Inhibiting GPI Anchor Biosynthesis in Fungi Stresses the Endoplasmic Reticulum and Enhances Immunogenicity

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Inhibiting GPI Anchor Biosynthesis in Fungi Stresses the Endoplasmic Reticulum and Enhances Immunogenicity

Catherine A. McLellan \(^{1,2}\), Luke Whitesell \(^{1}\), Oliver D. King \(^{3}\), Alex K. Lancaster \(^{1}\), Ralph Mazitschek \(^{4}\) and Susan Lindquist \(^{1,2,5}\)

Affiliations:

1. Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, Massachusetts 02142, USA

2. Howard Hughes Medical Institute, MIT, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA

3. Boston Biomedical Research Institute, 64 Grove Street, Watertown, MA 02472, USA

4. Center for Systems Biology, Massachusetts General Hospital, Richard B. Simches Research Center, 185 Cambridge Street, Suite 5.210, Boston, MA 02114, USA

5. Department of Biology, MIT, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA

Contact Information:

Susan Lindquist
Whitehead Institute
9 Cambridge Center
Cambridge, MA 02142
Tel: 617-258-5184
Fax: 617-258-7226
Email: Lindquist_admin@wi.mit.edu
Abstract

In fungi, the anchoring of proteins to the plasma membrane via their covalent attachment to glycosylphosphatidylinositol (GPI) is essential and thus provides a valuable point of attack for the development of antifungal therapeutics. Unfortunately, studying the underlying biology of GPI-anchor synthesis is difficult, especially in medically relevant fungal pathogens because they are not genetically tractable. Compounding difficulties, many of the genes involved in coupling GPI to proteins are essential. Here, we report the discovery of a new drug-like small molecule christened gepinacin (for GPI acylation inhibitor) which selectively inhibits Gwt1, a critical acyltransferase required for the biosynthesis of fungal GPI anchors. After delineating the target specificity of gepinacin using genetic and biochemical techniques, we used it to probe key, therapeutically relevant consequences of disrupting GPI anchor metabolism in fungi. We found that unlike all three major classes of antifungals in current use, the direct antimicrobial activity of this compound results predominantly from overwhelming stress to the endoplasmic reticulum. Gepinacin did not affect the viability of mammalian cells or inhibit their orthologous acyltransferase, which enabled its use in co-culture experiments to examine its effects on host-pathogen interactions. In isolates of Candida albicans, the most common fungal pathogen in humans, exposure to gepinacin at sub-lethal concentrations impaired filamentation and unmasked cell wall β-glucan to stimulate a pro-inflammatory cytokine response in macrophages. These results highlight Gwt1 as a promising antifungal drug target and define a useful probe for studying how disrupting GPI-anchor synthesis impairs viability and alters host-pathogen interactions in genetically intractable fungi.
Introduction

Fungi are a prominent cause of hospital-acquired infections that are becoming increasingly difficult to control (1). This disturbing trend is driven by the growing number of severely immunocompromised individuals in the population that has occurred as a result of advances in the management of cancer, organ transplantation, autoimmune disorders and HIV. Most fungus-related morbidity and mortality is caused by the pathogens Candida albicans and Aspergillus fumigatus, which remain costly to treat and extremely difficult to eradicate in the immunocompromised host. Candida species are currently the fourth leading cause of hospital-acquired bloodstream infection and kill up to 40% of their victims, while disseminated Aspergillus infections kill up to 80% of the patients they afflict (2, 3).

Fungal pathogens present a particular therapeutic challenge because as eukaryotes, they share many of the same basic molecular mechanisms that support the maintenance and proliferation of mammalian cells. As a consequence, the number of unique exploitable drug targets that have been identified in fungi remains very limited. Only three mechanistically distinct classes of anti-mycotic agents are in widespread clinical use for the treatment of systemic infections. The most widely deployed class, the azoles (e.g. fluconazole), inhibit the cytochrome P450 enzyme 14 α-demethylase. This blocks the conversion of lanosterol to ergosterol, the functional homolog of cholesterol in mammals. Ergosterol is an essential component of the fungal membrane and the selective fungistatic activity of the azoles results from their disruption of its biosynthesis. Ergosterol itself is the primary target of the oldest class of antifungals, the polyenes (e.g. amphotericin B) which selectively bind this sterol and directly disrupt fungal membrane integrity. The newest class of antifungals, the echinochandins (e.g. caspofungin), inhibits 1, 3 β-glucan synthase. This enzyme mediates an essential step in the production of glucan, the major structural component of the fungal cell wall. Unfortunately, high-grade resistance to
all three classes of antifungals occurs frequently in the clinical setting through molecular mechanisms that can involve both target-related mutations and increased transporter-mediated drug efflux. Clearly, to combat this mounting medical problem, effective new anti-fungal strategies are urgently needed.

Motivated by a long-standing interest in the basic biology of stress responses and how they enable diverse organisms to adapt and evolve, we identified a novel chemical structure that induces profound stress in the endoplasmic reticulum (ER) of fungi. Intrigued by its high fungal-selective activity against a broad range of medically relevant species, we sought to define its mechanism(s) of action through a combination of genetic and biochemical approaches. We found that this drug-like compound, which we here name gepinacin, specifically inhibits an essential step in the production of glycosylphosphatidylinositol (GPI)-anchors within the ER of fungi, but not mammalian cells. Guided by this insight, we used the compound as a probe to investigate how inhibiting this biosynthetic pathway disrupts protein homeostasis in fungi and alters key interactions between pathogen and host that are known to contribute to fungal virulence.

