

MIT Open Access Articles

Chemical Genomics-Based Antifungal Drug Discovery: Targeting Glycosylphosphatidylinositol (GPI) Precursor Biosynthesis

The MIT Faculty has made this article openly available. **Please share** how this access benefits you. Your story matters.

Citation: Mann, Paul A. et al. "Chemical Genomics-Based Antifungal Drug Discovery: Targeting Glycosylphosphatidylinositol (GPI) Precursor Biosynthesis." ACS Infectious Diseases 1.1 (2015): 59–72. © 2014 American Chemical Society

As Published: <http://dx.doi.org/10.1021/id5000212>

Publisher: American Chemical Society (ACS)

Persistent URL: <http://hdl.handle.net/1721.1/105537>

Version: Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

Terms of Use: Article is made available in accordance with the publisher's policy and may be subject to US copyright law. Please refer to the publisher's site for terms of use.



Chemical Genomics-Based Antifungal Drug Discovery: Targeting Glycosylphosphatidylinositol (GPI) Precursor Biosynthesis

Paul A. Mann,^{†,||} Catherine A. McLellan,^{‡,§,||} Sandra Koseoglu,[†] Qian Si,[†] Elena Kuzmin,[#] Amy Flattery,[†] Guy Harris,[†] Xinwei Sher,[†] Nicholas Murgolo,[†] Hao Wang,[†] Kristine Devito,[†] Nuria de Pedro,[⊥] Olga Genilloud,[⊥] Jennifer Nielsen Kahn,[†] Bo Jiang,[†] Michael Costanzo,[#] Charlie Boone,[#] Charles G. Garlisi,[†] Susan Lindquist,^{‡,§} and Terry Roemer^{*,†}

[†]Merck Research Laboratories, 2015 Galloping Hill Road, Kenilworth, New Jersey 07033, United States

[‡]Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, Massachusetts 02142, United States

[§]Howard Hughes Medical Institute and Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, United States

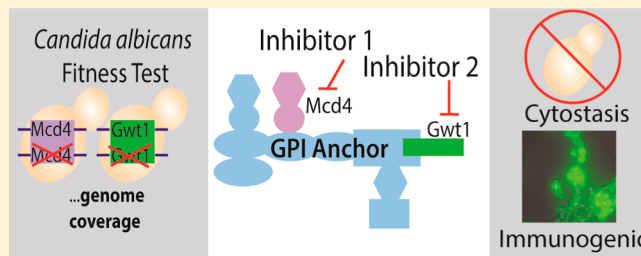
[#]Banting and Best Department of Medical Research, Terrance Donnelly Centre of Cellular and Biomedical Research, University of Toronto, Toronto, Ontario, Canada

[⊥]Fundación Centro de Excelencia en Investigación de Medicamentos Innovadores en Andalucía, Medina, Parque Tecnológico de Ciencias de la Salud, Avenida Conocimiento 34, 18016 Grenada, Spain

Supporting Information

ABSTRACT: Steadily increasing antifungal drug resistance and persistent high rates of fungal-associated mortality highlight the dire need for the development of novel antifungals. Characterization of inhibitors of one enzyme in the GPI anchor pathway, Gwt1, has generated interest in the exploration of targets in this pathway for further study. Utilizing a chemical genomics-based screening platform referred to as the *Candida albicans* fitness test (CaFT), we have identified novel inhibitors of Gwt1 and a second enzyme in the glycosylphosphatidylinositol (GPI) cell wall anchor pathway, Mcd4. We further validate these targets using the model fungal organism *Saccharomyces cerevisiae* and demonstrate the utility of using the facile toolbox that has been compiled in this species to further explore target specific biology. Using these compounds as probes, we demonstrate that inhibition of Mcd4 as well as Gwt1 blocks the growth of a broad spectrum of fungal pathogens and exposes key elicitors of pathogen recognition. Interestingly, a strong chemical synergy is also observed by combining Gwt1 and Mcd4 inhibitors, mirroring the demonstrated synthetic lethality of combining conditional mutants of *GWT1* and *MCD4*. We further demonstrate that the Mcd4 inhibitor M720 is efficacious in a murine infection model of systemic candidiasis. Our results establish Mcd4 as a promising antifungal target and confirm the GPI cell wall anchor synthesis pathway as a promising antifungal target area by demonstrating that effects of inhibiting it are more general than previously recognized.

KEYWORDS: glycosylphosphatidylinositol, GPI, GWT1, MCD4, *Candida albicans* fitness test, antifungal, yeast cell wall, chemical biology, next-generation sequencing, natural product



The need for novel antifungal agents is undeniable. *Candida albicans* persists as the principal clinically relevant fungal pathogen in the United States and remains the fourth leading cause of bloodstream infections despite decades of attention and therapeutic strategies designed to eradicate it.¹ The growing trend of *C. albicans* azole-resistance, as recently highlighted by the Centers for Disease Control, only underscores the need for new treatment options. Mortality associated with *Aspergillus fumigatus*, a second clinically important fungal pathogen, is also unacceptably high and exceeds 50% despite treatment with current standard of care (SOC) antifungal agents.² Compounding this problem, the antifungal drug armamentarium is restricted to only three basic classes of

agents, amphotericin B, azoles, and echinocandins, that target ergosterol in the plasma membrane, ergosterol biosynthesis, or synthesis of the fungal-specific cell wall polymer, β -1,3-glucan, respectively.^{2,3} Furthermore, each drug class possesses its own limitations (spectrum, administration, resistance, and/or toxicity), and with the exception of the echinocandins, was originally discovered and developed over 50 year ago.⁴

We developed the *C. albicans* fitness test (CaFT) assay as a genomics-based platform to screen synthetic or natural product libraries and identify target-specific inhibitors with antifungal

Received: November 6, 2014

Published: December 5, 2014

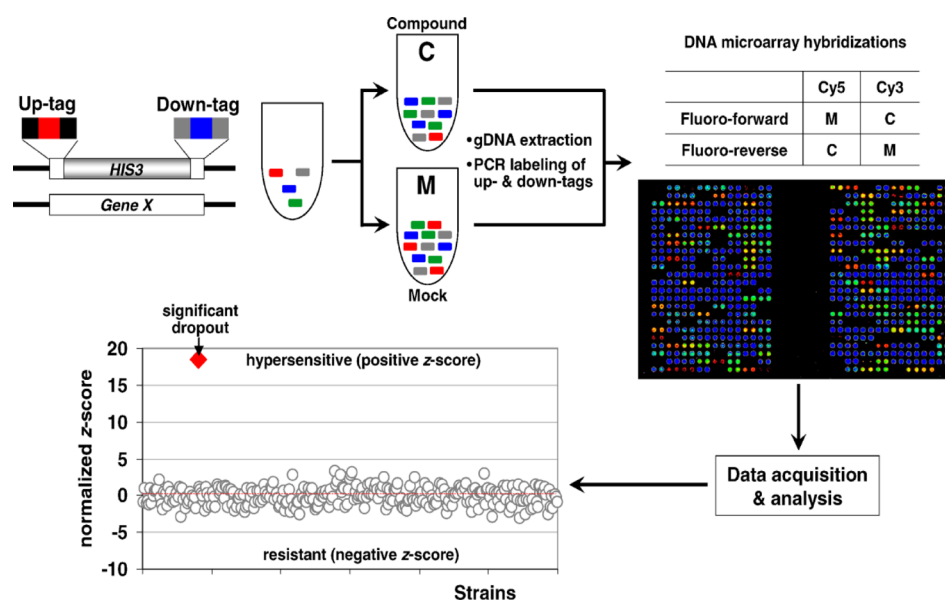


Figure 1. Individual *C. albicans* heterozygous deletion mutants contain two unique barcodes (red and blue boxes) flanking the deleted allele. The CaFT strain pool contains ~5400 heterozygote deletion mutants, comprising >90% genome coverage. Aliquots of the pool are treated with a sub-MIC of the growth inhibitory compound or mock treatment and grown for 20 generations. The relative abundance of each strain, which reflects their chemical sensitivity to the compound, is subsequently determined by DNA microarray analysis using PCR amplified barcodes of each heterozygote. The response of each heterozygote to the effects of the compound is then appraised by calculating a normalized Z score, with a positive value indicating hypersensitivity and a negative value reflecting resistance (or hyposensitivity) to the tested compound. See Xu et al. for details of Z-score calculation.⁶ In this example, the strain highlighted in red is uniquely hypersensitive to the compound (C) treatment and depleted from the pool versus the mock-treated (M) control.

drug-like properties (Figure 1).^{5–7} The assay is based on the principle of chemically induced haploinsufficiency, first described in the diploid yeast, *Saccharomyces cerevisiae*, where the deletion of one allele of a target gene renders the heterozygote deletion strain hypersensitive to bioactive inhibitors specific to the depleted target.^{8,9} As such, each strain expresses half the normal level of a particular gene product versus other heterozygote mutants or the wild type diploid strain, and, consequently, less inhibitor is required to inhibit the specific heterozygote that has been genetically depleted of the drug target. To maximize the likelihood of linking a target-selective inhibitor to its cognate target, the CaFT assay contains nearly 5400 unique heterozygote strains reflecting 90% *C. albicans* genome coverage.⁷ Each heterozygote strain also possesses two unique molecular barcodes (i.e., distinct 20 base pair strain-identifying DNA sequences). Consequently, all strains can be pooled and assayed in coculture, allowing multiplex screening of the entire heterozygote CaFT pool when challenged with a subminimum inhibitory concentration of a mechanistically uncharacterized bioactive compound or natural product extract. The relative abundance (or “fitness”) of each strain comprising the strain set exposed to drug versus mock treatment is then determined by PCR amplification and fluorescent labeling of all bar codes, microarray hybridization, and analysis. Knowledge of those genes specifically affecting an altered fitness to a particular bioactive agent provides important insight into the possible mechanism of action (MOA) of the inhibitory compound. To date, we have demonstrated the robustness of this screening paradigm, identifying novel whole cell active target-selective inhibitors to diverse cellular processes and biochemical pathways including mRNA processing,¹⁰ proteasome function,¹¹ and purine metabolism,¹² as well as cell wall β -1,3-glucan, protein, and fatty acid biosynthesis.^{7,13}

Here, we describe the discovery and characterization of two additional classes of antifungal compounds targeting specific steps in glucosylphosphatidylinositol (GPI) precursor biosynthesis. GPIs are endoplasmic reticulum (ER)-derived post-translational modifications broadly conserved in eukaryotes and serve as cell surface anchors for >100 cell wall proteins in yeast.¹⁴ GPI-anchored proteins function in diverse processes, including adherence, virulence, septation, and cell wall biogenesis. As such, GPI biosynthesis is essential for fungal growth.^{15,16} GPI proteins transit the secretory pathway and receive GPI anchor attachment *en bloc* by a transamidation reaction linking the GPI to their C-terminus.^{16,17} Following attachment, GPI proteins are secreted to the cell surface, where they may remain bound to the plasma membrane or, more often, cross-linked to β -1,6-glucan polymers of the cell wall.¹⁷ GPI biosynthetic enzymes and the precursor product itself (ethanolamine-P-6Man α 1–2Man α 1–6Man α 1–4GlcNH 2α 1–6-D-*myo*-inositol-1-phosphate-lipid, where the lipid is diacylglycerol, acylalkylglycerol, or ceramide) are largely conserved across eukaryotes. Important functional differences, however, exist in the pathway between fungi and mammalian cells, including Gwt1-dependent acylation of inositol and Mcd4-mediated ethanolamine phosphate (EtNP) addition to mannose 1 (Man1) of the GPI core, despite the existence of human homologues Pig-W and Pig-N, respectively.^{15,17}

