Enhancement of fludioxonil fungicidal activity by disrupting cellular glutathione homeostasis with 2,5-dihydroxybenzoic acid

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Abstract

The activity of fludioxonil, a phenylpyrrole fungicide, is elevated by coapplication of the aspirin/salicylic acid metabolite, 2,5-dihydroxybenzoic acid (2,5-DHBA). Fludioxonil activity is potentiated through a mitogen-activated protein kinase (MAPK) pathway that regulates osmotic/oxidative stress-responses. 2,5-DHBA disrupts cellular GSH (reduced glutathione)/GSSG (oxidized glutathione) homeostasis, further stressing the oxidative stress-response system. This stress enhances fludioxonil activity. 2,5-DHBA treatment also prevents tolerance of MAPK mutants resistant to fludioxonil.

Introduction

Many natural phenolic compounds are promising antifungal agents due to their ability to disrupt oxidative stress-response systems. When applied above antioxidant levels, phenolics are potent redox-cyclers that inhibit cell growth by interfering with cellular redox homeostasis (Guillen & Evans, 1994; Shvedova *et al.*, 2000).

Stress-activated signal transduction pathways serve as targets for fungal control (Smits & Brul, 2005). Fludioxonil, a phenylpyrrole fungicide, blocks the protein kinase involved in glycerol biosynthesis (Rosslenbroich & Steubler, 2000). Fungal mutants, such as those in *Cryptococcus neoformans* having defects in certain mitogen-activated protein kinase (MAPK) genes, are tolerant to fludioxonil (Kojima *et al.*, 2004, 2006). The emergence of such resistance in fungi, especially by natural mutation in the target gene, is a major human health issue (Moore *et al.*, 2000; Cowen *et al.*, 2001; Schoustra *et al.*, 2006). Approaches are needed to curtail the development of such tolerance or resistance to this and other antifungal agents.

2,5-Dihydroxybenzoic acid (2,5-DHBA; see Supplementary Fig. S1) is a cellular metabolite of salicylic acid (2-hydroxybenzoic acid), in turn a metabolite of aspirin (acetylsalicylic acid) (Forth *et al.*, 1987; Blacklock *et al.*, 2001). Aspirin metabolites have antimicrobial or antioxidant activities or induce apoptosis in cells grown on nonfermentable carbon sources, such as ethanol (Herrmann, 2003; Balzan *et al.*, 2004; Ashidate *et al.*, 2005; Sapienza & Balzan, 2005). Using the redox-active phenolic 2,5-DHBA, and its structural derivatives, it is shown how targeting fungal antioxidative signal transduction and/or stress-response systems, or genes downstream of the respective MAPK pathway, improves antifungal activity of fludioxonil. It is also shown how such treatments overcome resistance to fludioxonil found in certain MAPK mutants of *Aspergillus fumigatus*, a human pathogen causing invasive aspergillosis.

Materials and methods

Microorganisms and culture condition

Aspergillus fumigatus AF293, wild type, and *A. fumigatus* MAPK deletion mutants *sakA*Δ and *mpkC*Δ (Xue *et al.*, 2004; Reyes *et al.*, 2006) were grown at 37 °C on potato dextrose agar medium (PDA). *Aspergillus flavus* NRRL3357, *Aspergillus parasiticus* NRRL5862, *Aspergillus niger*