RESULTS AND DISCUSSION

In the course of high-throughput screening for small molecules that inhibit the function of heat-shock proteins, we encountered a false positive compound that possessed surprisingly broad anti-fungal activity. Under screening conditions, the compound caused swelling of the yeast, *Saccharomyces cerevisiae*, which caused it to score erroneously in a cell growth assay. Closer examination revealed that the compound was actually toxic to yeast. In contrast, no growth inhibition was seen when the compound was incubated with mammalian cells in culture. Intrigued by its marked fungal selectivity, we took advantage of the powerful genetic tools developed in the model fungal organism *S. cerevisiae* to identify the target of this small molecule and to understand the basis of its selective toxicity to fungi. Following target validation, we used this compound as a chemical probe to further characterize its mode
of action and explore the therapeutic implications of target inhibition in azole-resistant isolates of the far
less genetically tractable but more medically relevant fungal pathogen, *C. albicans*.

**Broad spectrum of highly selective antifungal activity**

The phenoxyacetanilide compound identified in our screen (Fig. 1a) inhibited the growth of
diverse yeasts and molds separated by approximately 800 million years of evolution, including *S.
cerevisiae, Candida glabrata, Candida albicans* and *Aspergillus terreus* (4). This suggested the
involvement of a mechanism widely conserved amongst fungi (Fig. 1b). Importantly, it inhibited the
growth of multiple isolates of the common fungal pathogen *C. albicans*. These had been chosen to
represent resistance to each of the three major classes of antifungals that are currently in clinical use
(Fig. 1c). The lack of cross-resistance and the absence of structural similarity to established antifungal
drugs suggested the compound might exert its activity through a novel mode of action. A complete lack
of cytotoxicity for mammalian cells suggested sufficient divergence of target and/or mechanism between
humans and fungi to provide a useful therapeutic index for eventual development of the chemotype as an
antimicrobial (Fig. 1d).

**Target Identification Using Yeast Genetics**

To determine the mechanism responsible for the antifungal activity of the compound, we utilized
the powerful genetic tools available in the model fungal organism *S. cerevisiae*. We screened both an
arrayed haploid over-expression library and a pooled heterozygous deletion library for enhancement or
suppression of toxicity. The over-expression library consisted of 5336 individual haploid strains arrayed
in 384-well plate format. With each strain expressing one open reading frame (ORFs), this library
covered approximately 90% of the yeast genome. (5). Our heterozygous deletion library consisted of a
pool of 5797 diploid strains in which one copy of approximately 95% of the ORFs in the *S. cerevisiae*
genome had been disrupted previously by targeted insertion of a bar-coded antibiotic resistance cassette.
Only one gene, GWT1 was recovered as a hit shared by both libraries. Gwt1 is essential for growth of S. cerevisiae under normal conditions and has previously been characterized as an acyltransferase that is critical for GPI-anchor biosynthesis (7). Having identified this putative target for the compound we christened the compound gepinacin for GPI acylation inhibitor. When GWT1 was over-expressed, it rescued gepinacin toxicity and when it was deleted, toxicity was enhanced. Furthermore, GWT1 was not identified as a hit for 8 unrelated compounds that were tested in parallel for other projects. To confirm the effect of GWT1 we constructed yeast strains engineered to express a wide range of GWT1 gene copies (Fig. 2a). Starting with a wild type diploid (Wt) strain that carries two genomic copies of GWT1, we introduced a low copy (CEN) plasmid expressing GWT1 or GFP (as a control). In parallel, the same plasmids were transduced into a heterozygous deletion strain (gwt1Δ/GWT1) which had only one genomic copy of GWT1. As expected, the toxicity of gepinacin inversely correlated with GWT1 copy number. High level expression of GWT1 driven by a two micron plasmid further decreased gepinacin activity in both genotypes (Supplementary Fig. 1). These genetic data establish Gwt1, or at least the pathway in which it acts, as the most functionally relevant target for the antifungal activity of gepinacin.

Biochemical validation of Gwt1 as target

Production of GPI-anchors begins on the cytoplasmic surface of the ER but is completed on the luminal side. Gwt1 (in yeast) or PIG-W (mammalian) acts at the first step on the luminal side of the ER, the acylation of glucosamine phosphatidylinositol (GlcN-PI) (Fig. 2b) (8) (9). To provide direct biochemical evidence that Gwt1 is the proximal protein target of gepinacin we performed in vitro acylation reactions using yeast membrane preparations. UDP[^3]H]GlcNAc was incubated with the membranes, the resulting lipid products were recovered by chemical extraction and fractionated by silica gel thin layer chromatography (TLC). The appearance of a phospholipase-C insensitive band indicated the production of the acylated product. Gepinacin inhibited the acylation of GlcN-PI at low micromolar
concentration (Fig. 2c) while a compound that was structurally very similar but biologically inactive did not (Fig. 1a, Supplementary Fig. 2).

Having demonstrated that the biochemical target of gepinacin is not restricted to fungi, we were interested in investigating the basis of its lack of toxicity to mammalian cells in culture. To determine if gepinacin remained active under mammalian cell culture conditions we cultured fungi (either an amphotericin B-resistant (Fig. 3a) or fluconazole-resistant (data not shown) isolate of *C. albicans*) and mammalian cells together. These co-culture experiments demonstrated profound inhibition of proliferation for both fungal strains under serum-containing cell culture conditions, with no effect on mammalian cells in the same well.

To determine whether the basis of fungal selectivity resided in the fungal protein itself, we constructed transgenic *S. cerevisiae* strains in which genomic deletion of the essential *GWT1* gene was rescued by heterologous expression of either the human or yeast genes (*PIG-W* or *GWT1*, respectively) thus confirming the highly conserved function of Gwt1/PIG-W. While gepinacin was inactive in the *PIG-W* expressing strain, it potently inhibited both cell growth and acyltransferase activity in the strain expressing the yeast gene (Fig. 3b, 3c). These results confirm the proximal determinant of gepinacin toxicity as the fungal Gwt1 protein and demonstrate highly species-selective activity for the compound.

