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Perspective

Confronting the Challenges of Natural Product-Based Antifungal Discovery

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Starting with the discovery of penicillin, the pharmaceutical industry has relied extensively on natural products (NPs) as an unparalleled source of bioactive small molecules suitable for antibiotic development. However, the discovery of structurally novel and chemically tractable NPs with suitable pharmacological properties as antibiotic leads has waned in recent decades. Today, the repetitive "rediscovery" of previously known NP classes with limited antibiotic lead potential dominates most industrial efforts. This limited productivity, exacerbated by the significant financial and resource requirements of such activities, has led to a broad de-emphasis of NP research by most pharmaceutical companies, including most recently Merck. Here we review our strategies—both technological and philosophical—in addressing current antifungal discovery bottlenecks in target identification and validation and how such efforts may improve NP-based antimicrobial discoveries when aligned with NP screening and dereplication.

Bioactive natural products (NPs) and their semisynthetic derivatives have historically provided an unparalleled source of therapeutic agents to treat infectious diseases. Indeed, approximately 80% of all available clinically used antibiotics are directly (or indirectly) derived from NPs (Newman et al., 2003). This success exploits the optimized characteristics of microbial-produced small molecules, namely their immense chemical diversity, intrinsic cell permeability, and target specificity versus that typically reflected in synthetic or combinatorial libraries. NPs reflect a reservoir of privileged chemical scaffolds which have been naturally selected by microbes to specifically interact with a diversity of biological targets in the environment, thereby providing a fitness advantage to the producing organism. Based on their target selectivity and intrinsic pharmacological properties, naturally produced small molecules also serve as critically important starting points in drug development. Whereas antibacterial discovery has yielded over a dozen distinct and clinically relevant chemical scaffolds (Walsh, 2003), all of which, excluding oxazolidinones, quinolones, and sulfonamides, are naturally derived, the breadth of antifungals is noticeably more limited and represented by only three structural classes, of which two (polyenes and echinocandins) are naturally derived and the third (azoles) is synthetic in origin (Ostrosky-Zeichner et al., 2010).

Despite an unmet medical need for more efficacious antifungal agents and the seemingly immense opportunity to address this by discovering novel antifungal leads from natural sources, a number of technical impediments remain (Koehn and Carter, 2005). Fermentation extracts from microbial cultures are highly complex mixtures of hundreds (if not thousands) of chemically uncharacterized components. Screening unfractionated materials in biochemical assays is therefore complicated by the unknown abundance of any target-specific inhibitor relative to other components in the mixture which can cause nonspecific inhibition. To address this, diverse chromatographic separation procedures are available to fractionate extracts; however, their application in large scale prior to primary assay screening is both time and resource consuming, particularly when applied on an industrial scale. Also at issue are the choice of the target selected, the extent of target validation, and the ease of adaptation to an assay format compatible for high-throughput screening. Although the full compendium of essential genes in the model yeast *Saccharomyces cerevisiae* has been identified (Winzeler et al., 1999), including orthologous genes in the human fungal pathogens *Candida albicans* (Roemer et al., 2003; Becker et al., 2010) and/or *Aspergillus fumigatus* (Hu et al., 2007), whether any individual gene product adapted for target-based screening is intrinsically "druggable" (i.e., amenable to chemical intervention) is far from certain (Payne et al., 2007). Finally, an important consideration in natural product screening is how to rapidly dereplicate the known and undesirable compounds from the novel desirable chemical matter suitable for antimicrobial lead development.

To address NP-based antimicrobial screening challenges, we and others have turned to chemical-genetic strategies first developed in baker's yeast (Baetz et al., 2004; Giaever et al., 1999, 2004; Lum et al., 2004) and more recently applied to the prominent human fungal pathogen *C. albicans* (Xu et al., 2007, 2009; Rodriguez-Suarez et al., 2007; Jiang et al., 2008). The overarching principle of this approach relies on a phenomenon known as chemically induced haploinsufficiency, where deletion of one allele of the target gene renders the heterozygous deletion strain hypersensitive to cognate inhibitors of the depleted drug

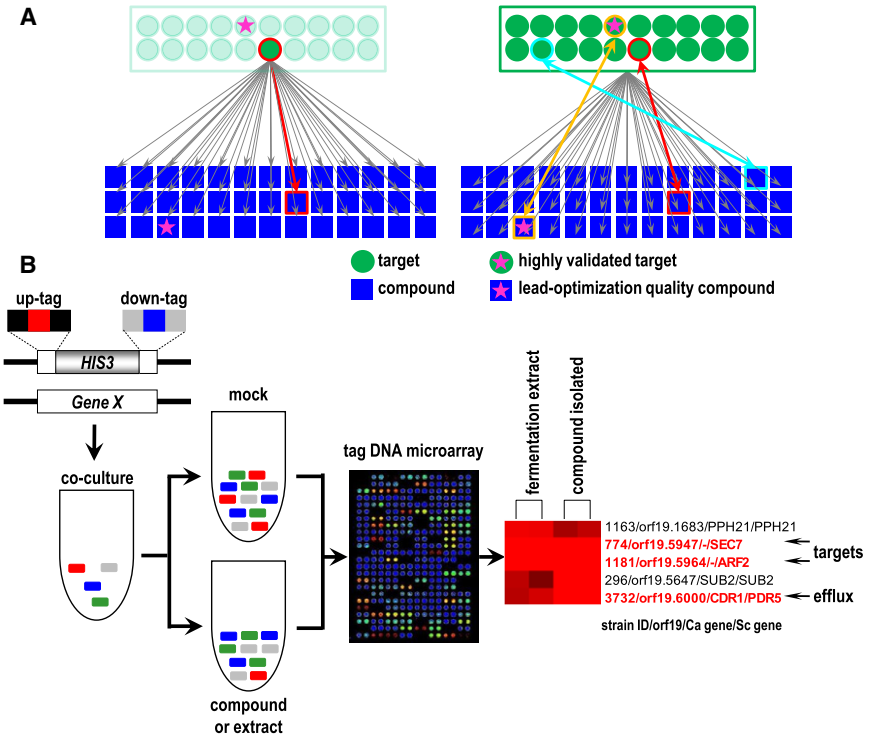

Figure 1. An Overview of the *C. albicans* FT

(A) Traditional target-based screening (left) relies on a somewhat subjective selection of a single target (dark green circle) among multiple alternative target choices (light green circles) to screen against a compound library (blue squares) and identify a cognate inhibitory lead (red arrow). FT screening (right) provides a compound-centric strategy to empirically identify druggable targets. Further, it provides an unbiased genome-scale, target-based whole-cell screening approach (dark green circles; boxed) to more fully link growth-inhibitory agents to such targets (blue arrow), including highly validated targets with promising "high-value" inhibitory lead molecules (yellow arrow) potentially omitted in single sequentially performed target-based screens.

(B) Individual heterozygote strains contain two unique barcodes, up (red) and down (blue) tags, flanked by two pairs of common primer sequences (black for all the up tags, gray for all the down tags) at the deleted allele. The FT is composed of either an ~2900 strain pool covering ~45% of the *C. albicans* genome (the early version we refer to as FT3K; see Xu et al., 2007) or the present version described here, containing ~5400 heterozygote strains (FT5K) and representing ~90% *C. albicans* genome coverage. Aliquots of the pool are treated with an inhibitory compound or NP extract at sublethal concentrations or a mock treatment over 20 population doublings. Provided the inhibitory agent acts through a target-specific MOA and the corresponding strain(s) is included in the assay, such strains (highlighted in red) display an altered growth rate (or fitness, hence the name of the assay) and are specifically depleted from the pool versus the untreated mock control. The relative abundance of each strain is subsequently dereplicated using DNA microarrays competitively hybridized with amplified and labeled barcodes from all strains grown under the two treatments. The response of each strain to the compound is appraised by a normalized z score, with a positive value indicating hypersensitivity and a negative value indicating a relative resistance (Xu et al., 2007). As the deleted allele is double barcoded, we selected one of the z scores with the higher absolute value for each strain in the overall analysis. Multiple experiments were analyzed by hierarchical clustering (cutoff value set at z scores with absolute values greater than 3.5 in at least two experiments) and results are displayed by heat maps. Shown is an FT profile of brefeldin A and an extract containing this NP, with the targets (*SEC7* and *ARF2*) and its principal efflux pump highlighted. The gene annotation was based on those that appear in the *Candida* Genome Database and consisted of (in this order): strain ID number, orf19 designation, *C. albicans* gene, and *S. cerevisiae* homolog. To simplify gene nomenclature, the *S. cerevisiae* ortholog name to all *C. albicans* genes is used throughout the review.

target (Shoemaker et al., 1996). Chemically induced growth phenotypes of individual mutants within a defined library of deletion mutants provide important clues as to the drug target and, therefore, the mechanism of action (MOA) of bioactive compounds (Lehár et al., 2008; Smith et al., 2010). Central to this "compound-centric" approach is the ability to screen bioactive agents across a comprehensive set of potential drug targets, thereby maximizing the likelihood of linking growth-inhibitory compounds to their cognate target and in so doing minimizing the inefficiencies of single, "target-centric" screening, which immensely restricts the available antimicrobial chemical space to a single chosen target of interest (Figure 1A). To perform such analyses comprehensively across a broad drug target set while using minimal amounts of material, strain-specific barcodes have been introduced to uniquely identify individual mutants and facilitate their parallel screening in a coculture (Shoemaker et al., 1996). Challenged with a nonlethal concentration of a single growth-impairing compound or an NP extract mixture, individual strains displaying an altered fitness (either reduced or enhanced) within this genetically defined population are identified by microarray-based methodologies (Figure 1B). Applying this fitness test approach, essential drug targets can be readily linked to their cognate inhibitors when screened against a comprehensive heterozygote deletion mutant strain collection, which could yield additional mechanistic information regarding drug uptake, metabolism, off-target activities, and/or export.

Recently, we demonstrated the utility of *C. albicans* fitness test (FT) screening to mechanistically annotate bioactive components within crude extracts and identified a new NP structural class, named parnafungins (Jiang et al., 2008; Parish et al., 2008; Overy et al., 2009; Adam et al., 2008; Sudhir et al., 2010). Genetic and biochemical data supported the FT-based prediction of their MOA as inhibitors of poly(A) polymerase-mediated mRNA processing (Jiang et al., 2008; Adam et al., 2008). Parnafungins exhibited potent broad-spectrum microbiological activity across medically relevant *Candida* and *Aspergillus* pathogens, were equipotent across existing antifungal drug-resistant *Candida* spp. (as expected for a new agent with novel antifungal MOA), and efficacious in a murine model of disseminated candidiasis—without displaying obvious issues of cytotoxicity (in vitro) or adverse effects (in vivo) (Jiang et al., 2008). Thus, parnafungins provide a clear validation of our NP screening approach to identify new and efficacious NPs with an unanticipated MOA and novel antifungal lead potential.

