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Identification of novel cell wall destabilizing antifungal compounds using a conditional *Aspergillus nidulans* protein kinase C mutant

Gabriel Mircus[1], Shelly Hagag[1], Emma Levdansky[1], Haim Sharon[1], Yona Shadkchan[1], Itamar Shalit[2] and Nir Osherov[1]*

[1]Department of Human Microbiology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel; [2]Schneider Children's Medical Center of Israel, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel

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***Objectives*: Despite the need for novel drugs to combat fungal infections, antifungal drug discovery is currently limited by both the availability of suitable drug targets and assays to screen corresponding targets. The aim of this study was to screen a library of small chemical compounds to identify cell wall inhibitors using a conditional protein kinase C (PKC)-expressing strain of *Aspergillus nidulans*. This mutant is specifically susceptible to cell wall damaging compounds under PKC-repressive growth conditions.**

***Methods*: The inhibitory effect of a library of small chemical compounds was examined *in vitro* using the conditional *A. nidulans* PKC strain and a panel of pathogenic fungal isolates. Microscopy was used to assess alterations to fungal ultrastructure following treatment.**

***Results*: Three 'hit' compounds affecting cell wall integrity were identified from a screen of 5000 small chemical compounds. The most potent compound, CW-11, was further characterized and shown to specifically affect cell wall integrity. In clinical isolates of *Aspergillus fumigatus*, CW-11 induces morphological changes characteristic of damage to the cell wall, including wall thickening and rupturing. Analysis of the susceptibility of *A. fumigatus* and *A. nidulans* cell wall and signalling pathway mutants to CW-11 suggests that its mode of action differs from that of the antifungals caspofungin and voriconazole.**

***Conclusions*: This work demonstrates the feasibility of using a conditional *Aspergillus* mutant to conduct a small-molecule library screen to identify novel 'hit' compounds affecting cell wall integrity.**

Keywords: drug screen, compound library, cell wall integrity pathway

Introduction

There are unmet needs for effectively treating and preventing infections caused by fungi. The number of life-threatening, invasive fungal infections has risen dramatically over the last 20 years.[1,2] The vast majority of these infections are due to species of *Candida*, *Aspergillus*, *Cryptococcus* and *Coccidioides*.[3] Today, 4% of all patients dying in modern tertiary care hospitals have invasive aspergillosis, whereas 2% of patients have invasive candidiasis.[2,4]

Treatments for invasive fungal infections remain challenging. There are four main classes of antifungal drugs in common clinical use—polyenes (e.g. amphotericin B), azoles (e.g. fluconazole, itraconazole, voriconazole and posaconazole), the newly introduced echinocandins (e.g. caspofungin) and allylamines (e.g. terbinafine). Of those, only the first three classes are used to treat systemic fungal infections. Despite a 40% yearly increase in sales of systemic antifungal drugs (2002–07) to approximately $5 billion/year, there are significant unmet needs in this market.[5] Several current treatments interact unfavourably with other medications, have resistance problems, a narrow spectrum of activity, limited formulation, are fungistatic as opposed to fungicidal and some are toxic.[6] This is primarily because fungi are eukaryotes and therefore share many of the same biochemical pathways and subcellular structures with mammalian cells. Therefore, most existing antifungals are not truly fungus specific. Only one new class of antifungals, the echinocandins, inhibits a fungus-specific target, glucan synthase, which polymerizes β(1,3)glucan—a major structural component of the fungal cell wall.[7] Indeed, the echinocandins exhibit an excellent

*Corresponding author: Tel: +972-3-640-9599; Fax: +972-3-640-9160; E-mail: nosherov@post.tau.ac.il

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safety profile and clinical efficacy. However, being relatively large, they cannot be administered *per os*. Also, they display a poorly understood attenuation of activity at higher concentrations.[7] Therefore, there is an urgent need to develop additional and novel antifungal drugs that inhibit fungal-specific targets such as the cell wall.

In fungi, protein kinase C (PKC) is a central enzyme involved in regulating the integrity of the cell wall. Reduced PKC activity results in hypersusceptibility to cell wall destabilizing compounds and drugs.[8] We recently generated an *Aspergillus nidulans* mutant (*alcA-PKC*) in which the PKC gene is regulated by the inducible promoter *alcA*.[9] When grown under derepressive conditions (in the presence of glycerol or ethanol), the mutant grows normally. However, under repressive conditions (in the presence of glucose), the mutant exhibits hypersusceptibility to the cell wall perturbing agents Congo Red and Calcofluor White (CFW), the echinocandin drug caspofungin and the PKC inhibitor staurosporine. Importantly, the *alcA-PKC* strain does not display hypersusceptibility to the antifungal drugs amphotericin B, which binds ergosterol and disrupts the fungal plasma membrane, or voriconazole, an inhibitor of ergosterol biosynthesis, which suggests that this strain is specifically susceptible to cell wall damaging agents.[9]

