Peptide-Like Nylon-3 Polymers with Activity against Phylogenetically Diverse, Intrinsically Drug-Resistant Pathogenic Fungi

Leslie A. Rank, Naomi M. Walsh, Fang Yun Lim, Samuel H. Gellman, Nancy P. Keller, Christina M. Hull

Department of Chemistry, University of Wisconsin—Madison, Madison, Wisconsin, USA
Department of Biomolecular Chemistry, School of Medicine and Public Health, University of Wisconsin—Madison, Madison, Wisconsin, USA
Department of Medical Microbiology and Immunology, School of Medicine and Public Health, University of Wisconsin—Madison, Madison, Wisconsin, USA

ABSTRACT  Understanding the dimensions of fungal diversity has major implications for the control of diseases in humans, plants, and animals and in the overall health of ecosystems on the planet. One ancient evolutionary strategy organisms use to manage interactions with microbes, including fungi, is to produce host defense peptides (HDPs). HDPs and their synthetic analogs have been subjects of interest as potential therapeutic agents. Due to increases in fungal disease worldwide, there is great interest in developing novel antifungal agents. Here we describe activity of polymeric HDP analogs against fungi from 18 pathogenic genera composed of 41 species and 72 isolates. The synthetic polymers are members of the nylon-3 family (poly-β-amino acid materials). Three different nylon-3 polymers show high efficacy against surprisingly diverse fungi. Across the phylogenetic spectrum (with the exception of Aspergillus species), yeasts, dermatophytes, dimorphic fungi, and molds were all sensitive to the effects of these polymers. Even fungi intrinsically resistant to current antifungal drugs, such as the causative agents of mucormycosis (Rhizopus spp.) and those with acquired resistance to azole drugs, showed nylon-3 polymer sensitivity. In addition, the emerging pathogens Pseudogymnoascus destructans (cause of white nose syndrome in bats) and Candida auris (cause of nosocomial infections of humans) were also sensitive. The three nylon-3 polymers exhibited relatively low toxicity toward mammalian cells. These findings raise the possibility that nylon-3 polymers could be useful against fungi for which there are only limited and/or no antifungal agents available at present.

IMPORTANCE  Fungi reside in all ecosystems on earth and impart both positive and negative effects on human, plant, and animal health. Fungal disease is on the rise worldwide, and there is a critical need for more effective and less toxic antifungal agents. Nylon-3 polymers are short, sequence random, poly-β-amino acid materials that can be designed to manifest antimicrobial properties. Here, we describe three nylon-3 polymers with potent activity against the most phylogenetically diverse set of fungi evaluated thus far in a single study. In contrast to traditional peptides, nylon-3 polymers are highly stable to proteolytic degradation and can be produced efficiently in large quantities at low cost. The ability to modify nylon-3 polymer composition easily creates an opportunity to tailor efficacy and toxicity, which makes these materials attractive as potential broad-spectrum antifungal therapeutics.

KEYWORDS  antifungal drug development, emerging pathogens, fungal disease, host defense peptide mimics, nylon-3 polymers

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Address correspondence to Samuel H. Gellman, gellman@chem.wisc.edu, Nancy P. Keller, npkeller@wisc.edu, or Christina M. Hull, cmhull@wisc.edu.
The pervasiveness of fungi and their impact on globally important processes can be seen in the evolution of the human immune system (1), the chemistry of soil (2), the genomes of plants (3, 4), and even the progression of climate change (5). Fungi interact extensively with plants, animals, bacteria, and other organisms; these relationships range from mutualists to saprotrophs to pathogens. The influence of fungi across a variety of biological processes has its roots in the massive degree of diversity within the kingdom of fungi. Fungi are characterized by varied morphologies, diverse growth strategies, and assorted nutrient acquisition capabilities. Though only roughly 100,000 species of fungi are accepted in the current taxonomy (6), it is estimated that there exist on the order of 1.5 million fungal species and that up to one-quarter of the world’s biomass is of fungal origin (7, 8). Our imperfect understanding of fungal diversity arises from the lack of taxonomically relevant morphological features and pleomorphisms of fungi, the difficulty or impossibility of culturing many fungi isolated from natural environments, and the inability to consistently identify diagnostic sexual structures in fungi grown in culture or observed in the environment. The great ecological success of fungi, either as filamentous or unicellular growth forms, is a testament to their diversity and functional importance in the global ecosystem.

Understanding the dimensions of fungal diversity has major implications for disease control, crop management, and decomposition of both recalcitrant organic and synthetic materials. For environmental, agricultural, and biomedical purposes, we need agents that target fungi broadly. The innate immune response to infection by fungi and other microbes includes production of host defense peptides (HDPs), and these molecules offer clues for development of synthetic antifungal agents. HDPs have diverse sequences. Many of these peptides are relatively short (10 to 50 amino acid residues) and adopt globally amphiphilic conformations, although the nature of these conformations is variable (α-helix versus β-sheet versus irregular) (9). HDP amphiphility is hypothesized to be critical for disruption of microbial membranes, which results in either microbial killing or inhibition of growth (other antimicrobial mechanisms of action have been proposed as well) (10, 11).

Since their discovery in the 1980s, HDPs and their synthetic analogs have been subjects of interest as potential therapeutic agents. The high cost of production and inherent toxicity to the host manifested by HDPs, however, have discouraged clinical development of these peptides thus far (9). The production challenge has encouraged evaluation of random copolymers with hydrophilic and lipophilic subunits for antimicrobial activity (12–18). Random copolymers can be synthesized in a more economical manner than can sequence-specific peptides.

Nylon-3 polymers have been studied as antibacterial agents since 2007, and more recently, examples with both antibacterial and antifungal activities have been reported (19–23). Nylon-3 polymers are synthesized via the anionic ring-opening polymerization of β-lactams. Use of racemic β-lactams leads to heterochiral polymer chains. The protein-like polypeptide backbone of nylon-3 polymers is believed to confer biocompatibility, while the presence of β-amino acid subunits renders these materials resistant to proteolytic degradation (24).

Three nylon-3 polymers have previously been shown to have activity against pathogenic fungi (20, 22). These polymers, designated MM-TM, DM-TM, and NM (Fig. 1), inhibit the growth of Candida albicans and Cryptococcus neoformans, pathogenic fungi with yeast morphology, as single agents. In contrast, these polymers were able to halt the growth of the pathogenic, filamentous Aspergillus fumigatus only when applied in combination with established azole-based antifungal agents; the polymers were not active as single agents. The activity of MM-TM, DM-TM, and NM against pathogenic yeasts led us to hypothesize that these polymers might be active against a wide range of unicellular yeasts, but not against filamentous fungi. The work described here presents a test of this hypothesis. We have undertaken, to our knowledge, the broadest phylogenetically susceptibility analysis of any antifungal compound to determine the extent of activity of the MM-TM, DM-TM, and NM polymers. We expanded our evaluation to include a diverse array of both filamentous and nonfilamentous species across
the fungal kingdom, including members of the Zygomycetes, Ascomycetes, and Basidiomycetes phyla. Surprisingly, most fungi tested, including those naturally resistant to current antifungal drugs, were sensitive to the nylon-3 polymers, which raises the possibility that nylon-3 polymers could be useful against pathogens for which there are only limited and/or no antifungal agents available at present.

