F901318 represents a novel class of antifungal drug that inhibits dihydroorotate dehydrogenase

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There is an important medical need for new antifungal agents with novel mechanisms of action to treat the increasing number of patients with life-threatening systemic fungal disease and to overcome the growing problem of resistance to current therapies. F901318, the leading representative of a novel class of drug, the orotomides, is an antifungal drug in clinical development that demonstrates excellent potency against a broad range of dimorphic and filamentous fungi. In vitro susceptibility testing of F901318 against more than 100 strains from the four main pathogenic *Aspergillus* spp. revealed minimal inhibitory concentrations of ≤0.06 μg/mL—greater potency than the leading antifungal classes. An investigation into the mechanism of action of F901318 found that it acts via inhibition of the pyrimidine biosynthesis enzyme dihydroorotate dehydrogenase (DHODH) in a fungal-specific manner. Homology modeling of *Aspergillus fumigatus* DHODH has identified a predicted binding mode of the inhibitor and important interacting amino acid residues. In a murine pulmonary model of aspergillosis, F901318 displays in vivo efficacy against a strain of *A. fumigatus* sensitive to the azole class of antifungals and a strain displaying an azole-resistant phenotype. F901318 is currently in late Phase 1 clinical trials, offering hope that the antifungal armamentarium can be expanded to include a class of agent with a mechanism of action distinct from currently marketed antifungals.

antifungal drug | *Aspergillus fumigatus* | mechanism of action | dihydroorotate dehydrogenase

A recent estimate puts the annual death toll from serious fungal infections at 1.5 million (1). As one of the four greatest killers, *Aspergillus* species are opportunistic human pathogens, particularly affecting immunocompromised persons, such as transplant recipients and those with hematologic malignancies. Invasive aspergillosis has a high mortality (30–90%) and is estimated to affect more than 200,000 people annually. Other diseases caused by *Aspergillus* species, including allergic bronchopulmonary aspergillosis (2) and chronic pulmonary aspergillosis (3), have a significant global impact, affecting millions of patients.

There has been a dearth of new drug classes developed to treat systemic fungal infections arriving in the clinic, with the most recent being the echinocandins, introduced in 2001. Only three other classes of antifungal drugs are currently available for the treatment of invasive fungal disease: polyenes (amphotericin B), azoles (e.g., voriconazole, posaconazole, and the recently licensed isavuconazole), and flucytosine (4). These agents work via a limited range of cellular targets. Echinocandins, such as caspofungin, inhibit β-(1,3)-glucan synthase, exploiting the most striking difference between the fungal cell and its human counterpart—the cell wall. Two antifungal drug classes target the cell membrane: azoles inhibit ergosterol biosynthesis, and polyenes disrupt fungal membranes via ergosterol binding. Flucytosine is a pyrimidine analog, converted to 5-fluorouracil within fungal cells, that disrupts DNA and RNA synthesis; however, owing to rapid development of resistance, it is used primarily in combination therapy.

Issues exist with current therapies, including overt toxicity, drug–drug interactions, variable pharmacokinetics, and increasing levels of drug resistance (5, 6). In particular, the development of resistance to the azole class of antifungals is concerning, because they are currently the sole orally available antifungal agent for the treatment of aspergillosis (7). Azole-resistant clinical isolates of *Aspergillus fumigatus* have been observed and isolated from patients worldwide, including Europe, the United States, Asia, Africa, Australia, and the Middle East (8, 9). Apparently exacerbated by the environmental use of azole fungicides in agriculture (10), reported rates of azole resistance have approached 30% at certain sites in Europe, with rates outside Europe varying between 0.6% and 11.2% (9).