NRRL326, *Aspergillus oryzae* FGSC A815, *Aspergillus ochraceous* NRRL 5175, *Aspergillus nidulans* A4 and *Penicillium expansum* NRRL 974 were cultured at 28 °C on PDA (See Table 1 and Supplementary Table S1 for sources of filamentous fungi; McCluskey, 2003). *Saccharomyces cerevisiae* wild-type BY4741 (*Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and selected deletion mutants were obtained from Invitrogen (Carlsbad, CA) and Open Biosystems (Huntsville, AL) as follows (See also Kim *et al.*, 2005): gene regulation mutants: *yap1Δ*, *msn2Δ*, *msn4Δ*, *hot1Δ*, *sko1Δ*, *rim101Δ*, *ure2Δ*; Transporter/assembly protein mutants: *flr1Δ*, *yor1Δ*, *pdr5Δ*, *vph2Δ*, *tfp1Δ/vma1Δ*; signal transduction mutants: *sho1Δ*, *sln1Δ*, *ste50Δ*, *ste20Δ*, *ypd1Δ*, *ssk1Δ*, *ptp2Δ*, *ptp3Δ*, *hog1Δ*, *hog4Δ*, *ssk22Δ*, *ssk2Δ*, *ste11Δ*; Antioxidation mutants: *ctt1Δ*, *cta1Δ*, *osr1Δ*, *trr1Δ*, *trr2Δ*, *tsa1Δ*, *grx1Δ*, *grx2Δ*, *trx1Δ*, *trx2Δ*, *glr1Δ*, *gsh1Δ*, *gsh2Δ*, *sod1Δ*, *sod2Δ*, *ahp1Δ*; and DNA damage control/energy metabolism mutants: *rad54Δ*, *sgs1Δ*, *acc1Δ*, *gpd1Δ*, *hor2Δ* (See http://www.yeastgenome.org for the description of each deletion mutant). Yeast strains were grown on YPD (1% Bacto yeast extract, 2% Bacto peptone, 110 μM glucose) or SG (0.67% Yeast nitrogen base w/o amino acids, 110 μM glucose with appropriate supplements: 180 μM uracil, 200 μM amino acids) medium at 30 °C without light. The fungicide fludioxonil and 2,5-DHBA/structural derivatives (i.e. acetylsalicylic acid, 2,3-, 2,4- and 3,4-DHBA; see Supplementary Fig. S1 for structures) were purchased from Sigma Co. (St Louis, MO). Each compound was dissolved in dimethyl sulfoxide (DMSO; absolute amount $< 20\,\mu L\,mL^{-1}$ media) before use.

Antifungal bioassays

The sensitivities of ascomycetous fungi were based on percent radial growth of treated fungal colonies compared with control colonies, receiving only DMSO. Assays were performed as follows: fungi ($\sim$200 spores) were diluted in phosphate buffered saline (PBS) and spotted on the center of PDA plates with or without antifungal compounds (see Tables and Figures). Growth was observed for 5–7 days. For testing the effects of the combined treatments, 2,5-DHBA was added to the growth medium, together with fludioxonil. For testing tolerance to fludioxonil and test compounds, the fungal sensitivities of *A. fumigatus* MAPK mutants (*sakAΔ* and *mpkCΔ*) were monitored by long-term exposure, up to 10 days.

The sensitivities of yeast strains were determined using a dilution bioassay, to further examine the fungal functional genomics of the mode of action of antifungal compounds (see Kim *et al.*, 2005, 2006). Briefly, $\sim$10^6 cells of the wild type and various deletion mutants of *S. cerevisiae*, cultured on YPD medium, were serially diluted 10-fold to 10^5-fold in SG liquid medium. Cells from each dilution of the respective yeast strain were spotted adjacently on SG agar medium incorporated with 2,5-DHBA (1–18 mM) and incubated at 30 °C. Results were recorded based on a designated value of the highest dilution where a colony became visible after 5–7 days of incubation, as follows: score 0, no colonies were visible from any of the dilutions (treatment highly antifungal); score 6, colonies were visible from all dilutions (no activity); score 1, only a colony from the undiluted cells

Table 1. Structure–activity relationship of effect of dihydroxybenzoic acid (DHBA) derivatives on growth of *Aspergillus fumigatus* and *Aspergillus flavus* (see Supplementary Table S1)*

Conc. (mM)	Control	Acetylsalicylic acid	2,3-DHBA	2,4-DHBA	2,5-DHBA	3,4-DHBA
A. fumigatus						
0	100	–	–	–	–	–
9	–	0	22	72	76	92
12	–	0	0	44	58	92
15	–	0	0	46	44	92
18	–	0	0	32	28	92
21	–	0	0	0	12	90
A. flavus						
0	100	–	–	–	–	–
9	–	60	88	92	80	96
12	–	0	84	80	74	96
15	–	0	0	72	60	96
18	–	0	0	42[†]	42	98
21	–	0	0	0	0	98

*Responses are percentage of radial growth compared with control colonies grown on potato dextrose agar (PDA) plates receiving only DMSO. Values are means of three replicates. SDs of all measurements are $< 3\%$, except where noted. For fungal assays, $\sim$200 spores were diluted in phosphate-buffered saline (PBS) and spotted onto the center of PDA plates containing test compounds and incubated at 37 and 28 °C for *A. fumigatus* and *A. flavus*, respectively, for 5 days. *Aspergillus flavus* NRRL3357 was obtained from National Center for Agricultural Utilization and Research, USDA, Peoria, IL (http://nrrl.ncaur.usda.gov/index.html).
[†]SD: 4%.

