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Identification and characterization of haemofungin, a novel antifungal compound that inhibits the final step of haem biosynthesis

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Abstract

Objectives—During recent decades, the number of invasive fungal infections among immunosuppressed patients has increased significantly, whereas the number of effective systemic antifungal drugs remains low and unsatisfactory. The aim of this study was to characterize a novel antifungal compound, CW-8/haemofungin, which we previously identified in a screen for compounds affecting fungal cell wall integrity.

Methods—The *in vitro* characteristics of haemofungin were investigated by MIC evaluation against a panel of pathogenic and non-pathogenic fungi, bacteria and mammalian cells in culture. Haemofungin mode-of-action studies were performed by screening an *Aspergillus nidulans* overexpression genomic library for resistance-conferring plasmids and biochemical validation of the target. *In vivo* efficacy was tested in the *Galleria mellonella* and *Drosophila melanogaster* insect models of infection.

Results—We demonstrate that haemofungin causes swelling and lysis of growing fungal cells. It inhibits the growth of pathogenic *Aspergillus*, *Candida*, *Fusarium* and *Rhizopus* isolates at micromolar concentrations, while only weakly affecting the growth of mammalian cell lines. Genetic and biochemical analyses in *A. nidulans* and *Aspergillus fumigatus* indicate that haemofungin primarily inhibits ferrochelatase (HemH), the last enzyme in the haem biosynthetic

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Transparency declarations

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pathway. Haemofungin was non-toxic and significantly reduced mortality rates of *G. mellonella* and *D. melanogaster* infected with *A. fumigatus* and *Rhizopus oryzae*, respectively.

Conclusions—Further development and *in vivo* validation of haemofungin is warranted.

Introduction

The incidence of life-threatening invasive fungal infections has risen significantly during the past 30 years.^{1,2} Most are caused by species of *Cryptococcus*, *Candida* and *Aspergillus*.³ It is estimated that invasive aspergillosis and candidiasis affect between 10% and 25% of all leukaemic and bone marrow transplant patients, with an alarmingly high mortality rate of ~50%.^{4,5} However, despite the growing needs, treatments for invasive fungal infections remain unsatisfactory, with existing classes of antifungals showing toxicity, narrow specificity, increasing resistance or limited formulation.⁶ Therefore, there is a pressing need to develop novel antifungals that inhibit fungus-specific targets such as the fungal cell wall.

We previously screened a diverse chemical library of 35000 drug-like molecules (ChemDiv, San Diego, CA, USA) to identify inhibitors of *Aspergillus fumigatus* growth.⁷ The resulting antifungal compounds were next tested for cell wall-damaging activity using the *alcA-PKC* mutant. Of these, one group, the CANBEFs, was described previously.⁷ In this report, we describe the detailed analysis of another compound identified in this screen, CW-8, which we named here haemofungin. It demonstrated cell wall-damaging properties, promising *in vitro* antifungal activity against a panel of pathogenic fungi and *in vivo* efficacy in insect models of fungal infection. Its mode of action, inhibition of haem biosynthesis, was elucidated.

Materials and methods

Strains and media

The strains used in this study are detailed in Table S1 (available as Supplementary data at *JAC* Online). Conidia were harvested in 0.2% (v/v) Tween 80, resuspended in double-distilled water (DDW) and counted with a haemocytometer. Moulds were grown in rich YAG medium containing 0.5% (w/v) yeast extract, 1% (w/v) glucose, 10 mM MgCl₂, supplemented with 0.1% (v/v) trace elements solution, and 0.2% (v/v) vitamin mix or in defined minimal medium (MM) containing 70 mM NaNO₃, 1% (w/v) glucose, 12 mM potassium phosphate pH 6.8, 4 mM MgSO₄, 7 mM KCl and trace elements. For MM containing glycerol, glucose was replaced with 0.2% (v/v) glycerol. Complete medium (CM) was prepared by adding 0.1% yeast extract, 0.2% peptone and 0.1% tryptone (all w/v) to MM. Yeasts were grown in YPD rich medium composed of 1% (w/v) yeast extract, 2% (w/v) peptone and 2% glucose (w/v). Bacteria were grown in LB broth composed of 1% tryptone, 0.5% yeast extract and 1% NaCl (all w/v).

Pan-fungal and bacterial screen

The fungal strains listed in Table S1 were tested for susceptibility according to CLSI standard M27-A3 or M38-A2 protocols, respectively.^{8,9} Identification of cell wall-active

antifungal compounds using the *Aspergillus nidulans alcA-PKC* mutant was performed as previously described.⁷

Cell culture

Hit compounds were assessed for toxicity to 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) as previously described.⁷ Cell viability was measured by the XTT assay kit (Biological Industries, Beit Haemek, Israel).

Microscopy and staining

The effect of the hit compounds on fungal ultrastructure was assessed by light and fluorescence microscopy after cell wall and vital staining as previously described.⁷ Colocalization studies were carried out by incubation of 8 h germinated conidia adherent on glass coverslips with 1 μ M MitoTracker Green FM (Life Technologies) for 1 h and haemofungin (2 μ M) for a further 15 min, both at room temperature. After two DDW washes, imaging was carried out on a Leica TCS SPF5 confocal microscope (excitation 488 nm, unmixing 500–650 nm and deconvolution).