Results

Discovery of F901318. With the aim of identifying new antifungal chemistries, a library of 340,292 small molecules was screened in vitro against *A. fumigatus*, and multiple chemical series with antifungal activity were identified. The initial hits in one such series, originally termed the "F3 series," were developed by a medicinal chemistry program that was driven by classical structure–activity relationships based on in vitro activity. This series was characterized by excellent in vitro potency against *Aspergillus* spp., but was devoid of activity against *Candida* spp. This unusual

Significance

New antifungal drugs that act via novel mechanisms are urgently needed to combat the high mortality of invasive fungal disease and the emergence of resistance to existing therapies. We describe the discovery, structure, activity, and mechanism of action of F901318, a new antifungal agent. A member of a novel class of antifungals, the orotomides, F901318 acts via inhibition of dihydroorotate dehydrogenase, an enzyme of de novo pyrimidine biosynthesis. F901318 is currently in clinical development for the treatment of invasive aspergillosis.

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Conflict of interest statement: J.D.O., G.E.M.S., N.B., S.d.P., A.J.K., D.L., and M.B. are employees of F2G Ltd. K.S.D. and M.J.B. are ex-employees of F2G Ltd. J.D.O., G.E.M.S., K.S.D., A.J.K., D.L., and M.B. are shareholders of F2G Ltd. W.W.H. has received research funding from Pfizer, Gilead, Astellas, AiCuris, and F2G, and has acted as a consultant and/or given talks for Pfizer, Basilea, Astellas, F2G, Nordic Pharma, Mayne Pharma, and Pulmocide. G.E.M.S., D.L., J.D.O., and M.B. are listed as inventors on patent WO2009130481. J.D.O., M.J.B., G.E.M.S., and M.B. are listed as inventors on patent WO200913379.

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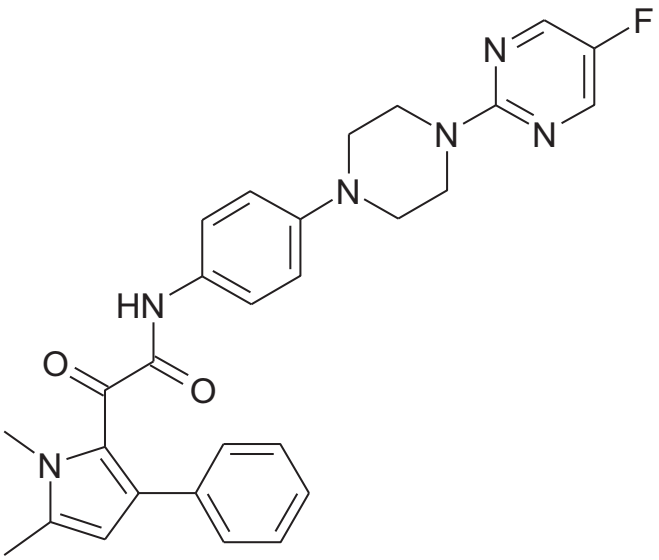

Fig. 1. Structure of F901318.

pattern perhaps explains why similar chemicals have not been found previously. Typically, antifungal screens have depended on first finding activity against *Candida*. Modifications to improve physicochemical properties, antifungal potency, pharmacokinetics, ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties, in vivo efficacy in infection models, and toxicology have led to the development of F901318 (Fig. 1). Antifungal susceptibility testing of F901318 using standardized techniques demonstrated its potent activities against clinical isolates of aspergilli, with minimum inhibitory concentrations (MICs) of <0.1 μg/mL against multiple strains of *A. fumigatus*, *Aspergillus terreus*, *Aspergillus niger*, and *Aspergillus flavus*, including isolates resistant to other antifungal agents (Table 1).

