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Communication

Octyl Gallate as an Intervention Catalyst to Augment Antifungal Efficacy of Caspofungin

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Abstract: Filamentous fungi such as *Aspergillus* spp. are opportunistic pathogens, which cause highly invasive infections, especially in immunocompromised individuals. Control of such fungal pathogens is increasingly problematic due to the small number of effective drugs available for treatment. Moreover, the increased incidence of fungal resistance to antifungal agents makes this problem a global human health issue. The cell wall integrity system of fungi is the target of antimycotic drugs echinocandins, such as caspofungin (CAS). However, echinocandins cannot completely inhibit the growth of filamentous fungal pathogens, which results in survival/escape of fungi during treatment. Chemosensitization was developed as an alternative intervention strategy, where co-application of CAS with the intervention catalyst octyl gallate (OG; chemosensitizer) greatly enhanced CAS efficacy, thus achieved ≥ 99.9% elimination of filamentous fungi in vitro. Based on hypersensitive responses of *Aspergillus* antioxidant mutants to OG, it is hypothesized that, besides destabilizing cell wall integrity, the redox-active characteristic of OG may further debilitate fungal antioxidant system.

Keywords: antioxidant system; cell wall integrity; chemosensitization; end point; fungi; small molecule

1. Introduction

Fungal infectious diseases, such as candidiasis, cryptococcosis or invasive aspergillosis caused by *Candida*, *Cryptococcus* or *Aspergillus*, respectively, are serious human health issues, since effective drugs, especially those for eliminating resistant pathogens, are often very limited [1 and references therein]. Therefore, there is continuous need to improve the efficacy of current antifungal drugs or discover/develop new intervention strategies. The cell wall integrity system of fungal pathogens could serve as an effective target of antimycotic drugs [2]. Genome and functional studies revealed that many genes in the cell wall integrity system of fungi are well conserved [3,4]. Caspofungin (CAS; **Figure 1**), like other echinocandins including micafungin and anidulafungin, is an antifungal lipopeptide drug. CAS inhibits the activity of β-1,3-D-glucan synthase in fungal cell wall integrity system, thus disrupts the synthesis of the cell wall component β-1,3-D-glucan [5 and references therein]. Echinocandins further lyse actively growing hyphal tips during filamentous fungal growth [5]. However, despite their utility, echinocandins generally cannot achieve complete inhibition of the growth of filamentous fungi [6], which results in pathogen survival during treatment.

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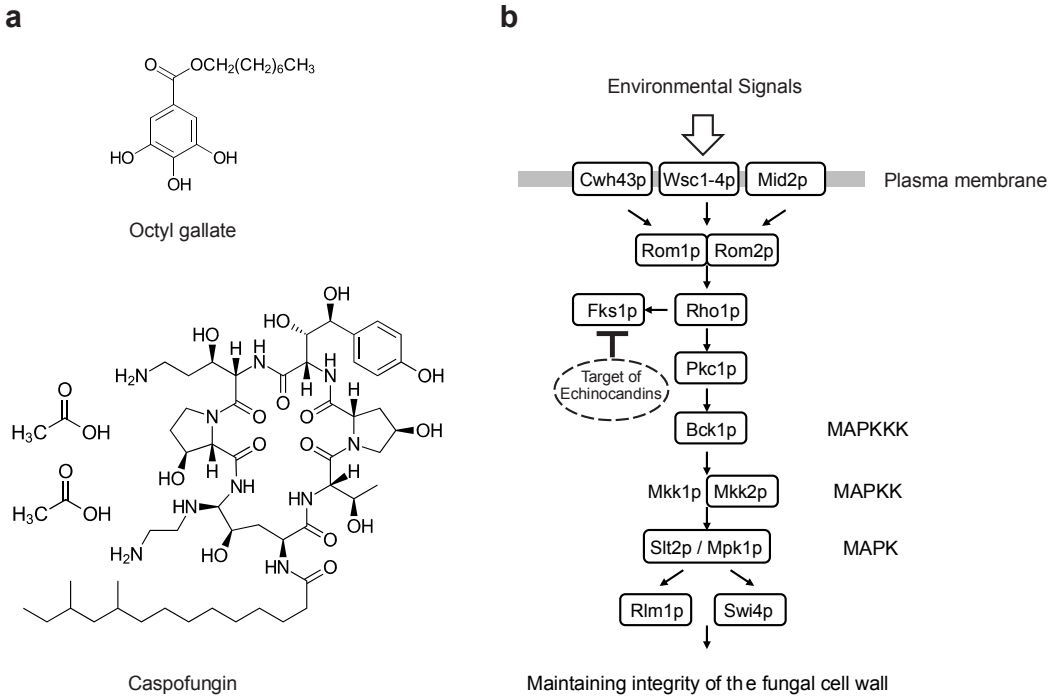

Figure 1. (a) Structures of octyl gallate (OG) and caspofungin (CAS); (b) Signal transduction pathway of fungi for maintaining cell wall integrity, viz., sensing the status of cell wall during growth and/or protecting the cell from environmental cues, such as external osmotic fluctuation (See [4,7] and references therein). See **Table S1** for the functions of proteins in the pathway, except: Rom1p, GDP/GTP exchange protein for Rho1p; Cwh43p, Putative sensor/transporter protein involved in cell wall biogenesis; Rho1p, GTP-binding protein, which regulates protein kinase C (Pkc1p) and the cell wall synthesizing enzyme β-1,3-D-glucan synthase (Fks1p; Target of echinocandins).