Here we review this *C. albicans* chemical genetics-based NP screening approach, which was performed at Merck over a 4 year period, to identify novel target-specific antifungal molecules potentially suitable for therapeutic development.

We describe our attempts to improve the end-to-end process of screening NPs, starting with the isolation of new and diverse microbial sources and eliciting induction of cryptic secondary metabolic pathways yielding new bioactive NPs. We also highlight a unique dereplication strategy to predict the presence of known or novel bioactive molecules within NP extracts by coupling LC-MS/FT-MS-based analytical chemistry methodologies and a reference set of known NPs with MOA annotation provided by genome-wide FT screening of unfractionated extracts. Miniaturized, high-throughput separation techniques based on a range of chromatographic techniques were adopted to rapidly isolate prioritized NP bioactivities. In illustrating our approach, we describe the identification and mechanistic characterization of a number of new and known NPs, including aspirochorine and a structurally unique but mechanistically related *Streptomyces* metabolite (yefafungin), a novel pentaene NP predicted to inhibit adenylate cyclase activity and/or cAMP regulation (campafungin), new 1,3-glucan synthase inhibitor analogs of the ergokonin and papulacandin structural classes, and the new proteasome inhibitors fellutamides C and D. Moreover, by integrating these strategies over the screening campaign exceeding 1800 bioactive extracts, we summarize the chemical and mechanistic diversity of NPs identified over this time frame, as well as prominent targets and pathways revealed to be druggable by NPs. In so doing, we share what we have learned through these efforts and discuss what we believe are significant issues that continue to impede NP-based antimicrobial discovery efficiencies.

Antifungal Target Identification and Validation

Pathogenic fungi, especially *Candida* species, have emerged as important and prevalent opportunistic pathogens infecting patients with compromised immunity, including transplant recipients, cancer patients, and those suffering from AIDS (Aperis et al., 2006). *Candida* species now account for 8%–9% of all bloodstream infections, and mortality rates (up to 40%) remain unacceptably high (Pfaller and Diekema, 2007). Indeed, fungal infections remain the fourth-leading cause of life-threatening infections in the hospital setting, and there remains an unmet medical need for more effective, broad-spectrum antifungal drugs (Pfaller and Diekema, 2007). Presently, there are only three classes of marketed antifungal drugs: amphotericin B and its various formulations that target the cell membrane; azoles (e.g., fluconazole, itraconazole, posaconazole, and voriconazole) that block ergosterol biosynthesis; and echinocandins (e.g., caspofungin, micafungin, and anidulafungin), which target cell wall biosynthesis (Ostrosky-Zeichner et al., 2010). However, each drug class has significant therapeutic limitations, ranging from toxicity (e.g., amphotericin B and, to a lesser extent, its liposomal formulations) to drug resistance (particularly azoles, whose clinical use exceeds 20 years) to limited routes of administration (e.g., echinocandins are restricted to i.v. use only).

To expand the antifungal drug armamentarium, considerable effort has been directed toward the discovery of new antifungal targets. As part of this effort, we and others have developed target validation technologies to extensively survey the *C. albicans* genome for essential genes and characterize their potential as antifungal targets (De Backer et al., 2001; Uhl et al., 2003; Roemer et al., 2003; Becker et al., 2010). Defining the compendium of *C. albicans* essential genes is warranted, as *C. albicans* remains the principal human fungal pathogen, its genome has been completely sequenced (Jones et al., 2004) and annotated (Braun et al., 2005), and extensive functional genomic studies in the related but nonpathogenic yeast *S. cerevisiae* provide a rational framework for predicting gene function in *C. albicans*. However, as *C. albicans* is an obligatory diploid, new tools were required to fulfill large-scale functional analysis of its genome. To this end, we developed a two-step *g*ene *r*eplacement *a*nd *c*onditional *e*xpression (GRACE) strategy using PCR methodologies (Roemer et al., 2003). One allele of the gene in question was first deleted to construct a heterozygous deletion mutant, and the expression of the remaining allele was conditionally controlled by replacing its native promoter with a tetracycline-regulatable promoter. Importantly, unlike historical approaches to identifying *C. albicans* essential genes which were limited to gene knockout and statistical analyses to infer essentiality (Noble and Johnson, 2007), the GRACE methodology enables direct comparative analysis of phenotypes under nonrepressing (in the absence of tetracycline) and repressing (in the presence of tetracycline) conditions. Demonstration of the GRACE methodology and its large-scale application to essential gene identification has been performed on approximately 35% of the *C. albicans* genome (Roemer et al., 2003). Importantly, by focusing on suspected orthologs of essential yeast genes, over 850 *C. albicans* genes were directly demonstrated to encode conserved essential functions in the pathogen. Further, the tetracycline-based conditional repression system extends to an in vivo infection setting (Rodriguez-Suarez et al., 2007; Jiang et al., 2008; Xu et al., 2009). To this end, over 100 new antifungal targets have been genetically validated as essential for establishing and maintaining an acute systemic candidiasis infection in immune-competent mice, thereby serving as suitable prophylaxis and acute infection targets for therapeutic intervention (Becker et al., 2010).

Extensive target identification and validation have also been performed in a second principal human fungal pathogen, *A. fumigatus* (Hu et al., 2007). Like *C. albicans*, the *A. fumigatus* natural sexual cycle is cryptic and not compatible with performing classical genetics to identify and validate essential genes (O'Gorman et al., 2009). As *A. fumigatus* is naturally haploid, a one-step promoter replacement strategy was developed in which a native nitrate-regulatable promoter (pNiiA) was used. Regulatable gene expression is achieved on nitrate- (inducing) or ammonium- (repressing) supplemented media and offers direct assessment of cell viability and terminal growth phenotypes (Hu et al., 2007). A systematic survey of 54 genes determined to be essential in yeast and *C. albicans* (Roemer et al., 2003) resulted in >70% predicted orthologs sharing an essential role in *A. fumigatus* viability. Similarly, this strategy also allows direct assessment of virulence in an aspergillosis model of systemic infection, where the in vivo nitrogen levels are sufficient to repress pNiiA (Hu et al., 2007; Rodriguez-Suarez et al., 2007; Jiang et al., 2008). Numerous independent large-scale gene deletion studies are also being performed in *C. albicans* (Noble et al., 2010) as well as other fungi, including *Cryptococcus neoformans* (Liu et al., 2008) and *Neurospora crassa* (Collopy et al., 2010). Collectively, these studies provide an unparalleled clarity to the increasingly broad assortment of

novel drug targets suitable for antifungal screening and potential drug discovery.

C. albicans FT-Based Screening Paradigm

With such an abundance of possible antifungal targets to screen, the fundamental question is, where to start? Our solution to this issue was to develop a *C. albicans* FT-based chemical-genetic approach to directly screen essentially *all* potential antifungal drug targets for cognate inhibitory compounds active against the pathogen. To facilitate this, we initially constructed 2868 distinct heterozygous deletion strains, each possessing two independent barcodes to uniquely identify each strain and facilitate their screening in coculture (Xu et al., 2007). These strains represent genes of greatest potential as antifungal targets, as they are (1) experimentally demonstrated as essential in *S. cerevisiae* (Winzeler et al., 1999; Giaever et al., 2002) and/or *C. albicans* (Roemer et al., 2003), (2) broadly conserved in *A. fumigatus* and in many cases demonstrated to be essential for growth (Hu et al., 2007), and/or (3) suggested to be required for cell growth based on their strong homology to genes conserved in metazoans. Initial demonstration of robust MOA determination by *C. albicans* FT screening was achieved by evaluating in the assay a broad collection of known antifungal compounds of NP or synthetic origin with well-characterized modes of action (Xu et al., 2007). These included azoles (various imidazoles and triazoles), amphotericin B, aureobasidin, brefeldin A, cytochalasin D, cerulenin, nocodazole (and related tubulin inhibitors), radicicol, tunicamycin, tubercidin, roridin A, verrucarin A, 5-fluorouracil, and 5-fluorocytosine. In this and subsequent studies, the mechanistic insights revealed by FT screening were largely congruent with the published MOAs and/or supported by independent biochemical and genetic data, which included important aspects of drug import, efflux, and metabolism (Xu et al., 2007, 2009; Rodriguez-Suarez et al., 2007).

Recently, we have expanded the *C. albicans* FT to include ~5400 double-barcoded heterozygotes (see Tables S1–S3 available online), thus with an ~90% genome coverage. This more extensive platform will more thoroughly identify target-specific inhibitors which could otherwise be missed in the earlier version of the assay. More importantly, we have constructed and included 125 double heterozygous deletion mutants of genes that are involved in cell wall biosynthesis and display synthetic lethal genetic interactions between orthologous genes in the yeast (Lesage et al., 2004). As we describe here, such strains provide elevated hypersensitivity to cell wall inhibitors and proved highly effective in identifying both known and new naturally produced 1,3-β-glucan synthase inhibitors.

FT-based screening on a genome-wide scale provides multiple advantages over traditional target-based screens. First, the FT facilitates a reverse genetic approach to linking traits (responses to inhibitory compounds) to preexisting mutations (heterozygous deletion strains), on a genome scale and directly in the pathogen. As such, target-based whole-cell assays are easily developed, scalable, and do not require functional understanding of the drug target. Second, drug resistance mechanisms, including drug efflux, permeability, and metabolism, can be identified early and in parallel to target identification of potential antifungal agents. Drug targets are also identified empirically and are biased toward those with intrinsic susceptibility to chemical inhibition, and thus are demonstrated to be druggable. Finally, target validation is performed in key fungal pathogens only subsequent to the identification of target-inhibitory compounds. In this way, *compound-target pairs* can be prioritized as antifungal drug leads according to their chemical attributes, the biological process they interdict, and their subsequent genetic target validation. Thus, a compound-centric focus is taken, whereby a comprehensive target set is screened across broad chemical diversity to identify antifungal agents that are both mechanistically and structurally novel.

C. albicans FT Screening of NP Extracts

Three fundamental issues that impede the efficient screening of NP extracts include (1) the significant natural products chemistry isolation and structure elucidation efforts required to identify the bioactive component within any extract, (2) the need to rapidly dereplicate known bioactive molecules from those that are novel early in this process, and (3) the time-consuming exercise of determining the compound's MOA. Recently, we and others have successfully demonstrated how FT profiling of unfractionated NP extracts can address each of these issues (Parsons et al., 2006; Jiang et al., 2008). We demonstrated the utility of the FT in mechanistically annotating and identifying target-specific bioactive compounds within microbial fermentation extracts that contained well-characterized bioactive compounds (Jiang et al., 2008). In nearly all cases, highly diagnostic profiles were obtained that reflected the purified NP's known MOA. Moreover, by constructing a broad reference set of FT profiles with pure, well-characterized NPs, a comparative analysis between new/uncharacterized extracts and the compendium of FT profiles derived from the NP reference set could be used to predict the presence of novel bioactive compounds within unfractionated extracts (Herath et al., 2009; Overy et al., 2009; Ondeyka et al., 2009).