**RESULTS**

**NM, MM-TM, and DM-TM are active at low concentrations against phylogenetically diverse yeasts.** To evaluate the antifungal activity of MM-TM, DM-TM, and NM against six species of vegetatively growing yeasts across three different genera, we used the CLSI M27-A3 broth microdilution method (Table 1) (25). Polymers were evaluated by measuring MICs (MIC$_{100}$s). For comparison, we also evaluated the commonly used antifungal drug fluconazole (FLC) in terms of MIC$_{50}$ (the MIC to halt 50% of growth per the CLSI M27-A3 standard). All of the yeasts tested were sensitive to all three of the nylon-3 polymers, including those strains resistant to azoles. The polymers were particularly effective against Cryptococcus spp. (Cryptococcus neoformans and Cryptococcus amylolentus) of the Basidiomycota phylum, exhibiting MIC$_{100}$ values from 2 to 4 μg/ml, which is comparable to or better than concentrations of fluconazole required to halt only 50% of fungal growth. MM-TM, DM-TM, and NM were also active against two genera within the Ascomycota phylum: Candida and Saccharomyces. Saccharomyces cerevisiae was particularly sensitive to all three nylon-3 polymers, with NM

**TABLE 1** MIC results for MM-TM, DM-TM, and NM against vegetatively growing yeasts

| Species                 | Isolate | Resistance | MIC$_{100}$ (μg/ml)$^a$ | FLU MIC$_{50}$ (μg/ml)$^b$ |
|-------------------------|---------|------------|-------------------------|----------------------------|
|                         |         |            | NM          | MM-TM | DM-TM |            | FLU |            | AMB$^d$ |            |            |
| *Saccharomyces cerevisiae* | W303    |            | 2           | 4     | 8     |            |     |            |         | <1         |
| *Cryptococcus neoformans* | CN1     |            | 4           | 4     | 4     |            |     | 4           |         | >64        |
|                         | CN2     | FLU        | 4           | 4     | 4     | >64        |     |             |         | >64        |
|                         | CN3     | FLU        | 4           | 4     | 4     | >64        |     |             |         | >64        |
| *Cryptococcus amylolentus* | CBS 6273 |            | 4           | 4     | 2     | 16         |     |             |         | >64        |
|                         | CBS 6039 |            | 4           | 4     | 4     | 16         |     |             |         | >64        |
| *Candida albicans*       | ATCC 90028 |            | 4           | 4     | 4     | 1          |     |             |         | >64        |
|                         | SCS314  |            | 8           | 16    | 16    | 0.25       |     |             |         | >64        |
|                         | CA3     | Azole      | 8           | 16    | 64    | >64        |     |             |         | >64        |
| *Candida krusei*         | QC$^e$  |            | 4           | 16    | 8     | 32         |     |             |         | >64        |
| *Candida auris*          | B11220  |            | 4           | 8     | 8     | 4          |     |             |         | >64        |
|                         | C54039  | AMB$^d$    | 8           | 16    | 16    | 16         |     |             |         | >64        |

$^a$*Candida lusitaniae* was tested previously (23) for MIC activity with MM-TM and shown to have an MIC$_{100}$ of 5 μg/ml.

$^b$MIC$_{100}$, MIC resulting in 100% reduction in growth.

$^c$FLU, fluconazole; MIC$_{50}$, MIC resulting in 50% reduction in growth.

$^d$AMB, amphotericin B.

$^e$QC, quality control.
were also active against Candida spp. were also susceptible to the polymers, with MIC 100 values for nylon-3 polymers tested. The remaining 12 species of pathogenicity of these three species is related to their relatively high resistance to the two strains of Conidia CBS 172.66 Aspergillus aculeatus May/June 2018 Volume 3 Issue 3 e00223-18

TABLE 2 MIC results for MM-TM, DM-TM, and NM against Aspergillus speciesa

| Species             | Cell type | Isolate   | MIC100 (µg/ml)b | NM | MM-TM | DM-TM | ITRAc |
|---------------------|-----------|-----------|-----------------|----|-------|-------|-------|
| Aspergillus fumigatus | Conidia   | AF293     | >64             | >64| >64   | >64   | <1    |
| Aspergillus fumigatus | Conidia   | CEA10     | >64             | >64| >64   | >64   | <1    |
|                     | Hyphae    |           | >64             | >64| >64   |       | 8–16  |
| Aspergillus flavus  | Conidia   | NRRL3357  | >64             | >64| >64   |       | <1    |
|                     | Hyphae    |           | >64             | >64| >64   |       | >64   |
| Aspergillus terreus | Conidia   | NCCB IH2626 | >64          | >64| >64   |       | <1    |
|                     | Hyphae    |           | >64             | >64| >64   |       | >64   |
| Aspergillus oryzae  | Conidia   | Rib40     | >64             | >64| >64   |       | 1     |
| Aspergillus parasiticus | Conidia   | Su-1      | >64             | >64| >64   |       | <1    |
| Neosartorya fischeri | Conidia   | CBS 544.65 | >64          | >64| >64   |       | >8    |
| Aspergillus nidulans| Conidia   | FGSCA4    | 16              | 8  | 8     | 8     | <1    |
|                     | Hyphae    |           | >64             | 64 | 32    | >64   |       |
| Aspergillus aculeatus | Conidia   | CBS 172.66 | >64          | >64| >64   | 8     | <1    |
| Aspergillus carbonarius | Conidia   | DT0115-B6 | 64             | 64 | 64    | 64    | <1    |
| Aspergillus wentii  | Conidia   | DT0136-E9 | >64             | 64 | 64    | 32    | <1    |
| Aspergillus sydowii | Conidia   | CBS 593.65 | >64           | >64| 64    | 64    | 2     |
| Aspergillus foetidus | Conidia   | CBS 106.47 | >64            | >64| 64    | 32    | 2     |
| Aspergillus zonatus | Conidia   | CBS 506.65 | 16             | 8  | 8     | <1    |       |
| Aspergillus niger   | Conidia   | CBS 113.46 | 64             | 32 | 32    | <1    |       |
| Aspergillus glaucus | Conidia   | CBS 516.65 | >64            | 64 | 32    | 2     |       |
| Aspergillus brasiliensis | Conidia   | CBS 101740 | 64           | 48 | 48    | 64    |       |
| Aspergillus clavatus | Conidia   | CBS 513.65 | >64          | >64| >64   | 64    |       |
| Aspergillus versicolor | Conidia   | CBS 795.97 | 16            | 16 | 8     | >1    |       |

aNote that the first six species (shown in boldface type) indicate that no reduction in growth was observed after incubation with nylon-3 polymer. The remaining 12 species (shown in lightface type) indicate that some reduction in growth was noted in response to MM-TM, DM-TM, and NM, even when the MIC100s were >64 µg/ml.
bMIC100, MIC resulting in 100% reduction in growth.
cITRA, itraconazole.

being the most active (MIC = 2 µg/ml) and DM-TM the least active (MIC = 8 µg/ml). Candida spp. were also susceptible to the polymers, with MIC100 values for Candida albicans and Candida krusei generally ranging from 4 to 16 µg/ml. The polymers were also active against Candida auris. Invasive and drug-resistant C. auris infections have been increasingly reported in health care facilities around the world (26, 27). The two strains of C. auris tested both showed sensitivity (MIC100s of 4 to 16 µg/ml) to all three polymers, with particular sensitivity to NM. Overall, yeasts were highly susceptible to the three nylon-3 polymers tested for efficacy, with NM being the most active polymer against yeasts.