We took advantage of the *alcA-PKC* strain to screen 5000 drug-like molecules from a diverse chemical compound library of small molecular weight compounds (Chemical Diversity Inc., San Diego, CA, USA) to identify cell wall inhibitors. This library was previously used successfully to identify MAP-kinase inhibitors in yeast.[10] First, we identified compounds that inhibit growth of wild-type *A. nidulans* in a 96-well liquid assay. The resulting antifungal compounds were then tested for their effect on the growth of the *alcA-PKC* mutant under derepressive and repressive conditions. The mutant exhibited hypersusceptibility to three cell wall active compounds under repressive conditions. The selectivity and mode of action of the most potent and specific compound, CW-11, was further investigated. We propose that this approach would be useful for large-scale screening for cell wall active compounds.

Materials and Methods

Strains and preparation of inocula

The strains used in this study are detailed in Table 1. Conidia were harvested in 0.2% (v/v) Tween 80, resuspended in double-distilled water (DDW) and counted with a haemocytometer. Strains were grown in minimal medium (MM) containing 70 mM $NaNO_3$, 1% (w/v) glucose, 12 mM potassium phosphate pH 6.8, 4 mM $MgSO_4$, 7 mM KCl, trace elements and 1.5% (w/v) agar (for MM agar plates). Minimal medium with glycerol (MMG) containing 0.2% (w/v) glycerol instead of glucose was used for growth of the *alcA-PKC* strain under derepressive conditions.

Screen for antifungal compounds

Aspergillus nidulans control wild-type strain R153 was grown in 96-well plates at a concentration of 10^4 conidia/mL in MM liquid medium. Each well was supplemented with 25 μM of a compound from a chemical compound library (Chemical Diversity Inc., San Diego, CA) composed of 5000 small drug-like molecules.

The compounds that completely inhibited fungal growth at 25 μM were selected for further characterization. To assess whether the selected compounds inhibit fungal growth by damaging the integrity of the cell wall, *A. nidulans* strain R153 and the conditional *alcA-PKC* mutant were grown in 96-well plates at a concentration of 10^4 conidia/mL in MM or MMG. The wells were supplemented with 2-fold dilutions of each selected compound. MICs (minimal inhibitory concentration: the lowest drug concentration to completely arrest germination and growth) and MECs (minimal effective concentration: the lowest drug concentration to cause visibly aberrant growth or a significant reduction of growth) were evaluated after 24 h incubation at 37°C, relative to non-treated wells. Caspofungin (Merck, NJ, USA) or voriconazole (Pfizer, NY, USA) were used as controls. Compounds for which the *alcA-PKC* mutant exhibited a ≥4-fold decrease in MIC and MEC when grown under repressive (+glycerol) conditions were determined as 'hit compounds'.

Pan-fungal and bacterial screen

The fungal and bacterial strains listed in Table 1 were grown in 96-well plates at a concentration of 10^4 conidia/mL in MM supplemented with the hit compounds, and antifungals in 96-well plates. MICs were evaluated after 24 h incubation at 37°C.

Cell culture

Hit compounds were assessed for toxicity towards mammalian cells using the human cancer cell line A549 (ATCC CLL 185), derived from a human lung carcinoma, and mouse embryo fibroblast cell line NIH-3T3 (ATCC CRL-1658).

The cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) in 10 cm tissue culture plates (Corning, Sigma-Aldrich, St Louis, MO, USA). Cells were incubated at 37°C, in 5.5% CO_2 and in a humidified atmosphere, and were routinely subcultured by trypsinization every 3–4 days. For MIC and MEC determination, cells were plated in 96-well plates at a concentration of 5×10^4 cells/well. After 24 h incubation, hit compounds were added and incubated again at 37°C in 5.5% CO_2 and in humidified atmosphere. After another 24 h incubation cell viability was measured by the XTT [2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide, disodium salt] assay (Biological Industries, Beit Haemek, Israel). XTT-based MICs and MECs were defined as the lowest drug concentrations to completely inhibit or significantly reduce dye formation, respectively.

Analysis of the susceptibility of mutant strains of Aspergillus fumigatus and A. nidulans to CW-11

To shed light on the mechanism of action of CW-11, we screened defined *A. fumigatus* and *A. nidulans* mutants for altered susceptibility to this compound. *A. fumigatus* and *A. nidulans* strains mutated in genes involved in cell wall biosynthesis and major signal transduction pathways were chosen. The MICs of CW-11 for these strains were evaluated as described above and compared with those of voriconazole and caspofungin.

