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

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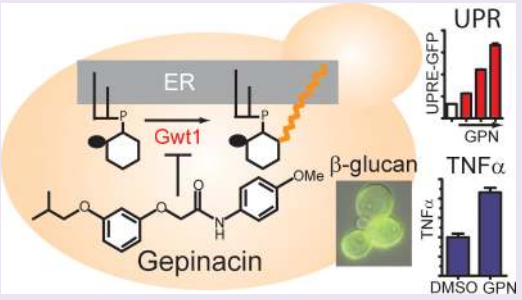
Supporting Information

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 in this pathway are essential in *Saccharomyces cerevisiae*. Here, we report the discovery of a new 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 its ability to induce overwhelming stress to the endoplasmic reticulum. Gepinacin did not affect the viability of mammalian cells nor did it inhibit their orthologous acyltransferase. This enabled its use in co-culture experiments to examine Gwt1's effects on host–pathogen interactions. In isolates of *Candida albicans*, the most common fungal pathogen in humans, exposure to gepinacin at sublethal concentrations impaired filamentation and unmasked cell wall β-glucan to stimulate a pro-inflammatory cytokine response in macrophages. Gwt1 is a promising antifungal drug target, and gepanacin is a useful probe for studying how disrupting GPI-anchor synthesis impairs viability and alters host–pathogen interactions in genetically intractable fungi.



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 antimycotic 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 homologue 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

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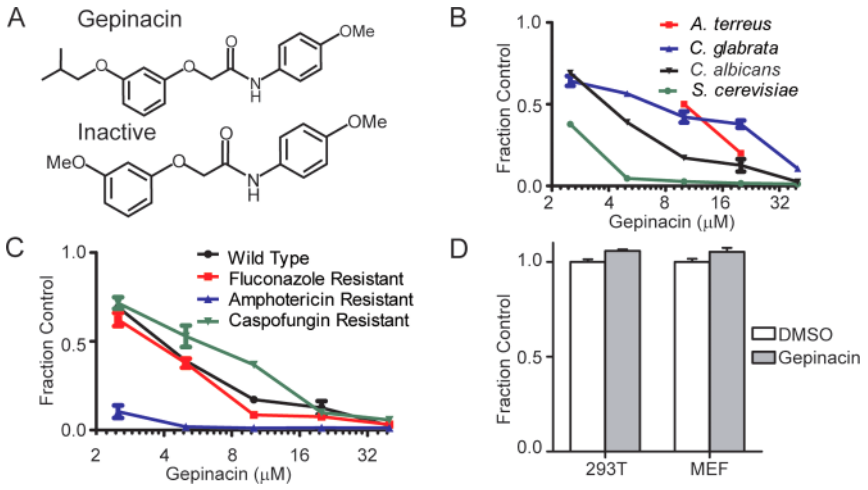

Figure 1. Gepinacin inhibits growth of evolutionarily distant fungi but does not affect mammalian cells. (A) Structures of gepinacin and a similar but inactive compound. (B) Antifungal susceptibility testing for an evolutionarily diverse group of fungi treated with gepinacin. For *A. terreus* the MIC50 and MIC80 are plotted. (C) Antifungal susceptibility testing for wild type *C. albicans* and strains resistant to the three major classes of antifungals 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 h after which relative viable cell number was measured by standard luciferase assay (Cell Titer-Glo, Promega).

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 antifungal 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 antifungal activity against a surprisingly diverse range of fungi. Under screening conditions, the compound induced 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*.