Applying a CaFT screening approach, the synthetic compounds, G365 and G884, are each predicted to inhibit Gwt1-mediated acylation of GPI precursors. Corroborating their proposed MOA, whole genome next-generation sequencing (NGS) of multiple G884 drug-resistant mutants isolated in the second yeast species, *S. cerevisiae*, reveals isolates faithfully contain single amino acid substitutions that map to *GWT1* and which are cross-resistant to the known Gwt1 inhibitor, gepinacin. Biochemical evidence also supports this view as,

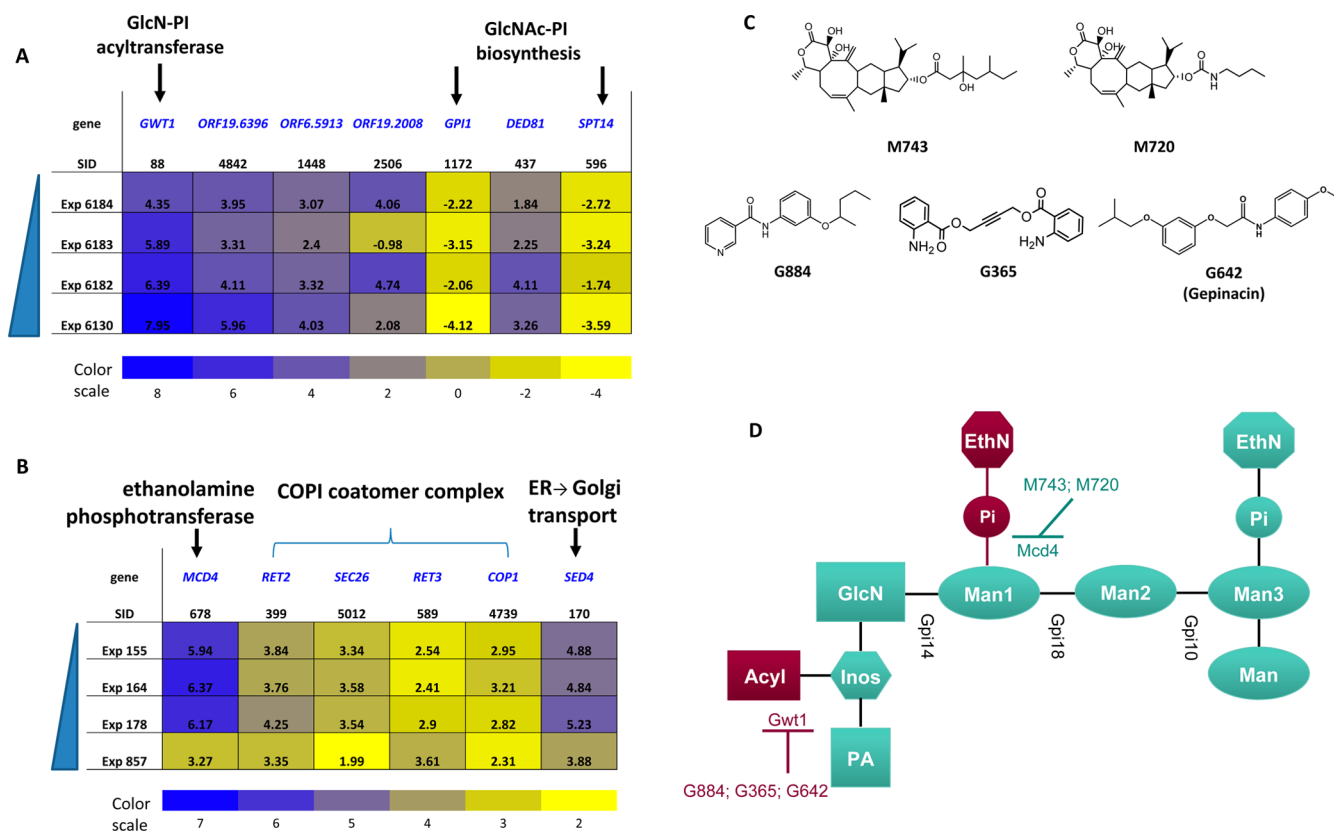


Figure 2. CaFT-based screening identifies GPI inhibitors. (A) CaFT summary of *C. albicans* heterozygote hypersensitivity to G884 across multiple drug concentrations. Note increasing strain sensitivities to G884 are displayed left to right (second row), based on the sum Z score across each independent CaFT experiment. Individual Z scores for each heterozygote strain are listed (rows 3–6), with Z scores highlighted in a color scale and heat map format. Note the *GWT1* heterozygote reproducibly displays greatest sensitivity to G884, and *GPI1* and *SPT14* heterozygotes display modest resistance to G884. (B) CaFT summary of *C. albicans* heterozygote hypersensitivity to M743 across multiple drug concentrations. Rank order of strain sensitivities and Z scores are highlighted as in (A). Note that the *MCD4* heterozygote displays greatest hypersensitivity to M743, whereas additional sensitive heterozygotes correspond to COPI coatomer subunits. (C) Chemical structures of G884, G365, M743, M720, and gepinacin. (D) Image of the *S. cerevisiae* GPI precursor and predicted enzymatic steps in precursor biosynthesis targeted by the above inhibitors.

like gepinacin, both inhibitors are shown in a cell-free system to deplete Gwt1-mediated acylation of GPI precursors in a dose-dependent manner. Similarly, we identify and mechanistically characterize the Mcd4-specific natural product inhibitor, M743, by CaFT screening unfractionated natural product extracts. M743 (previously named BE-049385A¹⁸ as well as YM3548¹⁹) is a terpenoid lactone ring-based natural product previously demonstrated to inhibit Mcd4 ethanolamine phosphotransferase activity.^{20,21} The striking hypersensitivity of the *mcd4* heterozygote and unique secondary profile to M743 provide a genome-wide prediction of the specificity and unique MOA of this agent in a whole cell context. Whole genome NGS of *S. cerevisiae* drug-resistant mutants to M743 and a highly related semisynthetic analogue (M720) corroborates this view as single missense mutations uniquely map to *MCD4*. Both *GWT1* and *MCD4* are essential for growth in yeast^{22,23} and, accordingly, cognate inhibitors of these targets display potent microbiological activity across multiple clinically relevant diverse pathogenic fungi. Unlike current antifungal agents, GPI biosynthesis inhibitors also expose β -1,3-glucan, an important agonist of Toll-like receptors (TLRs),²⁴ and induce TNF α secretion in mouse macrophage co-incubated with *C. albicans* drug-treated cells. Finally, we demonstrate that the Mcd4 inhibitor M720 provides significant efficacy in a murine infection model of systemic candidiasis and discuss the

potential of GPI inhibitors as a new mechanistic class of antifungal agents.

RESULTS AND DISCUSSION

CaFT Screening and GPI Inhibitor Identification. CaFT screening was performed against >1000 pure synthetic compounds within the Merck corporate library known to inhibit *C. albicans* growth at drug concentrations <20 μ M but for which their drug target and MOA were completely unknown. As a result of this unbiased screening approach, two compounds having CaFT profiles suggesting a GPI pathway-based MOA were identified and named G884 and G365, respectively (Figure 2A). Specifically, the *C. albicans* *GWT1* heterozygote (encoding a GlcN-PI acyltransferase involved in early GPI precursor synthesis²⁵) displays a unique and statistically significant hypersensitivity to G884 and G365 across a series of drug concentrations in a dose-dependent manner not previously seen despite extensive synthetic compound and natural product extract screening (Supporting Information, Figure S1A). G884 and G365 are structurally unrelated to each other (Figure 2C) as well as two recently described synthetic Gwt1 inhibitors, E1210²⁶ and gepinacin.²⁷ Interestingly, two additional heterozygotes corresponding to *GPI1* and *SPT14* (both of which participate in the preceding and first committed step in GPI biosynthesis, namely GlcNAc-PI biosynthesis¹⁷) display a significant and reproducible growth

advantage (i.e., enhanced fitness as judged by negative Z scores) to both G884 and G365, further implicating a GPI inhibitory MOA for these compounds. Confirmation of the altered chemical sensitivity phenotypes of each of the heterozygote deletion mutants identified by the CaFT assay was demonstrated directly by serial dilution and spotting heterozygotes onto drug-containing plates (Figure S2). Interestingly, the severity of drug sensitivity phenotypes among those heterozygotes reflecting these CaFT profiles not only matched between G884 and G365 but also gepinacin, reinforcing a possible common MOA shared by all three compounds.

The CaFT screening platform has also been extensively applied to screening unfractionated natural product extracts for target-selective antifungal agents.^{7,10,11} As part of this effort we independently identified M743 (a previously described antifungal natural product, YM3548^{18,19}) despite its presence in a complex mixture of additional compounds comprising the unfractionated extract. Its CaFT profile complements previous genetic and biochemical studies demonstrating YM3548 inhibits GPI biosynthesis by blocking Mcd4-mediated ethanolaminephosphate (EtNP) transferase activity of mannosyl transferase (Man1) of the GPI precursor.^{20,21,23} Specifically, a prominently reduced fitness of the *C. albicans* MCD4 heterozygote strain is specifically and reproducibly detected in the CaFT assay when challenged with M743-containing extracts, purified M743, or the M743 semisynthetic analogue, M720 (Figure 2B and Figure S3). MCD4 heterozygote hypersensitivity to M743 is directly demonstrated by serial dilution spot tests on drug-containing plates (Figure S2) and further corroborated by genetic knockdown of MCD4 expression using a conditional mutant under the control of a tetracycline-regulatable promoter (Figure S4). Importantly, hypersensitivity of the *C. albicans* MCD4 heterozygote to M743 and M720 is highly unique and not seen despite extensive screening of the strain, reinforcing Mcd4 as the likely drug target rather than a “promiscuous” mutant broadly hypersensitive to diverse antimicrobial compounds.

M743 and M720 also produce a highly unique secondary CaFT profile reflecting a number of additional hypersensitive heterozygote mutants implicated in the MOA of these agents (Figure 2B and Figure S3). Specifically, numerous strains heterozygous for subunits of the secretory coat protein complex I (COPI) including Cop1, Sec26, Ret2, and Ret3²⁸ as well as the COPII accessory factor, Sed4²⁹ demonstrate hypersensitivity to M743 and M720. Drug sensitivity phenotypes of each of these heterozygote strains were confirmed by serial dilution of each mutant on either M743 or M720 containing plates (Figure S2). Interestingly, each of these heterozygotes is predicted to be compromised in their function of retention and recycling of ER-localized membrane proteins containing a “KKXX” C-terminal retention sequence and of which Mcd4 is a member.^{23,30,31} Presumably, M743 and M720 hypersensitivity of these heterozygotes reflects their functional role in Golgi → ER retrograde transport and retrieval of ER-localized membrane proteins and is manifested indirectly by partially depleted Mcd4 levels in the ER (where early GPI synthesis events occur¹⁷) and concomitant mislocalization of the target to the Golgi. Accordingly, heterozygosity of genes involved in COPI-mediated retrograde transport may nonetheless produce M743 and M720 hypersensitive phenotypes that phenocopy heterozygosity of the actual (Mcd4) drug target (see below).

