Casposfungin Treatment of Aspergillus fumigatus Results in ChsG-Dependent Upregulation of Chitin Synthesis and the Formation of Chitin-Rich Microcolonies

Louise A. Walker,* Keunsook K. Lee, Carol A. Munro, Neil A. R. Gow
Aberdeen Fungal Group, School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, United Kingdom

Treatment of Aspergillus fumigatus with echinocandins such as casposfungin inhibits the synthesis of cell wall β-1,3-glucan, which triggers a compensatory stimulation of chitin synthesis. Activation of chitin synthesis can occur in response to sub-MICs of casposfungin and to CaCl2 and calcofluor white (CFW), agonists of the protein kinase C (PKC), and Ca2+-calcineurin signaling pathways. A. fumigatus mutants with the chs gene (encoding chitin synthase) deleted (ΔAfchs) were tested for their response to these agonists to determine the chitin synthase enzymes that were required for the compensatory upregulation of chitin synthesis. Only the ΔAfchsG mutant was hypersensitive to casposfungin, and all other ΔAfchs mutants tested remained capable of increasing their chitin content in response to treatment with CaCl2 and CFW and casposfungin. The resulting increase in cell wall chitin content correlated with reduced susceptibility to casposfungin in the wild type and all ΔAfchs mutants tested, with the exception of the ΔAfchsG mutant, which remained sensitive to casposfungin. In vitro exposure to the chitin synthase inhibitor, nikkomycin Z, along with casposfungin demonstrated synergistic efficacy that was again ΔAfchsG dependent. Dynamic imaging using microfluidic perfusion chambers demonstrated that treatment with sub-MIC casposfungin resulted initially in hyphal tip lysis. However, thickened hyphae emerged that formed aberrant microcolonies in the continued presence of casposfungin. In addition, intrahyphal hyphae were formed in response to echinocandin treatment. These in vitro data demonstrate that A. fumigatus has the potential to survive echinocandin treatment in vivo by ΔAfchsG-dependent upregulation of chitin synthesis. Chitin-rich cells may, therefore, persist in human tissues and act as the focus for breakthrough infections.

C. albicans, the protein kinase C (PKC), high-osmolality glycerol (HOG), and Ca2+-calcineurin signaling pathways all contribute to the regulation of transcriptional activation of chitin synthesis (23). In this organism, there are only four Chs enzymes: Chs2 and Chs8 (class I), Chs1 (class II), and Chs3 (class IV). Treatment of C. albicans with a combination of CaCl2 and calcofluor white (CFW) stimulated the Ca2+-calcineurin and PKC signaling pathways, respectively, and led to a 3–to 4-fold increase in chitin content. Strains of C. albicans that have elevated chitin contents were less susceptible to casposfungin than cells with wild-type chitin levels (19, 22, 24, 25).

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Address correspondence to Neil A. R. Gow, n.gow@abdn.ac.uk.
* Present address: Louise A. Walker, Molecular & Biomedical Sciences, University of Maine, Orono, Maine, USA.
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The \textit{A. fumigatus} cell wall is comprised of 20\% chitin, which is synthesized by eight Chs enzymes: \textit{A. fumigatus} ChsA (\textit{AfChsA}), \textit{AfChsB}, \textit{AfChsC}, \textit{AfChsD}, \textit{AfCamA} (\textit{AfChsE}), \textit{AfChsF}, \textit{AfChsG}, and \textit{AfCsmB} (2, 3, 26–29), which by sequence homology fall into different classes and have been characterized extensively via the analysis of single and multiple mutants. The class III and V to VII chitin synthase enzymes are specific to filamentous fungi. Disruption of single chitin synthase genes to create \textit{Af}
\textit{AfchsA} (class I), \textit{AfAfchsB} (class II), and \textit{AfAfchsC} (class III) mutants resulted in mild or no phenotypic growth effects compared to the wild type (26, 29–31). In contrast, hypoxia of the \textit{AfAfchsD} (class VI) mutant were shown to have an increase in chitin content, and the \textit{AfAfchsF} mutant had a 25\% reduction in chitin compared to the wild type (29). Disruption of the class V enzyme, \textit{AfCsmA}, resulted in an 80\% reduction in conidial chitin content (29), and disruption of \textit{AfCsmA} and \textit{AfCsmB} (class VII) resulted in hypersensitivity to caspofungin (28). The \textit{AfAfchsA} and \textit{AfAfchsB} mutants also had a defect in conidiation that may be abrogated by growth in osmotically stabilized media (28, 29, 32). A quadruple \textit{AfAfchsA} \textit{AfAfchsB} \textit{AfAfchsD} \textit{AfAfchsF} mutant was significantly attenuated in immunosuppressed mice (29). The \textit{AfAfchsG} single mutant and a quadruple \textit{AfAfchsA} \textit{AfAfchsC} \textit{AfAfchsB} \textit{AfAfchsG} mutant were hyperbranched and had reduced radial growth (26, 29). The \textit{AfAfchsA} \textit{AfAfchsC} \textit{AfAfchsB} \textit{AfAfchsG} mutant was also shown to have a reduction in conidiation, and conidia that were produced had a disorganized melanin layer on the surface which was attached loosely to the inner cell wall. \textit{AfChsG} was also shown to be required for \textit{in vitro} Chs enzyme activity and was involved in synthesizing chitin in the conidial wall (29). However, the quadruple \textit{AfAfchsA} \textit{AfAfchsC} \textit{AfAfchsB} \textit{AfAfchsG} mutant was as virulent as the wild type in a murine model of pulmonary aspergillosis (29). The double class III class V \textit{AfAfchsG} \textit{AfAfchsE} mutant had a 50\% reduction in chitin content compared to wild-type cells and a 95\% reduction in chitin synthase enzyme activity (31).