Microscopy and staining

The effect of the hit compounds on fungal ultrastructure was assessed by light and fluorescent microscopy following nuclear, cell wall and vital staining. *Aspergillus fumigatus* conidia at a concentration of 10^4 conidia/mL were incubated for 24 h at 37°C on glass coverslips in 24-well plates (Nunclon; Nalge Nunc, Roskilde,

Table 1. Strains used in this study

Strains	Genotype	Ref.	Source
A. nidulans strain R153	*wA2; pyroA4*		FGSC
A. nidulans strain GR-5	*wA3; pyrG89*; *pyroA4*		G.S. May
A. nidulans strain *alcA-PKC*	*wA2; pyroA4; pyrG89::pyr4 alcA(p)::pkcAΔp*	9	our lab.
A. fumigatus AF293/FGSC A1100	wild type (patient isolate)	17	FGSC
A. fumigatus strain #1	wild type (patient isolate)	18	I. Shalit
A. fumigatus strain #2	wild type (patient isolate)	18	I. Shalit
Aspergillus niger strain #1	wild type (patient isolate)	18	I. Shalit
A. niger strain #2	wild type (patient isolate)	18	I. Shalit
A. niger strain #3	wild type (patient isolate)	18	I. Shalit
Aspergillus flavus strain #1	wild type (patient isolate)	18	I. Shalit
A. flavus strain #2	wild type (patient isolate)	18	I. Shalit
A. flavus strain #3	wild type (patient isolate)	18	I. Shalit
A. flavus strain #4	wild type (patient isolate)	18	I. Shalit
Aspergillus terreus strain #1	wild type (patient isolate)	18	I. Shalit
A. terreus strain #2	wild type (patient isolate)	18	I. Shalit
Fusarium solani 603251	wild type (patient isolate)	19	I. Shalit
F. solani 600577	wild type (patient isolate)	19	I. Shalit
F. solani 600679	wild type (patient isolate)	19	I. Shalit
Fusarium oxysporum 600616	wild type (patient isolate)	19	I. Shalit
F. oxysporum 600711	wild type (patient isolate)	19	I. Shalit
F. oxysporum 601761	wild type (patient isolate)	19	I. Shalit
Rhizopus arrhizus 156	wild type (patient isolate)		I. Shalit
C. albicans ATCC 2901	wild type (patient isolate)		E. Segal
C. albicans ATCC 90028	wild type (patient isolate)		E. Segal
C. albicans 58455	wild type (patient isolate)		E. Segal
C. albicans 89122	wild type (patient isolate)		E. Segal
ΔAfuEcm33	*AF293.1: Δecm::pyr4; ΔpyrG*	20	our lab.
ΔPmt1	*CEA17 Δpmt1::pyrG; ΔpyrG*	21	C. Jin
ΔCnaA	*AF293.1: ΔcnaA::pyrG; ΔpyrG*	22, 23	W. Steinbach
ATCC 13073	TRB-sensitive *A. fumigatus* strain	24	D.S. Perlin
S678Y	FKS-S678Y	24	D.S. Perlin
ΔPkaA	*pabaA*, *yA2*, *ΔpkaA::argB*; *ΔargB::trpC*; *trpC801, veA1*	25	N.P. Keller
ΔMpkA	*pyrG89*; *ΔmpkA::pyrG*	26, 27	S. Osmani
ΔNikA	*pabaA1 yA2 ΔnikA::AfpyrG veA1*	28	J. Aguirre
ΔCalI11	*calI11; gmtA::NcPyr4; pyrG89; wA3; pyroA4*	29	T.W. Hill
ΔSwoA1	*swoA1; pyrG89; wA3; veA1*	30	M. Momany
ΔSwoM	*swoM1; pyrG89; pabaA6; veA1*	31	M. Momany
A. fumigatus G10 (CBS 144−89)	*niiA*	33	J.P. Latge
ΔchsG	*chsG; Ble* (phleomycin B phosphotransferase)	32	E. Mellado
ΔAgs1	*ags1; Hph* (hygromycin B phosphotransferase)	33	J.P. Latge
ΔGel2	*gel2; Ble*	34	J.P. Latge

FGSC, Fungal Genetics Stock Center.

Denmark) containing 1 mL MM/well in the presence of CW-11 (0.2 μg/mL). After 24 h, coverslips were used for microscopic analysis. Microscopy was performed with an Olympus BX-40 microscope (equipped for fluorescence with a UV filter and a FITC filter) at ×400 magnification. Images were recorded on an Olympus DP70 camera.