Synergy chequerboard assay

Chequerboard tests were performed in standard 96-well plates (Costar; Corning, Corning, NY, USA) according to CLSI M38-A2 microdilution methodology.⁸

Screening an *A. nidulans* overexpression genomic library for resistance-conferring plasmids

A library of *A. nidulans* transformants containing a genomic library cloned into the multicopy non-integrating vector pRG3-AMA1 of *A. nidulans*^{10,11} was screened for resistant strains. We have used this method to successfully identify the cellular target of two antifungal drugs.^{12,13} Transformation was undertaken by protoplasting as previously described.¹⁴ Screening was performed in the presence of 4 μ M haemofungin as described previously.⁷ Isolation of the resistance-conferring plasmids and identification of the resistance-conferring gene in the genomic DNA insert were undertaken as previously described.¹¹

Northern blot analysis

Freshly harvested *A. fumigatus* conidia were grown for 20 h at 37°C with shaking in liquid medium (MM or CM). Haemofungin (5 μ M) was added during the last 1 h. For northern blot analysis, RNA was isolated with TRI Reagent (Sigma) and peqGOLD Phase Trap (peqlab) reaction tubes; 10 μ g of total RNA was separated in formaldehyde-containing agarose gels, blotted onto Hybond-N⁺ membranes (Amersham Biosciences) and hybridized with digoxigenin-labelled probes. Hybridization probes were amplified by PCR with primers listed in Table S2.

Measurement of protoporphyrin IX (PPIX) levels

Freshly harvested *A. fumigatus* conidia were grown for 20 h at 37°C with shaking in YAG liquid medium. Haemofungin (2 µM) was added during the last 2 h. Samples were flash-frozen in liquid N₂, lyophilized overnight, ground to powder and dissolved in 1:1 methanol/DMSO. Chromatographic separation was carried out on an Agilent 1100 series HPLC fitted with a Phenomenex Gemini C18 110A (150×4.60 mm) 5 µm column. Next, 100 µL of sample in 1:1 methanol/DMSO was injected onto the column. The compound was eluted with a 20 min linear gradient (0–20 min) of 80% (0.1% TFA in H₂O), 20% acetonitrile to 100% acetonitrile followed by a 5 min isocratic elution (20–25 min) with 100% acetonitrile and finally a return to the initial conditions (25–32 min). The flow rate was 1.0 mL/min and the porphyrins were detected with a diode array detector at 398 nm.

In vivo antifungal activity: *Galleria mellonella* model

Groups of 10 caterpillars of the greater wax moth *G. mellonella* in the final instar larval stage, weighing 250–330 mg, were employed in all assays. Larvae were infected by injecting 10 µL of saline containing 1×10⁶ conidia of *A. fumigatus* strains Af293 or CEA10 into the haemocoel through the last proleg with a 50 µL Hamilton syringe. At 2 h post-infection, larvae were injected with 10 µL of saline containing haemofungin. Larval survival was assessed daily for up to 10 days post-treatment.

In vivo antifungal activity: *Drosophila melanogaster* model

Toll trans-heterozygotes (i.e. *Tl*^{-/-} flies) were generated by crossing flies carrying a thermosensitive allele of *Toll* (*Tl*⁶³²) with flies carrying a null allele of *Toll* (*Tl*^{-RXA}). The assay was performed as previously described.⁷ *Toll* flies infected with *Rhizopus oryzae* were fed with fly food containing 14.4 mg/mL haemofungin for 7 days. Survival was assessed until day 8 after infection.

Results

Screening for cell wall-destabilizing antifungal compounds

In a previously described screen for compounds with antifungal activity, we identified 16 ‘hit’ compounds that completely inhibited fungal germination and growth at a concentration of 25 µM.⁷ To identify potential cell wall-specific compounds, these ‘hits’ were further characterized in the *A. nidulans alcA-PKC* mutant. We have previously shown that this *PKC*-inducible strain exhibits hypersensitivity to cell wall-damaging compounds under repressing (MM-glucose) conditions, but not under inducing (MM-glycerol) conditions.^{15,16} One of the 16 compounds {CW-8/haemofungin; 5-chloro-3-ethyl-1-phenyl-2-[3-(1,3,3-trimethyl-2,3-dihydro-1*H*-indol-2-ylidene)prop-1-en-1-yl]-1*H*-1,3-benzodiazol-3-ium} (Figure 1a) displayed 4- and 8-fold hypersensitivity in MICs and minimum effective concentrations (MECs) under repressive conditions compared with inducing conditions for the *A. nidulans alcA-PKC* mutant (Table S3), supporting the conclusion that it affects the cell wall. Caspofungin, a known wall-perturbing drug, was used as a positive control and voriconazole as a negative control (no difference in MICs and MECs under repressing or inducing conditions). Haemofungin was therefore chosen for further analysis.

Haemofungin is active against most pathogenic fungi

Haemofungin was tested on a wide range of pathogenic fungal strains, mammalian cell lines and bacteria in culture (Table 1). Haemofungin was active against *Candida* spp. ($3.13 \mu\text{M} < \text{MIC} < 12.5 \mu\text{M}$), *A. fumigatus* and *Aspergillus niger* ($3.13 \mu\text{M} < \text{MIC} < 6.25 \mu\text{M}$) and *Fusarium* spp. ($3.13 \mu\text{M} < \text{MIC} < 12.5 \mu\text{M}$) as well as most *Rhizopus* spp. ($3.13 \mu\text{M} < \text{MIC} < 12.5 \mu\text{M}$). Haemofungin only markedly inhibited the proliferation of mammalian cells in culture at $25 \mu\text{M}$ (80% inhibition) and inhibited two of the four bacterial species tested, indicating that these compounds are not entirely fungus specific. The fungicidal activity of haemofungin was tested against *A. fumigatus* Af293, *Candida albicans* CBS 562 and *Saccharomyces cerevisiae* BY4741, respectively, exhibiting MFCs 2- to 4-fold higher than their MICs for these organisms.