The aims of this work were to determine whether treatment of \textit{A. fumigatus} with agents that increased chitin content affected susceptibility to caspofungin and to establish which Chs enzymes were important for the chitin upregulation in response to caspofungin. The data demonstrate that hypoxia with high chitin could survive caspofungin treatment and that this response was strongly \textit{AfCHSG} dependent.

\textbf{MATERIALS AND METHODS}

\textbf{Strains, media, and growth conditions.} \textit{A. fumigatus} strains used in this study are listed in Table 1. \textit{A. fumigatus} strains were maintained on Sabouraud dextrose (Sabdex) agar plates (1\% mycological peptone \text{[wt/vol]}, 4\% glucose \text{[wt/vol]}, and 2\% agar \text{[wt/vol]}). Antifungal agents. Cells were grown in RPMI 1640 supplemented with the following inhibitors: 2 \mu g/ml caspofungin (obtained from Edinburgh Royal Infirmary Pharmacy) and 2 \mu g/ml nikkomycin Z (Sigma-Aldrich, United Kingdom), which were dissolved in sterile water. In some experiments, \textit{A. fumigatus} was pretreated by growing in Sabdex broth containing 0.2 M CaCl\textsubscript{2} and 100 \mu g/ml CFW (Sigma-Aldrich, United Kingdom) for 8 h at 37°C with shaking at 200 rpm to elevate the chitin content of hyphal cells.

\textbf{Caspofungin sensitivity testing on solid medium.} Caspofungin was incorporated into RPMI 1640 agar plates at 2 \mu g/ml and 4 \mu g/ml. In some experiments, caspofungin was used in combination with 2 \mu g/ml nikkomycin Z. \textit{A. fumigatus} spores were collected and serially diluted to generate suspensions containing 1 \times 10\textsuperscript{6} to 1,000 spores/ml in sterile water. Plates were inoculated with 3-\mu l drops of each spore suspension and incubated for 48 h at 37°C.

\textbf{Antifungal susceptibility testing.} MICs were determined by broth microdilution testing using the CLSI (formerly NCCLS) guideline M38-A2 for filamentous fungi (33). Drug concentrations ranged from 0.032 \mu g/ml to 16 \mu g/ml of caspofungin. Caspofungin was serially diluted with sterile water in flat-bottomed 96-well plates. \textit{A. fumigatus} spores were collected from agar plates in phosphate-buffered saline (PBS) (Oxoid) plus 2\% (wt/vol) Tween 80 (Sigma) and inoculated in 11 mL 2\% RPMI 1640, and 200 \mu l of culture was added to each well. Plates were incubated for 48 h at 37°C. After incubation, each well was mixed thoroughly and optical density was read in a VersaMax tunable microplate reader (Molecular Devices, CA, USA) at 405 nm.

\textbf{Determination of dry weights of mycelia.} Dry weights of wild-type and \textit{AfAfchs} mutants were determined after 24 h growth in RPMI 1640 broth alone or supplemented with 2 \mu g/ml caspofungin. After incubation, cultures were collected and filtered through preweighed 0.45-\mu m filters. The filters containing \textit{A. fumigatus} strains were dried at 80°C for 24 h and were then pared to constant weight.

\textbf{Fluorescence microscopy.} After washing with sterile water to remove any excess medium, samples were fixed in 10\% (vol/vol) neutral buffered formalin (Sigma-Aldrich, United Kingdom) and examined by phase differential interference contrast (DIC) microscopy. Cells were stained with 25 \mu g/ml CFW to visualize chitin. All samples were examined by fluorescence microscopy using a Zeiss AxioPlan 2 microscope. Images were recorded digitally using the OpenLAB system (OpenLAB v4.04; Improvision, Coventry, United Kingdom) using a Hamamatsu C4742-95 digital camera (Hamamatsu Photonics, Hamamatsu, Japan).

\textbf{Electron microscopy.} Cultures were harvested by centrifugation, and the pellets were fixed in 2.5\% (vol/vol) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3) for 24 h at 4°C. Samples were encapsulated in 3\% (wt/vol) low melting point agarose prior to embedding in Spurr’s resin following a 24-h processing schedule on a Lynx tissue processor (Lynx Medical, Leeds, United Kingdom) using a Hamamatsu Photonics, Hamamatsu, Japan).

\textbf{Response of Aspergillus fumigatus to Caspofungin}
Caspofungin, after pregrowth with or without CaCl$_2$ and CFW, was titled water (dH$_2$O). Thin sections were poststained for 10 min with 5% aqueous uranyl acetate and with lead citrate for 4 min (37).

Time-lapse observations using microfluidics. The ONIX microfluidic perfusion system (CellASIC Corp., USA) was used to analyze the dynamic responses of $A. fumigatus$ cells when perfused with caspofungin-supplemented medium. According to the manufacturer’s instructions, the flow rate and exchanging solutions were controlled by pressure (pounds per square inch [psi]) using the ONIX FG software v2.6. Spores were diluted to $\sim 5 \times 10^6$ in Sabdex broth containing 5 µg/ml CFW to visualize chitin and applied to a microfluidics plate, Y04C (CellASIC Corp., USA). Approximately 50 spores were loaded into each chamber. Sabdex broth containing CaCl$_2$ and CFW was consistently perfused through the chamber with a flow rate of 4 lb/in$^2$ (10 µl/h) for 6 h. Then cells were treated with 32 µg/ml caspofungin for a further 6 h and grown in fresh medium for 8 h. Cells in the chamber were observed using a DeltaVision Core microscope (Image Solutions Ltd., Preston, United Kingdom). All images were taken using a CoolSNAP camera (Photometrics UK Ltd., London, United Kingdom). Image analysis was performed using ImageJ v1.45 free software (http://rsweb.nih.gov/ij/).