Based on these results, Merck aggressively implemented a large-scale work plan involving multidisciplinary teams across multiple research labs to provide microbial-derived extracts for FT screening as well as all associated follow-up activities required to identify and purify natural products to a single bioactive component and solve chemical structures of small molecules predicted to possess both structural and mechanistic novelty. Over the course of this research program, 1801 NP extracts with growth-inhibitory activity against *C. albicans* were evaluated by FT profiling. This includes 1231 fungal and 570 actinomycete extracts, of which over 770 were derived from fresh microbial isolates discovered internally to maximize their taxonomic and geographic diversity (Bills et al., 2009; Collado et al., 2007) and coupled with nutritional array methodologies to stimulate normally unexpressed secondary metabolic pathways to produce novel bioactive NPs (Bills et al., 2008).

The initial focus of the natural products chemistry group was to rapidly dereplicate known NPs that were present in the unfractionated extracts. Two approaches were taken to eliminate from consideration samples that contained known compounds at as early a stage as possible in this process. The unpurified samples were analyzed by a standardized LC-MS method in order to compare all extract components with the Merck historical database of isolated NPs. By this method, each peak in the chromatogram could be dereplicated with samples that had

been previously processed in our laboratories. All components from these extracts were compared by LC retention time, the UV spectrum of the component, and the apparent mass spectrum both in positive- and negative-ion modes (Genilloud et al., 2010). These analyses excluded NPs from subsequent FT profiling. Accordingly, LC-MS analysis would identify NPs without consideration for the assigned MOA by FT profiling. In a more focused second approach, NP dereplication libraries were constructed based on literature reports of antifungal components as well as internally discovered compounds with known MOAs. Each extract was analyzed by high-resolution (HR) LC-MS, and an ion-extraction algorithm was used to search for exact mass data that were consistent with the molecular weights of the known NPs. In this approach, only the library of NPs known to inhibit by the MOA obtained by FT profiling was considered for HR-LC-MS analysis. As this program progressed, these LC-MS-based dereplication approaches expanded as additional components could be added both to the internal Merck NP database as well as to the FT-annotated MOA listings.

Provided that no NP match was identified during dereplication at the extract stage, isolation efforts were prioritized based on the putative MOA obtained by FT profiling and initiated to separate the various components of the crude samples. Chromatographic processing of these extracts was used to identify more purified materials that maintained the original antifungal activities. Generally, hydrophobic polymeric resins, silica-based (reversed or normal phase) resins, countercurrent chromatography, and ion-exchange chromatography were used to isolate a purified component(s) over one or more isolation steps. Agar-based anti-*C. albicans* activity was used to rapidly screen chromatographic fractions for antifungal components. At times, multiple regions of antifungal activity were identified after chromatography. In this situation, FT profiling was used to determine the region of activity that was related to the desired MOA. Multiple antifungal components could be related to a family of NPs or distinct structural classes. Once a purified component was in hand, the FT profile of this material was related back directly to that seen with the original extract. Frequently, additional hypersensitive heterozygote FT strains were present in the overall profile at the purified component stage, further confirming the designated MOA. The isolated compounds were fully characterized by 1D and 2D NMR spectroscopic methods along with chemical derivatization when necessary in order to fully elucidate the structure of all novel antifungal NPs.

In total, over 60 isolation projects were completed in this screening campaign, identifying numerous novel and known compounds from this effort (Table 1). A survey of known target-specific inhibitors identified from this work validates our approach and reflects (in part) the achievable chemical and mechanistic diversity of bioactivities that exist in NP extracts. These include cholesterol-lowering molecules (lovastatin and hymeglusin), immunomodulators (rapamycin and wortmannin), and an extensive diversity of 1,3-β-glucan synthase cell wall inhibitors, from which the therapeutic antifungal agent Cancidas (commonly named caspofungin) is derived. In addition, numerous basic research chemical probes commonly used to study aspects of cellular biology were identified, including inhibitors of protein synthesis (sordarin and xylarin), actin cytoskeleton (cytochalasins), protein secretion (brefeldin A), fatty acid biosynthesis (cerulenin), and mRNA processing (cordycepin). Importantly, novel NPs with new MOAs of potential antifungal therapeutic utility were also identified. Below, we describe a small exemplary set of these compounds identified by FT-based screening, highlighting both the structural and mechanistic diversity of previously unknown bioactive NPs (Figure 2).

Aspirochlorine is a common secondary metabolite produced by *Aspergillus* species and is structurally related to the epidithiodioxopiperazine class of fungal toxins (Jordan and Cordiner, 1987). Unlike typical mycotoxins, however, aspirochlorine has been demonstrated to possess remarkable in vitro inhibitory selectivity toward fungal protein synthesis (*C. albicans* IC_{50} = 11 nM) over bacterial or mammalian (>100 μM) protein synthesis systems (Monti et al., 1999). Aspirochlorine was repeatedly identified during the course of our work based on a single strain depletion corresponding to *YEF3* (Figure 2B), a fungal-specific elongation factor of protein synthesis (Sturtevant, 2002). FT-based screening of aspirochlorine-containing fungal extracts (and reproduced using pure aspirochlorine) therefore provided a clear mechanistic profile reflecting the agent's highly selective MOA. Cross-referencing FT-MS spectra for the presence of aspirochlorine among extracts that yielded a *YEF3* FT signature provided robust dereplication of this bioactivity without requiring chemical isolation. Conversely, FT-MS failed to detect aspirochlorine in a streptomycete extract (ECC729), yielding a related FT profile with strain depletions corresponding to both *YEF3* and *SSC1*, a mitochondrial heat shock chaperone protein (Figure 2B). Following bioactivity-guided isolation and structural elucidation, a novel uridine analog ($C_{26}H_{48}N_4O_{13}P_2$; MW 686.63) containing a unique imidodiphosphate linkage and aliphatic side chain was identified (Figure 3A). The purified compound demonstrated potent activity against *C. albicans* (minimum inhibitory concentration [MIC] = 0.2 μg/ml) and, consistent with its FT profile, specifically inhibited protein synthesis in whole cells (Figure 3A). Based on these data, we have named the compound yefafungin, for *y*east *e*longation *f*actor *a*ffecting fungin.

Whereas the preceding examples highlight relatively simple FT profiles (e.g., one or two strain depletions reflecting the MOA of the bioactive NP), often more complex FT profiles were observed. Such profiles were deemed biologically significant provided the hypersensitive strains were highly reproducible over multiple screening replicates and, importantly, there was a clear functional relationship between those genes corresponding to the heterozygote strain set. One pertinent example is the fungal extract ECC1977, which produced a complex but reproducible profile spanning the original extract, the predominant bioactive fraction during isolation, and the purified antifungal compound (Figures 2A and 2C). After structure elucidation of this purified component ($C_{25}H_{34}O_4$; MW 398.55), it was determined to be a new analog of hamigerone (Breinholt et al., 1997) and, accordingly, named 12-deoxo-hamigerone (Figure 3B). Despite sharing hypersensitivity of the *TUB1* and *TUB2* (to a much less extent) heterozygotes, the overall FT profiles of 12-deoxo-hamigerone and its original extract were different from those of benomyl and nocodazole, known inhibitors of microtubule assembly, underscoring difference in chemical structures (and perhaps mechanistic detail) (Figure 2A). Nevertheless, the significant hypersensitivity of both *TUB2* and

TUB1 strains (α- and β-tubulins, respectively) indicates that 12-deoxo-hamigerone also perturbs microtubule assembly, as confirmed experimentally in a tubulin in vitro polymerization assay (Figure 3B). The compound was active against tubulin-containing eukaryotes (e.g., fungi such as *C. albicans* [MIC = 4 μg/ml] and *Trichophyton mentagrophytes* [MIC = 1 μg/ml]) as well as multiple mammalian cell lines (IC_{50} values ranging from 2 to 3 μg/ml), but had minimal appreciable activity against tubulin-lacking bacteria (e.g., *Staphylococcus aureus* [MIC > 32 μg/ml]).

More complicated—yet mechanistically significant—FT profiles can include distinct heterozygote strains displaying reproducible hyposensitivity (i.e., a fitness advantage and partial resistance to a bioactive compound) in addition to hypersensitive strains. They collectively provide a unique MOA signature of a bioactive agent. As exemplified by the fungal-derived NP extract ECC601 and the purified bioactive compound, their FT profiles highlighted two prominently hypersensitive heterozygote strains corresponding to *CYR1* (adenylate cyclase) and *PRS3* (phosphoribosylpyrophosphate synthetase, which synthesizes PRPP), as well as two hyposensitive strains heterozygous for *PDE2* (a phosphodiesterase which converts cAMP to AMP) and *BCY1* (the protein kinase A/PKA negative regulatory subunit) (Figure 2D). Remarkably, the chemical sensitivities of these heterozygotes were congruent with the functional relationship between the steps in the cAMP-dependent PKA pathway (inducing hyphal growth when activated) at which the corresponding gene products are involved, and suggested the bioactive compound affects the cellular cAMP levels. They also raised the possibility that the synthesis of cAMP is coupled with transfer of the pyrophosphate to PRPP, a step likely catalyzed by Prs3p (Figure 2D). Bioactivity-guided isolation led to the identification of a novel pentaene-containing compound ($C_{24}H_{30}O_4$; MW 382.50) with its chemical structure shown in Figure 3C. We name the compound campafungin, for *cAMP-a*ffecting *fungin*. According to the perceived MOA, campafungin inhibits Cyr1p and/or Prs3p, and hence the hypersensitivities of both corresponding heterozygous deletions. However, this inhibitory effect could be partially alleviated if the degradation of cAMP (by Pde2p) is partially impeded (hence the hyposensitivity of the corresponding strain), or the inhibitory effect of the regulatory subunit of the PKA (Bcy1p, to which cAMP binds and thereby activates the PKA) is quantitatively reduced (hence the hyposensitivity of the *BCY1* strain). Consistent with this hypothesis, campafungin effectively blocked the transition from yeast to hyphae of *C. albicans* (Figure 3C), as did the known PKA inhibitor PKI(11-22) (Castilla et al., 1998) (data not shown). However, unlike PKI (11-22), campafungin was partially suppressed by exogenous cAMP at a concentration near its MIC (200 μg/ml; Figure 3C). Conversely, for reasons unclear to us, suppression was not observed at low concentrations (e.g., 100 μg/ml; Figure 3C). As *CYR1* is not essential for viability of *C. albicans* (Rocha et al., 2001), our results suggest that Cyr1p is not the direct or only target of campafungin. The possibility remains that it inhibits Prs3p, which is essential for viability and hyphal growth (data not shown). Nevertheless, campafungin is an NP with dual antiproliferative and antihyphal activities.