Mechanism of Action Screen. Initially, owing to the method of discovery, the mechanism of action of this series was unknown. A combination of microbiological, genetic, and biochemical approaches were used to identify the target of this drug series. A genetic screen, similar to a multicopy suppressor screen, was performed to identify genes that, when present in multiple copies, provided resistance to F901318. This approach was previously validated for the antifungal drugs itraconazole and terbinafine, by demonstrating that the presence of additional copies of cytochrome P450 C-14 lanosterol α-demethylase and squalene epoxidase, respectively, led to resistance to those agents (11, 12). In the present study, *Aspergillus nidulans* spores that had been transformed with an *A. nidulans* genomic library carried by the autonomously replicating plasmid pAMA1 were exposed to F901318. Four independent resistant clones were obtained, pAMA1 DNA was isolated, and the genomic DNA insert was sequenced. All resistant clones contained inserts that mapped to the same region of chromosome I (Fig. S1*A*). Although sequence data from five genes was retrieved, only one gene—ANIA_05909—was intact in all four genomic fragments. This gene, named *pyrE* in *Aspergillus* spp., encodes the pyrimidine biosynthesis enzyme dihydroorotate dehydrogenase (DHODH; EC 1.3.5.2).

To confirm that extra copies of *pyrE* led to F901318 resistance, the recovered plasmid pAMA1_18.1 was treated with a bacterial transposon (Tn5) to disrupt either *pyrE* or a neighboring gene, ANIA_05910, and the resulting plasmids were transformed into *A. nidulans*. Strains carrying the intact pAMA1_18.1 or the ANIA_05910 disruptant exhibited resistance to F901318; however, on disruption of *pyrE*, the strain returned to wild-type (WT) levels of susceptibility to F901318 (Fig. S1*B*). This finding confirmed that extra copies of the gene encoding DHODH were responsible for the resistance to F901318, implicating DHODH as the target of the drug.

DHODH Is the Target of F901318. DHODH is an oxidoreductase that catalyzes the fourth step of the pyrimidine biosynthesis pathway (Fig. S2), the conversion of dihydroorotate to orotate. Confirmation that the drug disrupts pyrimidine biosynthesis was obtained after the addition of exogenous pyrimidines (uridine and uracil) to the media during susceptibility testing. A reversal of the antifungal effect of F901318 on *A. fumigatus* was observed, but only at millimolar concentrations of pyrimidines (5 mM and above; Fig. S3). Interestingly, human serum contains low levels of pyrimidines, estimated to be ∼15 μM (13), which are insufficient to reverse the effect of F901318 on *A. fumigatus* in vivo. Indeed, mutants of *A. fumigatus* (14), *Candida albicans* (15), *Histoplasma capsulatum* (16), and *Cryptococcus neoformans* (17), which are disrupted in pyrimidine biosynthesis, have exhibited attenuated virulence in animal models of infection, indicating that targeting pyrimidine synthesis is a valid antifungal strategy.

Biochemical evidence confirming that the target of F901318 was DHODH was obtained from in vitro enzyme assays that were set up with recombinant *A. fumigatus* DHODH using 2,6-dichloroindophenol as a redox indicator. F901318 inhibited *A. fumigatus* DHODH in a dose-dependent manner, with an IC_{50} of 44 ± 10 nM (n = 11; Fig. 2). DHODH is also present in mammals, although there is a low overall identity to *Aspergillus* DHODH (∼30%; Fig. S4). A known inhibitor of human DHODH, teriflunomide (18), which is used to treat multiple sclerosis in man, did not inhibit *A. fumigatus* DHODH in vitro. Species selectivity of F901318 was confirmed in an assay in which little inhibition of human DHODH was observed, whereas teriflunomide inhibited human DHODH, as expected. In fact, the IC_{50} value for F901318 against human DHODH was not reached at 100 μM, the highest concentration used in these experiments, indicating that F901318 was

Table 1. Antifungal potency of F901318 and other antifungal drugs against the major *Aspergillus* species