Antifungal chemosensitization is an intervention strategy, where co-application of a certain natural or synthetic compound, viz., chemosensitizer (intervention catalyst), with a commercial drug greatly enhances the efficacy of the drug co-applied [8]. The key advantage of chemosensitization is that, in contrast to combination therapy, which is a co-application of two or more commercial antimycotic drugs, a chemosensitizer itself does not have to possess a high degree of antifungal potency. However, chemosensitization not only enhances the antifungal efficacy of the drug co-applied, but also mitigates pathogen resistance to conventional drugs [8]. Therefore, chemosensitization-based intervention could complement current antifungal practices, such as combination therapy.

As a proof of concept, the effectiveness of antifungal chemosensitization [CAS + octyl gallate (OG; octyl 3,4,5-trihydroxybenzoic acid)] was investigated in the species of *Aspergillus* and *Penicillium* in this study (**Table 1**). Test strains belong to clinical and foodborne fungal pathogens, including environmental fungal contaminants. OG, an alkyl derivative of the natural product gallic acid (**Figure 1**), was investigated as a potent chemosensitizer (intervention catalyst) to enhance the efficacy of cell wall disrupting drug CAS. We recently determined that OG functioned as a safer, more effective preservative for consumer products [9].

Table 1. Filamentous fungal strains used in this study.

***Aspergillus* strains**	**Strain characteristics**	**Source**
A. fumigatus AF293	Human pathogen (aspergillosis), Parental strain, Reference clinical strain used for genome sequencing	[10]
*A. fumigatus sakA*Δ	Mitogen-Activated Protein Kinase (MAPK) gene deletion mutant derived from AF293	[10]

*A. fumigatus mpkC*Δ	MAPK gene deletion mutant derived from AF293	[11]
A. flavus 3357	Toxigenic (aflatoxin-producing), Human pathogen (aspergillosis), Reference strain for genome sequencing	NRRL[1]
A. flavus 4212	Toxigenic (aflatoxin-producing), Human pathogen (aspergillosis)	NRRL
A. parasiticus 2999	Toxigenic (aflatoxin-producing)	NRRL
A. parasiticus 5862	Toxigenic (aflatoxin-producing)	NRRL
***Penicillium* strains**	**Strain characteristics**	**Source**
P. expansum W1	Toxigenic (patulin-producing; parental strain)	[12]
P. expansum FR2	Fludioxonil resistant mutant derived from *P. expansum* W1	[12]
P. expansum W2	Toxigenic (patulin-producing; parental strain)	[12]
P. expansum FR3	Fludioxonil resistant mutant derived from *P. expansum* W2	[12]
P. glabrum 766	Environmental contaminant	NRRL
P. chrysogenum 824	Fleming's penicillin-producing strain	NRRL
P. griseofulvum 2159	Environmental contaminant	NRRL
P. italicum 983	Environmental contaminant	NRRL

[1] NRRL, National Center for Agricultural Utilization and Research, USDA-ARS, Peoria, IL, USA.

2. Materials and Methods

2.1. Chemicals

All chemicals including antifungal compounds [caspofungin (CAS), octylgallate (OG)] and culture media were procured from Sigma Co. (St. Louis, MO, USA). CAS and OG were dissolved in dimethylsulfoxide (DMSO; absolute DMSO amount: < 2% in media) before incorporation into culture media. Throughout this study, control plates (No treatment) contained DMSO at levels equivalent to that of cohorts receiving antifungal agents, within the same set of experiments.

2.2. Antifungal Bioassay: Saccharomyces cerevisiae

Susceptibility of the model yeast *S. cerevisiae* (See **Table S1**) was tested according to the protocol outlined by European Committee on Antimicrobial Susceptibility Testing (EUCAST) for yeasts [13]. Quantitative 96-well microtiter plate broth-dilution assays were performed in triplicate in liquid Synthetic Glucose (SG; Yeast nitrogen base without amino acids 0.67%, glucose 2% with appropriate supplements: uracil 0.02 mg/mL, amino acids 0.03 mg/mL) medium, where minimum inhibitory concentration [MIC; lowest concentration of compound showing no visible fungal growth in microtiter wells (200 µL per well)] was assessed after 24h at 30°C. Minimum fungicidal concentration (MFC; lowest concentration of compound showing ≥ 99.9% death of fungal cells inoculated, viz., achievement of ≥ 5 log fungal elimination) was determined after completion of MIC assays by spreading entire volumes of microtiter wells (200 µL) onto individual Yeast Peptone Dextrose (YPD; Bacto yeast extract 1%, Bacto peptone 2%, glucose 2%) recovery plates. Colony-forming units were counted after additional incubation of plates for 48h at 30°C.