An NP isolated from the actinomycete-derived extract ECC619 also produced an FT profile in which the MOA was reflected in both the hypersensitivity and hyposensitivity phenotypes of heterozygote strains. Two particularly responsive strains correspond to *DRE2*, encoding an essential Fe/S cluster protein involved in oxidative stress-induced cell death (Vernis et al., 2009), and *DIP5*, the dicarboxylic acid permease (Regenberg et al., 1998, 1999) (Figure 2E). As predicted by *DIP5* hyposensitivity, bioactivity-guided isolation identified a new amino acid analog ($C_6H_8N_2O_4$; MW 172.14) that we named dretamycin (*dre* two *a*mido*mycin*) (Figure 3D). Consistent with its FT profile, tetracycline-based repression of *DRE2* using the corresponding GRACE strain produced pronounced hypersensitivity to dretamycin versus control strains as well as marked resistance to dretamycin in the absence of tetracycline repression (Figure 3D). This is presumably due to overexpression of *DRE2* from the heterologous tetracycline promoter as compared to its endogenous promoter. Conditional repression of *DIP5* also faithfully reproduced the reduced whole-cell susceptibility to dretamycin as originally observed in the FT profile. Indeed, dretamycin import into the cell is likely mediated directly by *DIP5*, as the addition of exogenous substrates of this permease (including L-glutamate and L-aspartate) to the medium noticeably reduces dretamycin whole-cell inhibitory activity (Figure 3D).

NPs interdicting fungal cell wall processes such as glucan, chitin, and mannoprotein biosynthesis have long been viewed as a mechanistically privileged class of bioactive compounds analogous to the proven efficacy of β-lactam antibiotics. Indeed, the most recently approved class of antifungal agents (e.g., caspofungin, micafungin, and anidulafungin) represents semisynthetic derivatives of the echinocandin class of NPs. Significant effort was therefore directed to identifying new bioactivities targeting fungal cell wall biosynthesis and efficiently dereplicating novel natural products from those previously known. However, glucan synthesis inhibitors such as caspofungin do not elicit a pronounced and mechanistically unambiguous FT profile corresponding to heterozygote depletions of the 1,3-β-glucan synthase enzyme complex (Xu et al., 2007), which is composed of Fks1p (catalytic) and Rho1p (regulatory) subunits. To address this, double heterozygote mutant strains with elevated hypersensitivity to glucan synthesis inhibitors were constructed and supplemented to the FT assay. Genes were selected for this purpose according to their demonstrated mutant phenotype as either hypersensitive to caspofungin or synthetically lethal in combination with *FKS1* or *FKS2* mutations in *S. cerevisiae* (Lesage et al., 2004). Therefore, all resulting 125 *C. albicans* double heterozygote mutant strains were deleted of one *FKS1*, *FKS2*, *RHO1*, or *PKC1* allele in combination with deletion of one allele of a second gene (e.g., *FKS3*, *CHS5*, or *GAS1*) reported to genetically interact with the MOA of caspofungin. As we discuss in a later section, a broad diversity of known NP inhibitors of 1,3-β-glucan synthase was identified exclusively from fungal extract sources. New NPs sharing this MOA, however, were rarely discovered and were restricted to a new papulacandin analog (ECC3703; $C_{43}H_{56}O_{16}$; MW 828.90) and a desulfated ergokonin A analog (ECC3785; $C_{34}H_{53}NO_7$; MW 587.79; Vicente et al., 2001). FT profiles of their corresponding NP extracts highlight the typical MOA resolution achieved by including cell wall double heterozygote mutants in the FT assay (Figure 4).

Whereas NP inhibitors of fungal-specific targets such as Yef3p and Fks1p provide potential starting points as antifungal leads,

Table 1. Summary of *C. albicans* Fitness Test Profiling of Natural Products with Active Components Characterized

Original Extract ECC/Source[a]	CaFT Experiment ID[b]	MOA/Target/CaFT Profile[c]	Compound Isolated/ Active Component(s) Identified[d]
Natural Products that Have Been Published[e]			
577/F	See reference	RNA poly(A) polymerase, RNA cleavage/ polyadenylation complex	Parnafungin[f] (see Jiang et al., 2008; Parish et al., 2008)
3552/F	See reference	Sphingolipid biosynthesis	Phomafungin[f] (see Herath et al., 2009)
3684/F	See reference	Calmodulin and heat shock response (CMD1, CMP2, HSP82, SIS1)	Virgineone[f] (see Ondeyka et al., 2009)
Natural Products that Are Described in Figures 2 and 3 and in Table S1			
575/F	1477-3K, 2150-3K	Fungal-specific translation initiation factor YEF3	Aspirochlorine (exp. 2531-3K, 2532-3K)
729/A	2387-3K, 2488-3K	Fungal-specific translation initiation factor YEF3 (+SSC1)	Yefafungin[f] (exp. 2969-3K, 2971-3K)
1977/F	3012-3K, 3061-3K	Microtubule (TUB1, TUB2, CCT3, CCT8, BUB1)	12-deoxo-hamigerone[f] (exp. 1497, 2170)
601/F	2154-3K	cAMP-dependent protein kinase A pathway	Campafungin[f] (exp. 2544-3K, 2545-3K)
2567/F	222, 286	Proteasome	Fellutamides C and D[f] (exp. 1519, 1767)
619/A	1498-3K, 1499-3K	DRE2, DIP5	Dretamycin[f] (exp. 3115-3K, 3133-3K)
Natural Products that Are Described in Figure 4 and Table S2			
Known Glucan Synthase Inhibitors			
2406/F	227, 352	FKS1-related cell wall profile	Furanocandin
2410/F	314, 315	RHO1, PTP3 in FT3K (ECC1786)	Mulundocandin
2430/F	1164, 1211	FKS1-related cell wall profile	Papulacandins A and B
3301/F	957, 1148	FKS1-related cell wall profile	Pneumocandins
3444/F	297, 308	FKS1-related cell wall profile	Ergokonin A
3703/F	1405, 1674	FKS1-related cell wall profile	New papulacandin
3785/F	1603, 2174	FKS1-related cell wall profile	Novel desulfated ergokonin A
3787/F	1604, 2194	FKS1-related cell wall profile	Ascosteroside
3927/F	2003, 2028	FKS1-related cell wall profile	Ergokonin B
3979/F	2108, 2109	FKS1-related cell wall profile	Arundifungin
4442/A	3476, 3481	Not informative	Lydicamycin, 30-demethyllydicamycin
4631/F	3423, 3470	FKS1-related cell wall profile	WF11899A
Others			
Pure[g]	-	Translation elongation factor EF-2 (EFT1)	Sordarin (exp. 1252)
3659/F	1225, 1344	Translation elongation factor EF-2 (EFT1)	Xylarin
4534/F	3282, 3330	HMG1	Lovastatin
5828/F	4636, 4735	HMG1	Compactin (mevastatin)
3621/A	1170, 1250	TOR complex	Rapamycin
3675/A	1352, 1430	TOR complex	Wortmannin
3537/A	748, 1477	Sphingolipid biosynthesis	Lipoxomycins
3691/F	1437, 2956	Sphingolipid biosynthesis	Fumonsins
2922/A	599, 1065	DNA repair	Rachelmycin
3447/A	1203, 2535	DNA repair	Ravidomycin
3464/A	514, 517	DNA repair	Yatakemycin
3445/F	855, 1161	Fatty acid biosynthesis	Novel polyacetylene
3871/F	1807	Fatty acid biosynthesis	Cerulenin
3728/F	1561, 1658	ERG13, ergosterol biosynthesis	Hymeglusin
3755	1550, 1574	ERG24, ergosterol biosynthesis	Tyroscherin
Natural Products that Are Described in Table S3			
652/F	2232-3K, 2233-3K	Phosphoribosyl complex	11-desacetoxyl-wortmannin (exp. 2915-3K)
742/A	2361-3K, 2371-3K	Nucleoside analog (NNT1)	Toyocamycin
819/A	2413-3K, 2448-3K	Distinct complex profile	Oxanthromycin (exp. 2978-3K, 2979-3K)
2365/F	3793-3K, 3820-3K	ERG25, PDR16, SUB2	PF1163A

Table 1. *Continued*

Original Extract			
ECC/Source[a]	CaFT Experiment ID[b]	MOA/Target/CaFT Profile[c]	Compound Isolated/ Active Component(s) Identified[d]
2412/F	3251-3K, 3252-3K	MEX67	New compound[f]
2359/F	155, 178	MCD4, GPI biosynthesis	BE-04385A
2556/F	1221, 1350	IMD3, purine de novo synthesis	Mycophenolic acid
2662/F	989, 1155	TCA cycle	Conocandin (exp. 6268)
3112/F	1165, 1439	FAS1, fatty acid biosynthesis	Preussomerins
3232/F	789, 1548	Actin, ACT1, ARP2, ARP3	Cytochalasins
3420/F	465, 1592	SEC7, ARF2, TPO1	Tanzawaic acid G
3423/A	266, 1683	FOL2, folic acid metabolism, C1 pool	7-hydroxylguanine (exp. 4874, 4951)
3474/A	496, 1739	Nucleoside analog, ribosome biogenesis	Tubercidin
3489/F	1686, 3286	Signal peptidase complex (IRE1, SPC3, SEC11)	New compound,[f] structurally from cotransin
3536/F	747, 811	SNF-SWI1 transcription activation complex	Borolactin A
3551/F	3806, 3906	Complex, not informative	PF1140
3572/F	1082, 1248	RAD52, RFA1, DNA replication	Illudin (hydroxybovistol dimer, exp. 6207, 6265)
3689/F	1434, 1435	FKS1, AUR1, complex profile similar to phomafungin	Lipofungimide (exp. 3542, 3568)
3690/F	1378, 1408	ERG11, NCP1	Restricticin
3715/F	1471, 1537	Zinc homeostasis	Hyalodendrin
3721/F	4050, 4051	Nucleoside analog, RNA cleavage, and polyadenylation complex	Cordycepin
3722/F	1575, 1617	Pyruvate metabolism	Conocandin
3920/F	1913, 1960	U3 snoRNP, ribosome biogenesis	Homoarginine derivative of 3′-aminoadenosine (exp. 4977, 5213)
4164/F	2681, 2750	ADE8	Preussomerin MB7056B
4662/F	3387, 3733	SEC7, ARF2, CDR1	Brefeldin A
4743/A	3696, 3843	Distinct complex profile	TAN1254
4837/A	3797, 3837	ALG7, IAH1, RSB1	Tunicamycin