Drug	Parameter	*A. fumigatus* (n = 55)	*A. terreus* (n = 21)	*A. flavus* (n = 19)	*A. niger* (n = 19)
F901318	MIC mean, μg/mL	0.029	0.014	0.021	0.031
	MIC range, μg/mL	0.008–0.06	0.004–0.03	0.015–0.06	0.015–0.06
Amphotericin B	MIC mean, μg/mL	1.55	2.07	1.39	0.46
	MIC range, μg/mL	0.5–2	1–4	1–2	0.25–1
Caspofungin	MEC* mean, μg/mL	0.096	0.112	0.06	0.062
	MEC* range, μg/mL	0.06–0.12	0.06–0.12	0.06	0.06–0.12
Voriconazole	MIC mean, μg/mL	0.69	0.59	0.96	0.77
	MIC range, μg/mL	0.25–16	0.25–1	0.5–1	0.5–16

The MICs (in μg/mL) of F901318, amphotericin B, and voriconazole were determined for the *Aspergillus* spp. indicated (n, number of different strains tested). *For caspofungin, the MEC is shown, because growth is not completely inhibited with this drug. Data are displayed as the geometric mean of the MICs and the range of MIC from lowest to highest for the strains of a particular species.

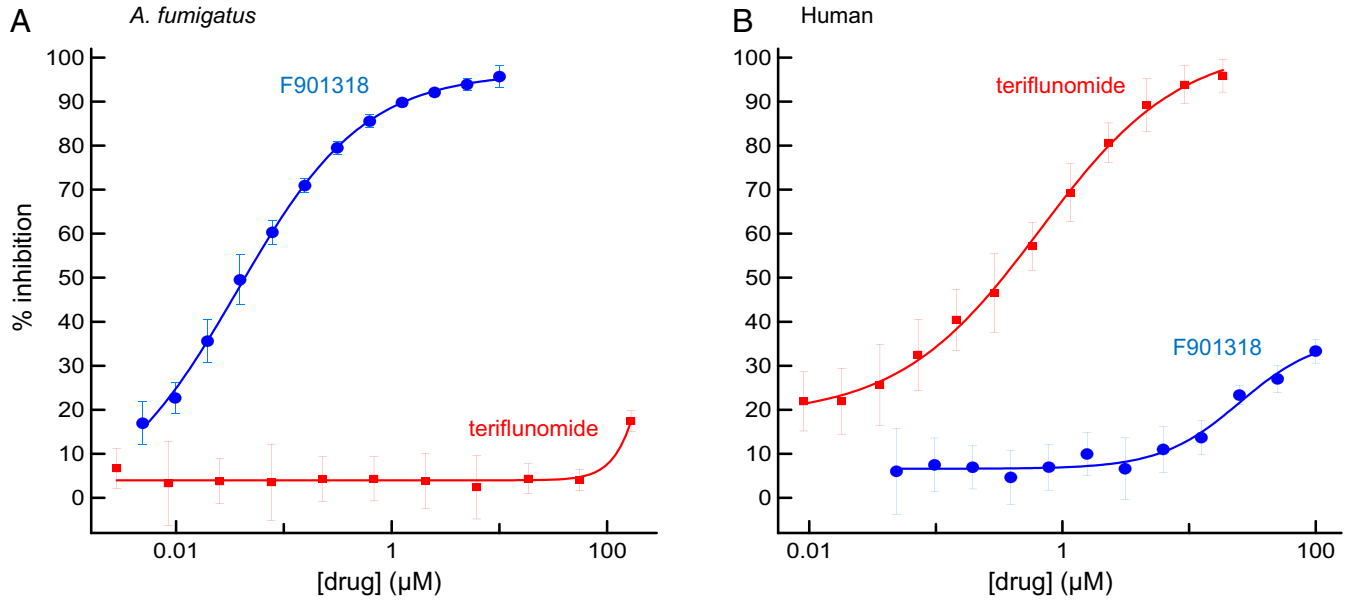

Fig. 2. F901318 inhibits *A. fumigatus* DHODH in vitro. Recombinant *A. fumigatus* DHODH (*A*) and human DHODH (*B*) were incubated in the presence and absence of varying concentrations of F901318 and teriflunomide. The activity of the enzymes was measured for each drug concentration, and the percent inhibition compared with no drug controls was calculated.