The Aspergillus genus is generally insensitive to nylon-3 polymers, NM, MM-TM, and DM-TM. Previous reports show that nylon-3 polymers are largely inactive against the pathogenic fungus Aspergillus fumigatus (22). To determine whether this resistance phenotype was specific to A. fumigatus, we assessed the antifungal activity of MM-TM, DM-TM, and NM against 18 different species within the Aspergillus genus. For 6 of the 18 Aspergillus species examined, none of the three polymers caused any decrease in hyphal growth relative to a no-treatment control (Table 2, boldface rows, rows 1 to 7). A. fumigatus, A. flavus, and A. terreus are considered to be the most pathogenic species of the Aspergillus genus (28). It seems possible that the high pathogenicity of these three species is related to their relatively high resistance to the nylon-3 polymers tested. The remaining 12 species of Aspergillus evaluated showed low levels of growth inhibition, with MIC100s ranging from 8 to >64 µg/ml, with the DM-TM copolymer demonstrating the most promising antifungal activity among the three nylon-3 polymers. While all 18 species of Aspergillus were tested according to CLSI...
M38-A2 methodology (conidia in liquid culture), four species, selected for their varied response to polymer, were also tested for susceptibility to polymer as hyphal clumps in liquid culture. MIC100 values against hyphal fragments were determined as the concentration of agent required to prevent hyphal growth, monitored as an increase in optical density at 600 nm (OD600), after incubation at 35°C for 48 h. Three of these species, *A. fumigatus*, *A. terreus*, and *A. flavus*, were highly resistant to polymer as conidia, and their hyphal fragments were also insensitive to polymer. The conidia of the fourth species of *Aspergillus* selected, *A. nidulans*, were susceptible to polymer, with MIC100 values ranging from 8 to 16 μg/ml; however, polymer activity was diminished when incubated with *A. nidulans* hyphal clumps (MIC100 values of 32 to 64 μg/ml). As expected from precedent, the efficacy of itraconazole against *Aspergillus* species as hyphal clumps was also greatly diminished relative to treatment of conidia (MIC100, conidia ~1 μg/ml; MIC100, hyphae ~8 to 64 μg/ml) (29). Overall, nylon-3 polymers are ineffective against species within the *Aspergillus* genus, regardless of the life form assayed (conidia or hyphae) for sensitivity.

NM, MM-TM, and DM-TM are active against phylogenetically diverse filamentous fungi. On the basis of our observation of low sensitivity of *Aspergillus* spp. toward the polymers as single agents, we hypothesized that nylon-3 polymers were intrinsically less effective against filamentous fungi relative to yeasts. To test this hypothesis, we assessed MM-TM, DM-TM, and NM activity against 10 genera of filamentous fungi, following the CLSI M38-A2 methodology (conidia in liquid culture) or a modified CLSI method (hyphal fragments in liquid culture). Surprisingly, MM-TM, DM-TM, and NM were very active against phylogenetically diverse filamentous fungi, with MIC100 values of 4 to 8 μg/ml (Table 3). The polymers were much more effective against medically relevant *Rhizopus arrhizus* (a causative agent of mucormycosis), the emerging pathogen *Paecilomyces variotii* (30), and *Fusarium oxysporum* isolates (which, along with *A. flavus*, is a serious agent of keratitis [31]) than they were against *Aspergillus* spp. Notably, all three nylon-3 polymers were active against *Scedosporium* spp., both *Scedosporium apiospermum* and *Scedosporium prolificans*. The latter species is an emerging fungal

### TABLE 3  MIC results for MM-TM, DM-TM, and NM against filamentous fungi

| Species                        | Cell type  | Isolate   | MIC100 (μg/ml) | NM | MM-TM | DM-TM | POS/VOR/ITRA | FLU |
|-------------------------------|------------|-----------|----------------|----|-------|-------|--------------|-----|
| *Penicillium expansum*        | Conidia    | d1        | >64            | >64| 64    | 1     | (I)          |     |
|                               | Hyphae     |           | >64            | >64| 64    | >64   | (I)          |     |
| *Talaromyces marneffei*       | Conidia    | FRR2161   | 64             | 32 | 8     | 0.125 | (I)          |     |
|                               | Hyphae     |           | >64            | >64| 64    | >64   | (I)          |     |
| *Paecilomyces variotii*       | Conidia    | QC        | 4              | 8  | 4     | 0.125 | (V)          |     |
| *Fusarium oxysporum*          | Conidia    | FO1       | 4              | 8  | 8     | 4     | (V)          |     |
| *Fusarium oxysporum*          | Conidia    | FO2       | 8              | 8  | 8     | 4     | (V)          |     |
|                               | Conidia    | FO3       | 4              | 8  | 4     | >16   | (V)          |     |
| *Scedosporium apiospermum*    | Conidia    | SA1       | 2              | 8  | 4     | 1     | (V)          |     |
| *Scedosporium apiospermum*    | Conidia    | SA2       | 4              | 4  | 4     | 1     | (V)          |     |
| *Scedosporium prolificans*    | Conidia    | LP1       | 4              | 4  | 4     | >16   | (V)          |     |
|                               | Conidia    | RA1       | 16             | 8  | 4     | 0.5   | (P)          |     |
|                               | Conidia    | RA2       | 8              | 8  | 8     | 0.5   | (P)          |     |
|                               | Conidia    | RA3       | 8              | 8  | 8     | 0.5   | (P)          |     |
| *Pseudogymnoascus destructans*| Conidia    | ATCC MYA-4855 | 4–8 | 2–4 | 2–4 | 0.13 | (I)          |     |
| *Trichophyton rubrum*         | Hyphae     | ATCC 28188 | >64            | 64 | 16    | 0.5   | (I)          |     |
| *Trichophyton tonsurans*      | Hyphae     | CBS 112818 | 16             | 8  | 8     | 0.13  | (I)          |     |
| *Microsporum canis*           | Hyphae     | UW10      | 16             | 8  | 16    | <0.13 | (I)          |     |
| *Filobasidiella depauperata*  | Conidia    | CBS 7855  | >64            | >64| >64   | 1     | (I)          | 32  |
|                               | Hyphae     |           | >64            | >64| >64   | >64   | (I)          | 32  |

*a* MIC100, MIC resulting in 100% reduction in growth.

*The MICs for posaconazole (POS), voriconazole (VOR), or itraconazole (ITRA) are shown followed by the drug abbreviation in parentheses: I, itraconazole; V, voriconazole; P, posaconazole.

*FLU*, fluconazole.

*QC* assays conducted in SD medium, rather than RPMI 1640.

*QC*, quality control.
pathogen of both immunocompetent and immunocompromised individuals that is intrinsically resistant to most antifungal drugs (voriconazole [VOR] MIC100 \( < 16 \) g/ml); *S. prolificans* infections are often fatal (32).

For the dermatophytes *Trichophyton tonsurans*, *Trichophyton rubrum*, and *Microsporum canis*, MIC100 values were determined as the concentration of the agent required to halt hyphal growth, monitored as an increase in OD600, after 4 days of incubation at 29°C in RPMI 1640. Mixed efficacy of the nylon-3 polymers was observed across the three species of dermatophytes tested. The highest levels of antifungal activity were observed against *T. tonsurans* and *M. canis* (8 to 16 g/ml), and the lowest levels were observed against *T. rubrum* (16 to 64 g/ml) (Table 3). The MIC100 of the dermatophyte *Pseudogymnoascus destructans* was evaluated after 7 days of incubation of conidia at 12°C in RPMI 1640. The activity against *P. destructans*, the causative agent of white nose syndrome in bats (33), is encouraging because there are limited options for preventing the spread of this devastating pathogen. The facility with which nylon-3 structure can be varied could provide opportunities for the development of topical agents with high specificity for particular fungi.

Strikingly, nylon-3 polymers were ineffective against *Filobasidiella depauperata* (MIC100 \( > 64 \) g/ml [Table 3]), which is closely related to *C. neoformans* and grows exclusively as filaments (34). Resistance of *F. depauperata* to nylon-3 polymers was surprising because of the close phylogenetic relationship between *C. neoformans* and *F. depauperata* and the consistent sensitivity of *C. neoformans* isolates. *F. depauperata* spores and hyphae were both strongly resistant to polymer in SD medium (synthetically defined, minimal medium) (35); this species of fungi did not grow in the RPMI 1640 growth medium specified by CLSI methods. *C. neoformans JEC20* spores and yeasts were both sensitive to all three polymers in SD medium (see Tables S1 and S2 in the supplemental material). As this level of inactivity against germinating conidia was reminiscent of the results observed with *Aspergillus* species, we asked whether the nylon-3 polymer MM-TM could act synergistically with an azole to inhibit germination of *F. depauperata* conidia, as had previously been observed with various *A. fumigatus* isolates. The copolymer MM-TM exhibited strong synergy (ΣFIC [fractional inhibitory concentration] index value of 0.04) with itraconazole against the azole-sensitive strain CBS 7855, resulting in \( > 20 \)-fold decreases in azole and polymer MIC100s when the polymers are given in combination (Table 4). The MM-TM copolymer was previously shown to act synergistically with azole-sensitive strains of *A. fumigatus* (22). Overall, all three nylon-3 polymers demonstrated strong activity against filamentous fungi spanning the fungal tree of life, with the notable exceptions of *F. depauperata* and the *Aspergillus* genus.