[a] Microbial sources: F, fungus; A, actinomycete.
[b] Underlined are the experiments with an early version of *Candida albicans* Fitness Test (CaFT) (FT3K).
[c] See Figures 2 and 4 and Tables S1–S3.
[d] If the isolated compounds were tested in the CaFT, the corresponding experiment numbers are given in parentheses.
[e] The CaFT results (z scores) are not included in Supplemental Information.
[f] Novel structures described here or manuscript in preparation.
[g] Pure compound was used.

bioactive compounds may possess alternative therapeutic development potential provided their proposed MOA reflects targets (e.g., Tub1p, Cyr1p) which are conserved in man. The fungal extract ECC2567 is a case in point. It generated an FT profile indicative of the proteasome being the target of bioactivity (Figure 2F). The proteasome is a large, multisubunit, proteolytic complex that degrades ubiquinylated proteins to ensure protein homeostasis and regulation across diverse cellular processes (Park et al., 2010). Accordingly, the proteasome is essential for eukaryote cell growth and viability, and inhibitors of this process, including bortezomib (VELCADE), have been developed for anticancer therapy (Adams, 2004; Kisselev, 2008). Remarkably, FT profiling of ECC2567 as well as the purified bioactivity reproducibly identified nine hypersensitive strains, corresponding to four α (structural) and five β (catalytic) subunits of the 20S core particle (Figure 2F). Moreover, ECC2567 and its purified bioactivity inhibited proteasome activity in vitro using a *C. albicans* whole-cell extract (Figure 3E), and this inhibitory activity was reproduced using a human 20S proteasome assay (data not shown). ECC2567 bioactivity isolation and structural elucidation revealed two highly related lipopeptide aldehydes with chemical formulas of $C_{28}H_{51}N_5O_8$ (MW 585.37) and $C_{29}H_{53}N_5O_8$ (MW 599.39) and differing only in the C-terminal amino acid of valine or leucine, respectively (Figure 3E). Based on their structural similarity to the recently characterized NP proteasome inhibitor fellutamide B (Hines et al., 2008), we named these new natural products fellutamide C and fellutamide D, respectively. Considering their MOA and significantly higher antiproliferative activity across multiple human cell lines (IC_{50} values range between 0.05 and 1.5 μM) than fungal pathogens (MIC values of 4–32 μg/ml across *Candida* spp.), fellutamides C and D may provide additional chemotherapeutic starting points for oncology or other recently identified therapeutic applications for proteasome inhibitors (Kisselev, 2008).

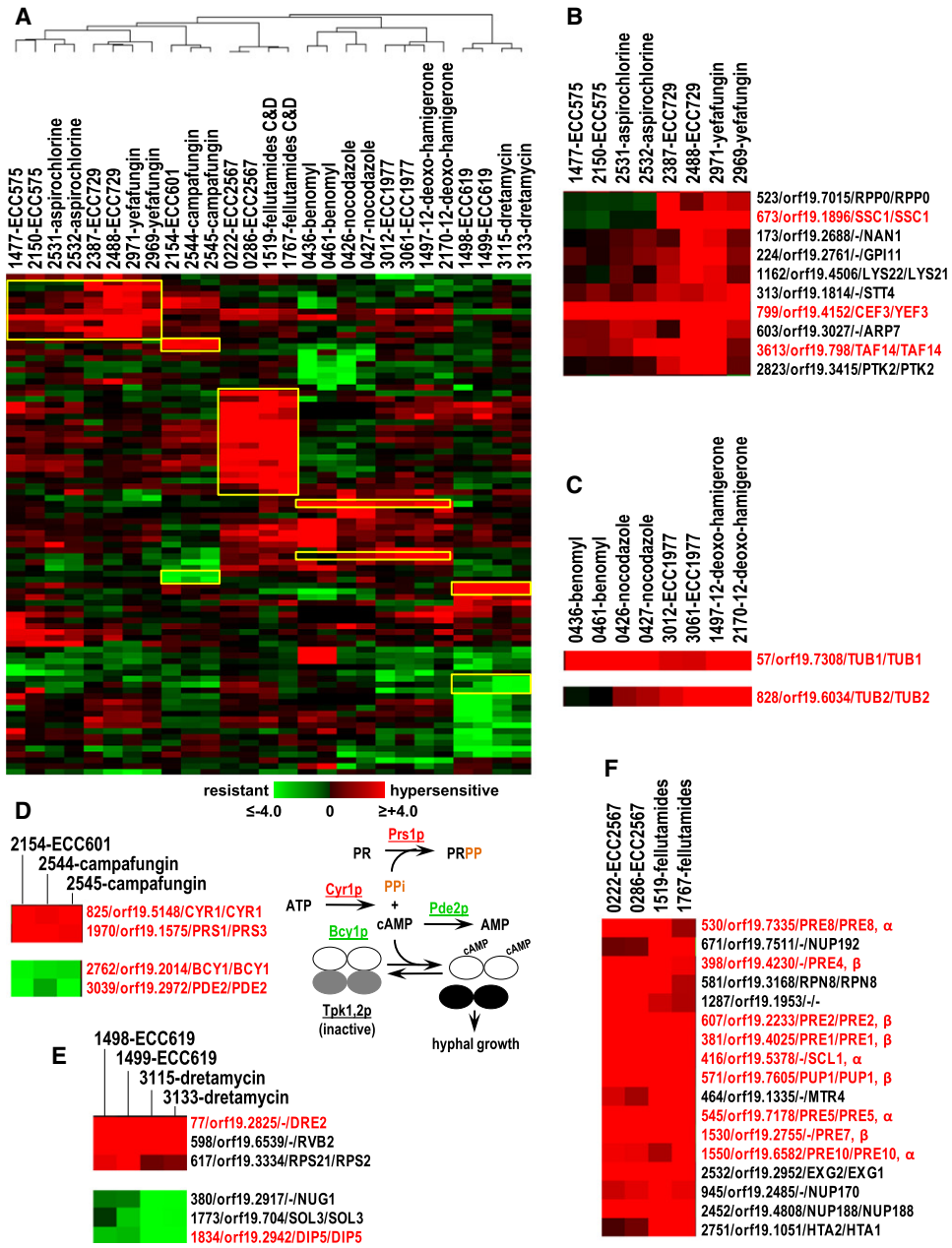

Figure 2. FT Profiling of Microbial Fermentation Extracts and Active Compounds Isolated

(A) For each extract (ECC) and compound, two independent fitness test experiments were selected, with the exception of ECC601. The normalized z scores of all strains in each experiment and the corresponding inhibitory concentration (IC) are listed in Table S1. For those extracts and compounds that were tested in the FT5K, only strains that were also presented in the 3K version were included in analysis. In the hierarchical clustering (by centroid linkage; Eisen et al., 1998), strains were selected based on their absolute values of z scores ≥ 3.5 in at least two FT experiments, with results displayed by heat map. The experiment numbers and the scale are indicated at the top and at the bottom of the heat map, respectively. Highlighted by yellow boxes and arrows are clusters of heterozygous deletion strains whose significant hypersensitivity or resistance is indicative of the MOAs of tested extracts and compounds.

(B–F) Clusters of strains that displayed significant hypersensitivity or resistance to specific compounds and the source extracts of aspirochlorine and yefafungin, both affecting fungal-specific translation factor Yef3p (B), 12-deoxo-hamigerone, a microtubule-perturbing agent (C), campafungin, affecting the cAMP-dependent PKA pathway (D), dretamycin, a Dre2p-affecting amino acid analog requiring the Dip5p dicarboxylic acid permease for cell permeability (E), and fellutamides C and D, inhibitors of the 26S proteasome (F). The inset in (D) illustrates the part of the cAMP-PKA pathway (highlighted in red and green, for heterozygote hypersensitivity and resistance) that was detected in the fitness test.

Surveying Target-Specific Chemical Diversity with NP Extracts from 2005 to 2008

Table 1 summarizes a broad subset of NP crude extracts profiled in the *C. albicans* FT that yielded discernable MOAs (with three exceptions). In most cases, follow-up HR-LC-MS identified the known NPs whose targets and/or MOAs were reinforced in the FT profiles, as indicated by significant and reproducible hypersensitivities of the heterozygotes corresponding to the targets.

In other cases, when HR-LC-MS failed to match the bioactivities suggested by the FT profiles to any known NPs in our database, full-scale isolation was promptly initiated. For known NPs, FT profiles can provide a strong chemical-genetic hypothesis reflecting their characterized modes of action, as determined by significant and reproducible hypersensitivities of strains corresponding to the target genes. For example, lovastatin- or compactin-containing extracts faithfully resulted in hypersensitivity of the *HMG1* heterozygote (Figures 4A and 4F). Similarly, other known NPs targeting other enzymes of the ergosterol biosynthetic pathway, including hymeglusin (Erg13p; Figures 4A and 4G), restricticins (Erg11p), and PF1163A (Erg25p), were routinely identified based on the principal depletion of the corresponding heterozygote strains (Table 1; Table S2). Although the MOA of tyroscherin remains uncertain (Tae et al., 2010) it too may interdict this pathway, as *ERG24* (and to a lesser extent, *ERG2*) heterozygotes were consistently the prominent depletion strains across multiple fungal extracts dereplicated to contain this natural product (Figures 4A and 4K). Despite robust demonstration that the FT correctly identifies each of these mechanistically distinct—but ergosterol pathway-specific—inhibitors from crude extracts, structurally novel bioactive compounds that interdict ergosterol biosynthesis were not discovered.

More broadly, new and previously discovered NPs targeting a wide range of cellular processes were identified. These include NPs targeting the biosynthesis of sphingolipids (e.g., lipoxamycins and fumonsins [Figures 4A and 4I], and phomafungin discovered through our study [Herath et al., 2009]), fatty acids (e.g., cerulenin and polyacetylenes [Figures 4A and 4H], and preussomerins), as well as protein synthesis (e.g., xylarin [Figures 4A and 4E], as well as sordarin [Figure 4E], aspirochlorine [Figure 2B], and at least one novel inhibitor, yefafungin [Figures 2A, 2B, and 3A]), 3′ mRNA processing (e.g., cordycepin and parnafungins) (Jiang et al., 2008), and rRNA biogenesis (a new homoarginine derivative of 3′-aminoadenosine). More broadly, additional hits likely targeting nuclear export (ECC2412), protein translocation in the endoplasmic reticulum (cotransin and a new lipopeptide derived from ECC3489), target of rapamycin (TOR) (wortmannin and rapamycin) (Figures 4A and 4D), adenylate cyclase signaling (campafungin) (Figures 2A, 2D, and 3C), and the new fellutamide inhibitors of the proteasome (Figures 2A, 2F, and 3E) were also identified by FT profiling. Importantly, the FT approach "rediscovered" several known compounds that had not been previously characterized by past Merck NP discovery efforts (Table 1), reiterating the efficiency of the approach.