Haemofungin at a concentration of up to $25 \mu\text{M}$ did not induce sheep red blood cell haemolysis even after 24 h of incubation, indicating it does not function as a membrane-disrupting agent (data not shown). Haemofungin was highly active in various media, including MM, rich YPD or YAG, and defined cell-culture medium RPMI 1640 or DMEM, with or without 10% serum (data not shown).

Haemofungin causes morphological changes characteristic of damage to the cell wall of *A. fumigatus*

Further characterization of the effects of haemofungin on fungal structure was carried out on *A. fumigatus* strain Af293. The effect of haemofungin on cell wall polysaccharide deposition, ultrastructure and viability was determined by transmission electron microscopy (TEM), calcofluor white (CFW) staining and DiBAC staining of dead cells, respectively. After 24 h of growth in the presence of subinhibitory concentrations of haemofungin, the Af293 strain displayed abnormalities in cell wall ultrastructure, characterized by swelling and lysis of cell bodies and abnormal thickening and fragmentation of the outer cell wall (Figure 1b). Similar swelling was seen in *S. cerevisiae* yeast cells in the presence of MIC levels of haemofungin (data not shown). CFW chitin staining demonstrated that haemofungin increased the deposition of chitin in the cell wall of the swollen cell bodies and along the hyphae (Figure 1c). DiBAC vital staining revealed numerous dead (fluorescing) hyphal segments after haemofungin treatment, in contrast to the untreated control (Figure 1d).

Haemofungin and caspofungin interact synergistically

We hypothesized that the mode of action of haemofungin differs from that of existing antifungals and that consequently they might display beneficial synergy when combined. Therefore, haemofungin was tested for interaction with the antifungal drugs caspofungin (an inhibitor of glucan synthase), voriconazole (an inhibitor of ergosterol biosynthesis) and amphotericin B (a membrane-disrupting compound) and with the protein kinase C inhibitor staurosporine (which blocks the cell wall integrity pathway) by means of a checkerboard modification of the guidelines presented in CLSI document M38-A.17 Whereas there was no interaction between haemofungin combined with voriconazole, amphotericin B or staurosporine, there was a synergistic interaction between haemofungin and the cell wall inhibitor caspofungin (FICI = 0.27; Table S4). These results suggest that both haemofungin

and caspofungin damage the cell wall, leading to synergy or alternatively that drug entry is enhanced when they are combined.

Overexpression of *AN7752* in *A. nidulans* confers resistance to haemofungin

We screened an *A. nidulans* high-copy vector overexpression library plated under the selection of haemofungin at 4 μM ($2\times$ MIC). Eleven resistant colonies (MIC=8 μM) were identified and the multicopy library vector isolated from all of them. Retransformation of 3 of these 11 plasmids into haemofungin-susceptible *A. nidulans* resulted in resistance (MIC of haemofungin=8 μM compared with MIC=2 μM for a control *A. nidulans* strain transformed with the empty library vector). The insert in all three plasmids was sequenced and contained several ORFs; only one of them, *AN7752*, was shared by all three inserts (Figure 2a), identifying it as a likely candidate for being the gene responsible for resistance to haemofungin. Furthermore, transposon mapping revealed that disruption of *AN7752* resulted in loss of resistance to haemofungin. *AN7752* is an uncharacterized ORF in *A. nidulans* with many known orthologues in other fungi (Figure 2b). The *S. cerevisiae* orthologue is *YOR176W/HEM15*, a confirmed ferrochelatase, a mitochondrial enzyme catalysing the eighth and final step in the haem biosynthetic pathway.

Addition of haemin attenuates haemofungin-induced growth inhibition

We reasoned that if ferrochelatase is the primary target of haemofungin in fungal cells, addition of haemin, a downstream product of haemin biosynthesis, would block haemofungin-induced inhibition. In contrast, addition of PPIX, the ferrochelatase substrate, or aminolaevulinic acid (ALA), produced in the first step of haemin biosynthesis would not. We therefore tested the effect of adding increasing concentrations of haemin, PPIX or ALA to broth microdilutions of haemofungin in a 96-well plate with 5000 *A. nidulans* conidia per well (Table S5). Specifically, the addition of haemin rescued the growth of *A. nidulans* in the presence of haemofungin. Addition of 25 $\mu\text{g}/\text{mL}$ haemin increased the haemofungin MIC 8-fold from 4 to 32 μM , whereas addition of PPIX or ALA did not. These results strengthen the hypothesis that haemofungin is an inhibitor of ferrochelatase activity. Similar results were seen with *A. fumigatus* (Af293), *S. cerevisiae* (BY4741) and *C. albicans* (CBS 562), suggesting that haemofungin works in a similar way in these fungi (data not shown). Further analyses described below were undertaken on *A. fumigatus*, because, unlike *A. nidulans*, it is a significant human fungal pathogen.

Haemofungin increases expression of key haem biosynthesis genes

Fungal haem biosynthesis is a tightly coordinated process in which the end product haemin represses the expression of key genes in the pathway.¹⁸ We predicted that haemofungin treatment, by decreasing endogenous haem levels, would activate the transcription of genes in the fungal haem biosynthetic pathway. To test this, we grew *A. fumigatus* conidia overnight at 37°C in liquid culture (CM or MM) and then added 5 μM haemofungin for another 1 h of incubation. Total RNA was prepared and analysed by northern blotting for expression of *hemA*, *hemB*, *hemE*, *hemF* and *hemH* (Table S2). The results revealed increased levels of *hemA*, *hemE* and *hemH* mRNA in the presence of haemofungin in both CM and MM. *hemF* mRNA was only increased in the presence of haemofungin in MM. The most affected gene, *hemA*, encodes ALA synthase, the first committed and rate-limiting

enzyme in haem biosynthesis.¹⁸ Together, these findings imply that a reduction in haem levels caused by haemofungin leads to the up-regulation of key haem biosynthetic genes (Figure 3).