(10^6 cells) was visible, etc. Thus, each unit of numerical difference was equivalent to a 10-fold difference in the sensitivity of the yeast strain to a test compound or treatment.

Results

2,5-DHBA inhibits fungal growth by disrupting cellular glutathione homeostasis

Whether 2,5-DHBA was protective (i.e. had antioxidative activity) or was fungicidal depended upon dose and whether chemically induced oxidative stress was present. Lower doses of 2,5-DHBA relieved oxidative stress in *S. cerevisiae* in accordance with prior evidence showing that 2,5-DHBA had antioxidant activity (Ashidate *et al.*, 2005). In a preliminary dilution bioassay, wild type and *sod2Δ*, a strain lacking mitochondrial superoxide dismutase (Mn-SOD), recovered 10 to 10 000-fold from oxidative stress induced by exposure to hydrogen peroxide (H_2O_2; 1, 2 or 3 mM; data for 2 mM H_2O_2 shown, Fig. 1) when treated with 1–3 mM 2,5-DHBA. However, when not exposed to H_2O_2, the growth of these yeast strains decreased progressively when exposed to 3–18 mM 2,5-DHBA. As a result, it was concluded 2,5-DHBA has antifungal activity when cells are not under oxidative stress, presumably through disruption of cellular redox homeostasis.

Next, the antifungal activity of 2,5-DHBA was tested against 46 additional yeast deletion mutants, using the dilution bioassay. These mutants were previously identified as lacking various genes in the oxidative stress response pathway (see Materials and methods; Kim *et al.*, 2005). Of these 46 strains, *ure2Δ* (putative glutathione transferase mutant), *vph2Δ* (vacuolar H(+)-ATPase assembly mutant), *ste20Δ* (protein ser/thr kinase mutant), *glr1Δ* (glutathione reductase mutant), *gsh1Δ* (γ-glutamylcysteine synthetase mutant), *gsh2Δ* (glutathione synthetase mutant), *sod1Δ* (Cu/Zn superoxide dismutase mutant), *sod2Δ* (Mn superoxide dismutase mutant), *hog1Δ* (MAPK mutant) and *hog4Δ* (MAPK kinase mutant) were 10–1000 times more sensitive, than the wild type, when exposed to 6–18 mM 2,5-DHBA. Based on this observation using *S. cerevisiae* as a model system, it was concluded that these 10 genes, or their orthologs, played relatively more significant roles, than other fungal genes, in responding to or tolerating toxic levels of 2,5-DHBA.

Supplementation of GSH at 0.1 mM resulted in almost complete recovery of the *glr1Δ* strain to 2,5-DHBA-induced toxicity, whereas supplementation of GSSG had no effect (Fig. 1). This response in recovery of the *glr1Δ* strain to GSH and not GSSG suggested that 2,5-DHBA may play a role in disrupting regulation of reduced vs. oxidized levels of glutathione in the cell (see Discussion).

2,5-DHBA enhances fludioxonil activity in wild type and MAPK mutants of fungi

Next, responses of *A. fumigatus sakAΔ* and *mpkCΔ*, mutants derived from AF293 (wild type), to treatment with
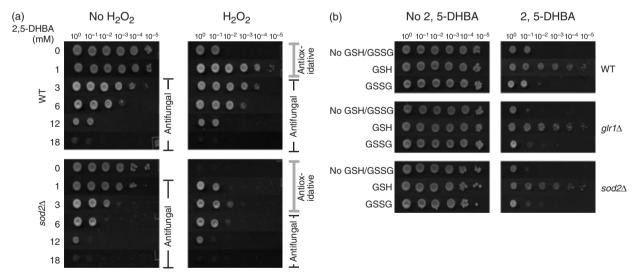