2.3. Antifungal Bioassay: Filamentous Fungi

To determine the level of compound interactions, namely chemosensitizing activity of OG to CAS, in filamentous fungi (**Table 1**), triplicate checkerboard bioassays (0.4×10^4 to 5×10^4 CFU/mL) were performed in 96-well microtiter plates at 28 or 35°C, depending on types of strains, using a broth microdilution method in RPMI 1640 medium (Sigma Co.) according to the protocol described by the Clinical and Laboratory Standards Institute (CLSI) M38-A [14]. MICs of antimycotic

compounds, alone or in combination, were assessed after 48h. MFCs of CAS and OG, alone or in combination, were determined following the completion of MIC analysis by spreading entire volumes of microtiter wells (200 µL) onto individual potato dextrose agar (PDA) recovery plates, and culturing for additional 48h. Compound interactions, namely Fractional Inhibitory Concentration Indices (FICIs) and Fractional Fungicidal Concentration Indices (FFCI) for determining CAS + OG synergism for "growth inhibitory" and "fungal death", respectively, were calculated as follows: FICI or FFCI = (MIC or MFC of compound A in combination with compound B/MIC or MFC of compound A, alone) + (MIC or MFC of compound B in combination with compound A/MIC or MFC of compound B, alone). Levels and types of compound interactions between antimycotic agents were defined as: synergistic (FICI or FFCI ≤ 0.5) or indifferent (FICI or FFCI > 0.5 - 4) [15]. If preferred, the Isenberg's [16] methodology could be substituted in parallel determinations of synergism, where compound interactions were: synergistic (FICI ≤ 0.5), additive (0.5 < FICI ≤ 1), neutral (1 < FICI ≤ 2), or antagonistic (FICI > 2).

2.4. Statistical Analysis

Statistical analysis (student's *t*-test) was performed according to "Statistics to use" [17], where $p < 0.05$ was considered significant.

3. Results and Discussion

3.1. Octyl gallate (OG) perturbs fungal cell wall integrity system: S. cerevisiae bioassay

To determine whether OG could target cell wall integrity system of fungi, OG susceptibility of eleven mutants of the model yeast *S. cerevisiae*, where genes in cell wall integrity mitogen-activated protein kinase (MAPK) pathway were systematically deleted (**Figure 1**, **Table S1**), was initially examined. *S. cerevisiae* is a useful model system for identifying antifungal agents and their gene targets in view that: (1) many genes in *S. cerevisiae* are orthologs of genes of fungal pathogens [18], and (2) *S. cerevisiae* gene deletion mutant collections have proven to be very useful for genome-wide drug-induced haploinsufficiency screen to determine drug mode of action [19-21]. OG is a generally recognized as safe (GRAS) reagent [22] and, thus, is currently used as an antioxidant added to food. OG is also known to inhibit the growth of bacterial pathogens, such as *Staphylococcus aureus* [23] and dairy isolates of *Enterococcus faecalis* expressing different virulence factors [24].

Results showed that *bck1*Δ [MAPK kinase kinase (MAPKKK) mutant] and *slt2*Δ (MAPK mutant) were the most sensitive mutants to OG (viz., both MICs and MFCs = 25 µM; Mean MICs and MFCs for other yeast strains = 47 and 50 µM, respectively; **Table data not shown**). We further observed that CAS + OG chemosensitization could lower dosages of CAS and OG to achieve ≥ 99.9% fungal death, where *slt2*Δ required much less dosages of each reagent (CAS: 0.25 µg/mL, OG: 12.5 µM), when compared to the wild type (CAS: 2.00 µg/mL, OG: 25.0 µM) (**Figure 2**)].

The *bck1*Δ and *slt2*Δ previously exhibited hypersensitivity to cell wall perturbing agents including CAS [25], and therefore, have been serving as screening tools for identifying new cell wall disrupting drugs [25]. Thus, hypersensitive response of *bck1*Δ and *slt2*Δ to OG indicates OG could target fungal cell wall system.