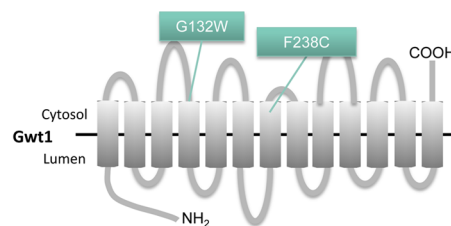
Drug Resistance-Based Mechanism of Action Studies. To genetically corroborate CaFT profiles that predict Gwt1 as

the drug target of G884, three stable independently derived drug-resistant mutants to G884 (G884^R) were isolated in a haploid *S. cerevisiae* strain. In each case, the minimal inhibitor concentration (MIC) of G884 was increased 8-fold (MIC = 128 μg/mL) in the G884^R isolate versus the parental wild type strain (Figure 3A). Whole genome NGS revealed that each

A

compound	mechanism	wild type S288c	G884_M1 S288c	G884_M3 S288c	M720_O4 S288c	BY4700 Apr15	M720_M1 Apr15	M720_M8 Apr15	M743_N5 Apr15	M743_N8 Apr15
G365	GWT1	> 64	> 64	> 64	> 64	> 64	> 64 (8)	> 64 (2)	> 64 (8)	> 64 (8)
G884	GWT1	32 (16)	128	128	32 (16)	16 (8)	32 (16)	32 (8)	32 (16)	64 (16)
G642 gepinacin	GWT1	2	128	4	4	2	2 (1)	2 (1)	2 (1)	2 (1)
M720	MCD4	4 (2)	4	8 (4)	64	0.063 (0.032)	16 (4)	8 (4)	32 (4)	8 (4)
M743	MCD4	1	2 (1)	2	64	0.063 (0.032)	2 (1)	2 (1)	64 (8)	8 (2)
AmB	polyene	0.5	0.5	0.5	0.5	0.5	0.25	0.5	0.25	0.5
FLZ	azole	64	64	16	16	2	2 (1)	2 (1)	2 (1)	2 (1)
CSP	echinocandin	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.008
Protein sequence		wt	Gwt1-G132W	Gwt1-F238C	Mcd4-P810Q	wt	Mcd4-P810L	Mcd4-G792C	Mcd4-Q679P	Mcd4-F800L
DNA sequence		wt	G394T	T713G	C2429A	wt	C2429T	G2374T	A2038C	T2388C

B



C

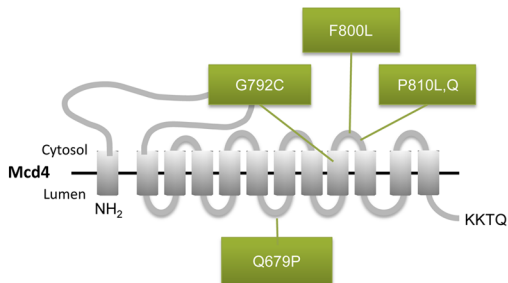


Figure 3. *S. cerevisiae* drug resistant mutant isolation and characterization of GPI inhibitors. (A) Summary of Gwt1 and Mcd4 drug resistant amino acid substitution mutants and altered susceptibility to GPI inhibitors versus control antifungal agents, amphotericin B (AmB), fluconazole (FLZ), and caspofungin (CSP). Specific amino acid substitutions and causal nucleotide mutations are shown. Note G884^R mutations were isolated using wild-type (wt) strain S288c, whereas M743^R and M720^R mutants were isolated from a *ptr5Δ* strain otherwise isogenic to BY4700. Also note cross-resistance is specifically observed between M743 and its semisynthetic analogue, M720, among Mcd4 amino acid drug-resistant isolates as well as between G884 and gepinacin with Gwt1-G132W. (B) G884^R amino acid substitutions (boxed) map to Gwt1. Predicted topology of *S. cerevisiae* Gwt1 is shown as recently determined with C-terminal sequence residing in cytosol.³³ (C) M743^R and M720^R amino acid substitutions (boxed) map to Mcd4. Predicted topology of *S. cerevisiae* Mcd4 is shown as previously described,²³ with the KKTQ C-terminal sequence residing in the lumen of the ER.

G884^R isolate maintains an amino acid substitution mutation corresponding to Gwt1-G132W or Gwt1-F238C (Table 2B). As no additional nonsynonymous mutations were identified in the genome of two independent G884^R isolates (Table 2B), we conclude these mutations are causal for the observed drug resistance phenotype. Gwt1-G132W and Gwt1-F238C mutations map to the fourth transmembrane domain (TMD, of which nine are predicted²²) or a highly conserved central region of Gwt1, respectively (Figure 3B) Interestingly, a similar

Table 1. Microbiological Spectrum^{ab}

compd	MOA	MB2865	Ca2323	Ca1055	ATCC22019	MY1396	ATCC6258	MY1381	S288c	MF5668
		<i>S. aureus</i>	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. parapsilosis</i>	<i>C. lusitaniae</i>	<i>C. krusei</i>	<i>C. glabrata</i>	<i>S. cerevisiae</i>	<i>A. fumigatus</i>
G365	GWT1	>128	4 (2)	4 (2)	2	>128	>128	>128	>128	4 (2)
G884	GWT1	128	4	4	8	64	128	32 (16)	16	128 (64)
G642 gepinacin	GWT1	>128	4 (2)	4 (2)	2	4	>128	4 (2)	1 (0.5)	(16)
M720	MCD4	>128	0.5 (0.25)	0.5 (0.25)	0.5	0.5	1 (0.5)	0.25	1 (0.5)	0.5 (0.25)
M743	MCD4	>128	0.5	0.5 (0.25)	0.5 (0.25)	1	1 (0.5)	0.5	1 (0.5)	0.25 (0.125)
AmB	polyene	>128	0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.5
FLZ	azole	>128	0.5 (0.25)	>128	1	1	32	8 (4)	4	>128
CAS	candin	>128	0.063	0.25	0.5	0.25	0.5	0.25 (0.125)	0.25	(0.016)

^aAll MIC values are represented as $\mu\text{g/mL}$. ^bMIC-50 values (drug concentrations required to inhibit growth 50%) are in parentheses.

Gwt1 amino acid substitution, Gwt1-G132R, is reported to confer drug resistance to the progenitor compound of E1210, named BIQ.²² Therefore, although G884 and E1210 are structurally unrelated, both compounds may share a common ligand binding site and inhibit Gwt1 activity in an analogous manner. Furthermore, the Gwt1-G132W mutant strain is highly cross-resistant to gepinacin (MIC shift >32-fold; Figure 3A), further suggesting a common Gwt1 inhibitory mechanism between each of these compounds. Drug resistance mapping of G365 was precluded by the fact that the compound only weakly inhibits *S. cerevisiae* growth despite its potent antifungal activity against *C. albicans*.

To obtain genetic evidence in support of MCD4 being the molecular target of M743, three alternative approaches to identifying M743^R mutants were performed in *S. cerevisiae*. As M743 appears to be a substrate of Pdr5-mediated drug efflux in yeast (Figure 3A), genetic studies to verify this compound as a cognate inhibitor of Mcd4 were first performed in a *pdr5Δ* strain background, from which seven independently derived M743^R isolates were identified. Following whole genome NGS, all M743^R isolates were determined to contain a single missense mutation causing amino acid substitution P810L ($n = 6$) or Q679P ($n = 1$; Figure 3C and Table 2A) in Mcd4. Drug resistance mutant selection was also performed using M720, with eight independently derived M720^R isolates recovered. In each case, whole genome NGS revealed only a single missense mutation in each resistor genome mapping to the MCD4 locus (including a new allele Mcd4-G792C), therefore demonstrating these Mcd4 amino acid substitution mutations as causal for M720 and M743 drug resistance. All MCD4 drug-resistant mutations map to the C-terminal region of the protein and lie within the C-terminal region of TMD11 (G792C) or within a cytosolic loop (Q679P) or ER luminal domain (F800L and P810L) based on the predicted topology of the protein (Figure 3C).²³ Notably, M720^R selection performed in the *pdr5Δ* strain background proved highly efficient in identifying target-based drug resistance as similar studies performed using a wild type *S. cerevisiae* strain identified only a single isolate containing a missense mutation in the drug target (Mcd4-P810Q) (Table 2A). All other M720^R isolates obtained from a wild type background contained distinct amino substitution mutations in Pdr1, a positive regulator of Pdr5, and are similar to previously described gain of function mutations to this master regulator of multidrug resistance.³² All M720^R and M743^R mutant strains were uniquely cross-resistant to the corresponding analogue, demonstrating these analogues maintain a common mechanism; none of the M720^R or M743^R mutants demonstrated cross-resistance to Gwt1p inhibitors (gepinacin, G884, or G365) or to a panel of clinically used antifungal drugs (Figure

3A). Collectively, these studies validate the CaFT as a robust reverse genetics platform to predict the MOA of bioactive agents, regardless of whether they are derived from natural product extract mixtures or exist as pure synthetic compounds. Furthermore, genetic identification of their cognate drug target by drug resistance selection in *S. cerevisiae* demonstrates their conserved MOA across yeast and *C. albicans*.

Gwt1 Inhibitors Selectively Block Acylation of Yeast Glucosamine Phosphatidylinositol (GlcN-PI) Biosynthesis. Gwt1 acylates GlcN-PI, an essential early precursor in GPI biosynthesis.^{25,33} To demonstrate *in vitro* the target-specific inhibitory effects of G884 and G365, Gwt1 acylation activity was evaluated following drug treatment in a cell-free system (see Methods for details).²⁷ Similar to the positive control compound gepinacin, G884 and G365 both inhibited acylation of GlcN-PI, unlike the negative control compounds, M743 and M720 (Figure 4A). Furthermore, G884 and G365 both inhibited acylation of GlcN-PI in a dose-dependent manner (Figure 4B). G884 *in vitro* inhibition of Gwt1 acylation activity also correlated strongly with its *S. cerevisiae* MIC ($\sim 16 \mu\text{g/mL}$), consistent with genetic evidence that the compound's whole cell activity is directly and specifically mediated through inactivation of Gwt1 function. Although G365 lacks yeast activity (*S. cerevisiae* MIC > 128 $\mu\text{g/mL}$), G365 similarly inhibited Gwt1 acylation activity ($\text{IC}_{50} \sim 20 \mu\text{g/mL}$) in this *in vitro* setting. Thus, consistent with CaFT data, G365 targets Gwt1 and its lack of yeast activity is not target-based but rather due to its species-specific activity (Table 1).

To further examine target selectivity of Gwt1 inhibitors, we took advantage of previously published transgenic yeast strains in which a *gwt1Δ* strain expresses either Gwt1 or the human orthologue, Pig-W.²⁷ Like gepinacin, G884 displayed appreciable (>10-fold) selectivity to its fungal target versus the human counterpart (Figure 4C), consistent with the minimal cytotoxicity data observed against multiple human cell lines, including HeLa cells (G884 $\text{IC}_{50} > 100 \mu\text{M}$) (Tables S1 and S2). Although similar studies to address G365 target selectivity could not be performed, again due to the lack of *S. cerevisiae* activity, cytotoxicity of the compound against HeLa cells is approximately 30-fold lower than that of cycloheximide, a positive control compound for this assay. Furthermore, minimal G365 cytotoxicity ($\text{IC}_{50} > 50 \mu\text{M}$) was detected against HepG2 or THLE-2 human liver immortalized cell lines (Table S2).