>2,200-fold more potent against the *A. fumigatus* enzyme. Thus, fungal DHODH was confirmed as the target of F901318, and despite the presence of a mammalian version of the enzyme, no target-based toxicity was predicted. On elucidation of the mechanism of action, the F3 series was renamed the orotomides, combining the mechanism (dihydroorotate) with the chemistry (α-ketoamide).

Further enzyme kinetic experiments revealed that F901318 is a reversible inhibitor of *A. fumigatus* DHODH (Fig. S5*A*) and is a competitive inhibitor with respect to the ubiquinone (coenzyme Q) cofactor, which functions as an electron acceptor in the reaction (Fig. S5*B*). This latter property is perhaps not unexpected, given that structural studies have shown that known inhibitors of human DHODH (teriflunomide and brequinar) (19) and the *Plasmodium falciparum* enzyme (DSM265) (20) bind in a region of the protein predicted to be a channel through which the ubiquinone enters the molecule from the inner mitochondrial membrane.

Structural Insights of F901318 Binding to *A. fumigatus* DHODH. In the absence of a crystal structure, we investigated the binding of F901318 to *A. fumigatus* DHODH by creating a homology model of *A. fumigatus* DHODH (Fig. S6) using the structural information provided by other class 2 DHODH enzymes, including the structure of human DHODH (19). F901318 and other members of the series were used to identify a likely binding mode. Key residues for binding were identified (Fig. 3*A*). Validation of the importance of two of these residues was obtained by mutagenesis of *C. albicans* DHODH. The WT *C. albicans* DHODH was not inhibited by F901318, but mutation of two residues, Phe_{162} and Val_{171}, to the residues predicted to occupy the same positions in the *A. fumigatus* enzyme, Val_{200} and Met_{209}, respectively, created a mutant *C. albicans* DHODH that was inhibited by F901318 (Fig. 3*B*). The IC_{50} of the mutant *C. albicans*_V_{162}_M_{171} was still ∼40-fold higher than the IC_{50} of F901318 against the *A. fumigatus* enzyme, indicating the existence of other important differences in DHODH between the two species; however, both of these residues are clearly important for inhibition. These data, along with the observed competition between F901318 and coenzyme Q in the in vitro assay, suggest that the orotomides bind in the "quinone channel," where ubiquinone enters the enzyme from the inner mitochondrial membrane, preventing reoxidation of the dihydroflavin mononucleotide ($FMNH_2$) cofactor essential for the reaction to proceed (Fig. S2).

Spectrum, Resistance, and in Vivo Efficacy of F901318. Although we observed no activity against *Candida* spp. and the zygomycetes in antifungal susceptibility testing, F901318 displayed excellent potency against a broad range of pathogenic filamentous and dimorphic fungi, including *Penicillium* spp., *Coccidiodes immitis*, *H. capsulatum*, *Blastomyces dermatitidis*, *Fusarium* spp., and the difficult-to-treat *Scedosporium* spp. This spectrum appears to be sequence-driven, given that the sensitive organisms were found to be grouped together in a phylogenetic analysis of DHODH (Fig. S7). This finding is consistent with observations that other DHODH inhibitors exhibit specificity of action owing to interspecies variations in the architecture of the hydrophobic channel in which the inhibitors are predicted to bind (19–21). Thus, the unique spectrum of F901318 is a reflection of the structure of the target, DHODH, and the binding mode of the orotomides.

We investigated the resistance to F901318 in *A. fumigatus* by repeated exposure to a concentration gradient of the drug on an agar plate and selection from the margins of growth. This was carried out for 50 passages, with no change in the MIC observed for the first 40 passages and only a modest increase thereafter (Fig. S8). In contrast, voriconazole exhibited an increase in the MIC between 10 and 15 passages. Based on these results, it appears that F901318 resistance is not easily induced in *A. fumigatus*.