**Deletion of Trafficking Adaptor Protein EMP24 Partially Suppresses Toxicity**

To identify mechanisms that might confer resistance we isolated two spontaneously arising gepinacin-resistant clones carrying mutations that partially suppressed gepinacin toxicity in *S cerevisiae* (Fig. 4a). Whole genome sequencing of these strains showed that resistance was due to two different mutations in the gene *EMP24* (Fig. 4b). This finding was recapitulated with targeted deletion of *EMP24* (Supplementary Figure 3).
The non-essential protein Emp24 is a component of a large multi-protein complex that regulates GPI-anchored protein transport and quality control (10). GPI-anchored proteins do not contain transmembrane domains. As an integral membrane protein, Emp24 specifically interacts with the anchor portion of GPI-anchored proteins monitoring the completion of their processing and assisting their incorporation into ER to Golgi transport vesicles (11). When GPI-anchored proteins are incompletely remodeled, Emp24 is thought to facilitate their return to the ER. The deletion or inactivation of EMP24 has been shown to allow GPI-anchored proteins to bypass this quality control step and to exit the ER (11). Deletion of EMP24 could suppress gepinacin toxicity in an analogous manner by relieving a trafficking block induced by the compound.

To determine if gepinacin does indeed alter trafficking and change Emp24 distribution we examined yeast in which GFP was fused in frame to the 3’ end of genomic EMP24. Emp24-GFP normally shows a classical ER distribution pattern consisting of well-defined circles in the interior of the cell with a fainter cortical ring and a few bright dots consistent with some Golgi localization. After overnight culture in gepinacin, however, Emp24-GFP dramatically re-localized into large, bright dot-like structures (Fig. 4c). This pattern indicates that the compound had induced severe disorganization of the ER, and/or a profound block in retrograde trafficking that would normally return Emp24 to the ER as part of its physiological cycling.

GPI-specific trafficking defects caused by gepinacin induce ER stress

To further pursue the effects of gepinacin on protein trafficking in the secretory compartment, we compared the effects of gepinacin on maturation of the sentinel proteins Gas1 and CPY. Both proteins undergo characteristic molecular weight changes as they are processed in transit through specific cellular compartments, a feature that has been used extensively to study protein trafficking in the secretory system. Gas1 travels through the ER where it acquires a GPI-anchor before translocating
to the Golgi and outward to the cell surface \( (12) \). CPY is not GPI-anchored but undergoes sorting and processing in the secretory pathway before ending up in the yeast vacuole \( (13) \). In actively growing wild type yeast, the mature (m), Golgi-processed form of Gas1 constitutes the predominant species, relative to the precursor (p) form found in the ER \( (\text{Fig. 5a}) \). This distribution was inverted following exposure to gepinacin. In contrast, the processing of CPY which is not GPI-anchored was identical in control and gepinacin-treated cells. This confirms a highly restricted effect of Gwt1 inhibition on the trafficking of GPI-anchored proteins. The control compound, tunicamycin, a natural product that blocks production of all N-linked glycoproteins, alters the processing of both proteins as expected \( (14) \).

The specific effects of gepinacin on GPI-anchored proteins provided a highly selective tool to investigate the impact of inhibition of inositol acylation on protein homeostasis within the ER. Culture for 5 hours in gepinacin induced a massive, concentration-dependent activation of the unfolded protein response (UPR) as monitored by the GFP reporter construct (UPRE-GFP), similar in extent to the effect produced by tunicamycin \( (\text{Fig. 5b}) \). Activation of the UPR was not seen when yeast were treated with representative compounds from the three major classes of anti-fungals: amphotericin B, caspofungin and fluconazole \( (\text{Supplementary Figure 4}) \). As expected based on its target selectivity, gepinacin did not perturb ER function in mammalian cells as monitored by induction of BIP, an ER-resident chaperone and classical marker of the UPR in mammalian cells \( (\text{Supplementary Figure 5}) \) \( (15, 16) \). Deletions of \textit{HAC1} and \textit{IRE1} which are essential components of the UPR activation pathway in yeast, both greatly increased the toxicity of gepinacin \( (\text{Fig. 5c}) \). As a positive control, similar enhancement of tunicamycin toxicity was seen in association with deletion of these genes \( (\text{Supplementary Figure 6a}) \). In contrast, toxicity of the conventional antifungal fluconazole which targets ergosterol biosynthesis was completely unaffected by these UPR-disabling deletions \( (\text{Supplementary Figure 6b}) \) \( (17) \).
Gwt1 Inhibition Blocks Filamentation

GPI-anchoring of proteins is conserved in all eukaryotes. However, major differences in the utilization of this post-translational modification between species provide an attractive point of attack in developing new antimycotics. In fungi, unlike mammalian cells, GPI-anchored proteins become covalently linked to β-1, 6 glucan following translocation to the cell surface which helps maintain integrity of the cell wall. In addition, their presence at the cell surface permits GPI-anchored proteins in fungi to play important roles in adhesion, filamentation and sensing of the environment. Also important to pathogenesis, they provide a heavily glycosylated and phosphorylated outer coat to shield fungi from recognition and attack by the immune system of the mammalian hosts they invade (18, 19).