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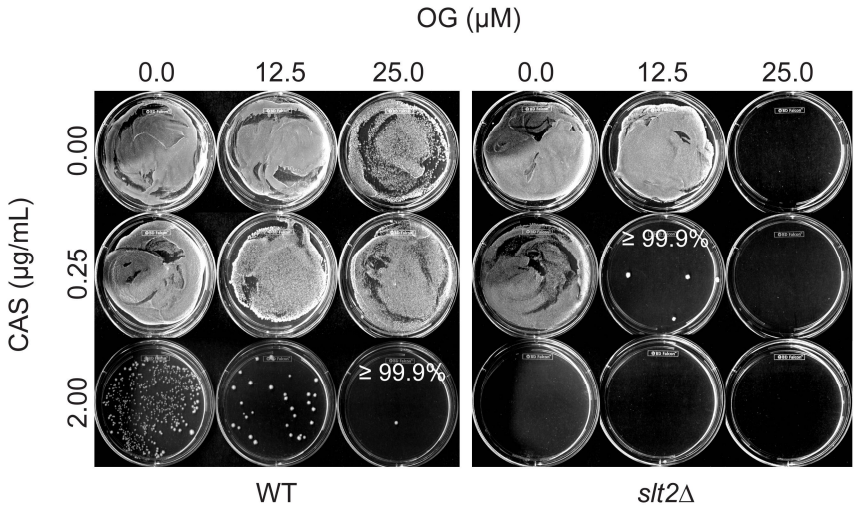

Figure 2. Exemplary chemosensitization (CAS + OG) test in *S. cerevisiae* wild type (WT) and *slt2*Δ strains. Results shown here are the determination of MFCs, after MIC measurement in 96-well microplates, of antifungal agents (The ≥ 99.9% indicates achievement of ≥ 99.9% fungal death). Note that *slt2*Δ required much lower dosages of CAS and OG to achieve ≥ 99.9% fungal death, when compared to the WT (See text).

3.2. Octyl gallate (OG) enhances the efficacy of caspofungin (CAS): Filamentous fungi bioassay

Antifungal chemosensitization (CAS + OG) was then investigated in filamentous fungal pathogens (*Aspergillus*, *Penicillium*). For FICIs in *Aspergillus*, "synergistic" FICI values (i.e., FICI ≤ 0.5) were found between OG and CAS for *A. flavus* 3357 and *A. parasiticus* 2999 (**Table 2**). Although there was no calculated synergism, as determined by "indifferent" [15] or "additive" interactions [16], there was enhanced antifungal activity of OG and CAS also in other *Aspergillus* strains, which was reflected in lowered MICs of OG or CAS (FICIs = 0.6 to 1.0) when two compounds were co-applied (**Table 2**). Of note, "synergistic" FICI values (FICI ≤ 0.5) were determined for most *Penicillium* strains tested (FICIs = 0.3 to 0.5). The only exception was *P. griseofulvum* 2159, where FICI was 0.6 (**Table 2**). Therefore, results indicated that *Penicillium* species were more susceptible to OG-mediated chemosensitization than the *Aspergillus* strains examined.

Table 2. Antifungal chemosensitization of octyl gallate (OG; mM) to caspofungin (CAS; μg/mL) tested against filamentous fungi. Synergistic FICIs and FFCIs (≤ 0.5) are shown in bold characters.[1]

	Compounds	MIC alone	MIC combined	FICI	MFC alone	MFC combined	FFCI
A. fumigatus AF293	CAS	128	32	0.8	128	64	1.0
	OG	0.2	0.1		0.4	0.2	
*A. fumigatus sakA*Δ	CAS	128	8	0.6	128	64	1.0
	OG	0.2	0.1		0.2	0.1	
*A. fumigatus mpkC*Δ	CAS	128	8	0.6	128	64	0.8
	OG	0.2	0.1		0.4	0.1	
A. flavus 4212	CAS	128	64	1.0	128	64	1.0
	OG	0.2	0.1		0.4	0.2	
A. flavus 3357	CAS	128	2	**0.5**	128	128	2.0
	OG	0.4	0.2		1.6	1.6	
A. parasiticus 5862	CAS	128	64	1.0	128	128	2.0
	OG	0.4	0.2		1.6	1.6	
A. parasiticus 2999	CAS	128	4	**0.5**	128	64	0.6
	OG	0.4	0.2		1.6	0.2	
Mean, *Aspergillus*[2]	CAS	128.00	33.20	0.8	128.00	89.60	1.4

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	OG	0.32	0.16		1.12	0.76	
t-test[3]	CAS	-	$P < 0.001$	-	-	$P < 0.05$	-
	OG	-	$P < 0.05$		-	$P < 0.5$	
P. expansum W1	CAS	128	32	**0.5**	128	32[4]	0.8
	OG	0.2	0.05		1.6	0.8	
P. expansum FR2	CAS	128	32	**0.5**	128	32	0.8
	OG	0.2	0.05		1.6	0.8	
P. expansum W2	CAS	128	32	**0.5**	128	32	0.8
	OG	0.2	0.05		1.6	0.8	
P. expansum FR3	CAS	128	32	**0.5**	128	32	0.8
	OG	0.2	0.05		1.6	0.8	
P. glabrum 766	CAS	128	16	**0.3**	128	32	**0.3**
	OG	0.2	0.025		1.6	0.05	
P. italicum 983	CAS	64	16	**0.5**	64	16	0.8
	OG	0.2	0.05		0.4	0.2	
P. griseofulvum 2159	CAS	128	8	0.6	128	16	**0.4**
	OG	0.2	0.1		0.8	0.2	
P. chrysogenum 824	CAS	128	16	**0.4**	128	32	**0.5**
	OG	0.2	0.05		0.2	0.05	
Mean, *Penicillium*[2]	CAS	117.33	20.00	**0.4**	117.33	26.67	0.6
	OG	0.20	0.05		1.03	0.35	
t-test[3]	CAS	-	$P < 0.001$	-	-	$P = 0.05$	-
	OG	-	$P < 0.001$		-	$P < 0.05$	
Mean, TOTAL[2]	CAS	122.18	26.00	0.6	122.18	55.27	1.0
	OG	0.25	0.10		1.07	0.54	
t-test[3]	CAS	-	$P < 0.001$	-	-	$P < 0.001$	-
	OG	-	$P < 0.001$		-	$P < 0.05$	