RESULTS

Morphological changes in $A. fumigatus$ hyphae in response to caspofungin treatment. The effect of caspofungin on the morphology of single germlings of $A. fumigatus$ that had been pregrown with CaCl$_2$ and CFW (Fig. 1B) compared to its effect on sham-treated cultures was examined in real time using a microfluidics system (Fig. 1A). Exposure of $A. fumigatus$ to 32 µg/ml caspofungin, after pregrowth with or without CaCl$_2$ and CFW, resulted in bursting of hyphal tips (Fig. 1). After lysis of hyphal tips, incidences of septum formation distal to the burst hyphal apices were observed (Fig. 1). Removal of caspofungin from the growth medium resulted in new apical growth of some of the burst hyphae (Fig. 1). The microfluidics chambers were then perfused with medium containing no drug. After 6 to 8 h, there was evidence of septum formation and of intrahyphal growth within dead cells (Fig. 1). Therefore, caspofungin induced tip lysis but did not sterilize cultures and sporadic septation and hyphal growth resumed in the continued presence of caspofungin.

Caspofungin treatment induces a compensatory increase in chitin content in $A. fumigatus$. $A. fumigatus$ conidia were germinated for 12 h in RPMI 1640 with and without caspofungin. Chitin levels were determined by staining hyphal tips with CFW and by measuring cell wall chitin content by high-pressure liquid chromatography (HPLC). Staining with CFW showed an increase in chitin in $A. fumigatus$ hyphae after treatment with caspofungin compared to untreated controls (Fig. 2A), as reported previously (38, 39). Treatment of $A. fumigatus$ with caspofungin resulted in the formation of short, stumpy, hyperbranched hyphae (Fig. 2A). Biochemical measurement of chitin content demonstrated that hyphae treated with 2 µg/ml caspofungin had a 2.5-fold increase in their cell wall chitin compared to untreated controls (Fig. 2B). The increase in chitin content of $A. fumigatus$ germings in response to caspofungin treatment was also examined in real time using a microfluidics perfusion system (Fig. 3). Treatment with caspofungin led to an initial lysis of most of the hyphal tips (Fig. 3b). However, following lysis of hyphal tips, an increase in chitin content within some hyphal regions distal to the sites of tip lysis was observed that was coincident with the resumption of growth of wide, hyperbranched hyphae (Fig. 3c). In some experiments, cultures exhibiting this adapted growth phenotype were perfused with 64 µg/ml nikkomycin Z in combination with caspofungin, but this did not inhibit growth (Fig. 3d).

Next, a series of $\Delta Afchs$ disruption mutants was stained with CFW to establish which CHS enzymes were required for the compensatory increase in chitin synthesis induced by exposure to caspofungin (Fig. 4i). Most untreated $\Delta Afchs$ mutants had similar chitin content to the wild type, with the exception of the $\Delta AfchsG$ mutant, which exhibited swollen cells with high CFW staining (Fig. 4i). Treatment with caspofungin resulted in a compensatory increase in chitin content in the individual $\Delta Afchs$ mutants compared to untreated strains, with the exception of strains lacking $AfCHSG$ (Fig. 4ii).

The class III chitin synthase, encoded by $AfCHSG$, is involved in the synergistic action of caspofungin and nikkomycin Z. Caspofungin has a fungistatic effect on the growth of $A. fumigatus$ and results in a compensatory increase in chitin synthesis (Fig. 2). Since chitin upregulation affected caspofungin efficacy, we assessed whether combinations of chitin synthase inhibitors and caspofungin acted synergistically. The addition of 2 µg/ml of the chitin synthase inhibitor nikkomycin Z did not affect growth on

FIG 1  Morphological changes in $A. fumigatus$ in response to caspofungin. $A. fumigatus$ spores were trapped in a microfluidics chamber and grown in Sabouraud plus 2% glucose for 6 h in the absence (A) or presence (B) of CaCl$_2$ and CFW. Then all cells were treated with 32 µg/ml caspofungin for another 6 h and grown in fresh medium for 8 h. Yellow arrowheads indicate intrahyphal hyphae. White arrowheads indicate newly formed septum. Scale bars = 20 µm.
RPMI 1640 agar, whereas the addition of 2 μg/ml caspofungin led to a dramatic reduction in colony size (Fig. 5A). Combined treatment with caspofungin and nikkomycin Z resulted in the formation of colonies with substantially reduced radial growth compared to treatment with caspofungin alone (Fig. 5A).