For nuclear 4′,6-diamidino-2-phenylindole (DAPI) staining, coverslips were fixed in 50 mM KH_2PO_4 pH 6.8, 5% (v/v) glutaraldehyde, 0.2% (w/v) Triton X-100 for 30 min at room temperature, stained with 50 μg/mL DAPI for 15 min, washed twice in PBS and analysed. For CFW staining, germlings were stained for 45 min at room temperature and in darkness with CFW (0.1 mg/mL in DDW). After staining, germlings were washed twice with PBS and analysed. For DiBAC [bis-(1,3-dibutylbarbituric acid)trimethine oxonol] staining of dead conidia and hyphae, cells were stained with DiBAC (2 μg/mL in 100 mM MOPS buffer, pH 7.0) for 1 h at room temperature, washed twice with PBS and analysed.

Results

Screen for cell wall destabilizing antifungal compounds

To identify compounds with antifungal activity, we screened 5000 drug-like molecules from a diverse chemical compound library (Chemical Diversity Inc.) (Figure 1A). Each compound was initially tested for general antifungal activity against wild-type

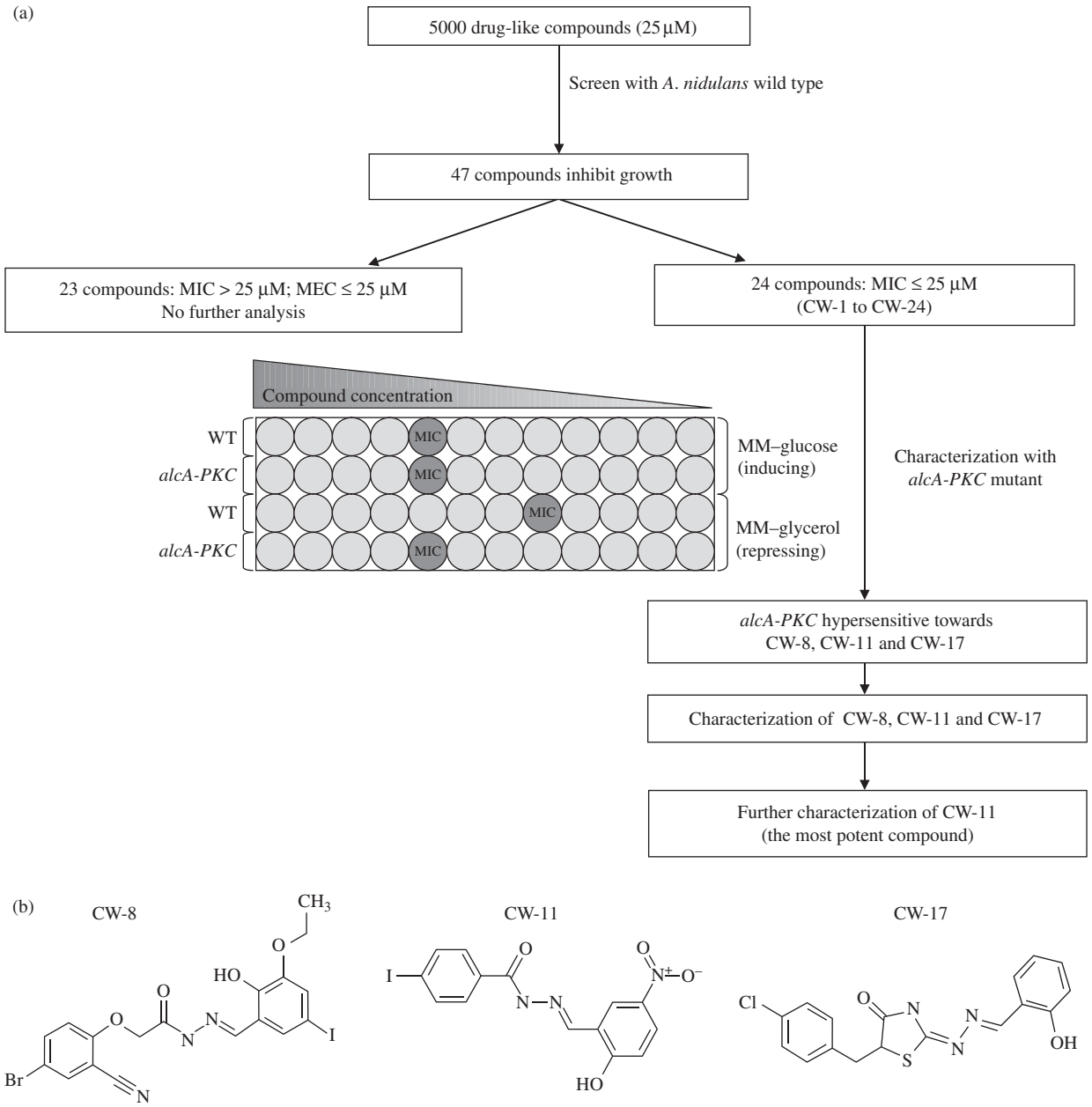

Figure 1. (a) Schematic outline of the steps used in the screen to identify cell wall damaging compounds. (b) Chemical structures of CW-8, CW-11 and CW-17.