[1] OG was tested at 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 mM, while CAS was examined at 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 μg/mL.

[2] Mean values were calculated by excluding mutant strains (*sakA*Δ, *mpkC*Δ, FR2, FR3).

[3] *t*-test, Student's *t*-test for paired data (combined; chemosensitization) was vs. mean MIC or MFC of each compound (alone; no chemosensitization) determined in strains. Statistical analysis was performed according to "Statistics to use" [17]', where $P < 0.05$ was considered significant.

[4] Achievement of 99.5% fungal death.

Regarding FFCIs in *Aspergillus*, enhanced fungicidality of CAS or OG was identified during chemosensitization (FFCIs = 0.6 to 1.0; additive [16]), despite no calculated synergism. For example, co-application of OG (0.1, 0.1 or 0.2 mM for *sakA*Δ, *mpkC*Δ or wild type, respectively) with CAS (64 μg/mL) achieved ≥ 99.9% fungal death of *A. fumigatus*, while individual treatment of each compound, alone, at the same concentrations allowed the survival of *A. fumigatus*. Of note, the *sakA*Δ and *mpkC*Δ (antioxidant signaling mutants) [10,11] were more susceptible to the chemosensitizer, viz., required lower concentration of OG compared to the wild type (**Table 2**; **Figure 3**). However, enhancement level of OG on CAS lethality was not high against these mutants when their MFC values were compared to that of wild type, indicating chemosensitizing capability of OG was at the level of lowering MICs (but not MFCs, thus fungistatic but not fungicidal) in *sakA*Δ and *mpkC*Δ. Meanwhile, no enhancement in fungicidality was identified in *A. flavus* 3357 and *A. parasiticus* 5862 during chemosensitization, even at the highest concentrations of either compound applied (FFCI = 2.0).

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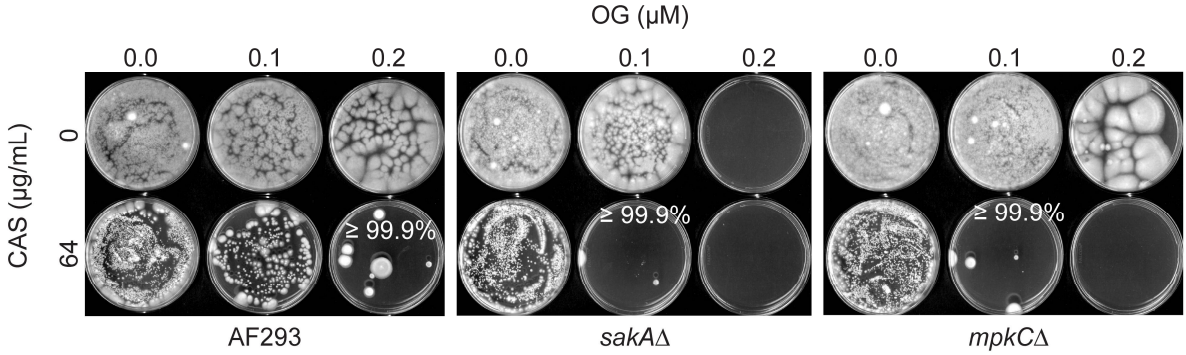

Figure 3. Chemosensitization test in *A. fumigatus* wild type (AF293), *sakA*Δ and *mpkC*Δ. Results shown here are the determination of MFCs of antifungal agents (CAS, OG) (The ≥ 99.9% indicates achievement of ≥ 99.9% of fungal death).

Synergistic FFCIs were also identified in *P. glabrum* 766, *P. griseofulvum* 2159 and *P. chrysogenum* 824 (FFCIs = 0.3 to 0.5) (**Table 2**; See also **Figure S1**). Although no calculated synergism was determined, there was enhanced antifungal activity of CAS and OG for the remaining *Penicillium* strains (FFCIs = 0.8, additive [16]) during chemosensitization. The *P. glabrum* 766 and *P. chrysogenum* 824 were the most susceptible strains to the chemosensitization, where synergism was found for both FICIs and FFCIs (**Table 2**; **Figure S1**). In general, *Penicillium* strains were more susceptible to CAS (viz., required lower concentration of CAS; 16 to 32 μg/mL) during chemosensitization, when compared to the *Aspergillus* strains (64 to 128 μg/mL CAS) (**Table 2**; **Figure S1**). The differences in susceptibility between *Penicillium* and *Aspergillus* to chemosensitization may be due to differences in cell wall sugar composition, such as mannose, galactose, galactofuranose, etc. [26,27]. Elucidation of precise mechanism of differential susceptibility of fungi to chemosensitization warrants future in-depth investigation.