Because of its role in tissue invasion, a key determinant of fungal virulence is the ability to switch between yeast and filamentous forms (20-22). To determine whether gepinacin impairs this important process in C. albicans, we used a series of three increasingly fluconazole-resistant clinical isolates that had been isolated over a two year period from an HIV patient treated with fluconazole, strains CaCi2, 8 and 17 (23). These strains were grown overnight with gepinacin or vehicle control in rich medium at 30° C, to maintain the cells in the yeast form. To induce morphogenic transformation they were then transferred to filamentation medium (Spider medium) at 37°C for 3 hours in the continued presence or absence of gepinacin (24). In the absence of gepinacin, all three drug-resistant clinical isolates underwent marked transformation to large macroscopic mats, readily visible to the eye. Because this process is so dependent on GPI-anchored proteins, however, a concentration of gepinacin that minimally reduced proliferation completely blocked the ability of all three strains to undergo such filamentation (Fig. 6a). The inability to form filaments was also apparent at the cellular level as demonstrated by the photomicrographs provided in Supplementary Figure 7. Comparable results were obtained when using serum-containing medium to induce filamentation instead of “Spider medium”.
Gepinacin also blocked filamentation on solid media using the well-characterized *S. cerevisiae* strain Sigma 1278B, which spontaneously grows as filamentous mats on agar substrates (Fig. 6b). Clearly the ability of gepinacin to inhibit conversion of fungi to their invasive filamentous forms is broadly relevant to diverse species and growth conditions with important implications for their pathogenicity in animal hosts.

**Gwt1 inhibition enhances immunogenicity**

We took advantage of the fungal selectivity of gepinacin to investigate another key determinant of fungal virulence, namely the ability to escape immune recognition. The large amount of β-glucan in the cell wall of fungi constitutes a potent pro-inflammatory stimulus, but it is normally masked by a mannoprotein coat (25). We predicted that disrupting GPI-anchor synthesis through inhibition of Gwt1 would unmask β-glucan leading to enhanced recognition of *Candida* by mammalian immune cells. Indeed, as revealed by immunostaining and fluorescence microscopy, sub-lethal concentrations of gepinacin did dramatically increase β-glucan presentation on the cell surface of *C. albicans* (Fig. 7a). Quantitation of this effect by flow cytometry confirmed a 4.5-fold increase in median channel fluorescence for gepinacin-treated candida compared to control-treated yeast. Such β-glucan exposure has been demonstrated previously for caspofungin, an echinochandin that perturbs cell wall synthesis. In contrast to gepinacin, however, the effect of caspofungin is limited to only filamentous forms of *C. albicans* (26). As an important functional consequence of increased β-glucan exposure, incubation of gepinacin-treated *C. albicans* with a mouse macrophage cell line (RAW264.7) more than doubled the secretion of the major pro-inflammatory cytokine TNFα by these professional antigen-presenting cells (Fig. 7b).
Gwt1 as a target for antifungal drugs

The biosynthesis of GPI anchors in fungi was first proposed as a potential antifungal drug target by Tsukahara et al (27). In an extensive screening effort, they identified 1-[4-butylbenzyl] isoquinoline (BIQ) as an inhibitor of the surface expression of GPI-anchored proteins (structure in Supplementary Figure 8a). GWT1 was subsequently cloned as a dosage-dependent suppressor of BIQ-induced phenotypes. Further discovery and optimization efforts by this group led to the synthesis of E1210, a potent and selective Gwt1 inhibitor with properties suitable for clinical development (structure in Supplementary Figure 8b). E1210 shows good activity in vitro and in mouse models against a broad spectrum of yeast and molds including medically relevant species of candida, aspergillus and fusarium (28, 29). Like gepinacin, it is non-toxic to mammalian cells at concentrations that far exceed those required for antifungal activity (30). The unusually high degree of selectivity for both gepinacin and E1210 arises from species-specific discrimination at the biochemical level of target inhibition and from the different roles that GPI-anchored proteins play at the biological level in fungi versus mammals.

Although the structurally distinct compounds gepinacin and E1210 were discovered through completely different screening strategies, they share key biological properties rooted in Gwt1, the molecular target they hold in common. Both compounds inhibit fungal proliferation, compromise cell wall integrity and impair the morphogenic filamentation program which is required for pathogenicity in animal hosts (30). Our discovery of a new chemotype that selectively inhibits the fungal protein Gwt1 highlights the suitability of this protein as a highly druggable, therapeutic target. This is noteworthy from the drug development perspective because Gwt1 and its close mammalian ortholog are multi-pass transmembrane proteins for which atomic-level structural information is not available to help guide medicinal chemistry efforts. In pursuing the work presented here, we have constructed gene-swapped yeast strains in which the sole source of essential acyltransferase activity for GPI anchor synthesis is
provided by either Gwt1 or its human ortholog PIG-W. These strains can now be used for SAR studies to optimize the potency and selectivity of the known chemotypes and also to efficiently screen for yet other compounds that preferentially inhibit the fungal enzyme or alternatively that inhibit the human enzyme to study its role in mammalian biology (9).

Using gepinacin as a probe in medically relevant pathogens for which few genetic tools are available, we have uncovered new consequences of Gwt1 inhibition with important clinical implications. First, Gwt1 inhibition profoundly stresses the fungal ER leading to critical dependence on activation of compensatory response pathways. Such dependence creates new liabilities for the organism that might be targeted in future work to synergistically enhance the antifungal activity of Gwt1 inhibition. Second, the cell wall compromise caused by Gwt1 inhibition in fungi not only exposes β-glucan on the cell surface, but enhances recognition by antigen-presenting cells and activation of a pro-inflammatory immune response. These effects would be expected to enhance clearance by host defense mechanisms and delay if not eliminate the emergence of resistance. In concert, these new insights further advance Gwt1 as a promising antifungal drug target and validate a useful new probe for studying the mechanisms by which inhibition of fungal GPI-anchor synthesis directly impairs viability and indirectly disrupts the complex process of pathogenesis.