Haemofungin causes accumulation of the precursor PPIX in *A. fumigatus*

We anticipated that if haemofungin inhibits HemH/ferrochelatase activity, its presence would result in the accumulation of porphyrin precursors and in particular its substrate PPIX. To test this, we grew *A. fumigatus* conidia overnight at 37°C in liquid YAG and then added 2 µM haemofungin for another 2 h of incubation. The haemofungin-treated mycelium became gradually reddish orange, suggesting that it was accumulating porphyrins (Figure 4a). HPLC analysis of mycelial extracts showed significant ($P = 0.03$) accumulation of PPIX in the presence of haemofungin (Figure 4b). Despite repeated attempts, using either HPLC analysis or commercial haemin assay kits, we were unable to reliably measure the levels of fungal haemin. Therefore, we could not demonstrate a parallel reduction in haemin levels after haemofungin treatment.

Haemofungin localizes in the mitochondrial membranes of *A. fumigatus*

While analysing the effects of haemofungin on fungal cells by means of fluorescence microscopy, we discovered that haemofungin fluoresces brightly in discrete subcellular regions under blue light (488 nm). We suspected that the fluorescing areas coincided with mitochondrial localization. To test this, we analysed by confocal fluorescence microscopy *A. fumigatus* hyphae incubated in the presence of both haemofungin and the mitochondria-specific dye MitoTracker Green. We found that haemofungin and MitoTracker fluorescence colocalize (Figure S1). Interestingly, HemH, the putative target of haemofungin, is a transmembrane protein localized in the mitochondrial membrane.¹⁸ These results suggest that haemofungin specifically accumulates in the immediate vicinity of its target, HemH, possibly enhancing its efficacy.

Haemofungin reduces mortality in *G. mellonella* larvae infected with *A. fumigatus* and in *D. melanogaster* T1 flies infected with *R. oryzae*

Toxicity of haemofungin injected into *Galleria* larvae indicated that it was non-toxic up to a concentration of 22.7 mg/kg (Figure 5a). To test the efficacy of haemofungin in treating invasive aspergillosis in *Galleria* larvae, groups of 10 larvae were infected with 5×10^6 *A. fumigatus* conidia per larva and, treated with 1.4, 2.8, 5.7, 11.4 or 22.7 mg/kg haemofungin or 2 mg/kg amphotericin B (positive control), or PBS for the untreated group. Treatment with amphotericin B at 2 mg/kg increased larval survival ($P = 0.03$). The two highest doses of haemofungin (11.4 and 22.7 mg/kg) caused increased larval mortality (data not shown). Encouragingly, the efficacy of 5.7 mg/kg haemofungin was similar to that of the gold standard antifungal amphotericin B at 2 mg/kg ($P = 0.03$). Haemofungin demonstrated a clear dose-dependent increase in efficacy as the dose was increased from 1.4 to 2.8 to 5.7 mg/kg (equivalent to 2.5, 5 and 10 µM, respectively) (Figure 5b).

Haemofungin was not toxic to *Toll* flies fed food containing up to 14.4 mg/mL of the compound (Figure 5c). Haemofungin was effective in treating *Toll* flies infected with *R. oryzae*. Flies were infected with *Rhizopus* conidia and fed with fly food containing 14.4

mg/mL haemofungin or regular fly food for 7 days. Results showed a significant difference in survival ($P=0.0002$) between flies infected with 1×10^6 conidia/mL of *Rhizopus* and fed with haemofungin-containing fly food (14.4 mg/mL) versus flies infected with 1×10^6 conidia/mL of *Rhizopus* and fed with regular fly food (Figure 5d).

Discussion

In this report, we characterized the activity and mode of action of haemofungin, a novel antifungal compound we identified in a screen of 35000 synthetic drug-like molecules in the pathogenic mould *A. fumigatus*.⁷ Haemofungin has not been previously described in the scientific literature as having antifungal activity.

In vitro, haemofungin had potent inhibitory activity. It damaged the morphology of the fungal cell wall, causing cell body swelling and lysis and completely inhibited growth of most pathogenic yeast and moulds at low concentrations (3.1–12.5 μM). Importantly, it was non-toxic and highly active *in vivo* in two insect models of fungal infection.

To determine the mode of action of haemofungin, we used an overexpression screen in *A. nidulans*.^{12,13} High-copy number expression of AN7752, homologous to the essential *S. cerevisiae* protein HemH/ferrochelatase, confers 4-fold increased haemofungin resistance in *A. nidulans*. This protein catalyses the last step of haem biosynthesis, the insertion of ferrous iron (Fe^{2+}) into PPIX and is located in the inner mitochondrial membrane with its active site facing the matrix space.¹⁸

Several more lines of evidence further supported the conclusion that haemofungin directly inhibits Hem15p/ferrochelatase activity: (i) addition of haem, the end product of the reaction, blocked the antifungal activity of haemofungin in *A. nidulans*, *A. fumigatus*, *C. albicans* and *S. cerevisiae*; (ii) the level of PPIX, the substrate of ferrochelatase, increased ~20-fold in haemofungin-treated *A. fumigatus* hyphae; (iii) the mRNA levels of the key *A. fumigatus* genes catalysing haem biosynthesis, *hemA*, *hemE* and *hemH* (the *A. fumigatus* homologue of *S. cerevisiae* *HEM15*), were increased in the presence of haemofungin, indicating that the cells were reacting to a shortage in available haemin by up-regulating the components of its biosynthetic pathway; and (iv) haemofungin specifically localized to the *A. fumigatus* mitochondrial membrane where HemH/ferrochelatase is found.