Pharmacokinetic studies in mice have identified good distribution of F901318 to tissues including the kidney, liver, and lung, with detection in the brain, albeit at lower levels, suggesting that the drug reaches key sites of infection. The efficacy of F901318 was demonstrated in a neutropenic murine model of invasive pulmonary aspergillosis. After infection with a well-characterized *A. fumigatus* strain (NIH 4215), survival was significantly improved by F901318 treatment (Fig. 4*A*). Treatment with the triazole drug posaconazole also increased survival after infection with this strain.

Mutations in the gene encoding the target molecule of the azole class of antifungal drugs, *Cyp51A*, that cause resistance have been identified. Several azole-resistant strains of *A. fumigatus* carry a combination of a tandem repeat in the promoter and a point

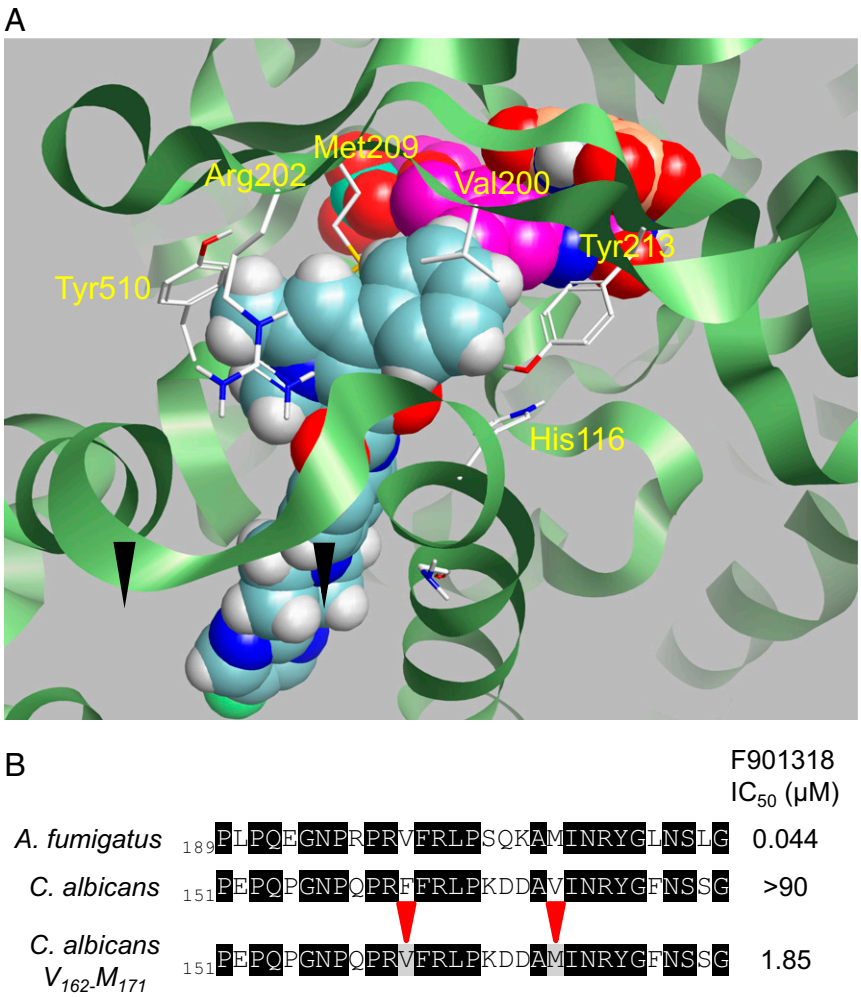

Fig. 3. (*A*) Binding of F901318 to *A. fumigatus* DHODH. A homology model of *A. fumigatus* DHODH was created, and the binding mode of F901318 (cyan) was estimated. The product orotate (orange) and the cofactor flavin mononucleotide (FMN; magenta) are also shown. Residues predicted to be close to the molecule are highlighted. (*B*) F901318 inhibits a mutant version, but not the WT version, of *C. albicans* DHODH. Recombinant *C. albicans* DHODH residues Phe_{162} and Val_{171} were mutated to Val and Met, respectively (their predicted equivalents in *A. fumigatus* DHODH). The IC_{50} of F901318 inhibition of the WT and mutant DHODH proteins is displayed in the right-hand column (*A. fumigatus* IC_{50}, $n = 11$, SD = 0.01 μM; WT *C. albicans* IC_{50}, $n = 7$, all seven replicates had an IC_{50} >90 μM; *C. albicans_V162_M171* IC_{50}, $n = 7$, SD = 0.91 μM).

mutation in the coding sequence. One such strain, *A. fumigatus* F16216, carrying the TR34/L98H mutation of *Cyp51A*, has proven to be resistant to multiple azole drugs, including itraconazole, voriconazole, and posaconazole (22). In vitro, *A. fumigatus* F16216 displayed no resistance to F901318, with an MIC of 0.03 μg/mL, which is comparable to the data presented in Table 1. In vivo, in the pulmonary aspergillosis model, *A. fumigatus* F16216 caused an infection that cannot be treated with posaconazole (Fig. 4*B*); however, F901318 therapy led to a significant increase in survival in this severe model, demonstrating that the different mechanism of action of the orotomides enables F901318 to overcome azole resistance caused by *Cyp51A* mutations.