NPs which inhibit cell wall biosynthesis have historically provided an important class from which potential antifungal drug leads were developed. Therefore, an important and quantifiable measure of our success relates to the diversity of NP inhibitors of glucan synthase identified by this project. In addition to a new papulacandin and a desulfated ergokonin A, a broad assortment of known echinocandins, pneumocandins, papulacandins, and acidic terpenoids was discovered (Table 1), a number of which (corynecandin, fusacandin, furanocandin, and mulundocandin) had never been previously identified at Merck. This success was based on the overall work plan as well as the addition of 125 distinct cell wall double heterozygous deletion mutants (e.g., *fks1/FKS1 gas1/GAS1*) corresponding to *S. cerevisiae* orthologs previously demonstrated to possess synthetic lethal growth effects in combination with *fks1*, *fks2*, or *fks3* mutants and/or hypersensitivity to caspofungin (Lesage et al., 2004). Double mutants encompassing heterozygous deletions of genes involved in both glucan synthase function and compensatory cell wall processes displayed elevated sensitivity to glucan synthase inhibitors compared to either single heterozygote. As such, their inclusion served to effectively identify glucan synthase inhibitors, with FT readouts reflecting extensive sets of cell wall double heterozygote strains unique to the glucan synthase profile (Figures 4A and 4B; Table S2). In fact, FT profiling identified glucan synthase inhibitors as the largest single class of mechanistically characterized bioactive NPs (n = 106), representing nearly 6% of the 1801 NP extracts screened (Figure 5A). As none of the above glucan synthase inhibitory NP classes were identified from actinomycetes extracts (n = 570), we conclude that secondary metabolism pathways responsible for their biosynthesis are either unlikely to be conserved in actinomycetes or are not expressed under the conditions used.

Based on the scale and particular sources of NP extracts examined, we assessed target-selective bioactive hit rates as well as their prominent targets (and/or interdicting pathway) and chemical diversity. FT screening of *C. albicans* growth-inhibitory NP extracts originating within our historical library (n = 673; 572 being fungal derived versus 101 extracts of actinomycetes origin) resulted in over 140 mechanistically annotated extracts warranting follow-up activities (Figure 5B). Thus, potential target-specific bioactive compounds were predicted from FT profiling at a hit rate of ~22%, far exceeding our observed hit rate for screening synthetic compounds with intrinsic antifungal activity (data not shown). FT-based screening of an external (to Merck) historical antifungal NP library for target-based bioactive compounds (n = 306; 260 of fungal origin versus 46 actinomycete-derived extracts) also yielded a similarly high hit rate (28%) to that of our internal historical library. These extremely high hit rates for target-selective agents within NP extracts likely reflect the well-accepted view that NPs are selected to provide highly specific target-modulating activities to benefit the producing organism. Conversely, target-specific inhibitors are rarely represented within synthetic compound libraries and (at best) are either manmade to interdict a small target set or inadvertently affect unintended targets.

Interestingly, particular cellular processes (e.g., cell wall, ergosterol, and fatty acid biosynthesis, as well as protein translation and actin dynamics) were disproportionately affected by bioactive NPs. In some instances, this likely reflects procedural biases for those extracts selected for screening or the nonrandom distribution of metabolic families among microorganisms. For example, roridin and verrucarin NPs are highly potent and display broad spectrum against *C. albicans* and *A. fumigatus*, as potency was one of the criteria used early on in the project to prioritize bioactive extracts for FT screening. However, these compounds are general mycotoxins of considerable toxicity and were not pursued beyond our initial MOA determination studies during FT profiling which support their presumed target being eukaryotic initiation factor (eIF) -mediated translation (Xu et al., 2007). In other instances, namely cell wall/glucan synthase and ergosterol inhibitors, their predominant representation cannot be similarly explained (such

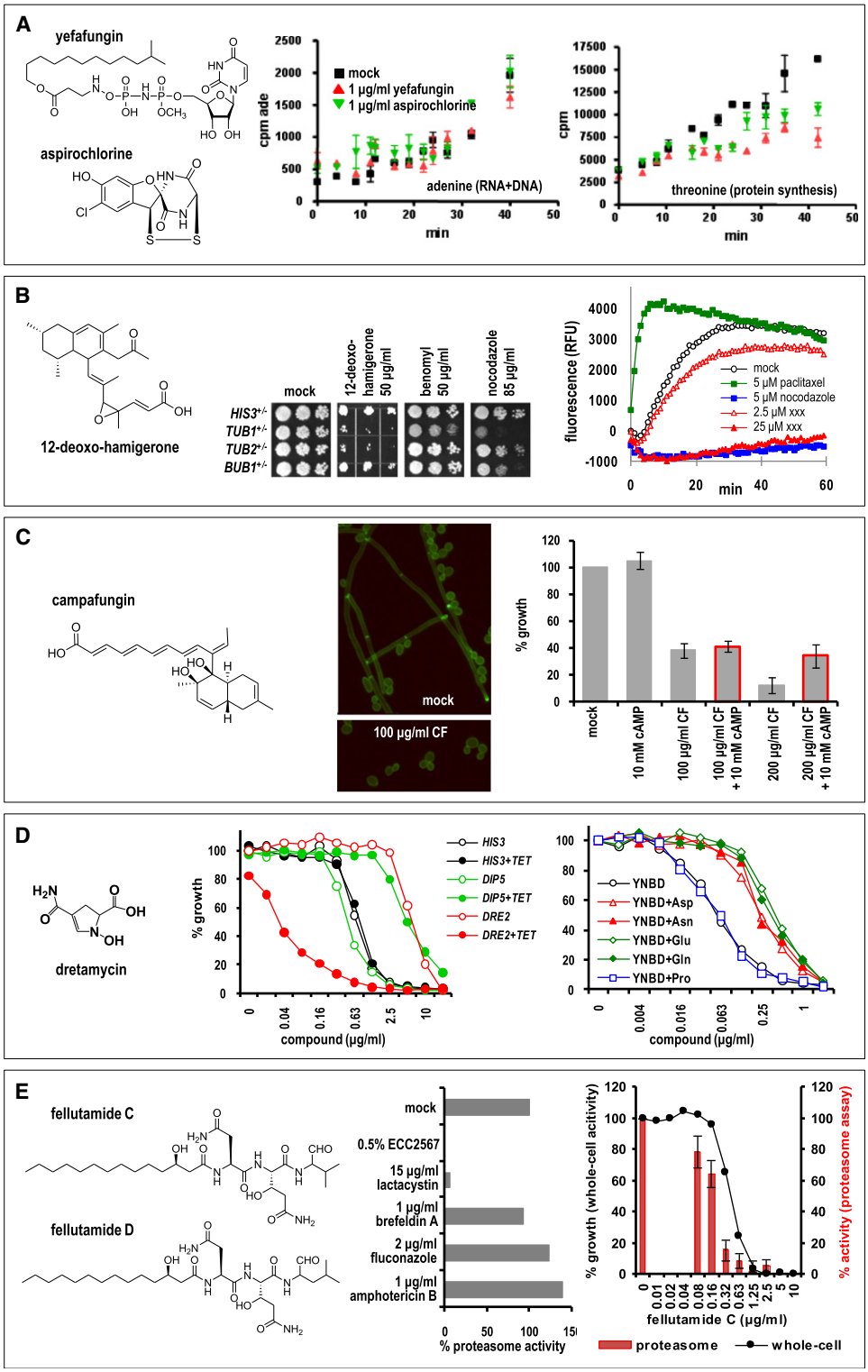

Figure 3. Chemical Structures and MOA Characterization of Representative Natural Products Isolated in This Study

(A) Aspirochlorine and yefafungin. The wild-type *C. albicans* cultures (in exponential growth) were labeled with [^{3}H]adenine (middle) or [^{14}C]threonine (right) for ~40 min in the presence or absence (mock, black squares) of 1 μg/ml yefafungin (red triangles) or aspirochlorine (green triangles). A portion of each culture was removed at different time points as indicated, and the incorporation of radioactive precursor was then determined. The incorporation of radioactive threonine was impaired by both aspirochlorine and yefafungin, suggesting inhibition of protein synthesis. Shown are averages of three measurements, with bars indicating standard deviations.

(B) 12-deoxo-hamigerone. Spot tests were performed as described elsewhere (Xu et al., 2007) with selected heterozygotes at the compound concentrations indicated. Note that both the *TUB1* and *TUB2* strains were hypersensitive to 12-deoxo-hamigerone. In vitro microtubule assembly assays were performed

bioactive NPs are not generally highly potent and are present in relatively low titer within crude extracts) and likely reflects their natural abundance as microbial secondary metabolites. Although it is possible that this may too reflect a screening bias, for example the superior resolving power of the FT assay to robustly identify such mechanisms, this seems an unlikely explanation because other equally identifiable mechanistic profiles for other NP classes (e.g., rapamycin, aspirochlorine, fellutamides, and cordycepin) were identified infrequently. Based on the apparent predominance of glucan synthase inhibitors we identified within bioactive NP extracts as well as their desirable MOA in targeting cell wall synthesis, one wonders whether this likely reflects the basis for their early discovery and advanced development as the most recently marketed class of NP-derived antifungal agents (Ostrosky-Zeichner et al., 2010). Interestingly, this observation extends to synthetic compounds and azole-based antifungal drugs in clinical use. The most abundant MOA gleaned by FT screening of synthetic compound libraries (~15% of all bioactive synthetic compounds tested) reveals the azole target *ERG11* (plus *NCP1* and *CDR1* often represented in the profiles; Xu et al., 2007), and these cognate inhibitors possess common imidazole or triazole pharmacophores. Thus, Fks1p and Erg11p appear intrinsically druggable to abundant NP and synthetic compound classes, respectively, potentially biasing their predominance as antifungal agents in drug development. Moreover, each of these antifungal drug classes was discovered and developed based on their intrinsic microbiological activity including antifungal spectrum and potency, rather than by target-based screening. In fact, elucidating their drug target and MOA was only achieved well after their antifungal activities were discovered. Therefore, an FT-based approach, or analogous genome-scale screening strategies, are likely required to survey more broadly the antifungal target space for less prominent compound/target interactions and from which novel antifungal leads will be discovered.