The ability to synthesize haemin is essential for cell survival. Haemin is a cofactor in the respiratory cytochromes *a-c*, the cytochrome P450 enzymes involved in sterol biosynthesis and in ligninases, peroxidases and catalases.¹⁸ In yeast, seven of the eight haem biosynthetic pathway genes (except *HEM14*) are essential. In *A. niger*, *hemH* is essential but growth can be partially restored by the addition of haemin. Interestingly, the *hemH* ferrochelatase null *A. niger* mutant is remarkably similar in phenotype to haemofungin-treated *A. fumigatus*, displaying both swollen cell bodies and PPIX accumulation.¹⁹

Previous attempts to develop antifungals targeting haemin biosynthesis have yielded the plant-derived alkaloid sampangine with broad, non-selective antiproliferative and antifungal activities, apparently by hyperactivating *HEM4* and inhibiting haem biosynthesis.²⁰ In contrast, haemofungin exerted its antifungal activity by inhibiting HemH/ferrochelatase, a

target that to date has only been inhibited by non-specific chemicals such as 3,5-diethoxycarbonyl-1,4-dihydrocollidine.²¹ Cell death following inhibition of haem biosynthesis is primarily attributed the accumulation of porphyrin intermediates that are toxic.²⁰ The cell wall damage, swelling and lysis that we observed after treatment with haemofungin may be a secondary effect due to porphyrin poisoning.

HemH/ferrochelatase is well conserved between fungi and humans with ~54% identity at the amino acid level, suggesting it is not an ideal antifungal target. However, this is not an insurmountable obstacle to further development of more selective inhibitors: fungal ERG11, the target of the antifungal azoles, shares ~40% identity with human lanosterol 14 α -demethylase, but nevertheless, fungus-specific azoles have been developed. Higher specific for haemofungin-like compounds could be achieved by generating scaffold derivatives and identifying lead compounds with greater selectivity for fungal ferrochelatase. In its present form, haemofungin may prove useful as a research tool for inducing rapid changes in haemin flux in living cells. It should also be tested in cancer cells that synthesize haemin at higher levels due to their increased metabolic requirements.

In summary, we have identified and characterized a novel broad-spectrum antifungal compound, haemofungin, which interferes with the process of haem biosynthesis. Further characterization in mammalian models of infection is needed to determine its potential.

Supplementary data

Refer to Web version on PubMed Central for supplementary material.

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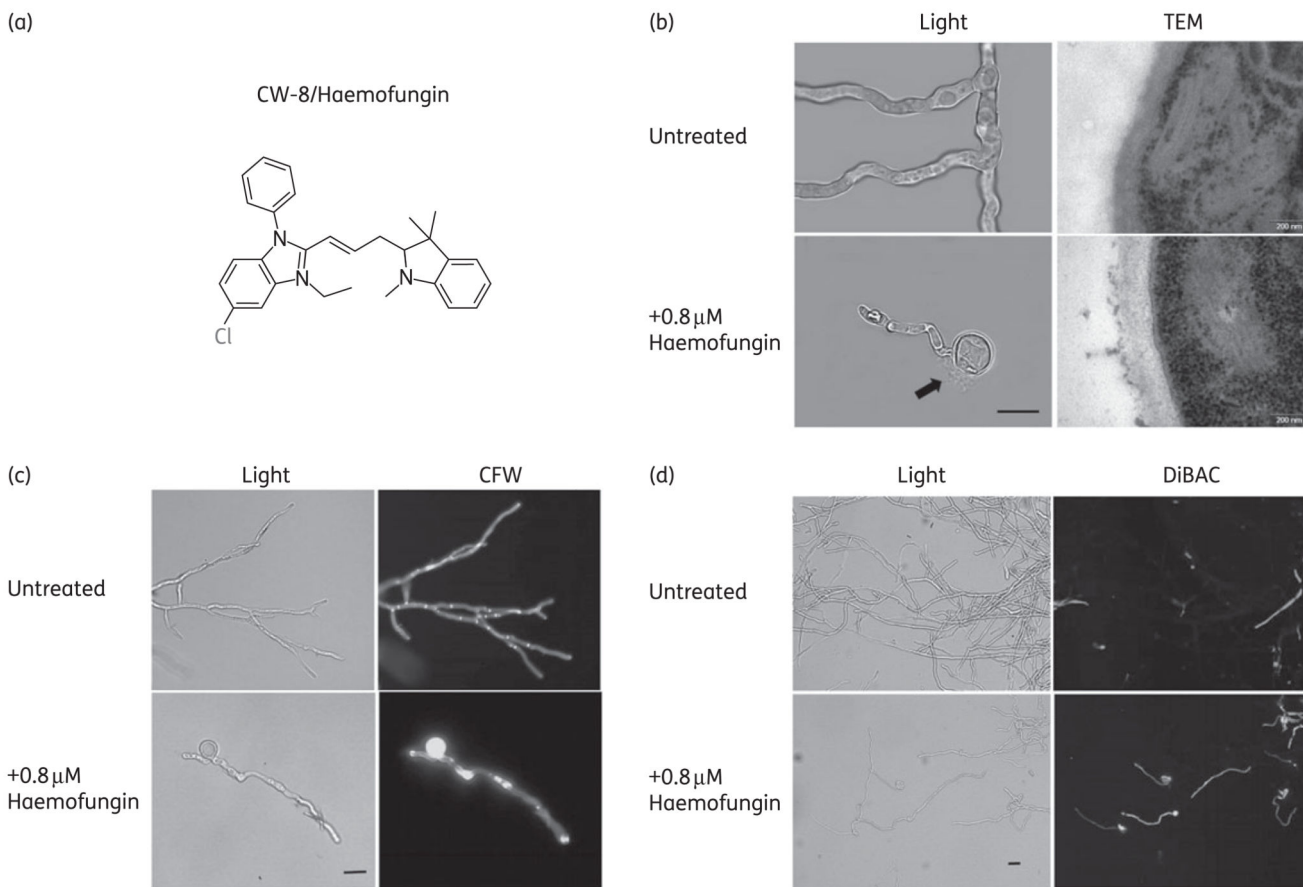