3.3. Octyl gallate (OG) debilitates antioxidant mutants during chemosensitization

The mode of antifungal action of OG has been discussed in prior studies, where: (i) OG interrupts or disorganizes the lipid bilayer-protein interface in fungal cells [28], and (ii) OG functions as a pro-oxidant (redox-active oxidative stressor), thus triggers cytotoxicity in fungi [29]. We speculate that, in addition to destabilizing cell wall integrity, disruption of cellular components by pro-oxidant characteristic of OG could also be one mechanism of action for the enhancement of CAS activity during OG-mediated chemosensitization.

For example, the *A. fumigatus sakA*Δ and *mpkC*Δ antioxidant mutants were more susceptible to chemosensitization (OG + CAS) compared to the wild type (See above). Redox-active compounds, such as benzo derivatives or sulfur-containing compounds, could function as potent redox-cyclers in microbes, and thus, inhibit pathogen growth by interfering with cellular antioxidant systems, redox homeostasis or the function of redox-sensitive macromolecules [30,31]. Therefore, it is postulated that, in addition to destabilizing cell wall integrity system, the redox-active OG (chemosensitizer) can further debilitate the susceptibility, viz., defects in ameliorating oxidative stress and/or disruption of cellular redox homeostasis, of the antioxidant mutants during chemosensitization. From the pathogens' perspective, intact antioxidant signaling system, such as MAPK pathway, plays an important role for fungal defense against the OG-mediated chemosensitization.

Notably, previous studies showed that, in addition to cell wall integrity system, another signaling pathway, viz., "antioxidant" MAPK system, also plays an important role in fungal susceptibility to cell wall interfering agents (See below). In principle, a functionally intact antioxidant MAPK system is required for achieving fungicidal effects of cell wall disrupting drugs, while mutations in the system result in resistance to the drugs. For instance, the antioxidant MAPK pathway mutants of *S. cerevisiae*, such as *hog1* (MAPK) or *pbs2* (MAPK kinase; MAPKK) mutants,

exhibited tolerance to cell wall-interfering agents [32-34]. A similar type of drug tolerance was also observed in *Candida albicans* [35]. Fungal dialogs between "antioxidant" and "cell wall integrity" MAPK pathways have been well documented recently [36]. Identification of precise mechanism or cellular target(s) of OG during chemosensitization warrants future study.

5. Conclusions

In conclusion, chemosensitization could be an effective antifungal intervention strategy (See also [37]). OG, a safe, alkyl derivative of natural benzoate, possesses a potential to serve as an antimycotic chemosensitizer when co-applied with CAS. This potential appears to be greatest with *Penicillium* strains. OG-mediated chemosensitization, as presented here, can sensitize cell wall integrity and antioxidant systems of filamentous fungi, and thus can lower effective doses of toxic antifungal agents (such as CAS), leading to coincidental lowering of environmental and health risks. The use of safe chemosensitizers as intervention catalysts that debilitate filamentous fungal pathogens could be a viable approach for pathogen control. Future in vivo studies are necessary to determine potential application of chemosensitization for therapeutic settings.

Supplementary Materials: The following are available online at www.mdpi.com/link, Table S1: The model yeast *Saccharomyces cerevisiae* strains used in this study, Figure S1: Chemosensitization test in *A. flavus* 4212, *A. parasiticus* 2999, *P. italicum* 983 and *P. glabrum* 766

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Author Contributions: JHK conceived, designed and performed research including data analysis and interpretation, literature search, and wrote the manuscript. KLC performed antifungal bioassays and prepared the figures. LWC, Research Leader, directed the research.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. McCarthy, M.W.; Kontoyiannis, D.P.; Cornely, O.A.; Perfect, J.R.; Walsh, T.J. Novel agents and drug targets to meet the challenges of resistant fungi. *J. Infect. Dis.* **2017**, *216*, S474-S483. doi: 10.1093/infdis/jix130.
2. Beauvais, A.; Latgé, J.P. Membrane and cell wall targets in *Aspergillus fumigatus*. *Drug Resist. Updat.* **2001**, *4*, 38-49. doi: 10.1054/drup.2001.0185.
3. Fuchs, B.B.; Mylonakis, E. 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* **2009**, *8*, 1616-1625. doi: 10.1128/ec.00193-09.
4. Fujioka, T.; Mizutani, O.; Furukawa, K.; Sato, N.; Yoshimi, A.; Yamagata, Y.; Nakajima, T.; Abe, K. MpkA-Dependent and -independent cell wall integrity signaling in *Aspergillus nidulans*. *Eukaryot. Cell* **2007**, *6*, 1497-1510. doi: 10.1128/ec.00281-06.
5. Perlin, D.S. Mechanisms of echinocandin antifungal drug resistance. *Ann. N. Y. Acad. Sci.* **2015**. doi: 10.1111/nyas.12831.
6. Walker, L.A.; Lee, K.K.; Munro, C.A.; Gow, N.A. Caspofungin treatment of *Aspergillus fumigatus* results in ChsG-dependent upregulation of chitin synthesis and the formation of chitin-rich micro-colonies. *Antimicrob. Agents Chemother.* **2015**. doi: 10.1128/aac.00862-15.
7. Cowen, L.E.; Steinbach, W.J. Stress, drugs, and evolution: the role of cellular signaling in fungal drug resistance. *Eukaryot. Cell* **2008**, *7*, 747-764. doi: 10.1128/ec.00041-08.