METHODS

Materials. Gepinacin, acetamide, N-(4-methoxyphenyl)-2-[3-(2-methylpropoxy) phenoxy, CAS registry 304692-07-7 was purchased from Ryan Scientific, Inc. The inactive compound, CAS registry 3922235-70-0 was purchased from Scientific Exchange, Inc.

Plasmids. Harvard Institute of Proteomics FLEXGene Saccharomyces cerevisiae (yeast) ORF collection (pBY011 expression vector) (5) and PIG-W entry clone HsCD00295253 were obtained from the Dana Farber/HCC DNA Resource Core. All other gateway vectors used are available from Addgene (31).
The reporter for the unfolded protein response, pRS304-4XUPRE-GFP, was a gift from Peter Walter (UCSD).

**Fungal Strains.** Archives of all strains were maintained in 25% glycerol at -80°C. A complete list of all strains and construction details for the humanized yeast strain are provided in the supplement.

**Mammalian Cell Lines.** 293T, NIH 3T3, and the RAW264.7 murine macrophage line were purchased from ATCC. Primary MEF were generated from day 13.5 mouse embryos using standard techniques.

**Antifungal Susceptibility Testing.** Sensitivity to gepinacin was determined in 96-well, flat bottom microtiter plates using a modification of the broth microdilution protocol NCCLS M27-a and RPMI as culture medium. Fungi were inoculated at ~ $10^3$ cells per ml and incubated for 48 hours at 37°C after which plates were sealed, shaken and the optical density read at 600 nm (OD$_{600}$) on a Tecan Safire 2 plate reader. All samples were run in duplicate or triplicate and assays were repeated at least once. Results are expressed as fraction of growth in the absence of compound and the mean and standard deviation are plotted. For the mold *A. terreus*, inoculum was $10^4$ cells per ml and plates were incubated at 35°C for 48 hours in the dark. Plates were then visually scored as specified by CLSI document M38-A2 to determine the MIC50 and MIC80.

**Mammalian Cell Toxicity Testing.** For co-culture experiments, confluent layers of NIH 3T3 cells were established in 6-well plates. The following day, the medium was replaced with RPMI1640+10%FBS containing the amphotericin-resistant strain CaAmphR at $10^3$ colony forming units per ml and 40 µM Gepinacin or an equivalent amount of DMSO. Co-cultures were incubated for 24 hours at 37°C in a micro-isolator box. Images were acquired on a Nikon Eclipse TS100 microscope at 40X.

**Genetic Screening.** Flexgene plasmids were transformed in parallel into *S. cerevisiae* strain BY4741. The resulting library was arrayed in 384-well plates, grown to saturation in selective media, diluted and
replicated using a Tecan EVO robot. Gepinacin (20 µM) or an equivalent amount of DMSO in SGal-URA-NH4 media was added to duplicate plates before incubation in a humidified chamber at 23°C in the dark. Plates were sealed, shaken, and read at 24 hours and then 4 more times over the next 2 days. Data analysis details are provided in the supplement. The heterozygous deletion collection was pooled and grown at 30°C in SGal-CSM with shaking to an OD$_{600}$ of approximately 1-2 and then diluted to an OD$_{600}$ of 0.05 in fresh media containing DMSO or 1.25 µM gepinacin. This was repeated 4 times with dilutions every ~ 12 hours. Genomic DNA preparation, PCR and chip hybridization were done as previously described (6, 32, 33). All hits were re-tested by growth assay in 384-well plate format and the relevant deletion confirmed by PCR with deletion specific primers.

**In vitro Acylation Assays.** Experiments were performed using a previously published protocol (7) with the following modifications; UDP[$^3$H]GlcNAc was used instead of [$^{14}$C] and TLC plates were imaged by autoradiography. Lipid extracts were treated overnight with phosphatidylinositol-specific phospholipase C to confirm that the band identified as GlcN-(acyl)PI was resistant to cleavage (data not shown).

**Filamentation.** For liquid assays, C. albicans strains were grown overnight in YPD with DMSO or 5 µM gepinacin. After dilution to an OD$_{600}$ of 0.1 in Spider media, growth was continued for an additional 3 hours with agitation at 37°C in the presence of compound before transfer to a 24 well plate for imaging (34). Assays with S. cerevisiae strain Sigma 1278B on solid media were performed as previously described (24) except gepinacin was added directly to partially cooled plate media and colonies were imaged after growth for 6 or 9 days at 30°C.

**Protein trafficking analysis.** HA-tagged GAS1 was expressed in strain BY4741 CPY-GFP (Yeast GFP collection (35) under control of its own promoter using plasmid pCM-HA-GAS1. This construct was created as previously reported except in a gateway plasmid backbone (36). To assess effects on trafficking,
strains were incubated for 1 hour at 30°C with test compounds. Total cellular protein (5 µg) was
separated by electrophoresis (Invitrogen 8% gels) and transferred to nitrocellulose membranes. Blots
were hybridized with antibodies against HA (Covance), GFP (Roche) or CPY (Invitrogen). Sizes for the
various post-translationally modified products of Gas1HA2 and CPY have been previously reported
(36). CPY-GFP was used to facilitate discrimination of CPY processing steps because GFP is cleaved
from CPY in the yeast vacuole. The experiment, however, was also performed with untagged CPY with
the same results.

**Unfolded protein response.** The plasmid pRS304-4XUPRE-GFP was linearized and integrated into the
TRP1 locus of *S. cerevisiae* (W303) to construct a reporter strain as previously described (37, 38).
Reporter cells were grown to log phase and exposed to compound at 23°C for 5 hours before analysis.
GFP reporter induction was monitored on a Guava EasyCyte Plus cytometer. The average mean channel
fluorescence of duplicate samples was determined and the experiment was performed twice with similar
results.