The sensitivity of the ΔAfchs mutants to caspofungin was determined. All of the ΔAfchs mutants grew similarly to the wild type on RPMI 1640 alone, with the exception of the ΔAfchsG and ΔAfchsC ΔAfchsG mutants, which were viable but had reduced radial growth (Fig. 5B). Similarly, treatment with caspofungin led to a reduction in colony diameter, and the inhibition of growth was similar to wild-type cells for the majority of ΔAfchs mutants (Fig. 5B). Exceptions were the ΔAfchsG and ΔAfchsC ΔAfchsG mutants, which were hypersensitive to caspofungin (Fig. 5B). Treatment with 2 μg/ml caspofungin alone led to a 50% reduction in growth of wild-type cells, whereas treatment with 2 μg/ml nikkomycin Z alone led to a 30% reduction in growth (Fig. 5C). Combined treatment of caspofungin and nikkomycin Z at the same concentrations had an additive effect and resulted in a 75% reduction in growth of wild-type cells (Fig. 5C). When grown in RPMI 1640 alone, all ΔAfchs mutants exhibited growth that was comparable to the wild type, again with the exception of the ΔAfchsG and ΔAfchsC ΔAfchsG mutants, which had a 45% reduction in growth (Fig. 5C). Similarly, treatment of the ΔAfchs mutants with caspofungin resulted in a 35% reduction in growth that was comparable to that of the wild type. Again the exception was the ΔAfchsG and ΔAfchsC ΔAfchsG mutants, which demonstrated an 80% to 90% reduction in growth in the presence of caspofungin (Fig. 5C). The reduction in growth of the ΔAfchsG and ΔAfchsC ΔAfchsG mutants in the presence of caspofungin was comparable to the reduction in growth observed when wild-type cells were treated with a combination of caspofungin and nikkomycin Z (Fig. 5C). Therefore, the class III chitin synthase A/ChsG was most critically involved in the response to caspofungin treatment.

**FIG 2** Treatment with caspofungin leads to a compensatory increase in chitin content in *A. fumigatus*. The wild-type strain was grown at 37°C for 12 h in RPMI 1640 in the presence and absence of 2 μg/ml caspofungin. (A) CFW-stained fluorescent images; scale bars are 10 μm. (B) Cell wall chitin assays were performed three times on three biologically independent samples (average ± standard deviation [SD], n = 9). The asterisk indicates significant difference (P < 0.05) from untreated cells.

**FIG 3** Intrahyphal growth of *A. fumigatus* corresponds to areas of increased chitin content in response to caspofungin treatment. *A. fumigatus* spores were trapped in a microfluidics chamber and grown in Sabdex broth plus 2% glucose for 2 h. Cells were then treated with 32 μg/ml caspofungin for 6 h, followed by combination treatment with 32 μg/ml caspofungin and 64 μg/ml nikkomycin Z for a further 6 h. Yellow arrowheads indicate intrahyphal hyphae. DIC (top) and CFW fluorescent images (bottom). Scale bars = 10 μm.
Treatment with CaCl2 and CFW increases chitin content in A. fumigatus. Previously, treatment with CaCl2 and CFW was shown to increase the chitin content of C. albicans and reduce susceptibility to caspofungin (19, 23). To determine whether treatment with CaCl2 and CFW also increased the chitin content of A. fumigatus, spores were germinated in Sab dex broth with and without a combination of 200 mM CaCl2 and 100 g/ml CFW for 8 h. Hyphae that had germinated in the presence of CaCl2 and CFW had a 2-fold increase in cell wall chitin content, measured by HPLC, compared to that of the wild type (Fig. 6A). Ultrastructural analysis using transmission electron microscopy revealed that CaCl2- and CFW-treated hyphae had 45% thicker cell walls (Fig. 6Bii), which were chitin rich (Fig. 6Biv) relative to those of untreated hyphae (Fig. 6Bi and Biii).

FIG 4 AfCHSG is required for the compensatory increase in chitin content in response to caspofungin treatment. CFW-stained fluorescent images of the wild-type (WT) strain and ΔAfchs mutants in RPMI 1640 alone (i) or supplemented with 2 g/ml caspofungin (ii) after 12 h at 37°C. Scale bars = 10 μm.

FIG 5 Disruption of AfCHSG leads to hypersensitivity to caspofungin. (A) Plate dilution sensitivity tests of the A. fumigatus wild-type strain on RPMI 1640 agar alone (i) or supplemented with 2 μg/ml caspofungin (ii), 2 μg/ml nikkomycin Z (iii), or a combination (iv). (B) The ΔAfchs mutants were grown on RPMI 1640 agar with or without 2 μg/ml caspofungin. Plates were incubated for 48 h at 37°C. Spore numbers per spot are 5,000, 500, 50, and 5 spores, from left to right. (C) Dry weights of wild-type and ΔAfchs mutants were determined after 24 h growth in RPMI 1640 broth alone or supplemented with 2 μg/ml caspofungin. The wild-type strain was also treated with 2 μg/ml nikkomycin Z alone and in combination with caspofungin. Error bars are SD (n = 3, from three independent experiments). Asterisks indicate significant differences (P < 0.05) from untreated cells of the same genetic background, #, significant difference (P < 0.05) from the wild-type cells in the same growth conditions. Numbers represent percentages of growth inhibition compared to untreated cells of the same genetic background.
Afchs mutants were also treated with the CaCl₂ and CFW combinations, and their relative chitin contents were determined by staining with 25 µg/ml CFW. Treatment with CaCl₂ and CFW led to defined chitin-rich patches in all Afchs mutants at some hyphal tips and at the kinks and bends of hyphal cells. The CFW-rich patches were evident in multiple ΔAfchs null mutants, suggesting that no single CHS was responsible exclusively for the increase in chitin content resulting from caspofungin exposure (Fig. 7). However, in the wild-type strain, there was a more uniform increase in chitin staining along the hypha, perhaps indicating that some differences exist between the mutants and the wild type in the spatial deposition of chitin upon CaCl₂ and CFW cotreatment.

**Activation of the cell wall salvage pathway protects against caspofungin treatment.** To determine whether increased chitin content leads to reduced caspofungin susceptibility in _A. fumigatus_, conidia were first germinated in Sabdex broth with and without 200 mM CaCl₂ and 100 µg/ml CFW to elevate chitin, then hyphae were washed and exposed to caspofungin. Pregrowth with CaCl₂ and CFW enhanced the growth of the wild type and ΔAfchs mutants on control plates and led to reduced susceptibility to caspofungin in all strains tested, with the exception of strains lacking _AfCHSG_ (Fig. 8). When strains lacking _AfCHSG_ were grown with CaCl₂ and CFW prior to caspofungin treatment, the radial growth of their colonies was still reduced compared to that of the colonies grown without CaCl₂ and CFW pretreatment (Fig. 8).