8. Campbell, B.C.; Chan, K.L.; Kim, J.H. Chemosensitization as a means to augment commercial antifungal agents. *Front. Microbiol.* **2012**, *3*, 79. doi: 10.3389/fmicb.2012.00079.
9. Buckley, H.L.; Hart-Cooper, W.M.; Kim, J.H.; Faulkner, D.M.; Cheng, L.W.; Chan, K.L.; Vulpe, C.D.; Orts, W.J.; Amrose, S.E.; Mulvihill, M.J. Design and testing of safer, more effective preservatives for consumer products. *ACS Sustain. Chem. Eng.* **2017**, *5*, 4320-4331. doi: 10.1021/acssuschemeng.7b00374.
10. Xue, T.; Nguyen, C.K.; Romans, A.; May, G.S. A mitogen-activated protein kinase that senses nitrogen regulates conidial germination and growth in *Aspergillus fumigatus*. *Eukaryot. Cell* **2004**, *3*, 557-560.
11. Reyes, G.; Romans, A.; Nguyen, C.K.; May, G.S. Novel mitogen-activated protein kinase MpkC of *Aspergillus fumigatus* is required for utilization of polyalcohol sugars. *Eukaryot. Cell* **2006**, *5*, 1934-1940. doi: 10.1128/ec.00178-06.
12. Li, H.X.; Xiao, C.L. Characterization of fludioxonil-resistant and pyrimethanil-resistant phenotypes of *Penicillium expansum* from apple. *Phytopathol.* **2008**, *98*, 427-435. doi: 10.1094/PHYTO-98-4-0427.
13. Arendrup, M.C.; Cuenca-Estrella, M.; Lass-Flörl, C.; Hope, W. EUCAST technical note on the EUCAST definitive document EDef 7.2: method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts EDef 7.2 (EUCAST-AFST). *Clin. Microbiol. Infect.* **2012**, *18*, E246-247. doi: 10.1111/j.1469-0691.2012.03880.x.
14. Clinical and Laboratory Standards Institute (CLSI). *Reference method for broth dilution antifungal susceptibility testing of filamentous fungi: Approved standard–Second edition. CLSI document M38-A2.* Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2008; Vol. 28.
15. Odds, F.C. Synergy, antagonism, and what the chequerboard puts between them. *J. Antimicrob. Chemother.* **2003**, *52*, 1. doi: 10.1093/jac/dkg301.
16. Isenberg, H.D. *Clinical Microbiology Procedures Handbook*. 1 ed.; American Society for Microbiology: Washington, DC, 1992.
17. Kirkman, T.W. Statistics to use. Available online: http://www.physics.csbsju.edu/stats/ (accessed on 23 April 2018).
18. Kim, J.H.; Campbell, B.C.; Yu, J.; Mahoney, N.; Chan, K.L.; Molyneux, R.J.; Bhatnagar, D.; Cleveland, T.E. 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.* **2005**, *67*, 807-815.
19. Lee, A.Y.; St.Onge, R.P.; Proctor, M.J.; Wallace, I.M.; Nile, A.H.; Spagnuolo, P.A.; Jitkova, Y.; Gronda, M.; Wu, Y.; Kim, M.K., *et al.* Mapping the cellular response to small molecules using chemogenomic fitness signatures. *Science* **2014**, *344*, 208-211. doi: 10.1126/science.1250217.
20. Norris, M.; Lovell, S.; Delneri, D. Characterization and prediction of haploinsufficiency using systems-level gene properties in yeast. *G3 (Bethesda)* **2013**, *3*, 1965-1977. doi: 10.1534/g3.113.008144.
21. Parsons, A.B.; Brost, R.L.; Ding, H.; Li, Z.; Zhang, C.; Sheikh, B.; Brown, G.W.; Kane, P.M.; Hughes, T.R.; Boone, C. Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways. *Nat. Biotechnol.* **2003**, *22*, 62. doi: 10.1038/nbt919
https://www.nature.com/articles/nbt919 - supplementary-information.
22. U.S. Food and Drug Administration (FDA). (2011). Everything Added to Food in the United States. Available online: http://www.fda.gov/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/ucm115326.htm (accessed on 23 April 2018).