A. nidulans spores germinating in 96-well microtitre plates on defined liquid medium (MM) for 24 h of growth at 37°C. All compounds were tested at a single concentration of 25 μM. The wells were scanned by an inverted microscope at ×40 magnification. We identified 47 compounds that significantly inhibited fungal germination and growth at a concentration of 25 μM. Of those, 23 compounds partially inhibited growth (MIC > 25 μM; MEC < 25 μM) and 24 compounds completely inhibited fungal growth (MIC < 25 μM). The compounds in the latter group were selected for further characterization in the *alcA-PKC* mutant under inducing (MM + glycerol) and repressive (MM) conditions. Three of the 24 compounds (CW-8, 11 and 17) inhibited the *alcA-PKC* mutant at least 4-fold more potently when grown under PKC repressive conditions, which suggests that they function by perturbing (directly or indirectly) the integrity of the cell wall (Figure 1B and Table 2). Importantly, the *alcA-PKC* strain showed wild-type susceptibility to the compounds when grown in inducing medium (Table 2). Further characterization was performed on CW-11, because it was the most potent inhibitor of wild-type *A. fumigatus* on defined MM (MIC = 0.8 mg/L) and showed the largest change in MIC and MEC values in the *alcA-PKC* mutant upon shift from inducing to repressive conditions.

CW-11 is specifically active against fungi

The specificity of CW-11 *in vitro* was tested against a panel of fungal, bacterial, human (A549) or mouse (3T3) cells in culture

Table 2. MIC and MEC of CW-8, CW-11 and CW-17 (mg/L)

	CW-8				CW-11				CW-17			
	MM		MM + glycerol		MM		MM + glycerol		MM		MM + glycerol	
Strain	MIC	MEC	MIC	MEC	MIC	MEC	MIC	MEC	MIC	MEC	MIC	MEC
Wild-type	1.6	0.8	0.4	0.1	0.8	0.2	0.8	0.4	>25	0.1	>25	0.2
alcA-PKC	0.1	0.025	0.4	0.05	0.2	0.05	0.8	0.4	>25	0.0016	>25	12.5

(Table 3). CW-11 was effective against all the important mould pathogens including *Aspergillus* and *Fusarium* species, and to a lesser extent, *Rhizopus*. In these species, CW-11 caused characteristic swelling and cell lysis, indicating that damage to the cell wall had occurred (data not shown). The compound partially inhibited the growth of the pathogenic yeast *Candida albicans*. A noteworthy finding is that CW-11 did not affect bacterial proliferation and caused no damage to the cultured mammalian cell lines as measured by cell counting (data not shown) and XTT-based cell proliferation assays (Table 3).

CW-11 causes morphological changes characteristic of damage to the cell wall of A. fumigatus

Further characterization of the effects of CW-11 on fungal structure was performed on *A. fumigatus* strain AF293, originally isolated from a patient with invasive pulmonary aspergillosis and subsequently extensively used in laboratory studies.[11] The effect of CW-11 on cell wall polysaccharide deposition, nuclear localization and viability was determined by CFW, DAPI and DiBAC staining, respectively. Following 24 h of growth in the presence of MEC concentrations of CW-11, AF293 displayed a phenotype characterized by abnormal hyphal branching patterns, a thickened cell wall containing increased polysaccharide and swollen hyphal tips (Figure 2A, CFW). Nuclear localization appeared abnormal, with numerous nuclei localized in the swollen cell bodies and hyphal tips or unevenly distributed along the hyphae (Figure 2A, DAPI). DiBAC vital staining revealed numerous dead (fluorescing) hyphal segments (Figure 2A, DiBAC).

To further determine the effect of CW-11 on the cell wall ultrastructure, we analysed the cell wall of treated AF293 cells by transmission electron microscopy (TEM). Freshly harvested conidia from the AF293 strain were grown for 24 h at 37°C in the presence of CW-11 at MEC concentration. The results indicate that in the presence of CW-11, the AF293 strain displays defects in the ultrastructure of the cell wall characterized by abnormal thickening and fragmentation of the outer cell wall (Figure 2A, lower panel). Numerous cells showed partial or complete loss of cytosol (Figure 2B), which suggests that they had undergone a process of collapse and disintegration. This lytic process probably resulted from a weakening of the cell wall because of CW-11 activity.