Following the screening of preserved/frozen NP libraries, significant effort was directed at the isolation of new microbes (both fungal and actinomycete sources) and preparation of fresh NP extracts (Bills et al., 2009; Genilloud et al., 2010). Our reasoning for this was two-fold: (1) only the most stable secondary metabolites are preserved when frozen over a long period of time, and thus certain less stable but novel bioactivities may not be adequately represented; and (2) historical NP libraries may represent only a limited microbial diversity, whereas the isolation of fresh microbes biased toward unique taxonomic and geographic origins may enhance the screening diversity of secondary metabolites. A summary analysis of screening 773 such extracts (of which 350 are derived from fungal sources versus 423 actinomycete-derived extracts) is provided in Figures 5C and 5D. A similarly high FT profiling hit rate for mechanistically significant extracts was achieved (17%) to that of historical/static/frozen NP extract libraries. This hit rate was again biased toward extracts containing bioactivities targeting both cell wall and ergosterol biosynthesis. Two additional NP classes identified were nucleosides (e.g., toyocamycin and tubercidin) yielding FT profiles as previously published (Xu et al., 2007) and DNA-alkylating agents, which produced dramatic *RAD52*-based FT profiles, the latter of which include ravidomycin, rachelmycin, yatekemycin (Figures 4A and 4J), and a new illudin analog, ECC3572 (Table 1). Their common identification within extracts derived from fresh isolates likely reflected a greater microbial bias toward actinomycetes (all but the illudin analog are actinomycete-derived compounds) than we previously screened in historical libraries. In fact, nearly half of all hit extracts were represented in one of these two NP classes (Figure 5D). Surprisingly, the FT-based mechanism annotation hit rate among actinomycete extracts was markedly lower (9%) than that derived from all other screening sources. Moreover, we frequently encountered complex FT profiles that either lacked clear MOA insights (e.g., oxanthromycin, antibiotic TAN1254, or borolactin A) or reflected commonly encountered

according to manufacturers' instructions in the presence of 5 μM paclitaxel, 5 μM nocodazole, 2.5 and 25 μM 12-deoxo-hamigerone, or mock treatment. The microtubule assembly was completely inhibited by 25 μM 12-deoxo-hamigerone.
(C) Campafungin. Wild-type *C. albicans* cells were grown in RPMI in the absence (mock) or presence of 50, 100, and 200 μg/ml campafungin for 20 hr, and then examined for hyphal growth using aniline blue staining. At these concentrations, no hyphal growth was observed (middle, 100 μg/ml, and data not shown). The fungal cells underwent full hyphal growth with the mock treatment (middle). If the cultures were supplemented with 10 mM exogenous dibutyryl-cAMP, the growth inhibition at 200 μg/ml campafungin was partially restored (right). However, no suppression of campafungin at lower concentrations (100 μg/ml, right; 50 μg/ml, not shown) was observed. These results suggest that campafungin is both antiproliferative and antihyphal, and that the latter activity is mediated through the cAMP-PKA pathway. However, it is likely that Cyr1p (the adenylate cyclase) is not the direct or only target of campafungin. Shown are averages of three experiments, with bars indicating standard deviations.
(D) Dretamycin. Middle: The conditional shut-off strains of *DRE2* and *DIP5* were tested for changes in susceptibility to dretamycin under both nonpressing (open symbols) and repressing (100 μg/ml tetracycline, +TET, closed symbols) conditions. Consistent with the observed hypersensitivity of the *DRE2* heterozygote strain, the conditional shut-off strain was exceedingly hypersensitive to dretamycin under a wide range of concentrations under the repressing conditions, whereas it was resistant under the nonrepressing conditions (likely due to elevated expression from the heterologous *TET* promoter). On the contrary, the *DIP5* strain was resistant to dretamycin when its expression was repressed, consistent with the FT results. Right: In the minimal medium YNBD, the potency of dretamycin (against wild-type *C. albicans*) was increased. However, it was suppressed partially by (dicarboxylic) amino acids Asp, Asn, Glu, and Gln (but not by proline), consistent with the known amino acid substrate specificity of the *S. cerevisiae* Dip5p permease (Regenberg et al., 1999). Shown are averages of three experiments with coefficient of variation $\leq$ 15% (not shown). These results suggest that dretamycin is transported into the cell as a dicarboxylic amino acid by the cognate transporter Dip5p, and its bioactivity is partially suppressed by amino acids that compete for the same permease.
(E) Fellutamides C and D. Proteasome assay (Proteasome-Glo; Promega) was performed according to manufacturer's instructions with modifications using whole-cell extracts prepared from *C. albicans* grown in the exponential phase. In the first experiment (middle), the original extract and four additional reference compounds were tested at concentrations $\geq$ 2× MIC, with the exception of lactacystin (without whole-cell activity against *C. albicans*). Both ECC2567 (the original bioactive extract eliciting a proteasome-specific FT profile) and lactacystin (a known inhibitor of the proteasome) inhibited the in vitro proteasome activity of *C. albicans*. Fellutamide C was tested for dose-dependent inhibition in wild-type *C. albicans* and for in vitro proteasome activity (right). The results suggest that the whole-cell activity of fellutamide C is largely consistent with inhibition of proteasome activity. Note that only six concentrations were tested in the proteasome assay. Shown are averages of three experiments, with bars indicating standard deviations.
See also Table S4.

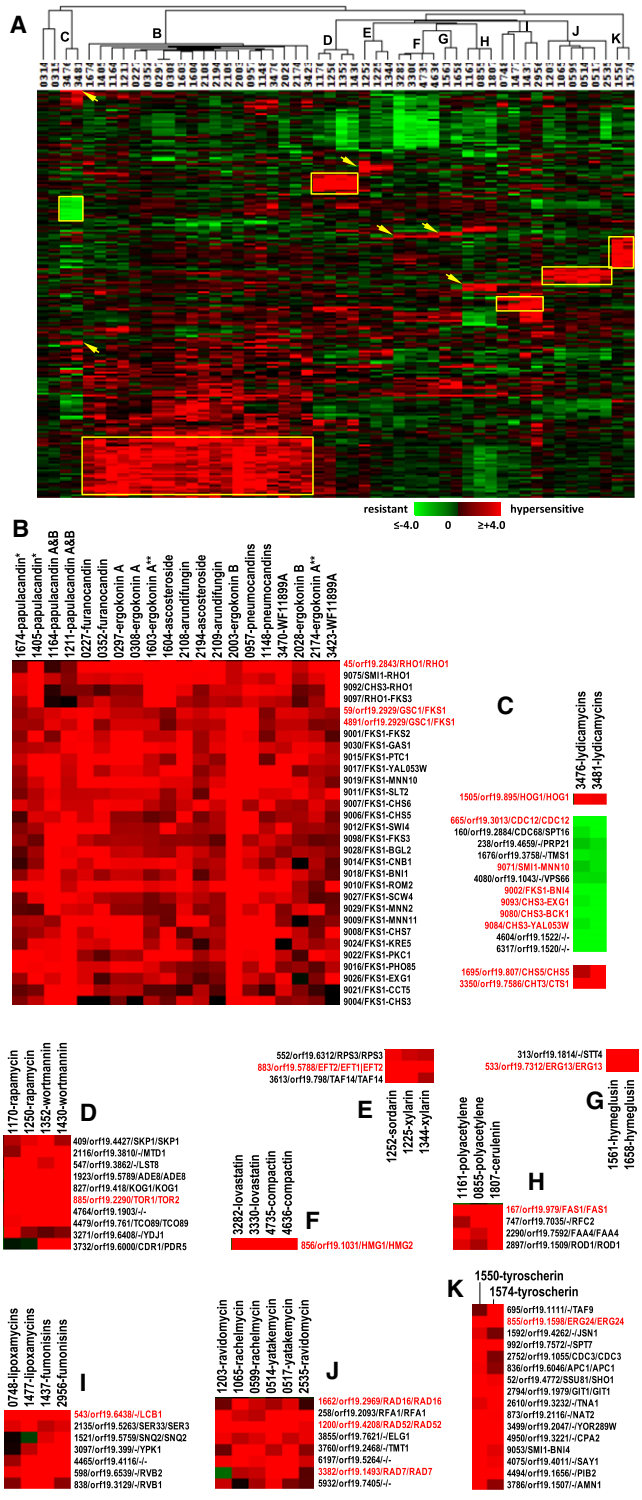

Figure 4. FT Profiling with Double Heterozygote Mutants Effecting Cell Wall Biosynthesis Robustly Identifies Glucan Synthase Inhibitors

(A) FT profiles of cell wall-acting bioactive extracts (clusters B and C) and non-cell wall-acting bioactive extracts (clusters D–K).

(B) FT profiles highlight 27 distinct double heterozygote strains (each deleted of one copy of *FKS1* or *RHO1*) displaying reproducible hypersensitivity across ten distinct fungal natural product extracts assayed in two independent experiments and whose subsequent bioactive elucidation identified known 1,3-β-glucan synthesis inhibitors or new analogs thereof (asterisks; see Table 1). Note that double heterozygote cell wall deletion strains are largely unresponsive to non-cell wall-acting bioactive NP extracts. Further, FT profiles robustly annotate alternative NP extracts containing structurally and mechanistically distinct bioactivities.

(C) A rare cell wall-related FT profile distinct from 1,3-β-glucan synthesis inhibitors derived from actinomycete extract ECC4442 and from which lyticamycins were isolated. Hypersensitive strains correspond to heterozygosity of *CTS1*, *CHS5*, and *HOG1*, which encode an endochitinase, chitin synthase III component, and MAP kinase involved in the high osmolarity stress response, respectively. Note prominent resistance among multiple cell wall mutant heterozygotes, including *NCE4* (a negative regulator of *CTS1*), as well as several double heterozygote cell wall mutants sharing chitin synthase (*CHS3*) heterozygosity.

(D) Rapamycin- and wortmannin-containing extracts produce highly related FT profiles reflecting their known MOA, with *TOR1* and the multiple components of the TOR complex (*KOG1* and *LST8*) uniquely responsive to these agents.

(E) Sordarin- and xylarin-containing extracts yielded a target-specific FT profile with *EFT1* heterozygote displaying prominent hypersensitivity (note that *RPS3* and *TAF14* heterozygotes contribute to the profile).

(F–I) Lovastatin and compactin, as well as hymeglusin (G), FT profiles capture their respective drug target, *HMG1* and *ERG13*. NP inhibitors targeting fatty acid biosynthesis (H; polyacetylenes and cerulenin) or sphingolipid biosynthesis (I; lipoxomycins and fumonsins) prominently affect their known targets, *FAS1* and *LCB1*, respectively.