Figure 1.

Haemofungin causes morphological changes characteristic of damage to the cell wall of *A. fumigatus*. (a) Molecular structure of haemofungin, a benzimidazole derivative. (b–d) Freshly harvested spores of *A. fumigatus* strain Af293 were incubated for 24 h in the presence of 0.8 μM haemofungin and analysed microscopically by light, fluorescence or TEM microscopy, CFW cell wall staining or DiBAC staining of dead cells. Cell swelling and lysis [arrow, (b), light microscopy], abnormal cell wall morphology (TEM) and increased CFW staining of wall polysaccharides reveal haemofungin-induced wall damage. DiBAC staining indicates partial cell death in discrete areas of the hyphae. Black bar = 10 μm.

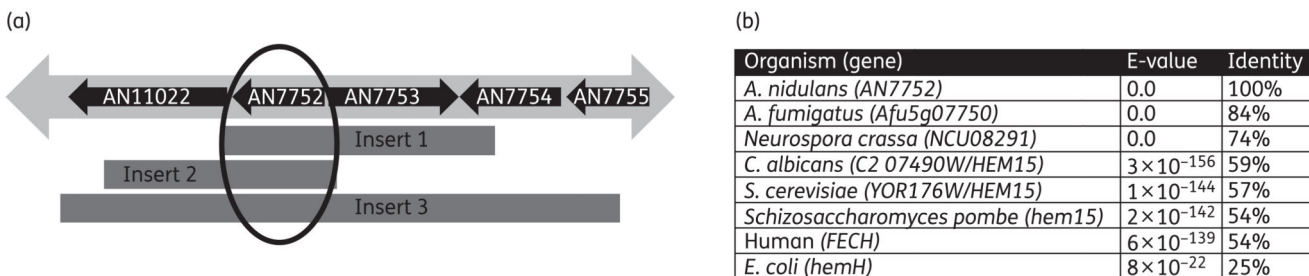


Figure 2.

Mapping and identification of the *A. nidulans* AN7752 gene encoding HemH/ferrochelatase that confers haemofungin resistance upon overexpression. (a) Schematic representation of the inserts in each of the three resistance-conferring high-copy plasmids. Only the gene AN7752 is shared by all three plasmids. (b) Amino acid similarity between AN7752 and homologues of select fungal, human and *Escherichia coli* ferrochelatase proteins according to pBLAST.