The caspofungin MECs for untreated and pretreated strains were measured in liquid RPMI 1640. In most cases, pregrowth with CaCl₂ and CFW led to a significant reduction in susceptibility to caspofungin (Table 2). Again, the exceptions were the ΔAfchsG and ΔAfchsC ΔAfchsG mutants, which retained their increased susceptibility to caspofungin even after pregrowth with CaCl₂ and CFW. Therefore, _AfCHSG_ was critical for the protective upregulation of chitin synthesis in _A. fumigatus_.

**DISCUSSION**

We show here that exposure to the echinocandin caspofungin can lead to the upregulation of chitin synthesis mediated by _AfChsG_ and subsequent survival and growth of aberrant chitin-rich hyperbranched hyphae. The importance of these observations is that this chitin-rich surviving biomass has the potential to act as a reservoir for regrowth of _Aspergillus_ mycelium following echinocandin treatment.

Different species of _Aspergillus_ have various susceptibilities to the echinocandins, and several examples of echinocandin resistance in _Aspergillus_ species have been reported (17, 40–48). Anidulafungin displays the greatest inhibition of growth across the _Aspergillus_ spp. compared to that of caspofungin and micafun-
that have reduced susceptibility to caspofungin (49). Point mutants have been highlighted by two classes of laboratory-generated mutants that were generated by cell wall digestion, followed by regeneration of spheroplasts on caspofungin-containing medium, disguised to be due to differences in cell wall composition (17). Potential mechanisms of resistance to caspofungin in *A. fumigatus* have been highlighted by two classes of laboratory-generated mutants that have reduced susceptibility to caspofungin (49). Point mutations within *AfFKS1* leading to an S678P amino acid substitution from a patient who failed caspofungin treatment has also been shown to be dependent on the *AfCNAA*-calcineurin and PKC signaling pathways, resulting in a 3- to 4-fold increase in chitin content (23).

In *C. albicans*, pregrowth of cells with a combination of CaCl2 and CFW activates the Ca2⁺-calcineurin pathway genes *AfCHSA* and *AfCHS* (21). Pretreatment with CaCl2 and CFW increases the caspofungin MEC (40, 45). Furthermore, there have been several reports of the emergence of anidulafungin-resistant molds in the clinic (40, 42, 44, 45).

In *C. albicans* and *A. fumigatus*, growth at high concentrations above the MIC of caspofungin, termed paradoxical growth, has been observed in vitro. Paradoxical growth occurs most commonly when *A. fumigatus* is treated with caspofungin, rather than miconafungin or anidulafungin, in vitro (38, 43). In *C. albicans*, cells demonstrating paradoxical growth were shown to have a 900% increase in chitin content, suggesting that the regrowth at high concentrations of caspofungin was due to an increase in chitin content (52). In *A. fumigatus*, addition of the calcineurin inhibitor, FK506, or deletion of genes from the calcineurin pathway abolished the occurrence of paradoxical growth (38). Rogg et al. (53) demonstrated that the Ca²⁺-calcineurin pathway was required for the transcriptional upregulation of *AfCHSA* and *AfCHSC* in response to caspofungin treatment. Likewise, chitin content and chitin synthase enzyme activity were also increased during paradoxical growth (38). A likely hypothesis is therefore that paradoxical effects arise because high levels of echinocandins are able to activate the cell wall salvage pathway(s) that promote chitin synthesis and cell survival.

The data presented here highlight the potential of increased chitin content as a mechanism of reduced susceptibility to caspofungin in *A. fumigatus*. Treatment of wild-type cells with caspofungin resulted in a 2.5-fold compensatory increase in chitin content. This supports the findings of previous studies where a different wild-type strain of *A. fumigatus* also demonstrated a compensatory increase in chitin content in response to treatment with all three echinocandins (21, 39). Here we show that disruption of the class II, III, and VI chitin synthase genes did not markedly affect the ability of *A. fumigatus* to increase chitin synthesis to compensate for the inhibition of β-1,3-glucan synthesis by caspofungin. Therefore, the remaining *AfCHS* genes may be deduced to be able to compensate for the loss of the class II, III, and VI chitin synthase enzymes by synthesizing sufficient chitin to protect the fungus from caspofungin. Supporting this, combined treatments of *A. fumigatus* with caspofungin and nikkomycin Z did not inhibit the compensatory increase in chitin content (21). The compensatory increase in chitin content of *A. fumigatus* in response to caspofungin treatment has also been shown to be dependent on the *AfCNAA*-calcineurin pathway genes *AfCHSA* and *AfCRZA* (21).

In *C. albicans*, pregrowth of cells with a combination of CaCl2 and CFW activates the Ca²⁺-calcineurin and PKC signaling pathways, resulting in a 3- to 4-fold increase in chitin content (23).