GPI Inhibitors Induce the Unfolded Protein Response. The modifications to the GPI anchor by Gwt1 and Mcd4 appear to be important for their forward transport and recognition by other enzymes in the pathway. This is indicated by the CaFT secondary profile for the Mcd4 inhibitors M743 and M720, which highlights aspects of ER to Golgi trafficking,

Table 2. Whole Genome NGS Mapping of GPI Inhibitor Drug Resistant Mutations to Their Cognate Target^a

A

BY4700 (<i>pdv5Δ</i>)	M720								M743						
	M1	M2	M3	M4	M5	M6	M7	M8	N1	N2	N3	N4	N5	N6	N7
<i>MCD4</i> (YKL165C) C2429T; P810L	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red
<i>MCD4</i> (YKL165C) T2398C; F800L	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
<i>MCD4</i> (YKL165C) G2374T; G792C	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
<i>MCD4</i> (YKL165C) A2036C; Q679P	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
<i>EMP65</i> (YER140W) A637C; K213Q	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black

B

Wild Type S288c	G884			M720				
	MA	MB	ME	O1	O2	O3	O4	O5
<i>GWT1</i> (YJL091C) T713G; F238C	Black	Black	Black	Black	Black	Black	Black	Black
<i>GWT1</i> (YJL091C) G394T; G132W	Red	Red	Black	Black	Black	Black	Black	Black
<i>PDR1</i> (YGL013C) G3142T; V1048F	Black	Black	Black	Black	Black	Black	Black	Black
<i>PDR1</i> (YGL013C) G2267C; C756S	Black	Black	Black	Black	Black	Black	Black	Black
<i>PDR1</i> (YGL013C) C1454G; S485W	Black	Black	Black	Black	Black	Black	Black	Black
<i>PDR1</i> (YGL013C) T914C; I305T	Black	Black	Black	Black	Black	Black	Black	Black
<i>MCD4</i> (YKL165C) C2429A; P810Q	Black	Black	Black	Black	Black	Black	Black	Black
YDR544C (dubious ORF) G167C; S56T	Yellow	Black	Black	Black	Black	Black	Black	Black
YDR544C (dubious ORF) G161C; S54T	Black	Black	Black	Black	Black	Black	Black	Black
YPL191C (Unknown function) A60C; L20F	Black	Black	Black	Black	Black	Black	Black	Black
<i>NEW1</i> (YPL226W) G1440T; R480S	Black	Black	Black	Black	Black	Black	Black	Black

^aHeat map summary of all nonsynonymous mutations identified by Illumina-based NGS (>100× genome coverage) of all independently isolated drug resistant mutants in the *pdv5Δ* strain, BY4700 (A) or a wild type S288C strain (B). Each column is a summary of all nonsynonymous mutations identified for a particular drug-resistant isolate; no additional nonsynonymous mutations were identified. Red, nonsynonymous mutation that maps to the predicted target *Mcd4* (inhibitors M743 and M720) or *Gwt1* (G884). Yellow, additional nonsynonymous mutations identified by NGS. Black, no change versus the parental wild type strain gene sequence. Nonsynonymous mutations mapping to the predicted drug target are causal for the drug resistance phenotype as they are faithfully identified in all M720^R and M743^R isolates from the *pdv5Δ* strain background as well as the G884^R wild type strain background. In several cases, M720^R isolates from the wild type starting strain background carry a *pdv5* missense mutation (rather than mapping to *Mcd4*), reflecting a bypass resistance mechanism. Base changes and resulting amino acid substitutions are shown.

and previous studies have shown that the deletion of *Mcd4* in yeast blocks the forward traffic of GPI-anchored proteins.³⁴ It has previously been shown that inhibition of *Gwt1* by gepinacin blocks the trafficking of GPI-anchored proteins and induces a large unfolded protein response (UPR) in the ER. Incubation of yeast for 3 h with either the *Gwt1* or *Mcd4* inhibitor compounds in this study induced a profound UPR as measured by the expression of GFP under the control of a UPR element (Figure 4D). In contrast, the UPR is not induced by the conventional antifungal agent fluconazole. These results indicate that inhibition of these targets has a strong effect on ER protein homeostasis.

Microbiological Evaluation of GPI Inhibitors. M743 displayed potent activity against all *Candida* species tested, including *C. albicans* (MIC = 0.5 μg/mL), *C. parapsilosis* (MIC = 0.5 μg/mL), *C. glabrata* (MIC = 0.5 μg/mL), *C. krusei* (MIC = 1.0 μg/mL), and *C. lusitanae* (MIC = 0.25 μg/mL) (Table 1). M743 also displayed remarkably potent activity against *A. fumigatus* (MIC = 0.25 μg/mL), paralleling the potency of caspofungin against this filamentous fungal pathogen. Broad anti-*Aspergillus* spp. activity was also noted on agar plates seeded with *A. fumigatus*, *A. niger*, or *A. terreus* and by measuring clear zones of inhibition where M743 was spotted on the surface of the plate (Figure S5). Conversely, G884, G365,

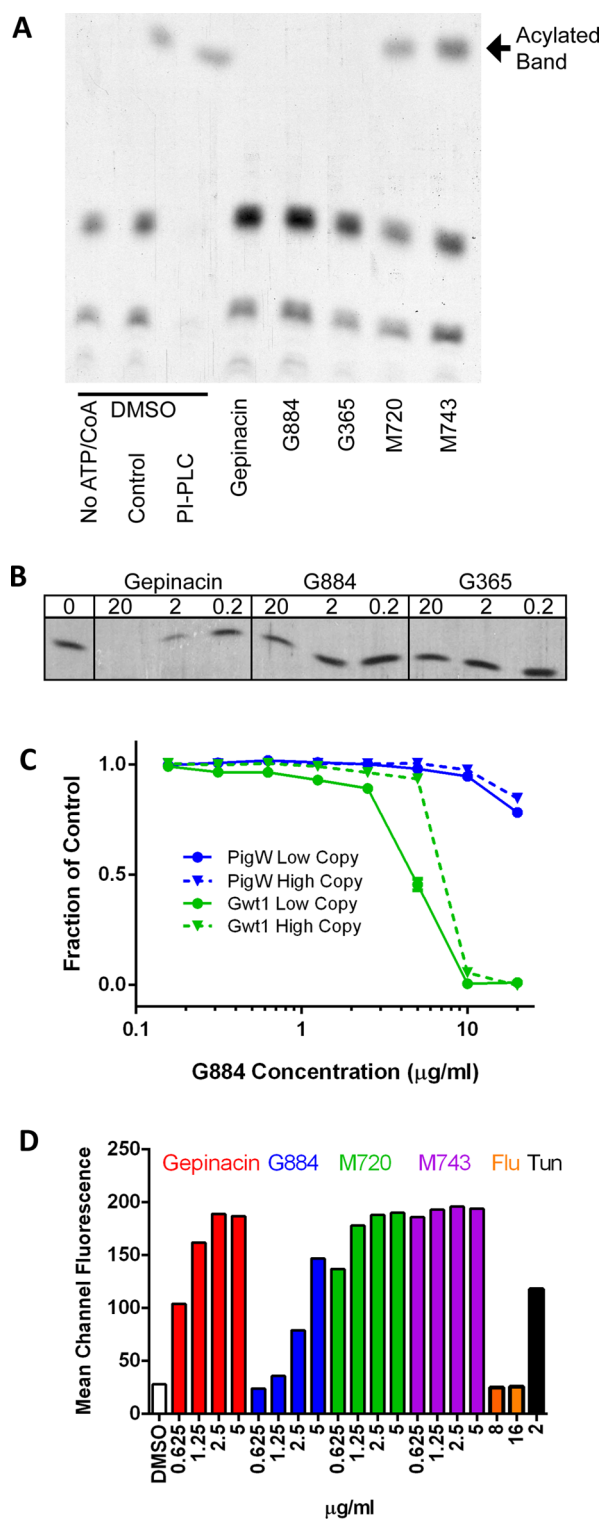


Figure 4. G884 and G365 inhibit Gwt1 acylation. (A) TLC of products of an *in vitro* acylation assay using membranes from *S. cerevisiae*. Controls show that in the absence of ATP and CoA the acylated band is not produced. In the presence of phospholipase C only the acylated band is preserved. Inhibitors of MCD4, M720, and M743 do not inhibit Gwt1-dependent acylation. The acylation reactions were incubated with 2 $\mu\text{g/mL}$ of gepinacin, M720, and M743 and 30 $\mu\text{g/mL}$ of G884 and G365. All GPI inhibitor MIC values for *S. cerevisiae* are listed in Table 1. (B) Dose response of the acylation inhibition by G884 and G365. The amount of compound in the reaction is given above the blot as $\mu\text{g/mL}$. (C) G884 preferentially inhibits the fungal enzyme, Gwt1. Growth curves of *S. cerevisiae* strains contain either the

Figure 4. continued

human enzyme Pig-W or the fungal enzyme, Gwt1, as their sole source of inositol acylating activity. The origin of replication for the low-copy plasmids is CEN and for the high-copy plasmids is 2 μm .²⁷ (D) Induction of the unfolded protein response in cells carrying a GFP reporter construct showing strong induction by inhibitors of Gwt1 and Mcd4. GFP expression was monitored by flow cytometry.

and the control compound, gepinacin, all possess similar antifungal potency against *C. albicans* (MIC = 4 $\mu\text{g/mL}$), and only G365 also displayed strong activity against *A. fumigatus* (MIC = 4 $\mu\text{g/mL}$; Table 1). No appreciable antibacterial activity for any of the above compounds was observed (*S. aureus* MIC \geq 128 $\mu\text{g/mL}$), reflecting the absence of orthologous drug targets and GPI biosynthesis in prokaryotes. Gwt1 inhibitors also lack obvious cytotoxicity at the highest drug concentration tested (with IC₅₀ values \geq 50 μM) against HeLa, HepG2, and THLE-2 human cell lines (Tables S1 and S2). Conversely, Mcd4 inhibitors displayed notable cytotoxicity against these cell lines, and only an approximate 10-fold therapeutic index (Tables S1 and S2). All GPI inhibitors tested (G884, G365, M743, and M720) also displayed a clear fungistatic terminal phenotype against *C. albicans* by standard kill curve analysis (Figure S6A), paralleling the demonstrated fungistatic nature of E1210.³⁵ Combining Gwt1 and Mcd4 inhibitors at 4 times the MIC of each agent, so as to chemically interdict GPI synthesis at two discrete steps in precursor synthesis, failed to convert their fungistatic effects to that of a fungicidal terminal phenotype (Figure S6B). Therefore, whereas small molecule inhibition of GPI biosynthesis affects fungal growth across diverse species, preliminary evidence suggests such agents are unlikely to achieve a broadly observed fungicidal terminal phenotype.

As we have highly selective and potent inhibitors to two distinct steps in GPI precursor biosynthesis, we tested whether pharmacological interdiction at multiple points in this pathway simultaneously achieves a synergist growth inhibitory effect. Indeed, substantial drug synergy was observed between each of the GPI inhibitor classes targeting Gwt1 and Mcd4 when examined by standard checkerboard analysis (Figure 5A,B). Fractional inhibitory concentration indices (FICI) as low as 0.25–0.313 were observed against *C. albicans* when G884 and M720 or G365 and M720 were combined (Figure 5C). Although no synergy between these compound classes was observed against *A. fumigatus*, strong chemical synergy between Gwt1 and Mcd4 inhibitors extends to *S. cerevisiae* (Figure 5C). On the basis of the observed synergy between Gwt1 and Mcd4 inhibitors, we predicted that *GWT1* and *MCD4* should exhibit a negative genetic interaction. Indeed, tetrad analysis revealed that *S. cerevisiae* yeast conditional mutants of *GWT1* and *MCD4* are synthetically lethal as double mutants, thus providing a clear genetic basis for the observed synergy of their cognate inhibitors (Figure 5D).