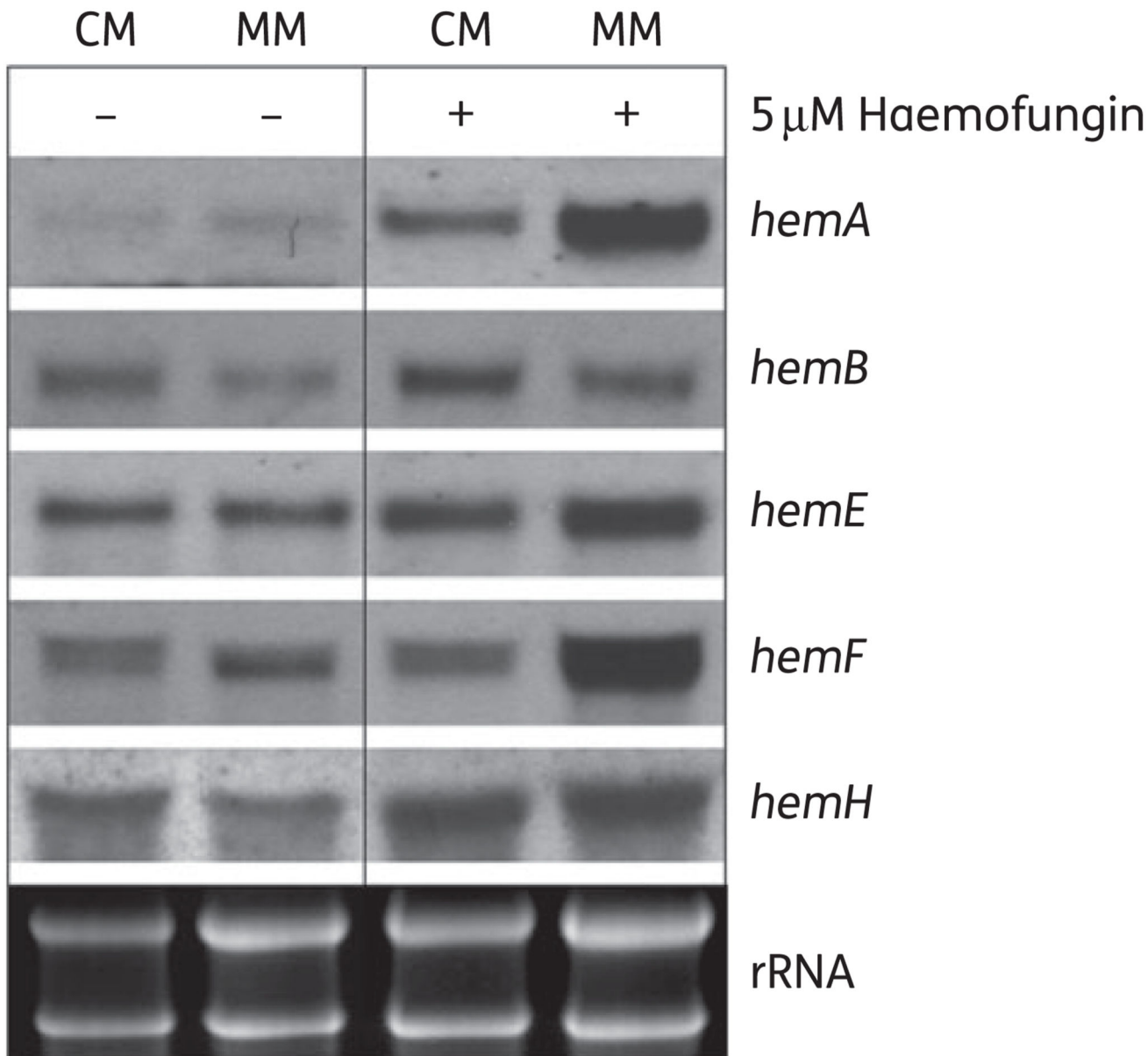


Figure 3.

Haemofungin induces the expression of key haem biosynthetic genes in *A. fumigatus*. *A. fumigatus* overnight culture grown in liquid medium (either CM or MM) was treated with 5 μ M haemofungin for 1 h. Total RNA was extracted and analysed by northern blotting with probes specific for the haem biosynthetic genes *hemA*, *hemB*, *hemE*, *hemF* and *hemH*. The expression of all genes except *hemB* was increased in the presence of haemofungin. Bottom panel: total RNA loading control.

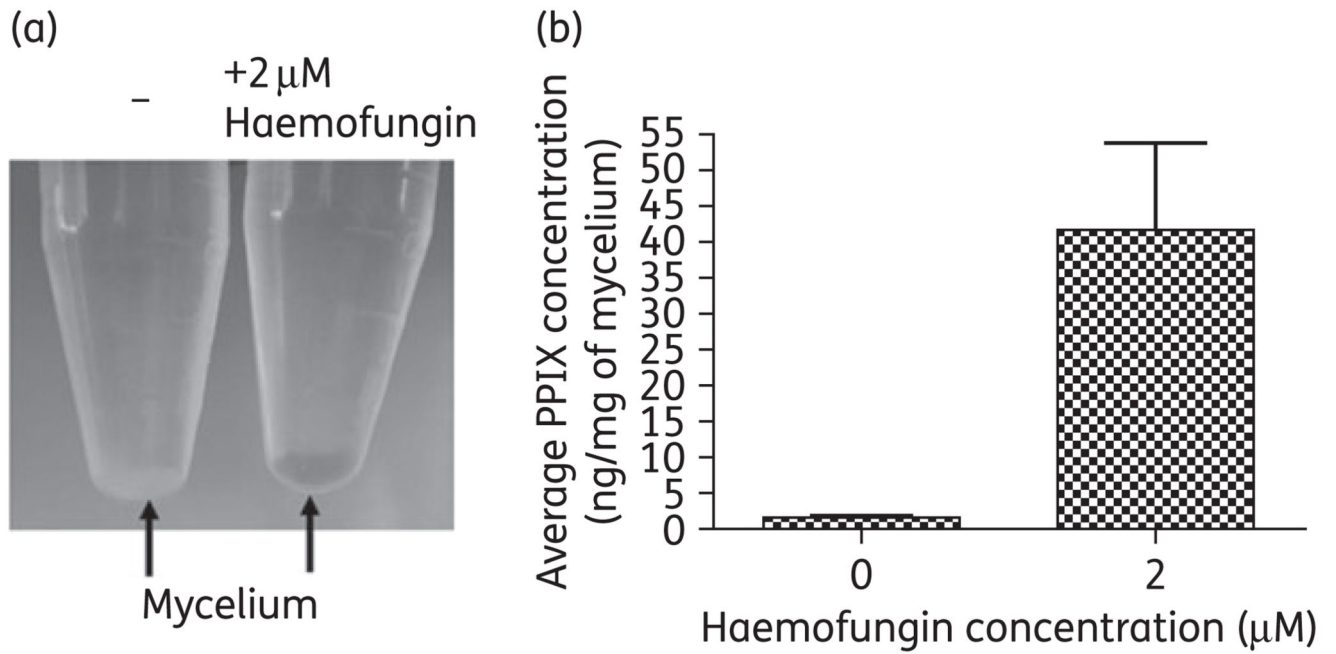
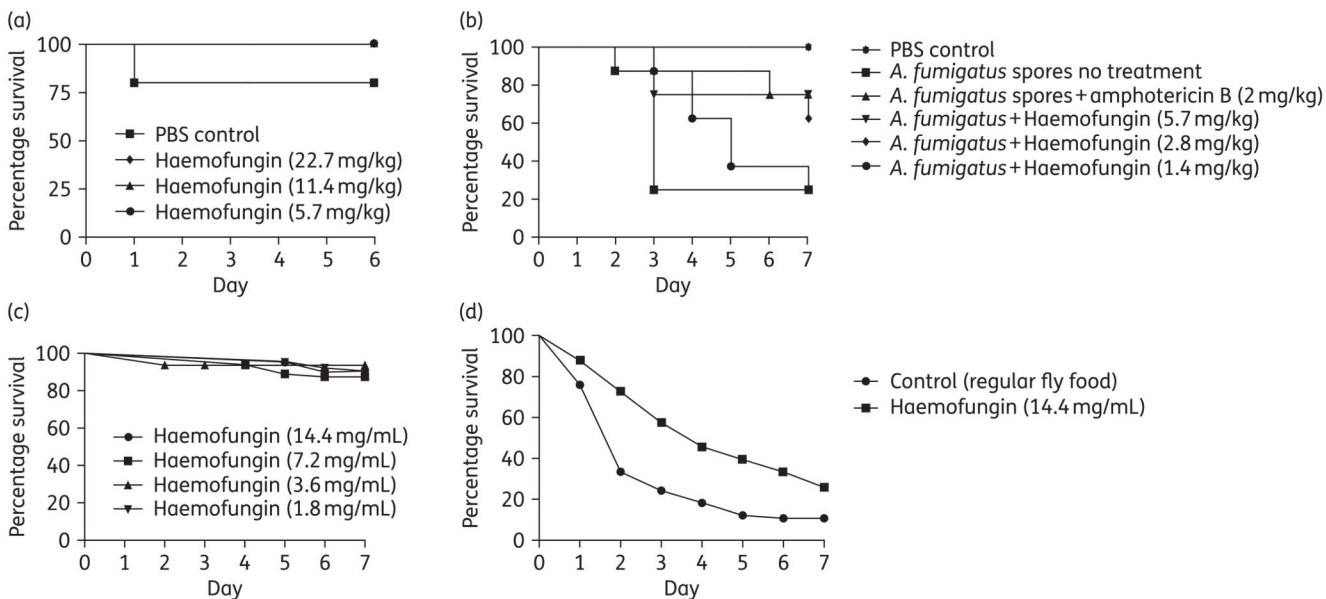


