens by using chemosensitizers.

Aspergillus strains are the aflatoxin biosynthesis genes, while novel gene targets for effective antifungal/antimycotoxigenic treatment remain undetermined. In this regard, *S. cerevisiae*, the genetically well-characterized model fungal system, could serve as a functional tool for effective control of mycotoxin-producing fungal pathogens. To date, *S. cerevisiae* has been a useful system for: examining mechanisms of toxicities of mycotoxins; disrupting mycotoxin production; and identifying the mode of action of antifungal natural products (Kim *et al.*, 2014b and references therein). The structural homology of genes involved in stress/defense response between *S. cerevisiae* and the aflatoxigenic *A. flavus* has also been identified (Kim *et al.*, 2014b and references therein).

With *S. cerevisiae*-based target validation, antifungal or antimycotoxigenic chemosensitization in fungal pathogens has been accomplished (Table 2). Noteworthy is that octyl gallate (OG) was found to be a better chemosensitizer to strobilurins, in comparison to the conventional chemosensitizer benzhydroxamic acid (See above) (Kim *et al.*, 2014a). While OG is currently used as a food additive (as an antioxidant) (www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=eafusListing, US Food and Drug Administration, accessed 18 May, 2015), this compound could also find a new use as an antifungal chemosensitizer (Molecular target: fungal cell wall integrity).

Certain benzaldehydes, in combination with phenylpyrroles (fludioxonil fungicide), also overcame tolerance of *Aspergillus* signaling mutants, *i.e.*, mitogen-activated protein kinase (MAPK) mutants, to fludioxonil (Figure 1). The benzo-derivative salicylaldehyde was also identified as a potent antifungal and anti-aflatoxigenic volatile agent that may have some practical application as a fumigant (Kim *et al.*, 2014b and references therein), thus improving the control strategy for food-borne fungal pathogens (Molecular target: superoxide dismutases, glutathione reductase).

Conclusions

Antimycotic chemosensitization could serve as an effective method for control of fungal pathogens. In a chemo-biological platform to enhance antimycotic susceptibility of fungi or to overcome fungal tolerance to conventional antimycotic agents, the model yeast *S. cerevisiae* could be a functional tool for identifying cellular targets of natural/synthetic chemical compounds. The model fungal system also enables the discovery of new uses for known compounds or the utilization of the newly developed compounds as chemosensitizing agents to enhance the efficacy of conventional antimycotic agents. Thus, chemo-biological approaches can lead to the development of novel antifungal intervention strategies, which enhance the efficacy of established microbe intervention practices and overcome antimycotic drug resistance.

There are several steps needed before the chemosensitization approach might be put into practice in production systems. First, chemosensitizers must be tested in the field to determine their environmental fate/effects, such as the breakdown caused by acidic/basic conditions, sunlight, metabolism, *etc.*, or possible volatilization or leaching into ground and surface water, *etc.* Second, chemosensitizers would need to be subjected to the same safety tests as pesticides when registered by the regulatory agencies, to demonstrate that they have no adverse effects to humans or the environment. However, by definition, it is considered that chemosensitizers can be labeled as “Inert ingredients”, rather than “Active ingredient”. Overall, a chemosensitization strategy can reduce costs, abate fungicide resistance, and alleviate environmental side effects associated with current antimycotic intervention strategies.

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Laura McConnell is an analytical chemist with more than 20 years experience in environmental science research with a focus on agricultural topics. She currently serves as a Senior Scientist and Environmental Fate Coordinator in the Environmental Safety Department of Bayer CropScience. She provides environmental chemistry expertise and interacts with scientists in the environmental fate laboratory, modelling, ecotoxicology, and regulatory groups.

Kathleen Chan is a research technician in the Foodborne Toxin Detection and Prevention Research Unit, Western Regional Research Center, Agricultural Research Service (ARS), US Department of Agriculture in Albany, California. She graduated from the Department of Botany, University of California, Berkeley, then joined ARS to perform research on biological control of invasive

weeds. Her current work focuses on preventing mycotoxin production, primarily aflatoxins, in edible crops. She provides antifungal bioassay and molecular biological expertise.

Jong Kim is a Research Molecular Biologist in the Foodborne Toxin Detection and Prevention Research Unit, Western Regional Research Center, Agricultural Research Service (ARS), US Department of Agriculture, Albany, California. His research focuses on the development of intervention strategies for control of mycotoxigenic and phytopathogenic fungi. He provides chemo-biological expertise, particularly in the identification of cellular targets, mechanisms of action and compound interaction, and participates in resistance management in collaboration with producers, industry and academia.

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