GPI Inhibitors Expose Cell Surface β -Glucan and Induce TNF α Secretion. Fungal cell wall β -glucans provide a powerful pro-inflammatory stimulus recognized by TLRs to induce a host immune response and combat infection.^{24,36} However, the β -glucan composition of the cell wall is naturally masked by surface mannoproteins and GPI-anchored proteins, thus offering a mechanism to evade immune detection.³⁶ As *C. albicans* cells treated with gepinacin display substantial changes in cell wall composition resulting in surface-exposed β -(1–3)-

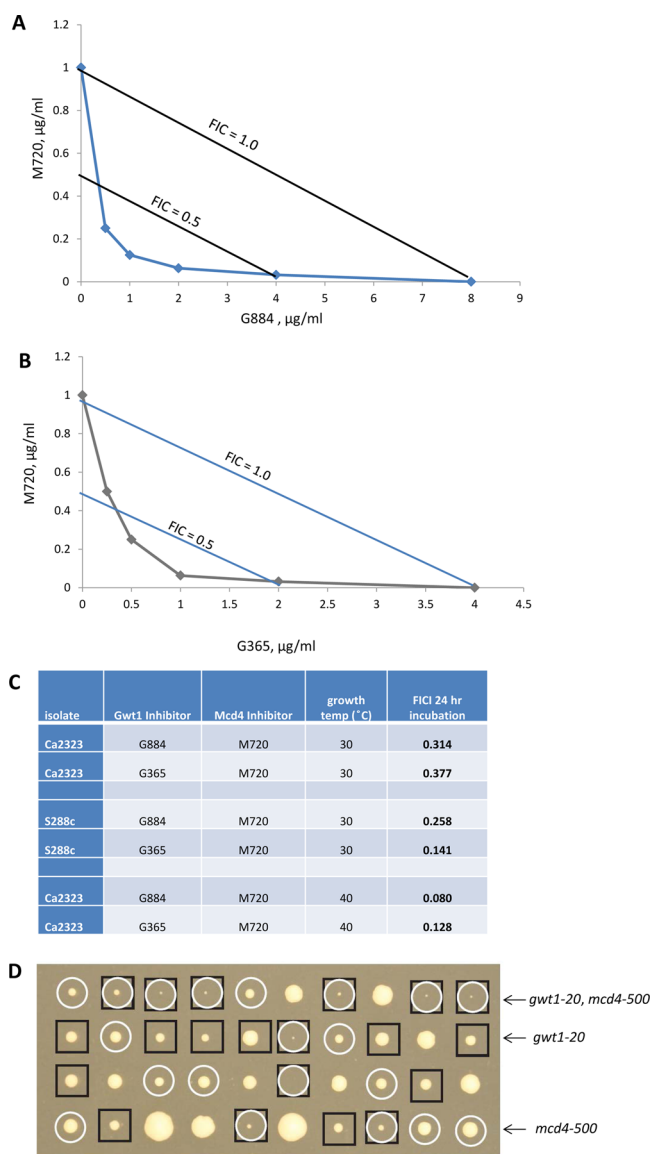


Figure 5. *GWT1* and *MCD4* display a synthetic lethal genetic interaction and cognate inhibitors possess highly synergistic antifungal activity. (A) Drug synergy of GPI inhibitor combinations G884 and M720 or (B) G884 and M720 determined by standard checkerboard analysis against *C. albicans* strain 2323. Drug concentrations and fractional inhibitory concentrations (FIC) used to evaluate synergy are indicated. Chemical synergy is achieved provided the sum FIC of the two agents (referred to as the FIC index, or FICI) is ≤ 0.5 . FICI = 0.5 is indicated by the diagonal blue line. (C) Tabular summary of the FICI values of Gwt1 and Mcd4 inhibitors tested against *C. albicans* and *S. cerevisiae*. (D) *GWT1* and *MCD4* exhibit a synthetic lethal genetic interaction in yeast. Spore progeny of a *gwt1-20::natMX4/GWT1*, *mcd4-500::kanMX4/MCD4* double-heterozygous diploid strain dissected onto synthetic complete (SC) medium and incubated at 30 °C for 4 days. Large colonies are identified as wild-type; smaller colonies are either *gwt1-20* or *mcd4-500* haploids (depending on drug resistance marker; natR, (black square); kanR, (white circle)), and microcolonies maintaining both markers are *gwt1-20*, *mcd4-500* double mutants.

glucan sufficient to induce the pro-inflammatory cytokine TNF α ,²⁷ we tested whether these observations are specific to gepinacin or instead more broadly reflect a GPI pathway depletion phenotype. Indeed, immunostaining and immunofluorescence microscopy of *C. albicans* cells treated at sub-MIC

levels with Gwt1 inhibitors (G365 and G884) and Mcd4 inhibitors (M743 and M720) all exhibited elevated immunoreactivity by a β -(1–3)-glucan antibody equal to or greater (e.g., M720) than achieved by gepinacin drug treatment (Figure 6A). Conversely, β -(1–3)-glucan antibody was completely unreactive to mock-treated cells or the control antifungal agent, fluconazole, under identical conditions tested (Figure 6A). Importantly, failure to observe β -(1–3)-glucan antibody immunoreactivity in fluconazole-treated cells strongly argues that the effect we detect is GPI pathway-based rather than an indirect consequence of dead or lysed cells releasing β -(1–3)-glucan. Moreover, elevated exposure of β -(1–3)-glucan led to significant (2–2.5-fold) increases in secreted TNF α by mouse macrophages co-incubated with *C. albicans* cells when specifically drug-treated with all GPI inhibitors (Figure 6B). Thus, unlike that of other essential cellular processes targeted by current antifungal drugs, new agents targeting GPI biosynthesis may possess dual attributes in a therapeutic context, namely, antiproliferative properties against the pathogen as well as immune stimulatory effects by the host.

In Vivo Efficacy of M720 in a Murine Infection Model of Candidiasis. On the basis of the superior potency and spectrum of M743 versus G365 or G884, we chose to examine its potential efficacy in a murine infection model of candidiasis. However, M743 displays significant instability in mouse plasma, where its ester-linked side chain is completely released within 2 h of incubation, rendering the product inactive (MIC shift from 0.5 to >25 $\mu\text{g}/\text{mL}$) (Figure S7). To address this issue, the M743 esterase sensitive linkage was replaced with a carbamate linkage, yielding the compound M720, which displayed highly favorable stability in mouse plasma (Figure S7) without any loss in antifungal activity (Table 1). Importantly, CaFT profiles of M720 and M743 are indistinguishable (Figure S3), and M720^R analysis in yeast unequivocally identifies causal drug-resistant mutations mapping to *MCD4*, thus demonstrating M720 remains highly selective to its cognate target.

To evaluate M720 antifungal efficacy, *C. albicans* infected mice were administered M720 by intraperitoneal (ip) injection twice daily (bid) or once daily (qd) over a 2 or 4 day dosing regimen in an abbreviated candidiasis model where fungal burden was quantified within kidneys 4 days after infectious challenge. Fungal burden in sham-treated mice exceeded >6 \log_{10} CFU/g kidney. Conversely, in each M720 dosing regimen tested fungal burden was significantly reduced in a dose-dependent manner, with nearly a 2 \log_{10} reduction of *C. albicans* CFU/g kidney achieved at the 50 mg/kg dose (Figure 7). Importantly, no mice exhibited gross effects of toxicity, including ruffled fur, lethargy, tremors, or other gross effects in any of the M720 treatment groups. These data provide pharmacological demonstration that M720 provides a beneficial antifungal therapeutic effect in a systemic infection model of candidiasis and broadens the relevance of GPI biosynthesis as an important target pathway for developing new antifungal agents.

Here we have used a *C. albicans* genome-wide reverse genetics small molecule screening strategy (CaFT) combined with *S. cerevisiae* drug resistance mutant selection and whole genome NGS to identify and mechanistically characterize multiple antifungal inhibitors specifically targeting GPI precursor biosynthesis. G365 and G884 display potent anti-*Candida* activity and prominent *GWT1* depletion profiles by CaFT analysis reproduced by directly examining growth rates of *gwt1/GWT1* and other signature heterozygote strains with

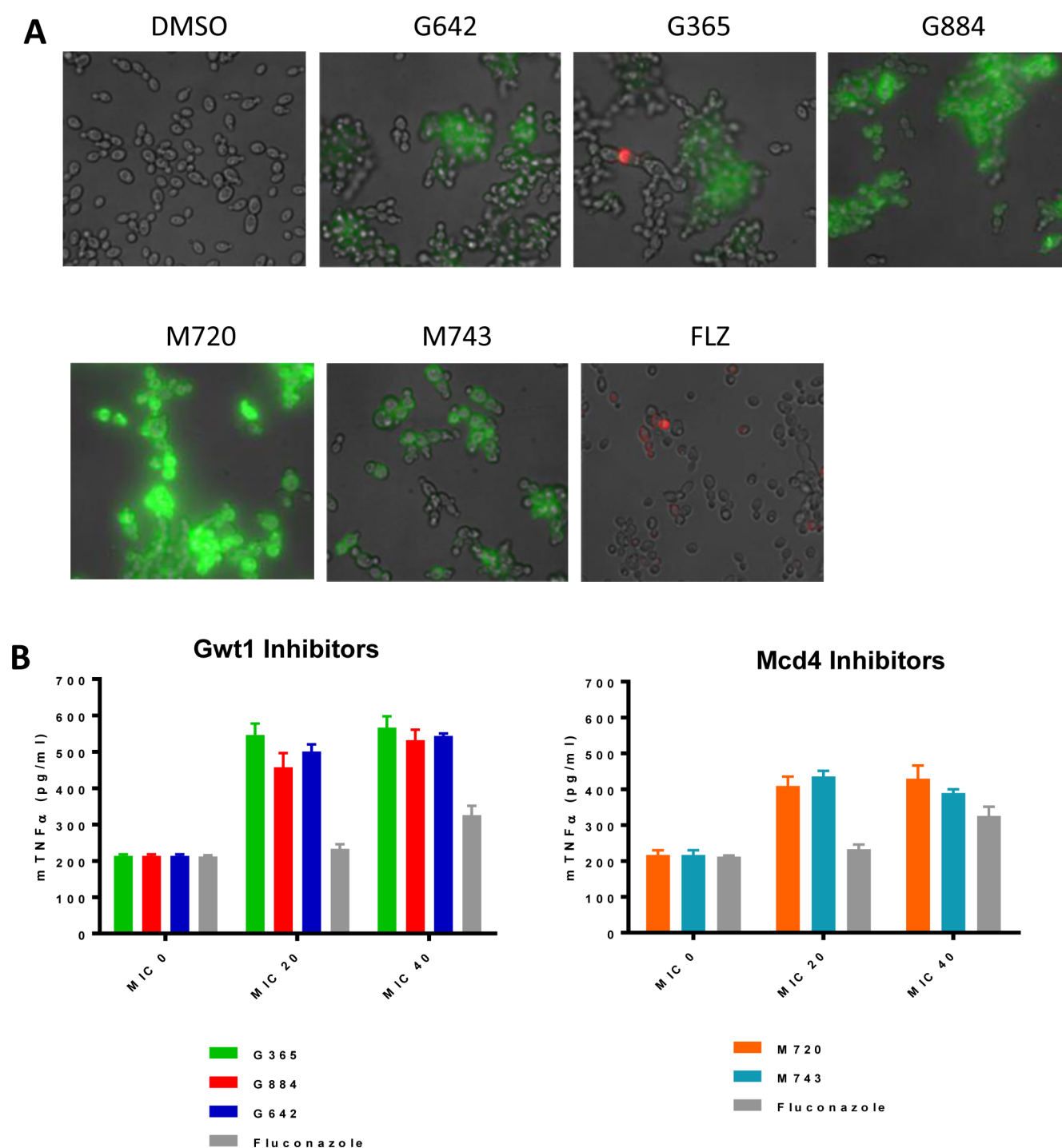


Figure 6. GPI inhibitors unmask cell surface β -glucan and induce TNF α secretion in macrophages. (A) Fluorescence photomicrographs of *C. albicans* and β -(1–3)-glucan (green) immunoreactivity with anti- β -(1–3)-glucan antibody following treatment with GPI inhibitors (G365, G884, M743, M720, and gepinacin), the control antifungal agent fluconazole (FLZ), or DMSO. All drug treatments were performed at a drug concentration equivalent to IC₄₀ to ensure immunoreactivity is not indirectly due to cell death. Propidium iodide staining confirms minimal cell death for all GPI inhibitors tested at their IC₄₀ concentration versus fluconazole. Images are merged fluorescence and phase contrast. (B) ELISA quantification of secreted TNF α by RAW264.7 macrophage co-incubated with *C. albicans* strain 2323^S treated at MIC₂₀ and MIC₄₀ values for each of the above agents.