Fig. 1. (a) Antioxidant/antifungal activities of 2,5-DHBA using a yeast bioassay based on 10-fold serial dilutions of yeast cultures placed sequentially on the growth medium. For testing the antioxidant activity of the compounds, different concentrations of hydrogen peroxide (H_2O_2; 1, 2, 3 mM) were applied to the culture medium and the antioxidant/antifungal effects of 2,5-DHBA were monitored as described in the text. The representative bioassays shown in this figure are of the 2 mM H_2O_2 treatment only. (b) Bioassay using strains of *Saccharomyces cerevisiae* showing that treatment with GSH (0.1, 0.5 mM) resulted in inducing recovery of cell growth (wild type, *glr1Δ*, *sod2Δ*) from 2,5-DHBA toxicity, whereas GSSG (0.1, 0.5 mM) did not induce a recovery. This shows 2,5-DHBA prevents reduction of GSSG to GSH. The representative bioassays shown here are of the 0.1 mM GSH/GSSG treatment.
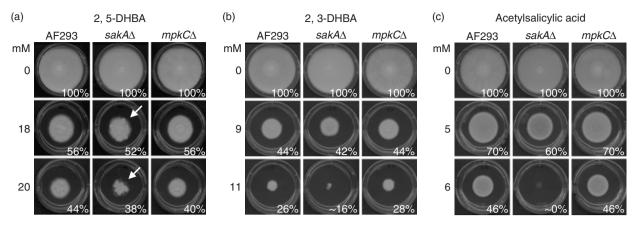
Fig. 2. Responses of *Aspergillus fumigatus* AF293, *sakA*Δ and *mpkC*Δ strains after 7 days of growth to treatment with (a) 2,5-DHBA, (b) 2,3-DHBA and (c) acetylsalicylic acid. Numbers equal per cent radial growth of fungi compared with controls; arrow, rough surface structure of colony; note that different amounts of each compound [20 mM (2,5-DHBA) > 9 mM (2,3-DHBA) > 6 mM (Ac-salicylic acid)] were necessary to achieve similar (~45%) radial growth in AF293, reflecting structure–activity relationship.

2,5-DHBA were examined. SakA and MpkC are orthologous proteins to Hog1p of *S. cerevisiae* (Xue *et al.*, 2004; Reyes *et al.*, 2006). The growth of AF293 and *mpkC*Δ was inhibited 12% to 44% with treatments of 2,5-DHBA at the concentrations tested (14–18 mM; Fig. 2). The *sakA*Δ strain was more sensitive, showing a 24% to 48% reduction in radial growth at the same concentrations of 2,5-DHBA. This strain also showed rough radial growth at concentrations > 18 mM. It is surmised that, like in *S. cerevisiae*, SakA plays a role in GSH homeostasis and, thus, tolerance to 2,5-DHBA (Fig. 2), as *sakA*Δ moderately recovered from 18 mM 2,5-DHBA when provided 0.5 mM GSH (~8% increase in radial growth, data not shown).

In comparison, the growth of *sakA*Δ in 6 mM acetylsalicylic acid was almost completely inhibited (Fig. 2), indicating that this compound (or its metabolite such as salicylic acid) is a more potent antifungal compound than 2,5-DHBA. The activity of structural derivatives of 2,5-DHBA on more species of aspergilli and *P. expansum* was further compared. Acetylsalicylic acid had the highest antifungal activity in all fungi tested. 2,3-DHBA and 2,4-DHBA/2,5-DHBA showed decreased toxicity, respectively (Table 1, Supplementary Table S1; See also Fig. 2). 3,4-DHBA had little antifungal activity, even at the highest concentration (21 mM). In a separate assay, benzoic acid (no –OH group on the aromatic ring; see Supplementary Fig. S1) had the highest antifungal activity, followed by 2-hydroxybenzoic (salicylic) acid and acetylsalicylic acid (Supplementary Table S2). These chemical activity-based responses indicate if the aromatic ring has additional substitutions of hydroxyl groups located away from the *ortho* position then antifungal activity decreases. Currently, further effort is being focused on other structural analogs of 2,5-DHBA, including benzoic acid and benzaldehyde derivatives, and 2,3-DHBA, which

has known antioxidant activity (Hayashi *et al.*, 1995). It is aimed to determine which of these shows the greatest activity alone, or as a fungicidal supplement, on a broad spectrum of fungal pathogens.

Certain fungi having MAPK mutations are tolerant to phenylpyrrole fungicides (Kojima *et al.*, 2004, 2006). Both *sakA*Δ/*mpkC*Δ MAPK mutants of *A. fumigatus* escaped fludioxonil toxicity (Fig. 3). However, it was found that coapplying 2,5-DHBA with fludioxonil prevented these mutants from developing tolerance to this fungicide (Fig. 3). Presumably, this tolerance is prevented because 2,5-DHBA targets genes downstream in these MAPK pathways targeted by fludioxonil and destabilizes GSH/GSSG homeostasis (Fig. 3).