Altered susceptibility of mutant Aspergillus strains to CW-11

To broaden our understanding of the mode of action of CW-11, we searched for genes whose deletion led to altered susceptibility to this compound. We selected *A. fumigatus* and

Table 3. MIC and MEC of CW-11 (mg/L)

	CW-11	
Fungus/strain	MIC	MEC
Aspergillus spp.		
A. fumigatus 293	0.4	0.2
A. fumigatus G10	1.6	0.4
A. fumigatus strain #1	0.4	0.2
A. fumigatus strain #2	0.4	0.1
A. niger strain #1	0.4	0.2
A. niger strain #2	0.4	0.2
A. niger strain #3	0.8	0.2
A. flavus strain #1	0.8	0.4
A. flavus strain #2	0.8	0.4
A. flavus strain #3	0.8	0.4
A. flavus strain #4	0.8	0.4
A. terreus strain #1	0.4	0.1
A. terreus strain #2	0.4	0.1
Fusarium spp.		
F. solani 251	>25	0.4
F. solani 577	0.4	0.1
F. solani 679	0.8	0.1
F. oxysporum 616	0.4	0.2
F. oxysporum 711	0.8	0.2
F. oxysporum 761	0.8	0.2
Rhizopus spp.		
R. arrhizus 156	>25	0.1
C. albicans		
C. albicans ATCC 2901	>25	0.4
C. albicans ATCC 90028	>25	0.2
C. albicans 58455	>25	0.4
C. albicans 89122	>25	0.8
Bacteria		
Escherichia coli	>25	>25
Staphylococcus epidermidis	>25	>25
Bacillus cereus	>25	>25
Human cells[a]		
A549	>25	>25
Mouse cells[a]		
3T3	>25	>25

[a]Viability of mammalian cells in culture was measured by the XTT assay.