altered susceptibility to each compound. Drug resistance mutant isolation and whole genome NGS of G884 resistors provide a genetic confirmation of the compound's hypothesized MOA as multiple independently derived G884^R mutants map to *GWT1* without any additional nonsynonymous mutations present in the genome of these isolates. As the Gwt1:G132W

mutant displays marked cross-resistance to gepinacin, these antifungal compounds likely share a common mechanism of inhibiting Gwt1p activity. Despite our inability to isolate drug-resistant mutants to G365 due to its poor activity against bakers' yeast (even a *pdr5* Δ strain; MIC > 64 μ g/mL), G365 and G884 share remarkably related CaFT profiles and

Efficacy of parenterally administered M720 and caspofungin against a disseminated *Candida albicans* MY1055 infection in DBA/2 mice

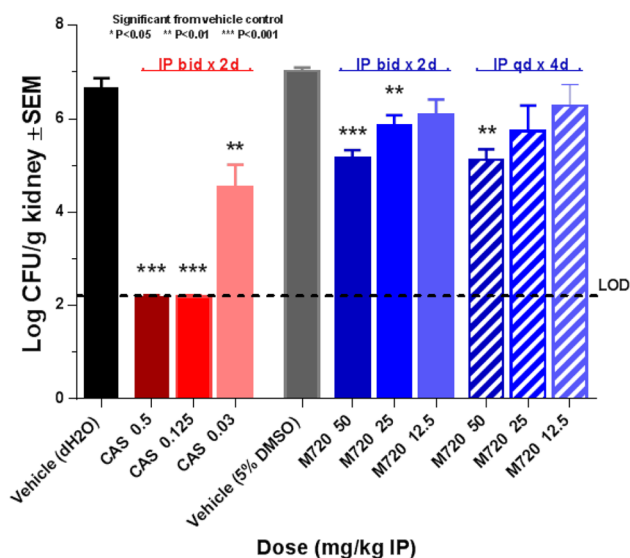


Figure 7. In vivo efficacy of M720 in a murine systemic infection model of candidiasis. DBA/2 mice were infected with *C. albicans* strain MY1055 and treated ip with M720 or caspofungin (CAS) at the indicated doses (mg/kg) either twice daily (bid) or once daily (qd). Kidneys were aseptically collected at 4 days after infectious challenge, and log reduction of colony-forming units (CFU) per gram of kidney tissue was calculated on the basis of kidney burden of vehicle-treated (5% DMSO or H₂O, respectively) control group. Note the limit of detection (LOD) is 1.5×10^2 CFU/g, as indicated by the dashed line. (*): $P < 0.05$, (**): $P < 0.01$, and (***) $P < 0.001$ significance versus vehicle control.

heterozygote strain sensitivities as observed for gepinacin. Importantly, both compounds also inhibit Gwt1-mediated acylation activity in a dose-dependent manner in a yeast cell-free system, thus unambiguously defining Gwt1 as the target of these agents.

CaFT screening of unfractionated natural product extracts also led to the identification of a second class of GPI inhibitors targeting Mcd4. Like the initial extract, M743 (the purified bioactive compound) as well as its semisynthetic analogue, M720, both revealed a striking *mcd4* heterozygote chemical sensitivity phenotype as well as highly related CaFT secondary profiles biologically relevant to the MOA of these agents. In support of their predicted MOA, whole genome NGS of numerous independently derived drug-resistant yeast isolates again identified a common missense mutation, in this case mapping to *MCD4*, thus demonstrating these mutations are causal for the drug resistance. Reciprocally, drug resistance mutant analysis reinforces the extent of mechanistic insight CaFT profiling can provide to study the MOA of growth inhibitory small molecules. Whereas inhibitors of distinct enzymes in a common pathway such as GPI biosynthesis might be expected (at best) to share highly related CaFT profiles suggestive of the pathway inhibited by these compounds, instead it was possible to differentiate proximal (i.e., molecular target Gwt1 or Mcd4) from distal (i.e., pathway and/or terminal phenotype) effects by these agents. Collectively, these results demonstrate how “togglng” between robust whole cell screening and MOA determination strategies best suited for different fungal species may be combined to

effectively identify and mechanistically characterize new target-specific growth inhibitory agents.

GPI inhibitors targeting multiple steps in GPI synthesis also provide valuable reagents to broadly explore the pharmacological consequences of interdicting this essential cellular process and examine whether the pathway is suitable for developing new antifungal agents. Gwt1-specific inhibitors are fungistatic, show potential antifungal spectrum, minimal cytotoxicity, and substantial specificity to their fungal target when tested directly in yeast expressing either Gwt1 or the human orthologue, Pig-W. Alternatively, whereas Mcd4 inhibitors are also fungistatic and demonstrate a striking potency and spectrum that parallels existing SOC antifungal drugs, notable cytotoxicity against multiple human cell lines was observed. On the basis of control antifungal agents similarly tested, the observed cytotoxicity of the M720 derivative of M743 appears somewhat between that of amphotericin B and fluconazole. Importantly, M720 displays significant efficacy in a murine infection model of candidiasis without any obvious host toxicity observed even at the highest dose tested. Indeed, the acute toxicity of M720 (i.e., the lethal dose of the drug for 50% of the mice treated (LD_{50})) is >300 mg/kg when administered IP; conversely, the LD_{50} of amphotericin B is only 4.5 mg/kg in mice. Unexpectedly, we also demonstrate that Gwt1 and Mcd4 inhibitors display strong synergistic antifungal activity, which is confirmed to be target-based as genetic studies in yeast demonstrate a synthetic lethal genetic interaction between *GWT1* and *MCD4*.

Intriguingly, *C. albicans* treated with any of the identified Gwt1 or Mcd4 inhibitors also led to cell wall alterations that specifically expose β -(1–3)-glucan that is normally masked from immune recognition by cell surface GPI-anchored mannoproteins.³⁷ As anti- β -glucan antibodies have been characterized in mouse and human sera,³⁸ GPI pathway inhibitors may therefore augment pathogen recognition by the immune system. This elevated exposure of β -(1–3)-glucan also led to a significant increase in secreted TNF α secretion by mouse macrophages co-incubated with *C. albicans* cells when specifically treated with all GPI inhibitors tested. These results suggest that an elevated immune response may also possibly exist in a therapeutic context and that inhibitors to other steps in GPI biosynthesis may similarly display a dual benefit: directly inhibiting growth of the pathogen at higher drug concentrations and at lower concentrations indirectly enhancing the host immune response. Accordingly, GPI inhibitors may also possess a unique advantage as combination agents to enhance therapeutic efficacy if paired with existing clinically used antifungals.

M743 was first described by Riezman and colleagues (named YW3548¹⁷) and Merck researchers (named BE49385A^{39,40}) as a novel terpenoid lactone-based natural product inhibitor of GPI biosynthesis in yeast. Initial studies suggested M743 likely inhibits Gpi10, responsible for the addition of the third mannose onto the GPI precursor.^{18,41} However, subsequent genetic and biochemical studies demonstrated *MCD4*, encoding a phosphoethanolamine transferase responsible for addition of phosphoethanolamine (EtNP) to C-2 of the first mannose (Man1) of the GPI precursor, and the mammalian orthologue, *Pig-n*, serve as the likely target of M743. In support of this conclusion, (i) yeast *mcd4-174* mutants as well as mammalian cell lines deleted of *Pig-n* accumulate a GPI precursor intermediate lacking EtNP addition to Man1 and which is identically detected in M743-treated cells,^{20,21,23} (ii) protozoa

lack such a modification to Man1 and are intrinsically resistant to this agent,^{18,42} and (iii) overexpression of *MCD4* suppresses the growth inhibitory effect of M743.²⁰ Drug resistance selection and whole genome NGS studies described here now provide an unequivocal genetic demonstration of *Mcd4* as the drug target to this agent.

As GPI biosynthesis is an essential process broadly conserved between fungi and higher eukaryotes, it may seem somewhat counterintuitive how inhibitors of this pathway would have potential therapeutic potential as novel antifungal agents for clinical use. However, the *Gwt1* inhibitor E1210 has recently entered antifungal clinical development,^{26,43} and potential cytotoxicity limitations appear mitigated by the high specificity of the agent to its fungal target versus its human counterpart. Gepinacin, G884, and G365 also display clear target selectivity and acceptable therapeutic index (>25-fold) between *C. albicans* and human cell lines, thus providing similarly desirable starting points. Therefore, like clinically successful azoles (whose human orthologues are cytochrome P450 enzymes), success in developing *Gwt1* inhibitors will ultimately require exquisite target selectivity. *Mcd4* inhibitors M743 and M720, on the other hand, appear to demonstrate a relatively narrow (~10-fold) therapeutic index against *C. albicans* and *A. fumigatus* versus human cell lines tested. It is also known that M743 is a potent inhibitor of GPI biosynthesis not only in yeast but mammalian cell lines as well.²⁰ Thus, the compound serves as a robust and well-validated chemical probe for chemical biology studies across diverse GPI-producing organisms. However, whether the fungal selectivity of M743 may be improved by medicinal chemistry optimization is unknown and not easily assisted by applying structure-based drug design due to the complex membrane topology of *Mcd4*. Considering *Mcd4* is demonstrated to be druggable, perhaps the identification of a new structurally distinct inhibitor series may be required, as recently reported using an analogous screening strategy developed in *S. cerevisiae*.⁴⁴

Regardless of the antifungal development potential of M743, a deeper understanding of the specific phenotypes associated with the loss of *MCD4* or *Pig-n* in yeast and mammalian cell lines offers a conceptual framework for considering new antifungal targets. For example, whereas gene knockout of yeast *MCD4* is lethal and *mcd4-174* temperature-sensitive mutants accumulate the GPI precursor intermediate Man2–Man1–GlcN–(acyl)PI prior to cell death at elevated temperature,²¹ deletion of *PIG-N* in a mouse F9 cell line is not essential.²⁰ Furthermore, full-length GPI polymers (but lacking EtNP attachment at Man1), and GPI-anchored cell wall proteins are correctly localized to the cell surface of *pig-n* deleted cells.²⁰ One particularly attractive hypothesis to explain this disconnect lies in the potential differences in substrate specificity between yeast and mammalian GPI enzymes that function downstream of the *Mcd4*-dependent EtNP–Man1 modification to continue subsequent steps in GPI precursor synthesis.^{20,21} In this case, the non-EtNP-containing GPI intermediate, Man2–Man1–GlcN–(acyl)PI, which accumulates by both genetic and pharmacological inactivation of *Mcd4* and mammalian *PIG-N*, may only adequately serve as substrate for the subsequent GPI mannosyl transferase enzyme in mammals (*PIG-B*) but not the yeast orthologue, *Gpi10*. Consistent with this view, heterologous expression of *PIG-B* suppressed loss of *MCD4* in yeast.²¹ Consequently, *Mcd4* inhibitors may exhibit antifungal-specific growth inhibitory activity by the failure of *Gpi10* (and not *PIG-B*) to sufficiently

recognize the non-EtNP containing substrate GPI intermediate, which would accumulate in drug-treated fungal cells. Such functional differences and alternate substrate specificities between fungal and human orthologues may considerably broaden the current antifungal target set and may have significant implications with regard to the antifungal spectrum and cytotoxicity of cognate inhibitors to such previously neglected drug targets.