Discussion

The present results, with both yeast and ascomycetous fungi, indicate that targeting the oxidative stress response system of fungi can augment the fungicidal activity of fludioxonil. In the present study, we used 2,5-DHBA to disrupt glutathione homeostasis, disrupting the oxidative stress response capabilities of the fungal cell. This was readily apparent in the bioassays using the yeast *glr1*Δ deletion mutant. *GLR1* encodes glutathione reductase, which converts GSSG to GSH. In *S. cerevisiae*, *GLR1* is a target gene of the Hog1p (MAPK) pathway (Rep *et al.*, 2001), for replenishing GSH from GSSG. However, *glr1*Δ and/or MAPK pathway mutants cannot reduce GSSG to GSH efficiently, resulting in higher sensitivity to 2,5-DHBA. Previous biochemical studies with *S. cerevisiae* showed that glutathione levels were greatly decreased by 2,5-DHBA, especially in *sod2*Δ mutants cultured on a nonfermentable carbon source (Balzan *et al.*, 2004; Sapienza & Balzan, 2005). Therefore,
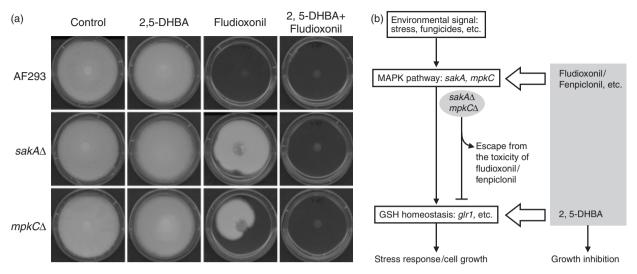
Fig. 3. Inhibiting tolerance (escape) of *sakAΔ* and *mpkCΔ* MAPK mutants of *Aspergillus fumigatus* to phenylpyrrole fungicides by coapplication of 2,5-DHBA. (a) Representative bioassays of wild type (AF293) and MAPK mutants with no treatment (Control), 2,5-DHBA (12 mM), fludioxonil (50 μM) and fludioxonil (50 μM)+2,5-DHBA (12 mM). Note that cotreatment of 2,5-DHBA prevents *sakAΔ* and *mpkCΔ* MAPK mutants from escaping fludioxonil toxicity. (b) Scheme showing where phenylpyrrole fungicides (e.g. fludioxonil) target MAPK signaling pathway genes. MAPK mutants escape toxicity by missing the signal stimulated by phenylpyrrole fungicides and, thus, avoiding the induced osmotic/oxidative stress response. Application of 2,5-DHBA disrupts cellular GSH homeostasis, which enhances the toxicity in the wild-type cells or helps prevent the escape of MAPK mutants from antifungal effects.

fungi having a defect in maintaining cellular GSH/GSSG balance, such as the *glr1Δ* mutant, will be sensitive to 2,5-DHBA. Moreover, targeting this system also prevents escape of MAPK mutants that regulate glutathione homeostasis. This was quite evident from our treatment of the *sakAΔ/mpkCΔ* MAPK mutants of *A. fumigatus* with 2,5-DHBA.

Several studies have shown the link between antifungal activities of certain fungicides and the response of the cellular antioxidative system. For example, a rapid decrease in glutathione was detected when *S. cerevisiae* cells were treated with sublethal concentrations of fungitoxic dimethyldithiocarbamic acid or thiram [bis(dimethylthiocarbamoyl) disulfide], suggesting that glutathione reductase (Glr1p) is crucial for fungal tolerance to these compounds (Elskens & Penninckx, 1997).

Strains of the rice blast fungus, *Pyricularia oryzae* (*Magnaporthe grisea*), having a mutation in the osmotic stress-responsive histidine kinase gene (*HIK1*), exhibited hyper-tolerance to three groups of fungicides: phenylpyrroles, dicarboximides and aromatic hydrocarbons (Motoyama *et al.*, 2005). This tolerance shows that signal transduction and MAPK pathways play roles where the osmotic stress pathway is a target of fungicides.