**NM, MM-TM, and DM-TM are active against dimorphic fungi.** Given the sensitivity of yeasts and most filamentous fungi to NM, MM-TM, and DM-TM, we hypothesized that dimorphic fungi would be sensitive to nylon-3 polymers. The CLSI M38-A2 broth macrodilution method was used to test all three polymers against *Coccidioides*, *Blastomyces*, and *Histoplasma* germinating conidia (36). Each of these dimorphic fungi represents a significant health threat for humans, and the dimorphism of these fungi is

| Test agent(s) | MIC100 (µg/ml) | Combination | ΣFIC index | FIC interpretation |
|---------------|---------------|-------------|------------|--------------------|
| MM-TM         | >64\(^{c}\)  | 1           | 0.04       | Synergistic        |
| Itraconazole  | 1             | <0.06\(^{d}\) |            |                    |

\(^{a}\)MIC after 72 h for the compound(s) as a single agent or for the combination of the compounds.

\(^{b}\)FIC, fractional inhibitory concentration.

\(^{c}\)The high off-scale MIC value, >64 µg/ml, was converted to the next highest concentration, 128 µg/ml, for calculation of the FIC index.

\(^{d}\)The low off-scale MIC value, <0.06 µg/ml, was converted to the next lowest concentration, 0.03 µg/ml, for calculation of the FIC index.
an important feature for their pathogenicity (37, 38). Activity was assessed based on 80% inhibition of growth (MIC₈₀ per the CLSI M38-A2 standard); the positive control for these studies was voriconazole (Table 5). MM-TM, DM-TM, and NM have MIC₈₀ values of <4 μg/ml for all strains of Histoplasma capsulatum, Blastomyces dermatitidis, and Coccidioides spp. assayed.

**TABLE 5** MIC results for MM-TM, DM-TM, and NM against dimorphic fungi

| Species            | Isolate | NM (μg/ml) | MM-TM (μg/ml) | DM-TM (μg/ml) | VOR (μg/ml) |
|--------------------|---------|------------|---------------|---------------|-------------|
| Coccidioides sp.   | Cocci1  | 1          | 2             | 2             | 0.25        |
|                    | Cocci2  | 1          | 2             | 2             | 0.125       |
|                    | Cocci3  | 0.5        | 1             | 1             | 0.06        |
|                    | Cocci5  | 2          | 2             | 1             | 0.125       |
|                    | Cocci6  | 1          | 1             | 1             | 0.25        |
|                    | Cocci7  | 1          | 1             | 1             | 0.125       |
|                    | Cocci8  | 1          | 1             | 1             | 0.125       |
|                    | Cocci9  | 1          | 2             | 2             | 1           |
|                    | Cocci10 | 1          | 1             | 1             | 0.06        |
| Blastomyces dermatitidis | BD1 | 1 | 2 | 1 | ≤0.03 |
|                     | BD2     | 1          | 2             | 1             | ≤0.03       |
|                     | BD3     | 1          | 2             | 1             | 0.5         |
| Histoplasma capsulatum | HC1     | 4          | 2             | 1             | ≤0.03       |
|                     | HC2     | 1          | 1             | 0.5           | 0.25        |
|                     | HC3     | 4          | 2             | 2             | 0.125       |
|                     | HC4     | 2          | 2             | 1             | 0.06        |
|                     | HC5     | 2          | 2             | 1             | 0.25        |
|                     | HC6     | 4          | 2             | 1             | 0.125       |
|                     | HC7     | 2          | 2             | 1             | 0.5         |
|                     | HC8     | 2          | 1             | 1             | 0.25        |
|                     | HC9     | 4          | 2             | 2             | 0.06        |
|                     | HC10    | 4          | 2             | 2             | 0.06        |

aMIC₈₀, MIC required to halt 80% of growth.

**NM, MM-TM, and DM-TM have moderate antifungal activity against Pneumocystis spp.** Pneumocystis spp. are known to cause lethal pneumonia in immunocompromised patients and are particularly associated with AIDS patients. Relatively little is known about Pneumocystis spp., ranging from its life cycle to survival strategies within mammalian hosts, since there currently exists no way to culture these species in a laboratory setting. Antifungal activity of the three polymers was assessed against cryopreserved and characterized Pneumocystis spp. Fungal viability in the presence of each polymer was measured using an ATP production assay (ATP-liteM assay) after 24, 48, and 72 h of polymer exposure. Calculating percent ATP reduction for all samples allowed us to determine 50% inhibitory concentrations (IC₅₀) (Table 6 and Tables S3 and S4). MM-TM and DM-TM exhibited moderate activity, with 72 h IC₅₀ values ranging from 2 to 5 μg/ml. The homopolymer NM was less active against Pneumocystis murina compared to Pneumocystis carinii, with 72-h IC₅₀ values of 15 and 3 μg/ml, respectively. These data illustrate that even noncanonical fungi with unusual adaptations and niches (Pneumocystis species were once thought to be protozoan parasites [39]) show sensitivity to nylon-3 polymers, expanding opportunities for the use of nylon-3 materials against diverse fungi.

**TABLE 6** IC₅₀ results for MM-TM, DM-TM, and NM against Pneumocystis spp.

| Species              | IC₅₀ (μg/ml) | Activity scale of day 3 IC₅₀ values (μg/ml) |
|----------------------|-------------|------------------------------------------|
| NM                   | MM-TM       | DM-TM                                   |
| Pneumocystis carinii | 3.4         | 4.5                                      | Moderate (1.0–9.99) |
| Pneumocystis murina  | 15          | 3.8                                      | Slight (10.0–49.9)  |

aIC₅₀, 50% inhibitory concentration.
**DISCUSSION**

Our data show that the nylon-3 polymers MM-TM, DM-TM, and NM are effective against a surprisingly broad spectrum of fungi (Fig. 2), with only low to moderate toxicity toward mammalian cells (see Text S1 and Tables S5 and S6 in supplemental material). The maximum likelihood phylogeny of fungal species used in this study (Fig. 2) shows that all three polymers showed roughly equivalent activity against highly diverse fungi across the fungal kingdom. Any strain for which polymer activity was not equivalent across all three polymers is indicated by the pound symbol. Any species assessed in this study in which a different strain was used to compose the phylogeny tree is indicated by an asterisk. The strain tested in a previous publication (11) is indicated by a caret. N/A, not available.
material). Here we were able to assess sensitivity of 18 pathogenic genera toward the nylon-3 chemotype, based on measurements with 41 species and 72 isolates. Visualization of our results in the context of fungal phylogeny indicates general sensitivity to the polymers, with notable exceptions in the Aspergillus clade and the basidiomycete Filobasidiella depauperata (Fig. 2). Overall, these data provide support for the idea that nylon-3 polymers could be useful as broad-action therapeutic agents.

Activity of the nylon-3 polymers was observed against several species of fungi with limited or ineffective treatment options. For instance, the nylon-3 polymers were active against Rhizopus arrhizus, one of the causative agents of mucormycosis, which is a life-threatening disease in both immunocompetent and immunocompromised patients (40). Depending on the predisease status of the patient and route of infection, mucormycosis may present in pulmonary, rhino-orbital-cerebral, cutaneous, gastrointestinal, or disseminated forms. Treatment of mucormycosis often requires administration of amphotericin B after surgical debridement of necrotic tissues (40). Even with rigorous treatment regimes, mortality rates are high (>40%), and amphotericin B toxicity is problematic for patients. The sensitivity of R. arrhizus to nylon-3 polymers suggests a new strategy to combat a challenging and deadly fungal disease for which current treatment options are highly limited (40).