**TABLE 2** Pretreatment with CaCl2 and CFW increases the caspofungin MEC against *A. fumigatus* strains

| *A. fumigatus* strain description | MEC (µg/ml) |
|----------------------------------|-------------|
| Untreated                        | Pretreated (CaCl2 and CFW) |
| Wild type                        | 0.5         | 4           |
| ΔchsC                            | 0.5         | 4           |
| ΔchsG                            | 0.064       | 0.064       |
| ΔchsD                            | 0.25        | 8           |
| ΔchsC ΔchsB                      | 0.25        | 4           |
| ΔchsC ΔchsG                      | 0.064       | 0.064       |
This elevation of chitin content renders \textit{C. albicans} cells less susceptible to caspofungin (19). Pregrowth of \textit{A. fumigatus} with CaCl$_2$ and CFW also resulted in an increase in chitin content, which led to reduced susceptibility to caspofungin. This increase in chitin content was not solely dependent on the class II, III, and VI chitin synthase enzymes because mutants lacking enzymes from these three classes still demonstrated an increase in chitin content. Despite this, the $\Delta$AfchsG and the $\Delta$AfchsC $\Delta$AfchsG mutants had no decrease in caspofungin susceptibility after treatment with CaCl$_2$ and CFW. Our results suggest that AFCHSG makes a major contribution to the ability of CaCl$_2$- and CFW-treated cells to grow in the presence of caspofungin. Mutants lacking AFCHSG may grow in 4 $\mu$g/ml caspofungin after CaCl$_2$ and CFW treatment compared to the other strains tested. The other Chs enzymes are therefore likely to make some contribution to the cell wall salvage mechanism, primed by CaCl$_2$ and CFW treatment, because mutants lacking AFCHSG still upregulated chitin production, even though this was not sufficient to decrease sensitivity to caspofungin. Therefore, increasing the cell wall chitin content is also a mechanism of tolerance to caspofungin in filamentous fungi such as \textit{A. fumigatus}.

Here we observed that treatment with caspofungin promoted intrahyphal growth within lysed hyphae, which may contribute to the ability of \textit{A. fumigatus} to survive caspofungin treatment. In other filamentous fungi, intrahyphal hyphae have been proposed to promote fungal survival in response to stress conditions (54). Supporting this, recent work has shown that compounds which inhibit septum formation act synergistically with caspofungin (39). However, this is the first example of the formation of intrahyphal hyphae as a response to echinocandin treatment.

\textit{A. fumigatus} activates a compensatory increase in chitin content in response to sub-MIC caspofungin treatment, which highlights the potential of combining chitin synthase inhibitors with the echinocandins for improved and/or broader spectrum therapy. In addition, the PKC, Ca$^{2+}$-calceinurin, and HOG signaling pathways have been shown to be required for the response of \textit{C. albicans} and \textit{A. fumigatus} to caspofungin and the \textit{in vitro} paradoxical growth phenomenon (19–21, 55, 56). Consequently, inhibitors of these pathways together with an echinocandin should be explored as possible combination therapies.

Chitin synthase inhibitors have been shown to enhance the activity of caspofungin and other echinocandins against a range of fungal pathogens (19, 57–60). Combination treatment with chitin inhibitors and the echinocandins may, therefore, add potency and increase the spectrum of activity of echinocandins to a range of filamentous fungal pathogens. For example, synergistic inhibition of \textit{Alternaria infectoria} with caspofungin and nikkomycin Z has been reported (61). Combination treatment of \textit{A. fumigatus} with nikkomycin Z and the echinocandins leads to enhanced killing \textit{in vitro} and results in the formation of swollen spores with aberrant walls, which are prone to lysis (21, 58, 62). Also, treatment with micafungin significantly prolonged host survival in systemic and pulmonary murine aspergillosis when combined with nikkomycin Z (63, 64).

This study illuminates the conserved clinical relevance of the cell wall compensatory mechanism in response to assaults that weaken the wall. A common feature of this mechanism is the activation of chitin synthesis, which involves multiple members of fungal chitin synthase families. These pathways are activated when fungi are exposed to sub-MIC echinocandins and may contribute to tolerance if drugs are administered suboptimally or there is reduced bioavailability. The ability to simultaneously block the synthesis of chitin and $\beta$-1,3-glucan represents a major opportunity for future strategies to augment the range and cidality of echinocandins.