The potential hazards associated with the use of conventional fungicides are an important human health issue. For example, Cereser *et al.* (2001) reported that thiram induces rapid oxidation of reduced glutathione in human skin fibroblasts, leading to oxidative stress and cell death. Radice *et al.* (2001) also showed that iprodione, a dicarboximide

fungicide, caused oxidative damage and decreased cellular GSH content to hepatocytes of steelhead trout, *Oncorhynchus mykiss*. This oxidative damage was not species specific, and showed potential widespread negative effects.

The present study showed that effective fungal control can be achieved by disrupting more than one molecular target in the antioxidative signal transduction/stress response system (e.g. MAPK pathway by fludioxonil and glutathione homeostasis by 2,5-DHBA). The present study also indicates that fludioxonil, mainly used as an agricultural fungicide, can be a useful screening tool if fungal isolates have a mutation in the MAPK gene. Such a screening assay would help identify the potential for development of fungal tolerance or resistance to the compound and could serve as a warning to use other or additional antifungals to prevent escape from phenylpyrrole toxicity.

In conclusion, natural phenolic compounds have the potential to serve as alternatives or supplements to commercial antimicrobials or as lead compounds to new agents. Certain phenolics disrupt cellular redox homeostasis by targeting the fungal antioxidative stress systems. It was shown how 2,5-DHBA, an aspirin metabolite, greatly improved the effectiveness of fludioxonil, a phenylpyrrole fungicide. Our results indicate that this improvement is from the ability of 2,5-DHBA to disrupt glutathione homeostasis, resulting in cellular GSH/GSSG imbalances. Supplementation with safe, natural compounds to augment the effectiveness of commercial fungicides or antifungal drugs lowers the dosages of commercial fungicides required for

effective control. Consequently, this lower dosage reduces environmental impact and risks to human health by lowering exposure to fungicides. Additionally, there is decreased cost in usage and potential for development of fungal resistance.

Acknowledgements

References

Ashidate K, Kawamura M, Mimura D, Tohda H, Miyazaki S, Teramoto T, Yamamoto Y & Hirata Y (2005) Gentisic acid, an aspirin metabolite, inhibits oxidation of low-density lipoprotein and the formation of cholesterol ester hydroperoxides in human plasma. *Eur J Pharmacol* **513**: 173–179.

Balzan R, Sapienza K, Galea DR, Vassallo N, Frey H & Bannister WH (2004) Aspirin commits yeast cells to apoptosis depending on carbon source. *Microbiology* **150**: 109–115.

Blacklock CJ, Lawrence JR, Wiles D, Malcolm EA, Gibson IH, Kelly CJ & Paterson JR (2001) Salicylic acid in the serum of subjects not taking aspirin. Comparison of salicylic acid concentrations in the serum of vegetarians, non-vegetarians, and patients taking low dose aspirin. *J Clin Pathol* **54**: 553–555.

Cereser C, Boget S, Parvaz P & Revol A (2001) Thiram-induced cytotoxicity is accompanied by a rapid and drastic oxidation of reduced glutathione with consecutive lipid peroxidation and cell death. *Toxicology* **163**: 153–162.

Cowen LE, Kohn LM & Anderson JB (2001) Divergence in fitness and evolution of drug resistance in experimental populations of *Candida albicans*. *J Bacteriol* **183**: 2971–2978.

Elskens MT & Penninckx MJ (1997) Thiram and dimethyldithiocarbamic acid interconversion in *Saccharomyces cerevisiae*: a possible metabolic pathway under the control of the glutathione redox cycle. *Appl Environ Microbiol* **63**: 2857–2862.

Forth W, Henschler D & Rummel W (1987) *Allgemeine und Spezielle Pharmakologie und Toxikologie*, Wissenschaftsverlag, Mannheim, Wien, Zurich.

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.

Hayashi K, Suzuki K, Kawaguchi M, Nakajima T, Suzuki T, Numata M & Nakamura T (1995) Isolation of an antioxidant from *Penicillium roquefortii* IFO 5956. *Biosci Biotechnol Biochem* **59**: 319–320.

Herrmann M (2003) Salicylic acid: an old dog, new tricks, and staphylococcal disease. *J Clin Invest* **112**: 149–151.

Kim JH, Campbell BC, Yu J, Mahoney N, Chan KL, Molyneux RJ, Bhatnagar D & Cleveland TE (2005) Examination of fungal

stress response genes using *Saccharomyces cerevisiae* as a model system: targeting genes affecting aflatoxin biosynthesis by *Aspergillus flavus* link. *Appl Microbiol Biotechnol* **67**: 807–815.