METHODS

Materials. All GPI inhibitors were synthesized by Merck Chemistry. Fluconazole, amphotericin B, and cycloheximide were purchased from Sigma-Aldrich. All fungal strains, including the CaFT heterozygote strain set, are from the Merck or Whitehead Institute culture collections and may be obtained upon request. For more information on the strains please see Table S3 in the Supporting Information.

CaFT Screening and GPI Inhibitor Identification. *C. albicans* heterozygote strain construction, CaFT DNA microarrays, experiments, and data analysis linking G884 and G365 to *GWT1* and linking M743 to *MCD4* were performed as previously described.¹³

Microbiological Evaluation of GPI Inhibitors. All fungal susceptibility testing was performed using RPMI media according to CLSI standard protocols, M27-A3 for yeasts and M38-A2 for *A. fumigatus*. Bacterial susceptibility was performed in Mueller–Hinton broth according to CLSI protocol M7-A7. Time–kill assays were performed over a 24 h course with *C. albicans* MY1055 in RPMI media using GPI compounds at 8 times the determined MIC. Amphotericin B was used at 0.5 and 2.0 times the MIC as a cidal control. For *Gwt1* and *Mcd4* inhibitor combinations, each single agent was used at 4 times the MIC. Duplicate cultures were sampled at T0, T2, T6, and T24 hours, diluted and plated on YPD agar, and incubated for 48 h at 37 °C. Plates were imaged on a NuTech Flash and Go colony counter (Neutec Group, Inc.) and the average viable colony-forming units was determined and compared to those of DMSO (0.3%) and AmB controls.

Measurement of TNF α Secretion. Measurements were performed as previously described.²⁷ Briefly, *C. albicans* strain Ca2323 was grown in YPD and treated overnight at the specified sublethal concentrations of antifungal or 0.2% DMSO. Cells were washed three times in 1 mL of water, counted, and resuspended in water at 1×10^7 cells/mL, maintaining the test concentration of the antifungal. Treated cells were incubated with mouse macrophage cell line RAW264.7 (ATCC) at a ratio of 10:1 yeast/macrophage in the presence of the drug. After 2 h, supernatant was collected and TNF α concentration was measured by ELISA (kit DY410, R&D Systems) according to the manufacturer's protocol.

β -Glucan Staining. *C. albicans* cells were grown in YPD overnight at the specified sublethal drug concentrations as described for the measurement of TNF α secretion. Cells were centrifuged and resuspended in 2% BSA/PBS and blocked for 30 min, then pelleted and resuspended in β -1,3-glucan antibody (1:100 in 2% BSA/PBS) and incubated at room temperature for 1 h. Cells were washed five times in phosphate-buffered saline (PBS), pelleted, and resuspended in goat anti-mouse Alexa-488 secondary antibody (1:100 in PBS containing 1 μ g/mL propidium iodide) and incubated at room temperature for 1 h. Cells were washed five times in PBS and transferred to a 384-well PDL-coated imaging plate (Griener). Ten-fold dilutions were transferred to ensure proper density for

visualization. Images were acquired on the BD Pathway 435 Bioimager using a 60× objective and transmitted light, Alexa 488, and propidium iodide channels. Antibody to β -1,3-glucan was purchased from Biosupplies Inc., Australia. Goat anti-mouse Alexa 488 secondary antibody and PI were purchased from Molecular Probes.

Mapping of Drug-Resistant Mutants to GPI Targets.

To obtain drug-resistant mutants, *S. cerevisiae* strain S288c was grown in YPD broth overnight, and 1×10^8 cells were spread on YPD agar plates containing 75 μ g/mL L884 or 60 μ g/mL M720. To obtain mutants in the efflux-deficient background, *S. cerevisiae* strain BY4700/ Δ *pdr5* was similarly grown and spread on 2 μ g/mL of M720 and M743. Plates were incubated at 30 °C for 72–96 h until resistors appeared. Following confirmation of reduced drug susceptibility, genomic DNA was prepared from each isolate using Qiagen's Blood and Tissue Genomic DNA Purification kit. Illumina-based next-generation sequencing was performed by Beijing Genomics Institute, Hong Kong, and all nonsynonymous mutations for each compound were identified and tabulated into heat maps.

Antifungal Efficacy. The *in vivo* efficacy of M720 was determined in a murine model of disseminated candidiasis. Briefly, groups of four or five DBA/2 mice weighing approximately 20 g were challenged intravenously with *C. albicans* MY1055 at 3.08×10^4 CFU/mouse. Mice were treated with M720 or vehicle control (5% DMSO in sterile water) ip twice daily (bid) for 2 days or once-daily (qd) for 4 days (four total doses) beginning immediately after infectious challenge. Caspofungin administered ip, bid \times 2 days (four total doses), was included as a positive control and was compared to sterile water vehicle control. At day 4 after challenge, mice were euthanized, and both kidneys were aseptically collected, weighed, homogenized in sterile saline, serially diluted, and plated onto Sabouraud's dextrose agar. Plates were incubated at 35 °C for 30–48 h and then colonies enumerated. *Candida* burden (CFU/gram kidney) in M720 or caspofungin treatment groups was compared to the relevant vehicle control, and significance was determined using the Student's paired two-tailed *t* test. Comparisons were considered significant at the $\alpha = 0.05$ level.

All procedures were performed in accordance with the highest standards for the humane handling, care, and treatment of research animals and were approved by the Merck Institutional Animal Care and Use Committee (IACUC).

In Vitro Acylation of Gwt1p Inhibitors. Assays were performed as described previously in a published protocol,²⁵ except that UDP[³H]GlcNAc was used instead of [¹⁴C] 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.

Mammalian Cytotoxicity Assessment. To assess potential mammalian cytotoxic effects of GPI inhibitors, two distinct cell proliferation assays were performed. One involved using the Click-iT EdU Alexa Fluor 488 HCS assay (kit C10351, Life Technologies) with minor modifications to the manufacturer's protocol. Images were captured and analyzed using an Acumen eX3 (TTP Labtech Ltd.) laser scanning cytometer. Hoechst 33342 (H3530, LifeTech) was used as a nuclear stain to facilitate enumeration of total cell number. Briefly, HeLa cells were seeded at 4000 cells/well in 384-well poly-D-lysine (PDL)-coated plates (Greiner, 781946) in 25 μ L of culture medium (Optimem I, Life Technologies) per well. A total of 20 2-fold

serial dilutions of GPI compounds, cycloheximide, or vehicle control were prepared and added (0.25 μ L per well) to obtain the desired working concentration. EdU was added to obtain a final concentration of 5 μ M. Cycloheximide was used as a toxic control compound. Percent EdU inhibition and cell count inhibition are calculated by normalizing against the DMSO control (maximum incorporation). Alternatively, the more traditional MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay, which measures mitochondrial metabolic activity, was performed using HepG2 and THLE-2 human cells lines over a 72 h period.

■ ASSOCIATED CONTENT

📄 Supporting Information

The following file is available free of charge on the ACS Publications website at DOI: 10.1021/id5000212.

Tables S1–S4 contain general strain information, mammalian cytotoxicity results, and expanded bacterial spectrum testing; Figures S1–S7 contain additional data describing CaFT analysis, heterozygote spot testing, *Aspergillus* zones of inhibition, time–kill assays, conditional mutant response, and Mcd4 inhibitor stability in plasma (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*(T.R.) E-mail: terry_roemer@merck.com.

Author Contributions

†P.A.M. and C.A.M. contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Carl Balibar and Juliana Malinverni for critically reading the manuscript and Luke Whitesell for technical assistance. This work was supported in part by a grant from Genome Canada and Genome Quebec.

■ ABBREVIATIONS

CaFT, *C. albicans* fitness test; GPI, glucosylphosphatidylinositol; MOA, mechanism of action; EtNP, ethanolamine phosphate; Man1, mannose 1; NGS, next-generation sequencing; TLRs, Toll-like receptors; COPI, coat protein complex I; MIC, minimal inhibitor concentration; GlcN-PI, glucosamine phosphatidylinositol; UPR, unfolded protein response; AmB, amphotericin B; FLZ, fluconazole; CSP, caspofungin; FIC, fractional inhibitory concentrations; SOC, standard of care

■ REFERENCES

- (1) Pfaller, M. A., and Diekema, D. J. (2007) Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* 20, 133–163.
- (2) Brown, G. D., Denning, D. W., Gow, N. A., Levitz, S. M., Netea, M. G., and White, T. C. (2012) Hidden killers: human fungal infections. *Sci. Transl. Med.* 4, 165rv13 DOI: 10.1126/scitranslmed.3004404.
- (3) Ostrosky-Zeichner, L., Casadevall, A., Galgiani, J. N., Odds, F. C., and Rex, J. H. (2010) An insight into the antifungal pipeline: selected new molecules and beyond. *Nat. Rev. Drug Discovery* 9, 719–727.
- (4) Roemer, T., and Krysan, D. J. Antifungal drug development: challenges, unmet clinical needs, and new approaches. (2014) *Cold Spring Harb Perspect. Med.* 4 (5), 10.1101/cshperspect.a019703.