All three nylon-3 polymers also showed efficacy against dermatophytes as preestablished hyphae. It is estimated that 20 to 25% of the world’s population has skin mycoses (41), and while these infections are often superficial and cosmetic in nature, they can have severe impact on quality of life. Topical treatment of dermatophyte infections relies mostly on two classes of antifungal drugs, the azoles (e.g., fluconazole and ketoconazole) and the allylamines (e.g., terbinafine and naftifine). However, there have been increasing incidences of resistant and refractory dermatophyte infections in the clinic, and daily dosages of terbinafine can reach 1,000 mg (42, 43). Nylon-3 polymers, with their ease of synthesis and low anticipated cost of production, could provide a viable option for treating such topical fungal infections.

Comparisons between sensitive and resistant genera may ultimately enable us to identify the origin(s) of sensitivity and to elucidate mechanisms of nylon-3 action. A particular opportunity to understand how nylon-3 polymers confer antifungal activity emerges from the observation that these materials were ineffective against Filobasidiella depauperata, despite the polymers’ strong activity against closely related Cryptococcus species. Since the polymers were active against other filamentous fungi, it is likely that properties not associated with filamentous morphology are responsible for this polymer resistance phenotype. Genetic comparisons of C. neoformans and F. depauperata may reveal insights into the mechanisms by which nylon-3 polymers exert their antifungal effects.

While all three nylon-3 polymers studied, NM, MM-TM, and DM-TM, had similar antifungal activities, subtle differences in activity profiles were noted throughout our phylogenetically broad analysis. NM was the most active polymer against the ascomycete yeasts Candida and Saccharomycyes, while DM-TM was the most potent polymer against filamentous fungi. Overall hydrophobicity represents one of the main chemical properties that varied among the polymers (NM < MM-TM < DM-TM). All subunits in NM bear a short cationic side chain. Therefore, this homopolymer therefore cannot display a large and well-defined hydrophobic surface, which is believed to be an important feature of host defense peptides (HDPs) and their synthetic mimics. The MM-TM and DM-TM copolymers, on the other hand, should manifest greater overall hydrophobicity relative to NM because there are one or two additional nonpolar CH₂ units in the MM and DM cationic subunits relative to NM, and each of the copolymers contains 20 to 30% of the entirely hydrophobic TM subunit. The copolymer expected to be most hydrophobic, DM-TM, was the most active among the three against filamentous fungi; however, this enhanced activity comes at the cost of increased mammalian cell toxicity relative to MM-TM or NM (Text S1 and Tables S5 and S6). The subtle differences in antifungal activity across the three polymers suggest that increased overall hydrophobicity may be required to enhance activity against filamentous fungi.
fungi, particularly of the Aspergillus clade. Polymers with increased hydrophobicity relative to those discussed here, however, may require additional design features to avoid enhanced toxicity toward eukaryotic cells. Here we present the activities of materials with a novel chemotype, nylon-3 polymers, against a varied array of fungi from across the fungal kingdom. The surprisingly broad spectrum of nylon-3 antifungal activities, including inhibition of difficult-to-treat human pathogens, offers promise for the development of polymeric compounds with therapeutically useful properties. The ease of synthesis and structural diversification of nylon-3 polymers provide a broad scope for future efforts to optimize activity against pathogenic fungi while limiting toxicity toward the host.

MATERIALS AND METHODS

Polymersynthesis and characterization. All polymers and monomers were prepared using previously reported methods (20–23). Please see the supplemental material for more information about polymer synthesis and characterization methods (Text S2 and Table S7).

CLSI M27–A3 protocol. The MIC_{100} endpoint of each antifungal agent was determined as the lowest concentration to inhibit 100% of fungal growth compared to the no-drug control. MIC_{100} values for all yeasts assayed (Table 7) were determined by the broth microdilution method according to the CLSI M27–A3 guidelines, with slight modifications (25). Briefly, fungal cells at a density of 0.5 × 10^{4} to 2.5 × 10^{4} cells/ml were incubated at 30 to 35°C in RPMI 1640 plus 0.145 M 3-[N-morpholino]propanesulfonic acid (MOPS) (pH 7.0) in 96-well plates with twofold serial dilutions of nylon-3 polymer or fluconazole (FLC) from 1 to 64 μg/ml. After 24 to 72 h, the optical density at 600 nm (OD_{600}) of each well was measured using a microplate reader. Wells containing fungal cells with no drug and wells containing only RPMI 1640 were used as positive and blank controls, respectively. Percent cell growth was determined as [(sample absorbance - blank absorbance) / (control absorbance - blank absorbance)] × 100%. All values reported represent the average MIC_{100} value for more than two biological replicates and two or more technical replicates each. The average MIC_{100} value consistently fell within a twofold serial dilution of the concentration of each experimental replicate. Any modifications to this protocol for a specific species is listed in Table 7.

Revised CLSI M38–A protocol for hyphae of filamentous fungi and dermatophytes. The MIC_{100} endpoint of each antifungal agent was determined as the lowest concentration to inhibit 100% of hyphal growth. MIC_{100} values for all filamentous fungi assayed were determined by the broth microdilution method according to CLSI M38–A guidelines, with modifications (44). For the dermatophytes, crushed hyphal fragments of Trichophyton tonsurans, Trichophyton rubrum, and Microsporum canis at a density corresponding to an OD_{600} reading of 0.05 were incubated at 29°C in RPMI 1640 plus 0.145 M MOPS, pH 7.0, in 96-well plates with twofold serial dilutions of nylon-3 polymer or with itraconazole (ITRA) from 1 to 64 μg/ml. After 96 h, the MIC of each well was measured by monitoring the changes in OD_{600} compared to the value at the 0-h time point. From the OD measurements, a difference in OD greater than 0.03 from the values at the 96- and 0-h time points was indicative of growth. All values reported represent the average MIC_{100} value for two biological replicates and six technical replicates each. The average MICs consistently fell within a twofold serial dilution of the concentration of each experimental replicate.

Pseudogymnoascus destructans at a density of 1 × 10^{4} spores/ml was incubated at 12°C in RPMI 1640 plus 0.145 M MOPS, pH 7.0, in 96-well plates with twofold serial dilutions of nylon-3 polymer or with ITRA from 1 to 64 μg/ml. After 96 h, the MIC of each well was measured by monitoring changes in OD_{600} compared to the value at the 0-h time point. From the OD measurements, a difference in OD greater than 0.01 from the values at the 120- and 0-h time points was indicative of growth. All values reported represent the average MIC_{100} value for two biological replicates and six technical replicates each. The average MICs consistently fell within a twofold serial dilution of the concentration of each experimental replicate.

A total of 1 × 10^{4} conidia/ml of Aspergillus fumigatus, Aspergillus terreus, Aspergillus flavus, or Aspergillus nidulans were inoculated into RPMI 1640 plus 0.145 M MOPS, pH 7.0 in 96-well plates and incubated at 37°C for 18 to 24 h to allow for growth of the extensive hyphal network. Hyphae were then treated with twofold serial dilutions of nylon-3 polymer or with ITRA from 1 to 64 μg/ml and allowed to incubate at 37°C. OD_{600} readings were obtained at 0, 24, and 48 h. From the OD measurements, a difference in OD greater than 0.05 from the values at the 48- and 0-h time points was indicative of growth. All values reported represent the average MIC_{100} value for two biological replicates and six technical replicates each. The average MICs consistently fell within a twofold serial dilution of the concentration of each experimental replicate.