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\textbf{REFERENCES}

1. Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC. 2012. Hidden killers: human fungal infections. Sci Transl Med 4:165sr13. http://dx.doi.org/10.1126/scitranslmed.3004404.
2. Mellado E, Auffauvre-Brown A, Specht CA, Robbins PW, Holden DW. 1995. A multigene family related to chitin synthase genes of yeast in the opportunistic pathogen \textit{Aspergillus fumigatus}. Mol Gen Genet 246:353–359. http://dx.doi.org/10.1007/BF00286608.
3. Latge JP. 2007. The cell wall: a carbohydrate armour for the fungal cell. Mol Microbiol 66:279–290. http://dx.doi.org/10.1111/j.1365-2958.2007.05872.x.
4. Latge JP. 2001. The pathobiology of \textit{Aspergillus fumigatus}. Trends Microbiol 9:382–389. http://dx.doi.org/10.1016/S0966-842X(01)02104-7.
5. Alcazar-Fuoli L, Mellado E, Astraurey-Izquierdo A, Cuenca-Estrella M, Rodriguez-Tudela JL. 2008. \textit{Aspergillus section Fumigati}: antifungal susceptibility patterns and sequence-based identification. Antimicrob Agents Chemother 52:1244–1251. http://dx.doi.org/10.1128/AAC.00942-07.
6. van der Linden JW, Snelders E, Kampinga GA, Rijnders BJ, Mattsson E, Debeets-Ossenkopp YJ, Kuijper EJ, Van Tiel FH, Melchers WJ, Verweij PE. 2011. Clinical implications of azole resistance in \textit{Aspergillus fumigatus}, The Netherlands, 2007–2009. Emerg Infect Dis 17:1846–1854. http://dx.doi.org/10.3201/eid1710.110226.
7. Lionakis MS, Lewis RE, Torres HA, Albert ND, Raad II, Kontoyiannis DP. 2005. Increased frequency of non-fumigatus \textit{Aspergillus} species in amphotericin B–or triazole-pre-exposed cancer patients with positive cultures for aspergilli. Diagn Microbiol Infect Dis 52:215–220. http://dx.doi.org/10.1016/j.diagmicrobio.2005.01.001.
8. Perkofer S, Lass-Florl C, Hell M, Russ G, Krause R, Honigl M, Gertner C, Aube felder J, Gastl G, Mitterbauer M, Willinger B, Knobl P, Resch G, Waldner R, Makrai A, Hartmann G, Girschikofsky M, Greil R. 2010. The Nationwide Austrian Aspergillus Registry: a prospective data collection on epidemiology, therapy and outcome of invasive mould infections in immunocompromised and/or immunosuppressed patients. Int J Antimicrob Agents 36:531–536. http://dx.doi.org/10.1016/j.ijantimicag.2010.08.010.
9. Verweij PE, Snelders E, Kema GH, Mellado E, Melchers WJ. 2009. Azole resistance in \textit{Aspergillus fumigatus}: a side-effect of environmental fungicide use? Lancet Infect Dis 9:789–795. http://dx.doi.org/10.1016/S1473-3099(09)70265-8.
10. Howard SJ, Harrison E, Bowyer P, Varga J, Denning DW. 2011. Cryptic species and azole resistance in the \textit{Aspergillus niger} complex. Antimicrob Agents Chemother 55:4802–4809. http://dx.doi.org/10.1128/AAC.00304-11.
11. Epaulard O, Villier C, Ravaud P, Chasidow O, Blanche S, Mamzer-Bruneel MF, Thiebaut A, Leccia MT, Lortholary O. 2013. A multistep voriconazole-related phototoxic pathway may lead to skin carcinoma: results from a French nationwide study. Clin Infect Dis 57:1842–1848. http://dx.doi.org/10.1093/cid/cit600.
12. Bowman JC, Hicks PS, Kurtz MB, Rosen H, Schatzme DM, Liberator PA, Douglas CM. 2002. The antifungal echinocandin caspofungin acetate kills growing cells of \textit{Aspergillus fumigatus} \textit{in vitro}. Antimicrob Agents Chemother 46:3001–3012. http://dx.doi.org/10.1128/AAC.46.3.3001-3012.2002.
13. Oddis FC, Brown AJ, Gow NA. 2003. Antifungal agents: mechanisms of action. Trends Microbiol 11:272–279. http://dx.doi.org/10.1016/S0966-842X(03)00117-3.
14. Kurtz MB, Heath IB, Marrinan J, Dreikorn S, Onishi J, Douglas C. 1994. Morphological effects of lipopeptides against Aspergillus fumigatus correlate with activities against (1,3)-β-glucan synthase. Antimicrob Agents Chemother 38:1480–1489. http://dx.doi.org/10.1128/AAC.38.7.1480.

15. Arakan S, Lozano-Chiu M, Paetznick V, Rex JH. 2001. In vitro susceptibility testing methods for caspofungin against Aspergillus and Fusarium isolates. Antimicrob Agents Chemother 45:327–330. http://dx.doi.org/10.1128/AAC.45.1.327-330.2001.

16. Espinel-Ingroff A. 2003. In vitro antifungal activities of anidulafungin and micafungin, licensed agents and the investigational triazole posaconazole as determined by NCCLS methods for 12,052 fungal isolates: review of the literature. Rev Iberoam Microl 26:121–136.

17. Imhof A, Balajee SA, Marr KA. 2003. New methods to assess susceptibilities of Aspergillus isolates to caspofungin. J Clin Microbiol 41:5683–5688. http://dx.doi.org/10.1128/JCM.41.12.5683-5688.2003.

18. Beauvais A, Bruneau JM, Mol PC, Buitrago MJ, Legrand R, Latge JP. 2001. Glucan synthesis of Aspergillus fumigatus. J Bacteriol 183: 2273–2279. http://dx.doi.org/10.1128/JB.183.7.2273-2279.2001.

19. Walker LA, Munro CA, de Bruijn I, Lenardon MD, McKinnon A, Gow NA. 2008. Stimulation of chitin synthesis rescues Candida albicans from echinocandin. PLoS Pathog 4:e1000040. http://dx.doi.org/10.1371/journal.ppat.1000040.

20. Cota JM, Grabinski JL, Talbert RL, Burgess DS, Rogers PD, Edlind TD, Wiederhold NP. 2008. Increases in S.12 expression and chitin content are associated with incomplete killing of Candida glabrata by caspofungin. Antimicrob Agents Chemother 52:1144–1146. http://dx.doi.org/10.1128/AAC.01542-07.

21. Fortwendel JR, Juvvadi PR, Pinchai N, Perfect BZ, Alspaugh JA, Perfect JR, Steinbach WJ. 2009. Differential effects of inhibiting chitin and 1,3-β-glucan synthesis in ras and calcineurin mutants of Aspergillus fumigatus. Antimicrob Agents Chemother 53:476–482. http://dx.doi.org/10.1128/AAC.00154-08.

22. Walker LA, Gow NA, Munro CA. 2013. Elevated chitin content reduces the susceptibility of Candida species to caspofungin. Antimicrob Agents Chemother 57:146–154. http://dx.doi.org/10.1128/AAC.01486-12.

23. Munro CA, Selvaginneri S, de Bruijn I, Walker L, Lenardon MD, Gersen B, Milne S, Brown AJ, Gow NA. 2007. The PKC, HOG and Ca2+ signalling pathways co-ordinately regulate chitin synthesis in Candida albicans. Mol Microbiol 63:1399–1413. http://dx.doi.org/10.1111/j.1365-2958.2007.05588.x.