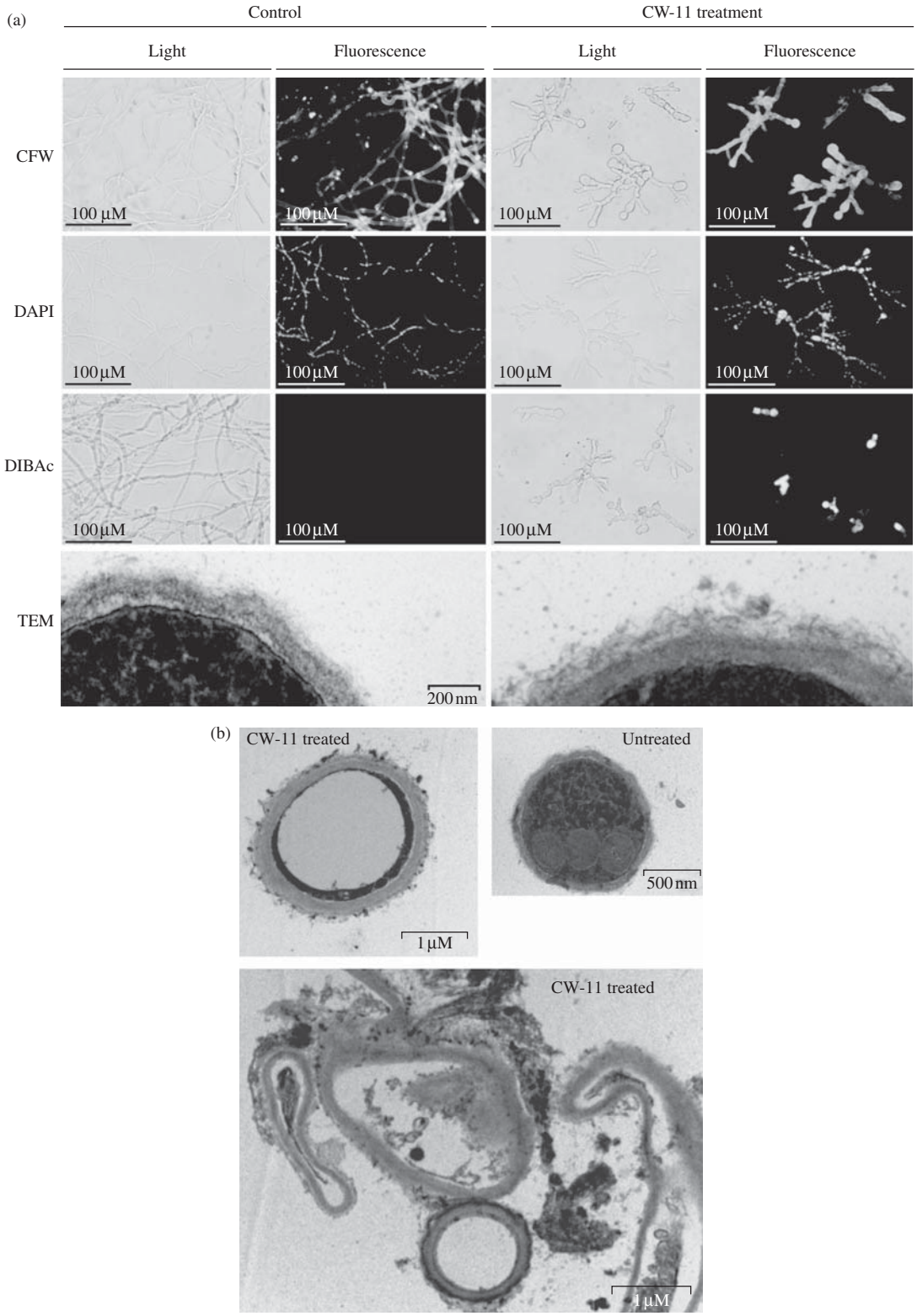

Figure 2. (a) CW-11 affects cell morphology, chitin deposition, nuclear distribution cell viability and cell wall structure of *A. fumigatus* AF293. AF293 spores were incubated at 37°C for 24 h with and without CW-11 (0.4 µg/mL). CW-11 caused alterations in the wall morphology and changes in chitin deposition as depicted by CFW staining (top panel). Nuclei, stained with DAPI in the second panel, are mislocalized and randomly distributed. In the third panel, DiBAC staining shows dead cells in the treated sample, whereas all untreated cells are alive. The bottom panel shows morphological changes in the cell wall following treatment with CW-11 as imaged by transmission electron microscope (TEM). (b) Defects in the cell wall ultrastructure of AF293 cells treated with CW-11. TEM of an untreated cell (top right) and of a treated cell (top left). The strains were grown for 16 h at 37°C on MM liquid medium with or without CW-11 (0.4 µg/mL) prior to fixation. Note the thickening of the cell wall of treated cells. Numerous cells lysed to form empty 'ghost' cells (lower panel).

Table 4. Mutant susceptibility to CW-11 in comparison with caspofungin or voriconazole (mg/L)

Species/strain	Gene deleted/annotation	Caspofungin		Voriconazole		CW-11	
		MIC	MEC	MIC	MEC	MIC	MEC
A. fumigatus							
AF293	parental strain	>12.5	0.1	0.8	0.4	0.8	0.2
ΔEcm33	GPI-anchored cell wall organization protein *AfuEcm33*[20]	>12.5	0.2	1.6	0.4	>12.5	0.2
ΔPmt1	protein mannosyltransferase[21]	>12.5	0.2	0.4	0.2	>12.5	0.2
ΔCnaA	calcineurin catalytic subunit *CnaA*[22,23]	>12.5	0.1	0.8	0.2	0.8	0.4
G10	parental strain	>12.5	0.025	3.125	0.8	1.6	0.4
ΔChsG	chitin synthase G subunit[32]	>12.5	0.003	0.4	0.2	>12.5	0.8
ΔAgs1	α-glucan synthase 1[33]	>12.5	0.0125	0.4	0.2	>12.5	>12.5
ΔGel2	β(1,3)-glucanosyltransferase[34]	>12.5	0.05	0.4	0.05	>12.5	>12.5
ATCC 13073	parental strain	>12.5	0.1	0.4	0.2	>25	3.1
S678Y	caspofungin resistant[24]	3.1	1.5	0.2	0.1	>25	3.1
A. nidulans							
R153	parental strain	>12.5	0.05	0.1	0.02	0.1	0.02
ΔPkaA	protein kinase A catalytic subunit[25]	0.8	0.4	0.1	0.02	0.8	0.2
ΔMpkA	mitogen-activated protein kinase *MpkA*[26,27]	0.01	0.006	0.05	0.02	0.006	ND
ΔNikA	histidine protein kinase[28,35]	0.8	0.2	0.2	0.05	1.6	0.4
ΔCalI11	GDP-mannose transporter (GMT)[29]	6.2	0.1	0.2	0.05	0.05	0.006
ΔSwoA1	protein *O*-mannosyltransferase *PmtA*[30]	6.2	0.05	0.2	0.006	0.2	0.01
ΔSwoM	phosphoglucose isomerase *SwoM*[31]	0.4	0.1	0.2	0.02	0.2	0.05

A. nidulans mutant strains deleted for genes involved in the major fungal wall biosynthesis and signal transduction pathways. The strains were tested for susceptibility to CW-11 in comparison with the antifungals voriconazole (an inhibitor of membrane ergosterol biosynthesis) and caspofungin (an inhibitor of glucan synthase and cell wall biosynthesis). Interestingly, increased resistance to CW-11 was found in *A. fumigatus* strains deleted in *ChsG* (chitin synthesis), *Ags1* (α-glucan synthesis), *Gel2* (1,3-β-glucanosyltransferase activity), *Ecm33* (involved in cell wall organization) or *Pmt1* (mannosylation of cell wall proteins) and in *A. nidulans* strains deleted in *PkaA* (cAMP signalling) or *NikA* (histidine kinase signalling) (Table 4). Increased susceptibility to CW-11 was found in *A. nidulans* strains deleted for the genes *MpkA* (MAP-kinase signalling) or *CalI* (GDP-mannose transport). Overall, the susceptibility profiles of the mutants to CW-11, voriconazole and caspofungin are markedly different, which suggests that CW-11 inhibits fungal growth through a different cellular target.