A total of 1 × 10^{4} conidia/ml of Penicillium expansum conidia were inoculated into RPMI 1640 plus 0.145 M MOPS, pH 7.0, in 96-well plates and incubated at room temperature for 24 to 48 h to allow for germination into established hyphae. Hyphae were then treated with twofold serial dilutions of nylon-3 polymer or with ITRA from 1 to 64 μg/ml and allowed to incubate further at room temperature. OD_{600} readings were obtained at 0, 24, and 48 h. After 48 h, the MIC of each well was measured by monitoring changes in OD_{600} in comparison to the value at the 0-h time point. From the OD measurements, a difference in OD greater than 0.05 from the values at the 48- and 0-h time point was indicative of growth. All values reported represent the average MIC_{100} value for two biological replicates and six technical replicates each. The average MICs consistently fell within a twofold serial dilution of the concentration of each experimental replicate.
| Species         | Isolate or strain | Source                                         | Description                                      | Antifungal testa |
|-----------------|-------------------|-----------------------------------------------|-------------------------------------------------|-----------------|
| S. cerevisiae   | W303 (ATCC 200060)| Lab strain, obtained from Catherine Fox lab    | leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 | CLSI M27-A3, 1 mM uracil |
| C. neoformans   | CN1               | Cerebrospinal fluid; clinical isolate         | Azole susceptible                               | CLSI M27-A3, tested by NIAID |
|                 | CN2               | Popliteal lymph node; clinical isolate        | Fluconazole resistant                            | CLSI M27-A3, tested by NIAID |
|                 | CN3               | Cerebrospinal fluid; clinical isolate         | Fluconazole resistant                            | CLSI M27-A3, tested by NIAID |
| C. amylolentus  | CBS 6273          | Insect frass isolate, obtained from Joseph Heitman lab |                  | CLSI M27-A3 |
|                 | CBS 6039          | Insect frass isolate, obtained from Joseph Heitman lab |                  | CLSI M27-A3 |
| C. albicans     | ATCC 90028 (CA1)  | Blood                                         | CLSI M27-A3, tested by NIAID                    |
|                 | CA2               | Blood                                         | Azole resistant                                 | CLSI M27-A3, tested by NIAID |
|                 | CA3               | Blood                                         | CLSI M27-A3, tested by NIAID                    |
| C. krusei       | QC                | CLSI QC isolate for susceptibility testing     | CLSI M27-A3, tested by NIAID                    |
| C. auris        | B11220            | Clinical isolate (Japan), obtained from David Andes lab |                  | CLSI M27-A3 |
|                 | CS4039            | Clinical isolate (Columbia), obtained from David Andes lab | Amphotericin B resistant                        | CLSI M27-A3 |
| Coccioides sp.  | Cocci1            | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
|                 | Cocci2            | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
|                 | Cocci3            | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
|                 | Cocci4            | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
|                 | Cocci5            | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
|                 | Cocci6            | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
|                 | Cocci7            | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
|                 | Cocci8            | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
|                 | Cocci9            | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
|                 | Cocci10           | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
| B. dermatitidis | BD1               | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
|                 | BD2               | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
|                 | BD3               | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
| H. capsulatum   | HC1               | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
|                 | HC2               | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
|                 | HC3               | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
|                 | HC4               | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
|                 | HC5               | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |
|                 | HC6               | Unknown; NIAID preclinical testing services   | CLSI M38-A2, tested by NIAID                    |

(Continued on next page)
replicates each. The average MICs consistently fell within a twofold serial dilution of the concentration of each experimental replicate.

**CLSI M38-A2 protocol.** The MIC\(_{100}\) endpoint of each antifungal agent was determined as the lowest concentration to inhibit 100% of hyphal outgrowth from conidia. The MIC\(_{80}\) endpoint of each antifungal

| Species | Isolate or strain | Source | Description | Antifungal test |
|---------|-------------------|--------|-------------|-----------------|
| A. fumigatus | CEA10 | Nancy Keller lab | | CLSI M38-A2, 35°C |
| A. flavus | NRRRL3357 | Nancy Keller lab | | CLSI M38-A2, 35°C |
| A. oryzae | Rib40 | JGI Genome Project | | CLSI M38-A2, 35°C |
| A. terreus | NCCB IH2624 | JGI Genome Project | | CLSI M38-A2, 35°C |
| A. parasiticus | Su-1 | JGI Genome Project | | CLSI M38-A2, 35°C |
| N. fischeri | CBS 544.65 | JGI Genome Project | | CLSI M38-A2, 35°C |
| A. nidulans | FGSCA4 | JGI Genome Project | | CLSI M38-A2, 35°C |
| A. ochraceus | CBS 172.66 | JGI Genome Project | | CLSI M38-A2, 35°C |
| A. carbonarius | DT0115-86 | JGI Genome Project | | CLSI M38-A2, 35°C |
| A. wentii | DT0136-89 | JGI Genome Project | | CLSI M38-A2, 35°C |
| A. sydowii | CBS 593.65 | JGI Genome Project | | CLSI M38-A2, 35°C |
| A. foetidus | CBS 106.47 | JGI Genome Project | | CLSI M38-A2, 35°C |
| A. zonatus | CBS 506.65 | JGI Genome Project | | CLSI M38-A2, 35°C |
| A. niger | CBS 113.46 | JGI Genome Project | | CLSI M38-A2, 35°C |
| A. glaucus | CBS 516.65 | JGI Genome Project | | CLSI M38-A2, 35°C |
| A. brasiliensis | CBS 101740 | JGI Genome Project | | CLSI M38-A2, 35°C |
| A. clavatus | CBS 513.65 | JGI Genome Project | | CLSI M38-A2, 35°C |
| A. versicolor | CBS 795.97 | JGI Genome Project | | CLSI M38-A2, 35°C |
| P. expansum | d1 | Apples from Israel in 2012 | | CLSI M38-A2, 29°C |
| T. marneffei | FRR2161, CBS 334.59, ATCC 18224 | ATCC | | CLSI M38-A2, 29°C |
| P. variotii | QC | | CLSI QC isolate | CLSI M38-A2, tested by NIAID |
| F. oxysporum | FO1 | Blood | Clinical isolate | CLSI M38-A2, tested by NIAID |
| F. oxysporum | FO2 | Bone | Clinical isolate | CLSI M38-A2, tested by NIAID |
| F. oxysporum | FO3 | Blood | Clinical isolate | CLSI M38-A2, tested by NIAID |
| S. apiospermum | SA1 | Toe | Clinical isolate | CLSI M38-A2, tested by NIAID |
| S. apiospermum | SA2 | Elbow tissue | Clinical isolate | CLSI M38-A2, tested by NIAID |
| L. prolificans | LP1 | Chest wound | Clinical isolate | CLSI M38-A2, tested by NIAID |
| R. arrhizus | RA1 | Nose tissue | Clinical isolate | CLSI M38-A2, tested by NIAID |
| R. arrhizus | RA2 | Tissue upper extremity | Clinical isolate | CLSI M38-A2, tested by NIAID |
| R. arrhizus | RA3 | Palate tissue | Clinical isolate | CLSI M38-A2, tested by NIAID |
| F. depauperata | CBS 7855 | Caterpillar isolate, obtained from Joseph Heitman lab | | CLSI M38-A2, SD was used as the growth medium during antifungal testing |
| T. rubrum | ATCC 28188 | Alana Sterkel lab; UW Department of Pathology and Laboratory Medicine | Clinical isolate; nail | CLSI M38-A with modifications |
| M. canis | UW10 | Karen Moriello lab; UW School of Veterinary Medicine | Wild-animal isolate; cat | CLSI M38-A with modifications |
| T. tonsurans | CBS 112818 | Theodore White lab, Broad Institute | Clinical isolate; cheek | CLSI M38-A with modifications |
| P. destructans | ATCC MYA-4855 | Jeffrey Lorch lab; U.S. Geological Survey | Wild-animal isolate; bat | CLSI M38-A with modifications |

*The antifungal test and modifications are given. NIAID, National Institute of Allergy and Infectious Diseases.