Kim JH, Campbell BC, Mahoney N, Chan KL & May GS (2006) Targeting antioxidative signal transduction and stress response system: control of pathogenic *Aspergillus* with phenolics that inhibit mitochondrial function. *J Appl Microbiol* **101**: 181–189.

Kojima K, Takano Y, Yoshimi A, Tanaka C, Kikuchi T & Okuno T (2004) Fungicide activity through activation of a fungal signalling pathway. *Mol Microbiol* **53**: 1785–1796.

Kojima K, Bahn YS & Heitman J (2006) Calcineurin, Mpk1 and Hog1 MAPK pathways independently control fludioxonil antifungal sensitivity in *Cryptococcus neoformans*. *Microbiology* **152**: 591–604.

McCluskey K (2003) The fungal genetics stock center: from molds to molecules. *Adv Appl Microbiol* **52**: 245–262.

Moore CB, Sayres N, Mosquera J, Slaven J & Denning DW (2000) Antifungal drug resistance in *Aspergillus*. *J Infect* **41**: 203–220.

Motoyama T, Kadokura K, Ohira T, Ichiishi A, Fujimura M, Yamaguchi I & Kudo T (2005) A two-component histidine kinase of the rice blast fungus is involved in osmotic stress response and fungicide action. *Fungal Genet Biol* **42**: 200–212.

Radice S, Ferraris M, Marabini L, Grande S & Chiesara E (2001) Effect of iprodione, a dicarboximide fungicide, on primary cultured rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquat Toxicol* **54**: 51–58.

Rep M, Proft M, Remize F, Tamas M, Serrano R, Thevelein JM & Hohmann S (2001) The *Saccharomyces cerevisiae* Sko1p transcription factor mediates HOG pathway-dependent osmotic regulation of a set of genes encoding enzymes implicated in protection from oxidative damage. *Mol Microbiol* **40**: 1067–1083.

Reyes G, Romans A, Nguyen CK & May GS (2006) The MAP kinase MpkC of *Aspergillus fumigatus* is required for utilization of sorbitol (Vp-29). 8th European Conference on Fungal Genetics. Vienna, Austria.

Rosslenbroich H-J & Steubler D (2000) *Botrytis cinerea* – history of chemical control and novel fungicides for its management. *Crop Prot* **19**: 557–561.

Sapienza K & Balzan R (2005) Metabolic aspects of aspirin-induced apoptosis in yeast. *FEMS Yeast Res* **5**: 1207–1213.

Schoustra SE, Debets AJ, Slakhorst M & Hoekstra RF (2006) Reducing the cost of resistance; experimental evolution in the filamentous fungus *Aspergillus nidulans*. *J Evol Biol* **19**: 1115–1127.

Shvedova AA, Kommineni C, Jeffries BA, Castranova V, Tyurina YY, Tyurin VA, Serbinova EA, Fabisiak JP & Kagan VE (2000) Redox cycling of phenol induces oxidative stress in human epidermal keratinocytes. *J Invest Dermatol* **114**: 354–364.

Smits GJ & Brul S (2005) Stress tolerance in fungi – to kill a spoilage yeast. *Curr Opin Biotechnol* **16**: 225–230.

Xue T, Nguyen CK, Romans A & May GS (2004) A mitogen-activated protein kinase that senses nitrogen regulates conidial

germination and growth in *Aspergillus fumigatus*. *Eukaryot Cell* **3**: 557–560.

Supplementary material

The following supplementary material is available for this article:

Fig. S1. Structures of benzoic acid derivatives used in this study. All chemicals used in this study [i.e. acetylsalicylic acid (aspirin), 2,3-, 2,4-, 2,5- and 3,4-dihydroxybenzoic acid (DHBA) and fungicide (fludioxonil)] were obtained from Sigma (St Louis, MO). All compounds were dissolved in dimethyl sulfoxide (DMSO; $< 20\,\mu\text{L mL}^{-1}$ media) for incorporation into culture media.

Table S1. Structure-activity relationship of effect of dihydroxybenzoic acid (DHBA) derivatives on growth of different aspergilli and *Penicillium expansum*.

Table S2. Minimum inhibitory concentration (MIC) of benzoic, salicylic and acetylsalicylic acid on growth of various aspergilli and *Penicillium expansum*.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2007.00682.x (This link will take you to the article abstract).

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