(J) FT profiles reflect DNA repair pathways (*RAD52*, *RAD16*, *RAD7*, and *RFA1*) among NP extracts containing DNA-alkylating agents, including ravidomycin, rachelmycin, and yatakemycin.

(K) FT hypersensitivity profile of tyroscherin includes its know target (*ERG24*) as well as a complex secondary "signature." As exemplified by the above examples, mechanistically distinct FT signatures containing both the drug target as well as biological pathway/process information provide key mechanistic annotation to warrant follow-up characterization and isolation of bioactivities among NP extracts.

See also Table S2.

polyenes and ionophores that inhibit growth by non-target-specific MOAs (e.g., disrupting plasma membrane and ion transport, respectively). We conclude that it strongly appears that fungal NP extracts are generally more likely to contain target-specific antifungal bioactivities (as based on clear *C. albicans* target-specific FT profiles) than those produced by actinomycetes. This may reflect a potential evolutionary selection for fungal secondary metabolites to chemically regulate aspects of fungal metabolism and/or function as growth-inhibitory agents of competing fungi in a common ecological niche.

Concluding Remarks

Despite our expectation that novel and druggable antifungal leads with unique MOAs, or "low-hanging fruit," should be robustly identified through our work plan, such discoveries proved rare. Why is this the case? Is this an issue of unknowingly screening a limited microbial diversity despite our best efforts to address this at the outset? Maybe a more limited biosynthetic potential exists to produce novel natural products than we assume from microbiota? We just do not know. However, even in the rare event when a new lead was identified, further development efforts proved challenging due to significant medicinal chemistry challenges of optimizing complex NP scaffolds. For example, parnafungins satisfied all the above requirements, including intrinsic efficacy of the starting NP (Jiang et al., 2008), yet essentially all attempted medicinal chemistry modifications of the NP abolished its activity. Potentially, this may be addressed by the recent report of a total synthesis of

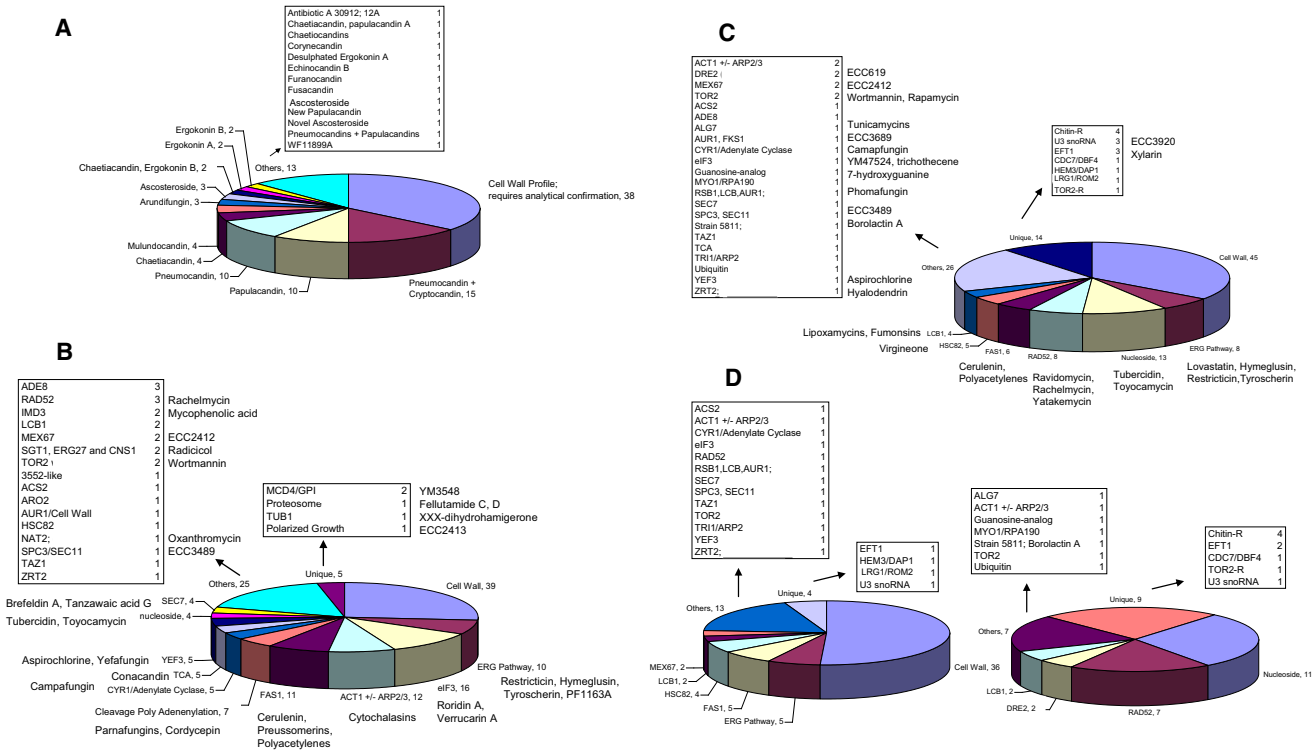

Figure 5. Summary of FT-Based NP Screening

(A) Summary of identified cell wall-specific NP inhibitors targeting 1,3-β-glucan synthesis. In total, 106 of 1801 (5.9%) NP extracts displaying (1) whole-cell *C. albicans* and/or *A. fumigatus* inhibitory activity and (2) tested in the FT produced profiles highly reflective of a cell wall mechanism of action as 1,3-β-glucan synthesis inhibitors. The relative frequency of particular glucan synthase inhibitor subclasses is graphically summarized. All but 38 extracts producing this FT profile were further characterized by UV spectra, retention time, LC-MS/MS, and/or HR-FT-MS to chemically identify the NP inhibitor. The subgroup labeled "others" reflects known glucan synthase inhibitors identified only once during the course of screening.

(B) Summary of NP-based FT profiles identified by screening the corporate historical antifungal NP library. Extracts (673) displaying whole-cell *C. albicans* and/or *A. fumigatus* inhibitory activity were screened within the FT, of which ~140 extracts (~22% hit rate) revealed specific and reproducible strain depletions reflecting a possible target-specific MOA. Prominent targets (or pathways) reflective of the proposed MOA for bioactive agents as well as the frequency they were observed from this library are indicated. Major mechanistic classes of inhibitory bioactive compounds target cell wall, ergosterol biosynthesis, protein synthesis/translation initiation (eIF), actin dynamics, and fatty acid biosynthesis. Representative known compounds within these subgroups are highlighted. Subgroups labeled "other" and "unique" reflect infrequent hits and those uniquely identified within this historical library, respectively. In cases where neither an NP common name or ECC number is given, these compounds are absent from Table 1.

(C) A summary of all target-specific NP extracts identified from FT screening using fresh microbial isolates and stimulating secondary metabolism by growth conditional nutritional array methods (Bills et al., 2008). Overall, 773 extracts annotated for displaying whole-cell *C. albicans* and/or *A. fumigatus* inhibitory activity were screened within the FT, of which 129 extracts (~17% hit rate) revealed specific and reproducible strain depletions reflecting a possible target-specific MOA. Major mechanistic classes of inhibitory bioactive compounds target cell wall and ergosterol biosynthesis, followed by nucleoside analogs and DNA-alkylating agents (likely due to the greater representation of actinomycetes among fresh microbial isolates screened). Note that eIF inhibitors (roridins and verucarrins) and actin inhibitors (cytochalasins) are not represented in this analysis, because they were easily dereplicated by LC-MS methods following their frequent identification during screening of the historical antifungal NP library.

(D) Summary of actinomycetes versus fungal-derived target-specific NP extracts identified from FT screening of in-house fresh microbial isolates. Major mechanistic classes of inhibitory bioactive compounds derived from fungi target cell wall and ergosterol biosynthesis, whereas actinomycetes are biased toward antifungal metabolites related to nucleoside analogs or DNA-alkylating agents. Note also that the hit rate for target-specific bioactive compounds from actinomycetes is <50% seen screening fungal-derived extracts. In part, this reflects the frequent identification of nonspecific growth-inhibitory metabolites (e.g., polyenes and ionophores) as well as undesirable modes of action (e.g., mitochondrial respiration inhibitors) common to actinomycetes and which could be effectively chemically dereplicated (e.g., LC-MS, UV spectra, etc.) and eliminated from FT analysis. As a collaborator's library yielded similar conclusions as screening our internal historical NP library (see B), a graphical summary of these results is not included.

parnafungins A and C (Zhou and Snider, 2010). In other instances, the starting NP scaffold failed to provide the intrinsic potency and spectrum necessary to justify exploratory chemistry resources to optimize their activity. Although such decisions are undoubtedly subjective, they are inevitable in a pharmaceutical company setting when choosing how best to invest medicinal chemistry resources across multiple competing therapeutic areas. We also conclude that the technological and philosophical rationale of our approach, although validated, is insufficient without accompanying innovative advances to expand accessible NP diversity for screening. For example, FT-based screening of NPs challenges historical metrics of screening throughput: rather than screening a single target across 1,000,000 compounds, thousands of potential targets can be screened in a single assay against a single extract containing hundreds of distinct NPs. Despite large-scale FT screening of NP extracts, we experienced a diminishing return in discovering novel target-specific inhibitors over time, regardless of the NP extract source (Figure 5). Our annotation of known and new target-specific inhibitors identified from fungi proved markedly

more diverse than that obtained from actinomycete sources. Therefore, an expanding NP screening diversity seems necessary. In part, this may be achieved by considering alternative NP sources such as plant or marine extracts. Advances in metagenomics (Lefevre et al., 2008; Li et al., 2009), novel approaches to culturing previously unculturable organisms (Kaeberlein et al., 2002; Lewis et al., 2010; D'Onofrio et al., 2010), or diversity-oriented synthesis using existing NP scaffolds (Marcaurelle and Johannes, 2008; Galloway et al., 2009) may also address this issue. Whether pharmaceutical companies can address these bottlenecks is also uncertain; hopefully, the biotechnology industry recognizes the substantial commercial opportunities in applying a reinvigorated basic research emphasis to these problems. The importance and increasing role of academic research facilities (e.g., Broad Institute) and granting institutions (e.g., NIH) in supporting NP research is also vital. Promoting public-private partnerships between these institutions and the pharmaceutical and biotechnology industries may provide an appropriate and sustainable model, perhaps a public and private consortium, with the right balance between the necessary scientific innovation and the accompanying financial cost dually required in supporting future NP-based antimicrobial drug discovery efforts. Indeed, the time for cooperation and collaboration between basic and industrial NP-based research has never been timelier.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four tables and can be found with this article online at doi:10.1016/j.chembiol.2011.01.009.

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