*UW, University of Wisconsin.

*CLSI M38-A with modifications to test established hyphae rather than conidia for susceptibility. See the paragraph on the revised CLSI M38-A protocol for hyphae of filamentous fungi and dermatophytes in Materials and Methods.
was determined as the concentration to inhibit 80% of fungal growth. MIC$_{100}$ or MIC$_{80}$ values for all filamentous or dimorphic fungi assayed (Table 7) were determined by the broth macrodilution method according to the CLSI M38-A2 guidelines, with slight modifications (36). Briefly, fungal cells at a density of $0.5 \times 10^6$ to $5 \times 10^6$ conidia/ml were incubated at 35°C in RPMI 1640 plus 0.145 M MOPS, pH 7.0, in 96-well plates with twofold serial dilutions of the nylon-3 polymer or with fluconazole (FLC), voriconazole (VOR), or itraconazole (ITRA) from 1 to 64 $\mu$g/ml. After 24 to 72 h, the MIC of each well was measured visually using a dissecting microscope. All values reported represent the average MIC$_{100}$ values for more than two biological replicates and two or more technical replicates each. The average MICs consistently fell within a twofold serial dilution of the concentration of each experimental replicate. Any modifications to this protocol for a specific species are listed in Table 7.

**ATP-liteM assay for Pneumocystis spp.** Cryopreserved and characterized *Pneumocystis carinii* isolated from rat lung tissue and *Pneumocystis murina* isolated from mouse lung tissue were distributed into triplicate wells of 48-well plates with a final volume of 500 $\mu$l at a final concentration of $5 \times 10^7$ nuclei/ml *P. carinii* and $5 \times 10^8$ nuclei/ml *P. murina*. Controls and compounds were added and incubated at 36°C and 5% CO$_2$. At 24, 48, and 72 h, 10% of the well volume was removed, and the ATP content was measured using PerkinElmer ATP-liteM luciferin-luciferase assay. The luminescence generated by the ATP content of the samples was measured by a BMG PolarStar optima spectrophotometer. A sample of each group was examined microscopically on the final assay day to rule out the presence of bacteria.

For 50% inhibitory concentration (IC$_{50}$) calculations, background luminescence was subtracted, and triplicate well readings of duplicate assays were averaged. For each day’s reading, percent reduction in ATP for all groups was calculated as follows: [(medium control — experimental value)/medium control] $\times$ 100. IC$_{50}$ Values were calculated using GraphPad Prism 6 linear regression program (Tables S3 and S4).

**Evolutionary analysis.** Multigene-based phylogeny between all fungal species depicted in Fig. 2 was constructed based on amino acid sequences of 14 genes (Table S8) identified in *Fusarium* spp. to be conserved for various energetic processes (45). Orthologous proteins for genes listed in Table S8 were identified in each species using HMMPER (Ensembl Fungi). Ten genes from Wiemann et al. (45) were omitted from the analysis due to lack of orthologous protein identity in one or more of the species tested. Resulting protein sequences for each of the gene were aligned to well-conserved regions using MAFFT (46) and trimmed using Gblocks (47) using default parameters. All positions containing gaps and missing data were eliminated. Trimmed alignments were then concatenated for each species, and a maximum likelihood (ML) phylogeny was inferred based on the JTT model (48) using MEGA7 (49) and viewed in FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

**Data availability.** All data are included in the article and supplemental files.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at [https://doi.org/10.1128/mSphere.00223-18](https://doi.org/10.1128/mSphere.00223-18).

**TEXT S1,** PDF file, 0.2 MB.

**TEXT S2,** PDF file, 0.1 MB.

**TABLE S1,** PDF file, 0.1 MB.

**TABLE S2,** PDF file, 0.1 MB.

**TABLE S3,** PDF file, 0.1 MB.

**TABLE S4,** PDF file, 0.1 MB.

**TABLE S5,** PDF file, 0.1 MB.

**TABLE S6,** PDF file, 0.1 MB.

**TABLE S7,** PDF file, 0.1 MB.

**TABLE S8,** PDF file, 0.1 MB.

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**REFERENCES**

1. LeibundGut-Landmann S, Wüthrich M, Hohl TM. 2012. Immunity to fungi. Curr Opin Immunol 24:449–458. [https://doi.org/10.1016/j.coi.2012.04.007](https://doi.org/10.1016/j.coi.2012.04.007).

2. Eastwood DC, Floudas D, Binder M, Majerczyk A, Schneider P, Aerts A, Asiegbu FO, Baker SE, Barry K, Bendiksby M, Blumentritt M, Coutinho PM, Cullen D, de Vries RP, Gathman A, Goodell B, Henrissat B, Ihrmark K, Kauserud H, Kohler A, LaButti K, Lapidus A, Lavin JL, Lee Y-H, Lindquist
E. Lilly W, Lucas S, Morin E, Murat C, Oguaia JA, Park J, Pisabarro AG, Riley R, Rosling A, Salamon A, Schmidt O, Schmutz J, Skrede I, Strobel S, Tien J, Wiebenga A, Xie X, Kues U, Hibbitt DS, Hoffmeister D, Högberg N, Martin F, Grigoriev IV, Watson KC. 2011. The plant cell wall-decomposing machinery underlies the functional diversity of forest fungi. Science 333:762–765. https://doi.org/10.1126/science.1205411.

3. Schulze-Leffert P, Panstruga R. 2003. Establishment of biotrophy by parasitic fungi and reprogramming of host cells for disease resistance. Annu Rev Phytopathol 41:641–667. https://doi.org/10.1146/annurev.phyto.41.061002.083300.

4. Jones JDG, Dangl JL. 2006. The plant immune system. Nature 444:323–329. https://doi.org/10.1038/nature05286.

5. Treseder KK. 2016. Model behavior of arbuscular mycorrhizal fungi: predicting soil carbon dynamics under climate change. Botany 94:1551–1557. https://doi.org/10.1139/bt15-0245.

6. Hibbett DS, Taylor JW. 2013. Fungal systematics: is a new age of enlightenment at hand? Nat Rev Microbiol 11:119–133. https://doi.org/10.1038/nrmicro2963.

7. Hawksworth DL. 1991. The fungal dimension of biodiversity: magnitude, significance, and conservation. Mycol Res 95:641–655. https://doi.org/10.1016/S0953-7562(09)80810-1.

8. Leeder AC, Palma-Guerrero J, Glass NL. 2011. The social network: deciphering fungal language. Nat Rev Microbiol 9:440–451. https://doi.org/10.1038/nrmicro2580.

9. Hancock REW, Sahl H-G. 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol 24:1551–1557. https://doi.org/10.1038/nbt1267.

10. Zasloff M. 2002. Antimicrobial peptides of multicellular organisms. Nature 415:389–395. https://doi.org/10.1038/415389a.

11. Wilmes M, Cammue BPA, Sahl H-G, Thevissen K. 2011. Antibiotic activities of host defense peptides: more to it than lipid bilayer perturbation. Nat Prod Rep 28:1350–1358. https://doi.org/10.1039/c1np00022e.

12. Lienkamp K, Madkour AE, Musante A, Nelson CF, Nüsslein K, Tew GN. 2008. Antimicrobial polymers prepared by ROMP with unprecedented selectivity: a molecular construction kit approach. J Am Chem Soc 130:9836–9843. https://doi.org/10.1021/ja801662y.

13. Uppu DSSM, Samaddar S, Hoque J, Konai MM, Krishnamoorthy P, Shome BR, Haldar J. 2016. Side chain degradable cationic–amphiphilic polymers with tunable hydrophobicity show in vivo activity. Biomacromolecules 17:3094–3102. https://doi.org/10.1021/acs.biomac.6b01057.