23. Rúa, J.; Fernandez-Álvarez, L.; de Castro, C.; del Valle, P.; de Arriaga, D.; Garcia-Armesto, M.R. Antibacterial activity against foodborne *Staphylococcus aureus* and antioxidant capacity of various pure phenolic compounds. *Foodborne Pathog. Dis.* **2011**, *8*, 149-157. doi: 10.1089/fpd.2010.0659.
24. Gutiérrez-Fernández, J.; García-Armesto, M.R.; Álvarez-Alonso, R.; del Valle, P.; de Arriaga, D.; Rúa, J. Antimicrobial activity of binary combinations of natural and synthetic phenolic antioxidants against *Enterococcus faecalis*. *J. Dairy Sci.* **2013**, *96*, 4912-4920. doi: 10.3168/jds.2013-6643.
25. Reinoso-Martín, C.; Schüller, C.; Schuetzer-Muehlbauer, M.; Kuchler, K. The yeast protein kinase C cell integrity pathway mediates tolerance to the antifungal drug caspofungin through activation of Slt2p mitogen-activated protein kinase signaling. *Eukaryot. Cell* **2003**, *2*, 1200-1210.
26. Parra, E.; Jiménez-Barbero, J.; Bernabe, M.; Leal, J.A.; Prieto, A.; Gómez-Miranda, B. Structural investigation of two cell-wall polysaccharides of *Penicillium expansum* strains. *Carbohydr. Res.* **1994**, *257*, 239-248. doi: https://doi.org/10.1016/0008-6215(94)80038-3.
27. Gow, N.A.R.; Latge, J.-P.; Munro, C.A. The fungal cell wall: structure, biosynthesis, and function. *Microbiol. Spectr.* **2017**, *5*. doi: doi:10.1128/microbiolspec.FUNK-0035-2016.
28. Fujita, K.; Kubo, I. Antifungal activity of octyl gallate. *Int. J. Food Microbiol.* **2002**, *79*, 193-201.
29. Sierra-Campos, E.; Valdez-Solana, M.A.; Matuz-Mares, D.; Velazquez, I.; Pardo, J.P. Induction of morphological changes in *Ustilago maydis* cells by octyl gallate. *Microbiology* **2009**, *155*, 604-611. doi: 10.1099/mic.0.020800-0.
30. Guillen, F.; Evans, C.S. Anisaldehyde and veratraldehyde acting as redox cycling agents for H_2O_2 production by *Pleurotus eryngii*. *Appl. Environ. Microbiol.* **1994**, *60*, 2811-2817.
31. Jacob, C. A scent of therapy: pharmacological implications of natural products containing redox-active sulfur atoms. *Nat. Prod. Rep.* **2006**, *23*, 851-863. doi: 10.1039/b609523m.
32. García-Rodriguez, L.J.; Durán, A.; Roncero, C. Calcofluor antifungal action depends on chitin and a functional high-osmolarity glycerol response (HOG) pathway: evidence for a physiological role of the *Saccharomyces cerevisiae* HOG pathway under noninducing conditions. *J. Bacteriol.* **2000**, *182*, 2428-2437.
33. Jiang, B.; Ram, A.F.; Sheraton, J.; Klis, F.M.; Bussey, H. Regulation of cell wall beta-glucan assembly: *PTC1* negatively affects *PBS2* action in a pathway that includes modulation of *EXG1* transcription. *Mol. Gen. Genet.* **1995**, *248*, 260-269.
34. Lai, M.H.; Silverman, S.J.; Gaughran, J.P.; Kirsch, D.R. Multiple copies of *PBS2*, *MHP1* or *LRE1* produce glucanase resistance and other cell wall effects in *Saccharomyces cerevisiae*. *Yeast* **1997**, *13*, 199-213. doi: 10.1002/(sici)1097-0061(19970315)13:3<199::aid-yea76>3.0.co;2-z.
35. Alonso-Monge, R.; Navarro-García, F.; Molero, G.; Diez-Orejas, R.; Gustin, M.; Pla, J.; Sánchez, M.; Nombela, C. Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. *J. Bacteriol.* **1999**, *181*, 3058-3068.
36. Rodríguez-Peña, J.M.; García, R.; Nombela, C.; Arroyo, J. The high-osmolarity glycerol (HOG) and cell wall integrity (CWI) signalling pathways interplay: a yeast dialogue between MAPK routes. *Yeast* **2010**, *27*, 495-502. doi: 10.1002/yea.1792.
37. Ogita, A.; Fujita, K.; Taniguchi, M.; Tanaka, T. Enhancement of the fungicidal activity of amphotericin B by allicin, an allyl-sulfur compound from garlic, against the yeast *Saccharomyces cerevisiae* as a model system. *Planta Med.* **2006**, *72*, 1247-1250. doi: 10.1055/s-2006-947203.