Highly Selective Antifungal Activity against a Diverse Range of Fungi. The phenoxyacetanilide compound identified in our screen (Figure 1a) inhibited the growth of diverse yeasts and molds separated by approximately 800 million years of evolution, including *S. cerevisiae*, *Candida glabrata*, *C. albicans*, and *Aspergillus terreus*.[4] This suggested the involvement of a mechanism widely conserved among fungi (Figure 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 (Figure 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. 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 (Figure 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 overexpression library and a pooled heterozygous deletion library for enhancement or suppression of toxicity. The overexpression library consisted of 5336 individual haploid strains arrayed in 384-well plate format. With each strain expressing one open reading frame (ORF), 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.[6] Only one gene, *GWT1*, was recovered as a hit shared

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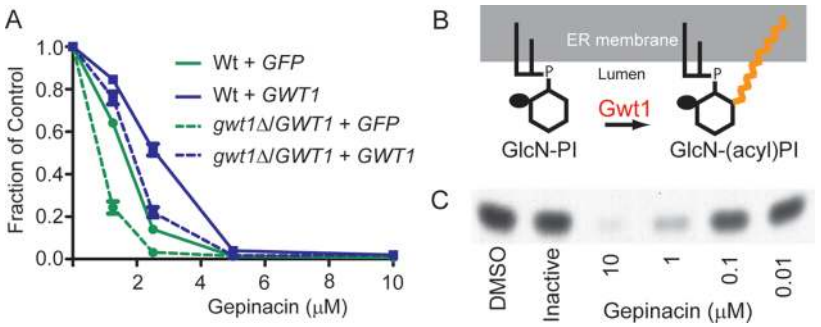

Figure 2. Gwt1 is the target of gepinacin. (A) Antifungal susceptibility testing of *S. cerevisiae* strains with graded levels of Gwt1 expression treated with gepinacin. Wild type diploids (Wt) or diploids with one copy of *GWT1* deleted (*gwt1*Δ/*GWT1*) were transformed with low copy (CEN) plasmids 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 analogue (10 μM). The structure of the inactive compound is shown in Figure 1.

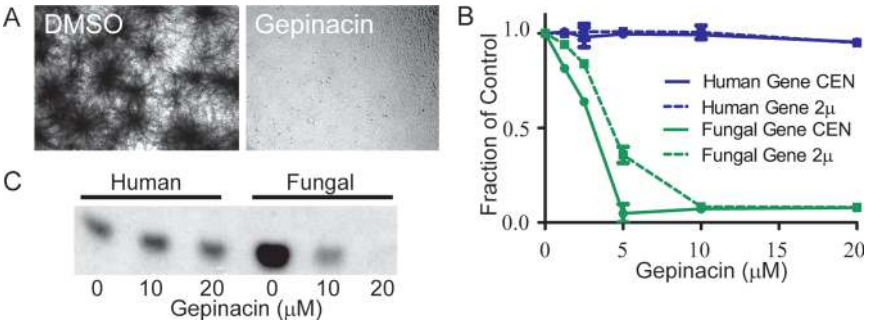

Figure 3. Gepinacin inhibits Gwt1 but not its human ortholog, PIG-W. (A) Representative photomicrograph of mammalian cells (NIH3T3) co-cultured with amphotericin-resistant *C. albicans* following addition of DMSO or 40 μM gepinacin. Photographs were taken after 24 h. DMSO-treated cultures have large, dense colonies of *C. albicans*, which are not present in gepinacin-treated cultures. (B) Antifungal 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, 2 μm 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.

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 overexpressed, 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 (Figure 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 (*gwt1*Δ/*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 2 μm plasmid further decreased gepinacin activity in both genotypes (Supplementary Figure 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 the 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) (Figure 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, and 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 (Figure 2c; full autoradiograph provided in Supplementary Figure 2, phospholipase-C control provided in Supplementary Figure 3), while a compound that was structurally very similar but biologically inactive did not (Figure 1a, Supplementary Figure 4).