14. Michl TD, Locock KE, Stevens NE, Hayouka Z, Chakraborty S, Lemke JJ, Hayouka Z, Chow C, Welch RA, Traven A, Haeusler M, Meager L, Griesser HJ. 2014. RAFT-derived antimicrobial polymeric ethers: elucidating the impact of end-groups on activity and cytotoxicity. Polym Chem 5:5813–5822. https://doi.org/10.1039/C4PY00652F.

15. Hu K, Schmidt NW, Zhu R, Jiang Y, Lai GH, Wei G, Palermo EF, Kuroda K, James S, Jones M, Karafa L, Karányi Z, Kato M, Keller N, Kelly DE, Kiel JAKW, Kim J-M, van der Klei U, Kils MF, Kovalchuk A, Krasevec N, Kubicek CP, Liu B, Maccabe A, Meyer V, Mirabito P, Miskei M, Mos M, Mullins J, et al. 2009. The 2008 update of the Aspergillus nidulans genome annotation: a community effort. Fungal Genet Biol 46(Suppl 1):S1–S52. https://doi.org/10.1016/j.fgb.2008.12.003.

16. van de Sande WW, Tavakol M, van Vianen W, Bakker-Woudenberg JA. 2010. The effects of antifungal agents to conidial and hyphal forms of Aspergillus fumigatus. Med Mycol 48:48–55. https://doi.org/10.1111/j.1365-3019.2009.00739.x.

17. Larkin E, Hager C, Chandra J, Mukherjee PK, Retuerto M, Salem I, Long L, Isham N, Kovanda L, Borroto-Esoda K, Wring S, Angulo D, Ghanmoum M. 2017. The emerging pathogen Candida auris: growth phenotype, virulence factors, activity of antifungals, and effect of SCY-018, a novel glucan synthesis inhibitor, on growth morphology and biofilm formation. Antimicrob Agents Chemother 61:e02396-16. https://doi.org/10.1128/AAC.02396-16.

18. Calvo-Chapmany A, Sharma C, Meis JF. 2017. Candida auris: a rapidly emerging cause of hospital-acquired multidrug-resistant fungal infections globally. PLoS Pathog 13:e1006290. https://doi.org/10.1371/journal.ppat.1006290.

19. Wortman JR, Gilsenan AM, Joardar V, Deegan J, Clutterbuck J, Andersen MR, Archer D, Bencina M, Braus G, Coutinho P, van Dohen H, Doonan J, Driessen AJM, Durek P, Espeso E, Fekete E, Filippi M, Estrada CG, Geysens S, Goitsmans G, de Groot PJW, Hansen K, Harris SD, Heinemck T, Höfling K, Henrikkson K, Hoffmann T, Horio T, Horuchi H, James S, Jones M, Karafa L, Karányi Z, Kato M, Keller N, Kelly DE, Kiel JAKW, Kim J-M, van der Klei U, Kils MF, Kovalchuk A, Krasevec N, Kubicek CP, Liu B, Maccabe A, Meyer V, Mirabito P, Miskei M, Mos M, Mullins J, et al. 2009. The 2008 update of the Aspergillus nidulans genome annotation: a community effort. Fungal Genet Biol 46(Suppl 1):S1–S52. https://doi.org/10.1016/j.fgb.2008.12.003.

20. van de Sande WW, Tavakol M, van Vianen W, Bakker-Woudenberg JA. 2010. The effects of antifungal agents to conidial and hyphal forms of Aspergillus fumigatus. Med Mycol 48:48–55. https://doi.org/10.1111/j.1365-3019.2009.00739.x.

21. Houbraken J, Verweij PE, Ali AJMM, Borman AM, Samson RA. 2010. Identification of Paecilomyces variotii in clinical samples and settings. J Clin Microbiol 48:2754–2761. https://doi.org/10.1128/JCM.00764-10.

22. Chang DC, Grant GB, O’Donnell K, Wannemuehler KA, Noble-Wang J, Rao CY, Robinson LM, Crowe SL, Sneed RS, Lewis FMT, Schaffzin JK, Kainer MA, Genese CA, Alfonso EC, Jones DB, 3.0.CO;2-R

23. Eskew EA, Todd BD. 2013. Parallels in amphibian and bat declines from pathogenic fungi. Emerg Infect Dis 19:379–385. https://doi.org/10.3201/jcid.1093.120707.

24. Rodriguez-Carres M, Findley K, Sun S, Dietrich FS, Heitman J. 2010. Morphological and genomic characterization of Filobasidiella depauperata: a homothallic sibling species of the pathogenic Cryptococcus species complex. PLoS One 5:e9620. https://doi.org/10.1371/journal.pone.0009620.

25. Sherman F, Hicks J. 1987. Methods in yeast genetics: a laboratory course manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

26. Clinical and Laboratory Standards Institute. 2008. Reference standard M27-A3. Reference method for broth dilution antifungal susceptibility testing of yeasts, 3rd ed. Clinical and Laboratory Standards Institute, Wayne, PA.

27. Sil A, Andrianopoulos A. 2014. Thermally dimorphic human fungal pathogens—polyphyletic pathogens with a convergent pathogenicity.
trait. Cold Spring Harb Perspect Med 5:a019794. https://doi.org/10.1101/cshperspect.a019794.

38. Boyce KJ, Andrianopoulos A. 2015. Fungal dimorphism: the switch from hyphae to yeast is a specialized morphogenetic adaptation allowing colonization of a host. FEMS Microbiol Rev 39:797–811. https://doi.org/10.1093/femsre/fuv035.

39. Cushion MT. 2010. Are members of the fungal genus Pneumocystis (a) commensals; (b) opportunists; (c) pathogens; or (d) all of the above? PLoS Pathog 6:e1001009. https://doi.org/10.1371/journal.ppat.1001009.

40. Sun H-Y, Singh N. 2011. Mucochromy: its contemporary face and management strategies. Lancet Infect Dis 11:301–311. https://doi.org/10.1016/S1473-3099(10)70316-9.

41. Havlickova B, Czaika VA, Friedrich M. 2008. Epidemiological trends in skin mycoses worldwide. Mycoses 51:2–15. https://doi.org/10.1111/j.1439-0507.2008.01606.x.

42. Dolton MJ, Perera V, Pont LG, McLachlan AJ. 2014. Terbinafine in combination with other antifungal agents for treatment of resistant or refractory mycoses: investigating optimal dosing regimens using a physiologically based pharmacokinetic model. Antimicrob Agents Chemother 58:48–54. https://doi.org/10.1128/AAC.02006-13.

43. Revankar SG, Nailor MD, Sobel JD. 2008. Use of terbinafine in rare and refractory mycoses. Future Microbiol 3:9–17. https://doi.org/10.2217/17460913.3.1.9.

44. Clinical and Laboratory Standards Institute. 2002. Approved standard M38-A. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Clinical and Laboratory Standards Institute, Wayne, PA.

45. Wiemann P, Sieber CMK, von Bargen KW, Studt L, Niehaus E-M, Espino JJ, Huß K, Michielse CB, Albermann S, Wagner D, Bergner SV, Connolly LR, Fischer A, Reuter G, Kleigrewe K, Bald T, Wingfield BD, Ophir R, Freeman S, Hippler M, Smith KM, Brown DW, Proctor RH, Münsterkötter M, Freitag M, Humpf H-U, Gündener U, Tudzynski B. 2013. Deciphering the cryptic genome: genome-wide analyses of the rice pathogen Fusarium fujikuroi reveal complex regulation of secondary metabolism and novel metabolites. PLoS Pathog 9:e1003475. https://doi.org/10.1371/journal.ppat.1003475.

46. Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30:3059–3066. https://doi.org/10.1093/nar/gkf436.

47. Castresana J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 17:540–552. https://doi.org/10.1093/oxfordjournals.molbev.a026334.

48. Jones DT, Taylor WR, Thornton JM. 1992. The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci 8:275–282. https://doi.org/10.1093/bioinformatics/8.3.275.

49. Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874. https://doi.org/10.1093/molbev/msw054.