Figure 4.

Treatment with haemofungin causes accumulation of PPIX in *A. fumigatus*. *A. fumigatus* overnight culture grown in YAG liquid medium was treated with 2 μ M haemofungin for 2 h. The mycelium was (a) spun down and directly viewed for accumulation of porphyrins and (b) lyophilized and analysed for PPIX content by HPLC (C18) column separation.

**Figure 5.**

Toxicity and efficacy analysis of haemofungin. For analysis of toxicity, *G. mellonella* larvae (a) were injected once with up to 22.7 mg/kg haemofungin and *Drosophila TI^{-/-}* flies (c) were fed with fly food containing up to 14.4 mg/mL haemofungin for 7 days. For analysis of survival, *G. mellonella* larvae (b) and *Drosophila TI^{-/-}* flies (d) were infected with *A. fumigatus* (Af293) or *R. oryzae* conidia and treated with up to 5.7 mg/kg and 14.4 mg/mL haemofungin, respectively. Treatment with amphotericin B was used as a positive control in *G. mellonella*.

Table 1

Activity spectrum of haemofungin against fungi, bacteria and mammalian cell lines

	MIC (μM)	MEC (μM)
<i>C. albicans</i> ATCC 2901	3.13	1.56
<i>C. albicans</i> ATCC 90028	3.13	1.56
<i>C. albicans</i> ATCC 18804/CBS 562	3.13	1.56
<i>Candida rugosa</i> 3929	6.25	3.13
<i>Candida parapsilosis</i> ATCC 22019	3.13	0.20
<i>Candida tropicalis</i> ATCC 20336	12.50	1.56
<i>Candida glabrata</i> 59343	6.25	0.78
<i>Candida krusei</i> ATCC 6258	3.13	1.56
<i>R. oryzae</i> 3465	>12.50	3.13
<i>Rhizopus microspores</i> 3484	3.13	0.78
<i>Rhizopus arrhizus</i> 176	12.50	6.25
<i>Fusarium oxysporum</i> 600711	12.50	0.20
<i>Fusarium solani</i> 603251	3.13	0.10
<i>F. solani</i> 600679	6.25	0.78
<i>A. fumigatus</i> ATCC 13073	6.25	0.20
<i>A. fumigatus</i> Af293	3.13	0.40
<i>Aspergillus flavus</i> strain #1	>12.50	0.20
<i>A. flavus</i> strain #2	>12.50	0.78
<i>A. niger</i> strain #1	3.13	1.56
<i>A. niger</i> strain #2	3.13	0.78
<i>A. niger</i> strain #3	6.25	1.56
<i>S. cerevisiae</i> A2	6.25	—
<i>S. cerevisiae</i> BY4741	6.25	—
<i>Escherichia coli</i>	>25	—
<i>Staphylococcus epidermidis</i>	0.78	—
<i>Staphylococcus aureus</i>	>25	—
<i>Bacillus cereus</i>	3.13	—
A549	25 (80%)	—
NIH-3T3	25 (80%)	—

MIC= the lowest drug concentration to completely arrest germination and growth; MEC= the lowest drug concentration to cause visibly aberrant growth or a significant reduction in growth.