Having determined the target of gepinacin in fungi, we were interested in examining gepinacin's effect on the human ortholog of Gwt1, PIG-W. To determine if gepinacin remained active under mammalian cell culture conditions, we cultured fungi (either an amphotericin B-resistant (Figure 3a) or fluconazole-resistant (data not shown) isolate of *C. albicans*) and mammalian cells together with 40 μM gepinacin. 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

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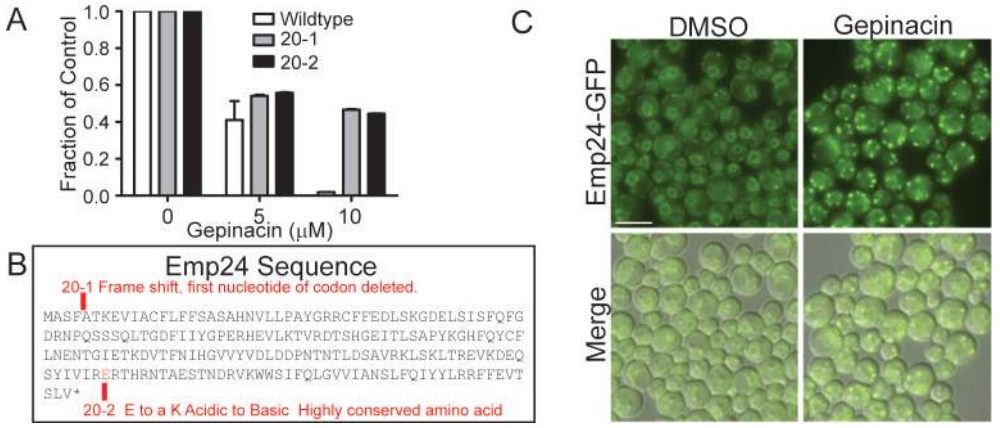

Figure 4. *EMP24* deletion decreases gepinacin toxicity. (A) Antifungal susceptibility testing of gepinacin using suppressor strains 20-1 and 20-2. (B) The sequence of *EMP24* in *S. cerevisiae* is shown with the location of mutations found by whole genome sequencing in the suppressor strains 20-1 and 20-2 indicated. (C) Micrographs showing the redistribution of Emp24-GFP after overnight incubation with 5 μM gepinacin. Green fluorescence and DIC images are merged in the lower panels. Scale bar = 5 μm.

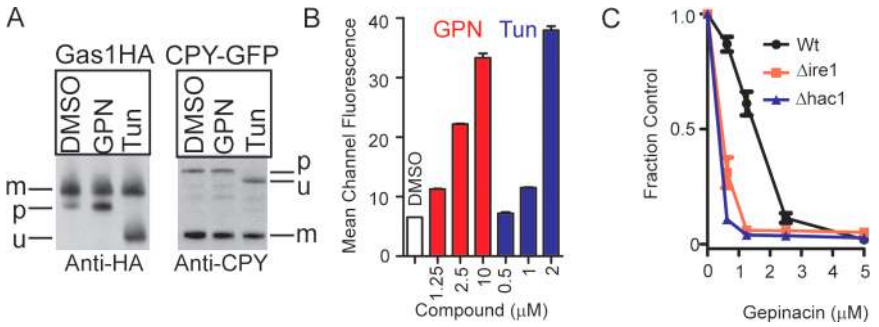

Figure 5. Gwt1 inhibition by gepinacin impairs GPI-anchored protein maturation and causes ER-related toxicity. (A) Immunoblots of lysates prepared from *S. cerevisiae* treated with gepinacin showing GPI-anchor-selective impairment of protein maturation. The unprocessed (u), precursor (p), and mature (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) Antifungal susceptibility testing of strains deleted for *IRE1* (activator of the UPR) or *HAC1* (effector of the UPR) showing their increased sensitivity to gepinacin.

same well. PIG-W (and GPI anchor biosynthesis) is not essential for mammalian cells in culture. Therefore, these experiments do not rule out the possibility that gepinacin inhibits PIG-W, but they do show that gepinacin remains active in this complex co-culture environment.