**β-glucan staining.** Overnight treatment and staining of *C. albicans* strain Sc5314 was performed as
previously described for caspofungin treatment using YPD media at 30°C to maintain growth in the
yeast form (39). Antibody to (1-3) β-D-glucan was obtained from Biosupplies (Australia). Cells were
propidium iodide (PI) stained to assess viability and only PI-negative cells were analyzed. Microscopy
was performed on a Nikon Eclipse microscope with a 100X oil objective.

**Macrophage stimulation and TNFα measurement.** Cultures of *C. albicans* Sc5314 cells were drug-
treated as described for β-glucan staining. After overnight incubation, cultures were washed
extensively, counted and added to cultures of the mouse macrophage cell line RAW264.7 at a
macrophage:yeast ratio of 1:2.5 in the continued presence of drug. After 2 hours supernatants were
harvested and TNFα concentration measured by ELISA using a kit according to manufacturer’s
instructions (DY410, R&D Systems).

Suppressor strains. Approximately 2 x 10^7 W303 MATa cells were spread on a YPD plate containing
20 µM gepinacin. Three colonies were recovered 5 days later. Of these, only two grew sufficiently for
further experimentation. Designated strains, 20-1 and 20-2, they were mated and sporulated. The
gepinacin-resistant phenotype segregated 2:2 in the progeny indicating that the mutation conferring
resistance was a single trait or more than one, but closely linked trait. The sensitivity of strains to
cycloheximide was the same as wild type suggesting that the resistant phenotype was target-related, not
efflux pump-mediated.

Genomic Sequencing Using an Illumina HiSeq platform, we performed whole genome shotgun (WGS)
sequencing of wild-type and gepinacin-resistant strains, obtaining two lanes of 76 base pair, paired-end
reads and one lane of 101 base pair, paired-end reads for each genome (raw reads are available via
NCBI under BioProject accession number PRJXXXXX). Depth of coverage averaged 100-fold. Details
of computational analysis are provided in the supplement.

Figure Legends

Figure 1. Gepinacin inhibits growth of a broad spectrum of fungi but does not affect mammalian
cells. (A) Structures of gepinacin and a similar but inactive compound. (B) Anti-fungal susceptibility
testing for an evolutionarily diverse group of fungi treated with gepinacin. For A. terreus the MIC50
and MIC80 are plotted. (C) Anti-fungal susceptibility testing for wild type C. albicans and strains
resistant to the three major classes of anti-fungals treated with gepinacin. (D) Mammalian cell toxicity
testing for proliferating human cells in culture (293T) or quiescent cells (mouse embryo fibroblasts).
Cells were treated with gepinacin (20 µM) for 48 hours after which relative viable cell number was measured by standard luciferase assay (Cell Titer-Glo®, Promega).

Figure 2. **Gwt1 is the target of gepinacin.** (A) Anti-fungal susceptibility testing of *S. cerevisiae* strains with graded levels of Gwt1 expression treated with gepinacin. Wild type diploids (Wt) or diploids with one copy of *GWT1* deleted (*gwt1Δ/GWT1*) were transformed with low copy (CEN) plasmids encoding *GWT1* or *GFP* (as a control). (B) Schematic of Gwt1 protein function in cells. Glucosamine (shown as a black circle) phosphatidyl inositol (GlcN-PI) is acylated (orange zigzag) by Gwt1 to become GlcN-(acyl)PI. (C) Autoradiograph depicting the relative amount of GlcN-(acyl)PI product formed in acylation reactions supplemented with various concentrations of gepinacin or an inactive analog (10 µM). The structure of the inactive compound is shown in Figure 1.

Figure 3. **Gepinacin inhibits Gwt1 but not its human ortholog, PIG-W.** (A) Representative photomicrograph of mammalian cells (NIH3T3) co-cultured with amphotericin-resistant *C. albicans* following addition of DMSO or gepinacin. Photographs were taken after 24 hours. DMSO-treated cultures have large, dense colonies of *C. albicans* which are not present in gepinacin-treated cultures (B) Anti-fungal susceptibility testing of *S. cerevisiae* strains in which the endogenous *GWT1* gene had been replaced by plasmid-driven expression of the human gene (*PIG-W*), or the fungal gene (*GWT1*). CEN plasmids are low copy, 2µ plasmids are high copy. (C) Autoradiograph depicting the relative amount of GlcN-(acyl)PI product formed in acylation reactions supplemented with gepinacin and using membranes prepared from the low copy plasmid strains presented in panel B. These results confirm that activity of the human enzyme is not inhibited by gepinacin.
Figure 4. **EMP24 deletion decreases gepinacin toxicity.** (A) Anti-fungal susceptibility testing of gepinacin using suppressor strains 20-1 and 20-2. (B) The sequence of EMP24 in *S. cerevisiae* is shown with the location of mutations found by whole genome sequencing in the suppressor strains 20-1 and 20-2 indicated. (C) Micrographs showing the redistribution of Emp24-GFP after overnight incubation with gepinacin. Green fluorescence and DIC images are merged in the lower panels. Scale bar; 5 µm.

Figure 5. **Gwt1 inhibition by gepinacin impairs GPI-anchored protein maturation and causes ER-related toxicity.** (A) Immunoblots of lysates prepared from *S. cerevisiae* treated with gepinacin showing GPI-anchor-selective impairment of protein maturation. The unprocessed (u), precursor (p) and mature (m) forms of the reporter proteins are indicated. The identity of the tagged reporter proteins (top of panel) and the antibodies used for their detection (bottom of panel) are also indicated. Treatment conditions are DMSO (0.05%), gepinacin (GPN, 10 µM) or tunicamycin (Tun, 10 µM). The same lysate was used for both blots. (B) Induction of the unfolded protein response in cells carrying a GFP reporter construct showing strong induction by gepinacin. GFP expression was monitored by flow cytometry. (C) Anti-fungal susceptibility testing of strains deleted for *IRE1* (activator of the UPR) or *HAC1* (effector of the UPR) showing their increased sensitivity to gepinacin.