- (5) Roemer, T., Jiang, B., Davison, J., Ketela, T., Veillette, K., Breton, A., Tandia, F., Linteau, A., Sillaots, S., Marta, C., Martel, N., Veronneau, S., Lemieux, S., Kauffman, S., Becker, J., Storms, R., Boone, C., and Bussey, H. (2003) Large-scale essential gene identification in *Candida albicans* and applications to antifungal drug discovery. *Mol. Microbiol.* 50, 167–181.
- (6) Xu, D., Jiang, B., Ketela, T., Lemieux, S., Veillette, K., Martel, N., Davison, J., Sillaots, S., Bachewich, C., Bussey, H., Youngman, P., and Roemer, T. (2007) Genome-wide fitness test and mechanism-of-action studies of inhibitory compounds in *Candida albicans*. *PLoS Pathog.* 3, e92.
- (7) Roemer, T., Xu, D., Singh, S. B., Parish, C. A., Harris, G., Wang, H., Davies, J. E., and Bills, G. F. (2011) Confronting the challenges of natural product-based antifungal discovery. *Chem. Biol.* 18, 148–164.
- (8) Shoemaker, D. D., Lashkari, D. A., Morris, D., Mittmann, M., and Davis, R. W. (1996) Quantitative phenotypic analysis of yeast deletion mutants using a highly parallel molecular bar-coding strategy. *Nat. Genet.* 14, 450–456.
- (9) Giaever, G., Shoemaker, D. D., Jones, T. W., Liang, H., Winzeler, E. A., Astromoff, A., and Davis, R. W. (1999) Genomic profiling of drug sensitivities via induced haploinsufficiency. *Nat. Genet.* 21, 278–283.
- (10) Jiang, B., Xu, D., Allocco, J., Parish, C., Davison, J., Veillette, K., Sillaots, S., Hu, W., Rodriguez-Suarez, R., Trosok, S., Zhang, L., Li, Y., Rahkhoodae, F., Ransom, T., Martel, N., Wang, H., Gauvin, D., Wiltsie, J., Wisniewski, D., Salowe, S., Kahn, J. N., Hsu, M. J., Giacobbe, R., Abruzzo, G., Flattery, A., Gill, C., Youngman, P., Wilson, K., Bills, G., Platas, G., Pelaez, F., Diez, M. T., Kauffman, S., Becker, J., Harris, G., Liberator, P., and Roemer, T. (2008) PAP inhibitor with in vivo efficacy identified by *Candida albicans* genetic profiling of natural products. *Chem. Biol.* 15, 363–374.
- (11) Xu, D., Ondeyka, J., Harris, G. H., Zink, D., Kahn, J. N., Wang, H., Bills, G., Platas, G., Wang, W., Szwczak, A. A., Liberator, P., Roemer, T., and Singh, S. B. (2011) Isolation, structure, and biological activities of fellutamides C and D from an undescribed *Metulocladosporiella* (Chaetothyriales) using the genome-wide *Candida albicans* fitness test. *J. Nat. Prod.* 74, 1721–1730.
- (12) Rodriguez-Suarez, R., Xu, D., Veillette, K., Davison, J., Sillaots, S., Kauffman, S., Hu, W., Bowman, J., Martel, N., Trosok, S., Wang, H., Zhang, L., Huang, L. Y., Li, Y., Rahkhoodae, F., Ransom, T., Gauvin, D., Douglas, C., Youngman, P., Becker, J., Jiang, B., and Roemer, T. (2007) Mechanism-of-action determination of GMP synthase inhibitors and target validation in *Candida albicans* and *Aspergillus fumigatus*. *Chem. Biol.* 14, 1163–1175.
- (13) Xu, D., Sillaots, S., Davison, J., Hu, W., Jiang, B., Kauffman, S., Martel, N., Ocampo, P., Oh, C., Trosok, S., Veillette, K., Wang, H., Yang, M., Zhang, L., Becker, J., Martin, C. E., and Roemer, T. (2009) Chemical genetic profiling and characterization of small-molecule compounds that affect the biosynthesis of unsaturated fatty acids in *Candida albicans*. *J. Biol. Chem.* 284, 19754–19764.
- (14) Richard, M. L., and Plaine, A. (2007) Comprehensive analysis of glycosylphosphatidylinositol-anchored proteins in *Candida albicans*. *Eukaryot. Cell.* 6, 119–133.
- (15) Pittet, M., and Conzelmann, A. (2007) Biosynthesis and function of GPI proteins in the yeast *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* 1771, 405–420.
- (16) Klis, F. M., Sosinska, G. J., de Groot, P. W., and Brul, S. (2009) Covalently linked cell wall proteins of *Candida albicans* and their role in fitness and virulence. *FEMS Yeast Res.* 9, 1013–1028.
- (17) Orlean, P., and Menon, A. K. (2007) Thematic review series: Lipid posttranslational modifications. GPI anchoring of protein in yeast and mammalian cells, or: how we learned to stop worrying and love glycosphospholipids. *J. Lipid Res.* 48, 993–1011.
- (18) Sütterlin, C., Horvath, A., Gerold, P., Schwarz, R. T., Wang, Y., Dreyfuss, M., and Riezman, H. (1997) Identification of a species-specific inhibitor of glycosylphosphatidylinositol synthesis. *EMBO J.* 16, 6374–6383.
- (19) Wang, Y., Dreyfuss, M., Ponelle, M., Oberer, L., Riezman, H. A. Tetracarboxycyclic sesterpene skeleton from the fungus *Codinaea simplex*. *Tetrahedron* 54, 6415–6426. [10.1016/S0040-4020\(98\)00322-6](https://doi.org/10.1016/S0040-4020(98)00322-6)
- (20) Hong, Y., Maeda, Y., Watanabe, R., Ohishi, K., Mishkind, M., Riezman, H., and Kinoshita, T. (1999) Pig-n, a mammalian homologue of yeast Mcd4p, is involved in transferring phosphoethanolamine to the first mannose of the glycosylphosphatidylinositol. *J. Biol. Chem.* 274, 35099–35106.
- (21) Wiedman, J. M., Fabre, A. L., Taron, B. W., Taron, C. H., and Orlean, P. (2007) *In vivo* characterization of the GPI assembly defect in yeast mcd4–174 mutants and bypass of the Mcd4p-dependent step in mcd4Delta cells. *FEMS Yeast Res.* 7, 78–83.
- (22) Tsukahara, K., Hata, K., Nakamoto, K., Sagane, K., Watanabe, N. A., Kuromitsu, J., Kai, J., Tsuchiya, M., Ohba, F., Jigami, Y., Yoshimatsu, K., and Nagasu, T. (2003) Medicinal genetics approach towards identifying the molecular target of a novel inhibitor of fungal cell wall assembly. *Mol. Microbiol.* 48, 1029–1042.
- (23) Gaynor, E. C., Mondésert, G., Grimme, S. J., Reed, S. I., Orlean, P., and Emr, S. D. (1999) MCD4 encodes a conserved endoplasmic reticulum membrane protein essential for glycosylphosphatidylinositol anchor synthesis in yeast. *Mol. Biol. Cell* 10, 627–648.
- (24) Dennehy, K. M., and Brown, G. D. (2007) The role of the beta-glucan receptor dectin-1 in control of fungal infection. *J. Leukoc. Biol.* 82, 253–258.
- (25) Umemura, M., Okamoto, M., Nakayama, K., Sagane, K., Tsukahara, K., Hata, K., and Jigami, Y. (2003) GWT1 gene is required for inositol acylation of glycosylphosphatidylinositol anchors in yeast. *J. Biol. Chem.* 278, 23639–23647.
- (26) Watanabe, N. A., Miyazaki, M., Horii, T., Sagane, K., Tsukahara, K., and Hata, K. (2012) E1210, a new broad-spectrum antifungal, suppresses *Candida albicans* hyphal growth through inhibition of glycosylphosphatidylinositol biosynthesis. *Antimicrob. Agents Chemother.* 56, 960–971.
- (27) McLellan, C. A., Whitesell, L., King, O. D., Lancaster, A. K., Mazitschek, R., and Lindquist, S. (2012) Inhibiting GPI anchor biosynthesis in fungi stresses the endoplasmic reticulum and enhances immunogenicity. *ACS Chem. Biol.* 7, 1520–1528.
- (28) Lee, M. C., Miller, E. A., Goldberg, J., Orci, L., and Schekman, R. (2004) Bi-directional protein transport between the ER and Golgi. *Annu. Rev. Cell Dev. Biol.* 20, 87–123.
- (29) Kodera, C., Yorimitsu, T., Nakano, A., and Sato, K. (2011) Sed4p stimulates Sar1p GTP hydrolysis and promotes limited coat disassembly. *Traffic* 12, 591–599.
- (30) Letourneur, F., Gaynor, E. C., Hennecke, S., Demolliere, C., Duden, R., Emr, S. D., Riezman, H., and Cosson, P. (1994) Coatamer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. *Cell* 79, 1199–1207.
- (31) Ma, W., and Goldberg, J. (2013) Rules for the recognition of dilysine retrieval motifs by coatamer. *EMBO J.* 32, 926–937.
- (32) Carvajal, E., van den Hazel, H. B., Cybularz-Kolaczowska, A., Balzi, E., and Goffeau, A. (1997) Molecular and phenotypic characterization of yeast PDR1 mutants that show hyperactive transcription of various ABC multidrug transporter genes. *Mol. Gen. Genet.* 256, 406–415.
- (33) Sagane, K., Umemura, M., Ogawa-Mitsuhashi, K., Tsukahara, K., Yoko-o, T., and Jigami, Y. (2011) Analysis of membrane topology and identification of essential residues for the yeast endoplasmic reticulum inositol acyltransferase Gwt1p. *J. Biol. Chem.* 286, 14649–14658.
- (34) Zhu, Y., Vionnet, C., and Conzelmann, A. (2006) Ethanolaminephosphate side chain added to glycosylphosphatidylinositol (GPI) anchor by mcd4p is required for ceramide remodeling and forward transport of GPI proteins from endoplasmic reticulum to Golgi. *J. Biol. Chem.* 281, 19830–19839.
- (35) Miyazaki, M., Horii, T., Hata, K., Watanabe, N. A., Nakamoto, K., Tanaka, K., Shirotori, S., Murai, N., Inoue, S., Matsukura, M., Abe, S., Yoshimatsu, K., and Asada, M. (2011) *In vitro* activity of E1210, a novel antifungal, against clinically important yeasts and molds. *Antimicrob. Agents Chemother.* 55, 4652–4658.

(36) Poulain, D., and Jouault, T. (2004) *Candida albicans* cell wall glycans, host receptors and responses: elements for a decisive crosstalk. *Curr. Opin. Microbiol.* 7, 342–349.

(37) Wheeler, R. T., and Fink, G. R. (2006) A drug-sensitive genetic network masks fungi from the immune system. *PLoS Pathog.* 2, e35.

(38) Ishibashi, K., Yoshida, M., Nakabayashi, I., Shinohara, H., Miura, N. N., Adachi, Y., and Ohno, N. (2005) Role of anti-beta-glucan antibody in host defense against fungi. *FEMS Immunol.* 44, 99–109 DOI: 10.1016/j.femsim.2004.12.012.

(39) Kushida, H., Nakajima, S., Uchiyama, S., Nagashima, M., Kojiri, K., Kawamura, K., and Suda, H. (1999) Antifungal substances BE-49385 and process for their production. U.S. Patent 5,928,910.

(40) Hirano, A., Sugiyama, E., Kondo, H., Suda, H., Ogawa, H., and Kojiri, K. (2001) Sesterterpene derivatives exhibiting antifungal activities U.S. Patent 6,303,797 B1.

(41) Sütterlin, C., Escribano, M. V., Gerold, P., Maeda, Y., Mazon, M. J., Kinoshita, T., Schwarz, R. T., and Riezman, H. (1998) *Saccharomyces cerevisiae* GPI10, the functional homologue of human PI3-B, is required for glycosylphosphatidylinositol-anchor synthesis. *Biochem. J.* 332, 153–159.

(42) McConville, M. J., and Ferguson, M. A. J. (1993) The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. *Biochem. J.* 294, 305–324.

(43) Hata, K., Horii, T., Miyazaki, M., Watanabe, N. A., Okubo, M., Sonoda, J., Nakamoto, K., Tanaka, K., Shirotori, S., Murai, N., Inoue, S., Matsukura, M., Abe, S., Yoshimatsu, K., and Asada, M. (2011) Efficacy of oral E1210, a new broad-spectrum antifungal with a novel mechanism of action, in murine models of candidiasis, aspergillosis, and fusariosis. *Antimicrob. Agents Chemother.* 55, 4543–4551.

(44) Lee, A. Y., St. Onge, R. P., Proctor, M. J., Wallace, I. M., Nile, A. H., Spagnuolo, P. A., Jitkova, Y., Gronda, M., Wu, Y., Kim, M. K., Cheung-Ong, K., Torres, N. P., Spear, E. D., Han, M. K., Schlecht, U., Suresh, S., Duby, G., Heisler, L. E., Surendra, A., Fung, E., Urbanus, M. L., Gebbia, M., Lissina, E., Miranda, M., Chiang, J. H., Aparicio, A. M., Zeghouf, M., Davis, R. W., Cherfils, J., Boutry, M., Kaiser, C. A., Cummins, C. L., Trimble, W. S., Brown, G. W., Schimmer, A. D., Bankaitis, V. A., Nislow, C., Bader, G. D., and Giaever, G. (2014) Mapping the cellular response to small molecules using chemogenomic fitness signatures. *Science* 344, 208–211.

■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on December 12, 2014, with an error in Figure 2. The corrected version reposted with the issue on January 9, 2015.