To determine whether gepinacin is selective for the fungal protein, we took advantage of the genetic tractability of *S. cerevisiae*. Gwt1 is essential in this organism, and the human protein is capable of fulfilling its essential function.[10] We constructed a pair of transgenic strains in which a deletion of the *GWT1* gene was rescued by heterologous expression of either the human or yeast genes (*PIG-W* or *GWT1*, respectively). 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 (Figure 3b,c, full autoradiograph provided in Supplementary Figure 5). These results definitively establish 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* (Figure 4a). Whole genome sequencing of these strains showed that resistance was due to two different mutations in the gene *EMP24* (Figure 4b). This finding was recapitulated with targeted deletion of *EMP24* (Supplementary Figure 6).

The non-essential protein Emp24 is a component of a large multiprotein complex that regulates GPI-anchored protein transport and quality control.[11] 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.[12] 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.[12] 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

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with a fainter cortical ring and a few bright dots consistent with some Golgi localization. After overnight culture in gepinacin, however, Emp24-GFP dramatically relocalized into large, bright dot-like structures (Figure 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.[13] CPY is not GPI-anchored but undergoes sorting and processing in the secretory pathway before ending up in the yeast vacuole.[14] 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 (Figure 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.[15]

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 h in gepinacin induced a massive, concentration-dependent activation of the unfolded protein response (UPR) as monitored by the GFP reporter construct (UPRE-GFP), which encodes GFP under the transcriptional control of the KAR2 unfolded protein response element. Reporter activation was similar in extent to the effect produced by tunicamycin (Figure 5b). Activation of the UPR was not seen when yeast were treated with representative compounds from the three major classes of antifungals: amphotericin B, caspofungin, and fluconazole (Supplementary Figure 7). As expected on the basis of 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 (Supplementary Figure 8).[16,17] Deletions of *HAC1* and *IRE1*, which are essential components of the UPR activation pathway in yeast, both greatly increased the toxicity of gepinacin (Figure 5c). As a positive control, similar enhancement of tunicamycin toxicity was seen in association with deletion of these genes (Supplementary Figure 9a). In contrast, toxicity of the conventional antifungal fluconazole, which targets ergosterol biosynthesis, was completely unaffected by these UPR-disabling deletions (Supplementary Figure 9b).[18]

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.[19,20]

Because of its role in tissue invasion, a key determinant of fungal virulence is the ability to switch between yeast and filamentous forms.[21−23] A previous report has shown that mature GPI-anchors are required for this process.[24] 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 2 year period from an HIV patient treated with fluconazole, strains CaCi2, 8, and 17.[25] 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 h in the continued presence or absence of gepinacin.[26] 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 (Figure 6a). The inability

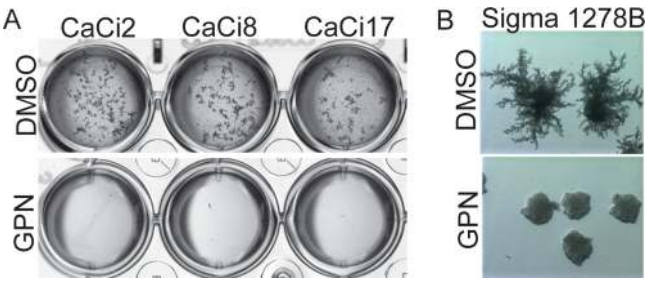

Figure 6. Gepinacin blocks filamentation in liquid and solid culture models. (A) Fluconazole resistant *C. albicans* strains were treated overnight with DMSO (0.025%) or gepinacin (GPN) (5 μM) in filamentation media and then imaged macroscopically. (B) *S. cerevisiae* strain Sigma 1278B was grown on filamentation-inducing plates containing DMSO (0.025%) or gepinacin (GPN) (5 μM) for 6 days and then imaged microscopically.

to form filaments was also apparent at the cellular level as demonstrated by the photomicrographs provided in Supplementary Figure 10. 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 (Figure 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.[27] In previous studies, mutation of *GWT1* was shown to change the cell wall composition such that the outer mannoprotein layer became thinner and the inner glucan layer became thicker.[28] 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, sublethal concentrations of gepinacin did dramatically increase β-glucan presentation on the cell surface of *C. albicans* (Figure 7a). Quantitation of this effect by flow