Figure 6. **Gepinacin blocks filamentation in liquid and solid culture models.** (A) Fluconazole resistant *C. albicans* strains were treated overnight with DMSO (0.025%) or gepinacin (GPN) (5 µM) in filamentation media and then imaged macroscopically. (B) *S. cerevisiae* strain Sigma 1278B was grown on filamentation-inducing plates containing DMSO (0.025%) or gepinacin (GPN) (5 µM) for 6 days and then imaged microscopically.
Figure 7. **Gepinacin treatment increases β-glucan exposure on the cell surface and enhances TNFα secretion from macrophages.** (A) Fluorescence photomicrographs depicting the β-glucan (green) immunoreactivity of *C. albicans* after treatment with DMSO or 5 µM gepinacin (GPN). Cells were also imaged with DIC and counter-stained with a viability marker. Representative fields of live cells are shown here. Scale bar; 5 µm. (B) Measurement of TNFα concentration in macrophage supernatant by ELISA. Supernatants were harvested after co-culture of RAW264.7 murine macrophages and *C. albicans* treated with DMSO or gepinacin at the concentrations indicated.

**Acknowledgements**

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**Supporting Information Available:** This material is available free of charge via the Internet.

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ACS Chemical Biology

Gwt1

ER

Gepinacin

β-glucan

UPR

GPN

TNFα

DMSO GPN

ACS Paragon Plus Environment
Figure 4: (A) Structure of Gepinacin. (B) Growth inhibition of A. terreus, C. glabrata, C. albicans, and S. cerevisiae by Gepinacin at different concentrations. (C) Fractional growth inhibition of various fungal strains by Gepinacin. (D) Comparison of growth inhibition between 293T and MEF cells treated with DMSO and Gepinacin.
Figure A: Graph showing the fraction of control for different concentrations of Gepinacin on Wildtype, 20-1, and 20-2.

Figure B: Emp24 Sequence:

<table>
<thead>
<tr>
<th>Emp24 Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-1 Frame shift, first nucleotide of codon deleted.</td>
</tr>
<tr>
<td>20-2 E to K: Acidic to Basic</td>
</tr>
<tr>
<td>Highly conserved amino acid</td>
</tr>
</tbody>
</table>

Figure C: Images showing the effect of DMSO and Gepinacin on Emp24-3FP expression.
A. 

B. 

C. 

150x49mm (300 x 300 DPI)
A. β-glucan images showing DMSO and GPN conditions.

B. Bar graph showing TNFα levels in response to N. Yeast treatment at different concentrations of GPN (DMSO, 5 μM, 10 μM).
SUPPORTING INFORMATION

Material and Methods

Fungal strains. Standard *S. cerevisiae* lab strains W303, BY4741, BY4742 and BY4743 were used for experiments. The *C. albicans* wild type strain in Figure 1 was CaI4, a standard laboratory strain (1). The *C. albicans* fluconazole-resistant strains CaCi-2, CaCi-8 and CaCi17 were generously provided by Ted White and originally collected by Spencer Redding and colleagues (2-4). The sequenced *C. albicans* wild type strain, Sc5314 (5) used in β-glucan experiments was a gift from John Perfect’s lab. CaAmphR, an amphotericin B-resistant strain is from ATCC (#20095). Caspofungin-resistant strain Cfr1 was derived by plating Sc5314 on caspofungin plates and isolating resistant colonies. *A. terreus* was purchased from ATCC (MYA-3633). The *C. glabrata* strain CgL5c was obtained from John Bennett’s lab. The YKO heterozygous diploid strain collection is from Open Biosystems and was generated in BY4743. Haploid deletion strains were from the Yeast Knock-out Deletion Collection and purchased from Invitrogen. The over-expression library was made by transforming BY4741 with the Harvard Institute of Proteomics Flexgene library.

Humanized yeast strain. Because *GWT1* deletion is lethal in *S. cerevisiae* strain BY4741, a haploid shuttle strain was constructed in which the genomic copy of *GWT1* was deleted and replaced by *GWT1* on a plasmid that also encoded the *URA3* gene and a G418- resistance gene. To construct this shuttle strain a gw1Δ/GWT1 heterozygous deletion mutant was transformed with pAGGPD416-GWT1, sporulated and dissected. A colony was selected that grew on both SD–URA and G418-containing plates. PCR with the deletion specific primers (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html) was performed to confirm the *GWT1* deletion. To change the source of *GWT*, this shuttle strain was transformed with plasmids containing *GWT1* (as a control) or PIG-W and the LEU2 auxotrophy gene followed by selection for growth on SD-LEU plates. Plasmids were constructed by conventional Gateway technology using a *GWT1* encoding entry vector and either pAG415GPDrccdB or pAG425GPDrccdB as the destination vectors. Positive colonies were selected and grown on 5-Fluoro-orotic acid (5-FOA)-containing plates to
promote loss of the pAGGPD416–*GW*T1 plasmid. The colonies from this plate were then tested for return of uridine auxotrophy to confirm pAGGPD416-*GW*T1 loss. The *PIG-*W expression vector was constructed by recombination in yeast as follows. The pAG415GPDccdB vector was cut with Xho1 and Xba1 and the larger piece was gel and column purified. The coding sequence of *PIG-*W was PCR amplified from the entry clone so that it would have ends facilitating recombination with the cut vector. The shuttle strain was then transformed with these two DNA fragments and plated on SD-LEU plates to select for recombinants. *PIG-*W plasmid was isolated from the final strain and sequence verified.