Discussion

Current therapeutic modalities for invasive aspergillosis, including the introduction of new agents such as caspofungin and voriconazole, are still associated with significant mortality.[12] Thus, new antifungal drugs, developed by mining the information rapidly accumulating from molecular studies, are urgently needed. Several previous studies have described the use of *Saccharomyces cerevisiae*,[13,14] *Candida albicans*[12,15] and *Neurospora crassa*[16] mutant strains to identify novel compounds with antifungal activity. Hu *et al.*[17] recently generated conditional promoter replacement mutants for 35 essential genes in *A. fumigatus*. One of those, in which *ERG11A* (encoding 14α-demethylase, the known target of azole-based antifungal drugs) is conditionally expressed, showed increased susceptibility to fluconazole but not to the unrelated compound tunicamycin under repressive conditions. However, the use of these *A. fumigatus* conditional mutants to identify novel antifungal compounds has not been described to date.

In this study, we used a conditional *alcA-PKC* mutant we have generated in *A. nidulans*[9] to identify small drug-like molecules that perturb the cell wall. This mutant is hypersensitive to cell wall damage only when grown in repressive medium containing glucose because under these conditions *PKC* transcription is down-regulated and the *PKC*-dependent cell wall integrity response is blocked. In contrast, on *alcA-PKC*-inducing medium (containing glycerol), the strain shows normal susceptibility to cell wall damage.[9] The novelty in this approach is that the *alcA-PKC* mutant allows us to differentiate between compounds that inhibit general fungal growth and those that inhibit cell wall integrity and function. An additional advantage of this approach is that screening is performed in intact fungal cells rather than in purified protein targets. This eliminates the isolation of compounds that cannot cross biological membranes or are cytotoxic.

We screened a library of 5000 randomly selected small molecules for compounds displaying enhanced antifungal activity against the *alcA-PKC* strain under repressive conditions. We identified three compounds (CW-8, 11 and 17). Interestingly, they are chemically related; all are aromatic substituted hydrazine (N–N) derivatives containing ortho-substituted phenolic and halogenated benzyl rings. None has been described previously in the scientific literature as having antifungal activity, which suggests that they are novel antifungal hit compounds.

The most potent of the three compounds, CW-11, was further characterized and shown to have several properties of a lead antifungal compound: (i) it specifically inhibits fungal growth and not bacterial or mammalian cell proliferation in culture; and (ii) microscopic analysis indicated that it causes extensive cell wall damage characterized by swelling, thickening and lysis.

To initially characterize the mode of action of CW-11, we tested its ability to inhibit *A. fumigatus* and *A. nidulans* mutant strains deleted for genes involved in fungal wall biosynthesis and signalling. Such 'chemical genomic profiling' has been used extensively in yeast, and is based on the premise that genes whose deletion affects drug susceptibility are directly or indirectly involved in the mode of action of the drug.[12] Our findings, based on the different susceptibility profiles of the mutant strains to caspofungin, voriconazole and CW-11, suggest that CW-11 functions through a different cellular mechanism. Surprisingly, we found that several of the mutants lacking genes involved in cell wall biogenesis (*ChsG*, *Ags1*, *Gel2*, *Ecm33)* showed *increased* resistance to CW-11. In the yeast *S. cerevisiae*, defects in cell wall biogenesis activate the PKC/mitogen activated kinase cascade, resulting in the activation of the cell wall integrity pathway and subsequent repair of the cell wall.[8] We hypothesize that the cell wall integrity (CWI)/PKC-dependent pathway in these mutants is activated due to their defective cell wall and that one of the components of this pathway is a target of CW-11. This is congruent with our finding that deletion of the mitogen activated kinase *MpkA*, a component of the CWI/PKC-dependent pathway, results in hypersusceptibility to CW-11. Although the precise mode of action of CW-11 awaits further investigation, we found, intriguingly, that its antifungal activity is abolished in rich media containing high (mM) levels of the amino acid cysteine (G. Mircus and N. Osherov, unpublished results).

In summary we provide a proof of concept for the long-standing idea of using inducible mutant strains to isolate antifungal hit compounds. We propose that screening for cell wall damaging antifungal compounds is far from being exhausted and should be continued with libraries composed of hundreds of thousands of compounds. Furthermore, the screen may be improved and refined by generating and screening additional conditional mutants in essential cell wall biosynthetic genes.

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Transparency declarations

None to declare.

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