Preclinical safety pharmacology and toxicology studies of F901318 have supported the progression and evaluation of this antifungal in Phase 1 oral and i.v. single and repeated dose trials.

Discussion

As highlighted by Denning and Bromley (23), the antifungal pipeline has failed to produce new antifungal drugs with mechanisms of action different from those of existing classes in the years since caspofungin was licensed in 2001. Many potential antifungal targets have been investigated, but translating these early stage projects into clinical candidates has proven elusive. This difficulty has mirrored the issues with target-based screening encountered in the antibacterial arena (24). In fact, a review of new mechanism, first-in-class medicines approved by the US Food and Drug Administration between 1999 and 2008 revealed that target-based screens were responsible for the discovery of only 3 of 10 drugs for infectious disease, with the majority discovered by phenotypic screening (i.e., "whole-cell screens" for antibiotics/antifungals) (25). The orotomides were discovered via a whole-cell screening approach, providing hits that were known to have antifungal activity from the start, but with no knowledge of mechanism of action. This classical approach was coupled with a genetic screen to identify the target of the drug, DHODH. A recent review of antifungal drug discovery suggested that similar approaches, taking advantage of genetic tools such as haploinsufficiency strain collections and new technologies such as next-generation sequencing, may accelerate the translation of antifungal chemistries toward the clinic (26).

Pyrimidines are essential to the cell, not just for the synthesis of DNA and RNA, but also for the formation of precursors for lipid and carbohydrate metabolism. For example, synthesis of the cell wall requires UDP-activated sugars at multiple stages, including