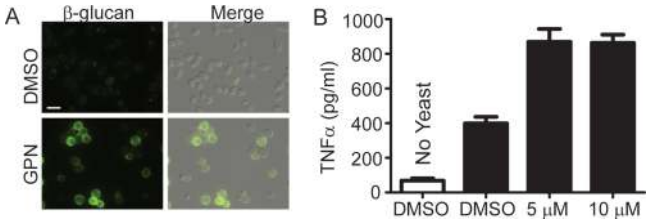

Figure 7. Gepinacin treatment increases β-glucan exposure on the cell surface and enhances TNFα secretion from macrophages. (A) Fluorescence photomicrographs depicting the β-glucan (green) immunoreactivity of *C. albicans* after treatment with DMSO or 5 μM gepinacin (GPN). Cells were also imaged with DIC and counterstained 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.

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*.[29] 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 (Figure 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.*[28] 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 10a). *GWT1* was subsequently cloned as a dosage-dependent suppressor of BIQ-induced phenotypes. Further discovery and optimization efforts by this group led to better antifungal candidates[30] and the synthesis of E1210, a potent and selective Gwt1 inhibitor with properties suitable for clinical development (structure in Supplementary Figure 11b). 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*.[31,32] E1210 has also been shown to decrease fungal adherence to polystyrene and inhibit biofilm formation. Like gepinacin, it is nontoxic to mammalian cells at concentrations that far exceed those required for antifungal activity.[10] 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 the cell wall, and impair the morphogenic filamentation program which is required for pathogenicity in animal hosts.[10] 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 multipass 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] Similar strains have also been created by N. Watanabe *et al.*[10]

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 also 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 decrease 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.[33] 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 Supporting Information.

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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 NCCLS broth microdilution protocol CLS M27-A3 and RPMI as culture medium. Fungi were inoculated at ~10^3 cells/mL and incubated for 48 h at 37 °C after which plates were sealed and shaken and the optical density was 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/mL and plates were incubated at 35 °C for 48 h 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 RPMI 1640 + 10% FBS containing the amphotericin-resistant strain CaAmphR at 10^3 CFU/mL and 40 μM gepinacin or an equivalent amount of DMSO. Cocultures were incubated for 24 h at 37 °C in a microisolator 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 h and then 4 more times over the next 2 days. Data analysis details are provided in the Supporting Information. 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 h. Genomic DNA preparation, PCR, and chip hybridization were done as previously described.[6,34,35] All hits were retested by growth assay in 384-well plate format, and the relevant deletion was 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 (Supplementary Figure 3).

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 h with agitation at 37 °C in the presence of compound before transfer to a 24-well plate for imaging.[36] Assays with *S. cerevisiae* strain Sigma 1278B on solid media were performed as previously described[26] with the exception that 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[37]) under control of its own promoter using plasmid pCM-*HA-GAS1*. This construct was created as previously reported except in a gateway plasmid backbone.[38] To assess effects on trafficking, strains were incubated for 1 h 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.[38] 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.[39,40] Reporter cells were grown to log phase and exposed to compound at 23 °C for 5 h 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.[41] 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 to yeast ratio of 1:2.5 in the continued presence of drug. After 2 h, supernatants were harvested, and TNFα concentration was measured by ELISA using a kit according to manufacturer's instructions (DY410, R&D Systems).

Suppressor Strains To Identify Mechanisms of Resistance. Approximately 2 × 10^7 W303 *MAT*a 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 PRJNA167645 (derived strains) and SRA accession numbers SRX118359 and SRX118360 (wild type). Depth of coverage averaged 100-fold. Details of computational analysis are provided in the Supporting Information.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ NOTE ADDED AFTER ISSUE PUBLICATION

The BioProject accession number in the Genomic Sequencing section has been updated. The revised version was re-posted on May 7, 2013.