**Protein lysis procedure.** Yeast were spun down, washed two times with H2O and lysed in ethanol containing phenylmethylsulfonyl fluoride. An equivalent volume of glass beads were added and after beating, samples were transferred to a -80°C freezer for an hour. The samples were then evaporated to dryness in a Savant SpeedVac concentrator. Solubilization buffer (200 ul of 2% SDS in 20 mM Tris HCl pH 6.8) was added to the dry beads which were vortexed and then boiled 5 min. Protein concentrations were determined by BCA assay (Pierce) and equal amounts of total protein were diluted into 5X reducing loading buffer (0.5 M DTT, 20 mM Tris HCl, 50 % Glycerol) for subsequent analysis by SDS-PAGE.

**Data analysis of over-expression screen.** Raw data for each gepinacin-treated 384-well plate was quantile normalized to achieve the same distribution as one of the DMSO replicates at the corresponding reading time. The R package *limma* (6) was then used to compute empirical Bayes moderated t-statistics for each gene. Genes were flagged as hits if the mean difference in normalized optical density scores between DMSO and gepinacin was at least 0.1 at any reading time, and if the p-value for this difference had an associated FDR (false discovery rate) of at most 0.2. Hits were re-tested and plasmids sequenced to confirm their identity.

**Analysis of sequencing data-** After quality control was performed on raw reads for each genome, we aligned the filtered reads against the *S. cerevisiae* reference sequence sacCer2, (June 2008 assembly, downloaded from UCSC on April 1, 2011: [http://hgdownload.cse.ucsc.edu/goldenPath/sacCer2/chromosomes/](http://hgdownload.cse.ucsc.edu/goldenPath/sacCer2/chromosomes/)) using the BWA aligner (7). For each of the strains, we called SNPs and indels with respect to the reference using *mpileup* from the *SAMtools*
package (8). To identify SNPs and indels unique to each of the resistant strains, we compared the parental strain to individual resistant lines. We used a combination of custom code and the Genome Analysis Toolkit (GATK) (9) to locate, and then rank by quality, the SNPs and indels detected in open reading frames that were present only in the suppressor strains. Alignments of the reads for the top ranked SNPs and indels were then visually inspected.

Supplementary Figure Legends

Supplementary Figure 1

*GWT1 copy number determines gepinacin sensitivity*. Anti-fungal susceptibility testing of wild type diploids (Wt) or diploids with one copy of GWT1 deleted (gwt1Δ/GWT1) transformed with high copy (2 micron) plasmids containing *GWT1* or *GFP* (as a control). The strains were treated with gepinacin for 48 hours and the growth was monitored by measuring the optical density at 600 nm (OD$_{600}$). The results are expressed as a fraction of the OD$_{600}$ measured in the absence of gepinacin.

Supplementary Figure 2

*A close structural analog of gepinacin does not inhibit growth*. Anti-fungal susceptibility testing of a fluconazole-resistant *C. albicans* strain under standard conditions. Yeast were incubated at 30° C for 24 hours with serial dilutions of the indicated compounds. Growth was monitored by OD$_{600}$ and is expressed as fraction of the growth observed in the absence of compound.

Supplementary Figure 3

*EMP24 deletion partially suppresses inhibition of growth by gepinacin*. Anti-fungal susceptibility testing of *S. cerevisiae* strains that are wild type or deleted for *EMP24*. Growth was assessed by measuring the OD$_{600}$ following gepinacin treatment for 48 hours at 30° C in 96-well plate format. Mean and standard deviation of triplicate determinations are shown.

Supplementary Figure 4

*Conventional anti-fungal drugs do not induce the UPR*. Induction of the unfolded protein response in cells carrying a GFP reporter construct was monitored by flow cytometry after exposure to serial dilutions
of the indicated compounds for 5 hrs. Strong induction by gepinacin and tunicamycin but not by conventional anti-fungal drugs is evident.

**Supplementary Figure 5**

_BIP level, an indicator of the UPR in mammalian cells, is not increased after gepinacin treatment._

Immunoblot of lysates prepared from human leukemia cells (K562) using an antibody to BIP after 28 hour exposure to tunicamycin (1 µg ml⁻¹), gepinacin (20 µM) or solvent vehicle (DMSO, 0.1%). Beta-actin was blotted as a loading control.

**Supplementary Figure 6**

_Compromising the UPR enhances tunicamycin toxicity but does not alter the toxicity of fluconazole._

Anti-fungal susceptibility testing of strains bearing deletion of _IRE1_ (activator of the UPR) or _HAC1_ (effector of the UPR) using either tunicamycin (A) or fluconazole (B). Growth was measured by OD₆₀₀ after 48 hour exposure to serial dilutions of the compounds as indicated. Results are expressed as a fraction of the OD₆₀₀ measured in the absence of gepinacin.

The mean and standard deviation of triplicate determinations are shown.

**Supplementary Figure 7**

_Gepinacin blocks morphogenic switching in _C. albicans_. Three drug-resistant strains of _C. albicans_ were treated with DMSO or gepinacin (5 µM) and induced to filament for 3 hours. DIC images were acquired at 60X.

**Supplementary Figure 8**

_Previously reported Gwt1 inhibitors are structurally distinct from gepinacin._ (A) BIQ (10) and (B) E1210 (11).

**References**


Wt + GFP

Wt + GWT1
gwt1Δ/GWT1 + GFP
gwt1Δ/GWT1 + GWT1

Gepinacin (µM)

Fraction of Control

0.0
0.2
0.4
0.6
0.8
1.0

0 2 4 6 8 10

Gepinacin (µM)