24. Mora-Montes HM, Netea MG, Ferwerda G, Lenardon MD, Brown GD, Mistry AR, Kullberg BJ, O'Callaghan CA, Sheth CC, Odds FC, Brown AJ, Munro CA, Gow NA. 2011. Recognition and blocking of innate immunity cells by Candida albicans chitin. Infect Immun 79:1961–1970. http://dx.doi.org/10.1128/IAI.01282-10.

25. Lee CM, Jacson MB, Jacobsen MD, Walker LA, Odds FC, Gow NA, Munro CA. 2012. Elevated cell wall chitin in Candida albicans confers echinocandin resistance in vivo. Antimicrob Agents Chemother 56:208–217. http://dx.doi.org/10.1128/AAC.00683-11.

26. Mellado E, Auffaure-Brown A, Gow NA, Holden DW. 1996. The Aspergillus fumigatus chsC and chsG genes encode class III chitin synthases with different functions. Mol Microbiol 20:667–679. http://dx.doi.org/10.1046/j.1365-2958.1996.5571084.x.

27. Mellado E, Specht CA, Robbins PW, Holden DW. 1996. Cloning and characterization of chsD, a chitin synthase-like gene of Aspergillus fumigatus. FEMS Microbiol Lett 143:69–76. http://dx.doi.org/10.1111/j.1574-6966.1996.tb08463.x.

28. Jimenez-Ortigosa C, Aimaniana V, Muszkietka L, Mouyna I, Al- seas D, Pire S, Beau R, Krappmann S, Beauvais A, Dufrene YF, Roncoro C, Latge JP. 2012. Chitin synthases with a myosin motor-like domain control the resistance of Aspergillus fumigatus to echinocandins. Antimicrob Agents Chemother 56:6121–6131. http://dx.doi.org/10.1128/AAC.00752-12.

29. Muszkietka L, Aimaniana V, Mellado E, Gribaldo S, Alcicar-Fuoli L, Szweczyk E, Prevost MC, Latge JP. 2014. Deciphering the role of the chitin synthases Chs1 and Chs2 in the in vivo and in vitro growth of Aspergillus fumigatus by multiple gene targeting deletion. Cell Microbiol 16: 1784–1805. http://dx.doi.org/10.1111/cmi.12326.

30. Borgia PT, Iartchouk N, Riggle PJ, Winter KR, Koltin Y, Bulawa CE. 1996. The chsB gene of Aspergillus nidulans is necessary for normal hyphal growth and development. Fungal Genet Biol 26:193–203. http://dx.doi.org/10.1006/fgb.1996.0035.
Candida albicans: possible role of cell wall integrity and calcineurin pathways. Antimicrob Agents Chemother 49:5146–5148. http://dx.doi.org/10.1128/AAC.49.12.5146-5148.2005.

Sadowsky-Losica H, Shwartzman R, Lahat Y, Segal E. 2008. Antifungal activity against Candida albicans of nikkomycin Z in combination with caspofungin, voriconazole or amphotericin B. J Antimicrob Chemother 62:635–637. http://dx.doi.org/10.1093/jac/dkn216.

Steinbach WJ, Cramer RA, Jr, Perfect BZ, Henn C, Nielsen K, Heitman J, Perfect JR. 2007. Calcineurin inhibition or mutation enhances cell wall inhibitors against Aspergillus fumigatus. Antimicrob Agents Chemother 51:2979–2981. http://dx.doi.org/10.1128/AAC.01394-06.

Ganesan LT, Manavathu EK, Cutright JL, Alangaden GJ, Chandrasekar PH. 2004. In-vitro activity of nikkomycin Z alone and in combination with polyenes, triazoles or echinocandins against Aspergillus fumigatus. Clin Microbiol Infect 10:961–966. http://dx.doi.org/10.1111/j.1469-9891.2004.00996.x.

Stevens DA. 2000. Drug interaction studies of a glucan synthase inhibitor (LY 303366) and a chitin synthase inhibitor (nikkomycin Z) for inhibition and killing of fungal pathogens. Antimicrob Agents Chemother 44:2547–2548. http://dx.doi.org/10.1128/AAC.44.9.2547-2548.2000.

Fernandes C, Anjos J, Walker LA, Silva BM, Cortes L, Mota M, Munro CA, Gow NA, Goncalves T. 2014. Modulation of Alternaria infectoria cell wall chitin and glucan synthesis by cell wall synthase inhibitors. Antimicrob Agents Chemother 58:2894–2904. http://dx.doi.org/10.1128/AAC.02647-13.

Chiou CC, Mavrogiorgos N, Tillem E, Hector R, Walsh TJ. 2001. Synergy, pharmacodynamics, and time-sequenced ultrastructural changes of the interaction between nikkomycin Z and the echinocandin FK465 against Aspergillus fumigatus. Antimicrob Agents Chemother 45:3310–3321. http://dx.doi.org/10.1128/AAC.45.12.3310-3321.2001.

Clemons KV, Stevens DA. 2006. Animal models testing monotherapy versus combination antifungal therapy: lessons learned and future directions. Curr Opin Infect Dis 19:360–364. http://dx.doi.org/10.1097/01.qco.0000235163.70678.59.

Luque JC, Clemons KV, Stevens DA. 2003. Efficacy of micafungin alone or in combination against systemic murine aspergillosis. Antimicrob Agents Chemother 47:1452–1455. http://dx.doi.org/10.1128/AAC.47.4.1452-1455.2003.