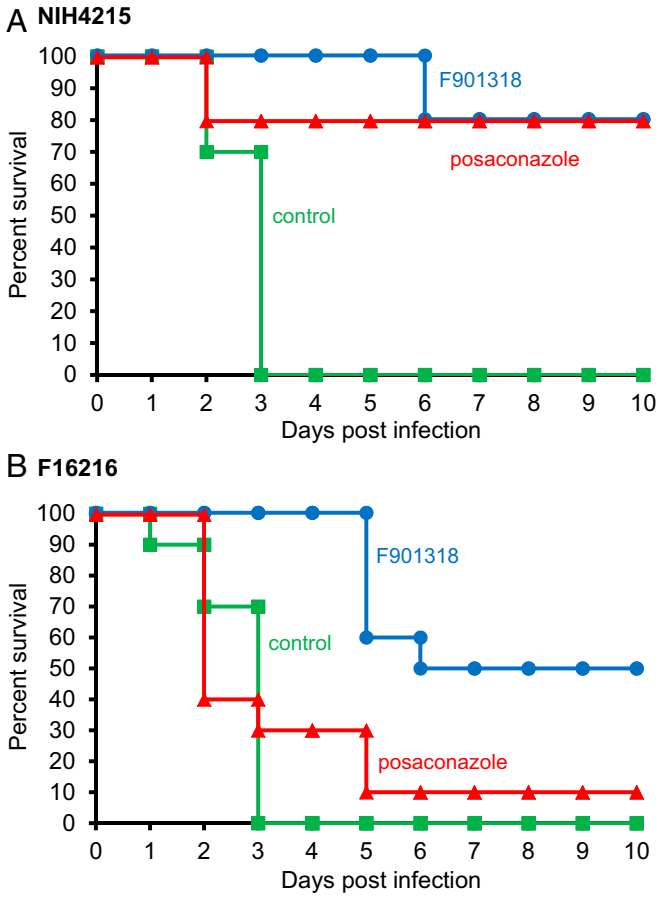

Fig. 4. In vivo efficacy of F901318 in a murine model of *A. fumigatus* infection. Groups of 10 immunosuppressed mice were infected intranasally with *A. fumigatus* NIH 4215 (*A*) or the azole-resistant *A. fumigatus* F16216 (*B*) conidia on day 0. Treatment with F901318 (15 mg/kg i.v., three times daily; blue circles), posaconazole (7.5 mg/kg orally, once daily; red triangles), or control (green squares) was initiated at 6 h postinfection. Kaplan–Meier curves of surviving mice in each group were plotted. After infection with NIH 4215, F901318 treatment significantly improved survival compared with controls ($P < 0.001$, Mantel–Haenszel test). After infection with F16216, F901318 treatment significantly improved survival compared with controls ($P < 0.001$) and compared with posaconazole treatment ($P < 0.005$).

UDP-glucose for β-(1,3)-glucan synthesis. Pyrimidines are synthesized in the de novo pyrimidine biosynthesis pathway (Fig. S2), in which DHODH is a key enzyme, but they also can be scavenged by fungi from the environment via the salvage pathway. The pyrimidine salvage pathway appears to be inefficient for *A. fumigatus*, however (Fig. S3). In animal models of infection, pyrimidine biosynthesis mutants from several pathogenic fungi are highly attenuated for virulence, including studies on *A. fumigatus* (14), *C. albicans* (15), *H. capsulatum* (16), and *C. neoformans* (17). In *Saccharomyces cerevisiae*, a *ura3* deletion strain lacking the orotidine-5′-phosphate (OMP) decarboxylase enzyme of pyrimidine biosynthesis was unable to survive in vivo, exhibiting a decrease in the competitive index versus a WT or reconstituted strain observed after just 4 h (27). In *C. albicans*, *URA3* has been commonly used as a selectable marker, but concerns have been raised that in some virulence studies, the ectopic expression of *URA3*, leading to reduced OMP decarboxylase activity, had a greater effect on virulence compared with the disruption of the target gene of interest (28). Thus, the evidence in the literature supports the targeting of pyrimidine biosynthesis as a valid antifungal strategy.

Identifying DHODH as the target of the orotomides has helped explain the spectrum of antifungal activity that we observed. F901318 has activity against numerous pathogenic filamentous and dimorphic fungi, including *Aspergillus* spp., *H. capsulatum*, *Blastomyces dermatitides*, and *C. immitis*, along with the difficult-to-treat *Scedosporium prolificans*. These F901318-susceptible organisms group together on the phylogenetic tree of DHODH (Fig. S7), whereas DHODH from *Candida* spp., *C. neoformans*, and the human and *Plasmodium* enzymes are more distantly related while still classified as class 2 DHODH enzymes. DHODH from the zygomycota, such as *Rhizopus* and *Mucor*, align more closely with class 1A DHODHs, cytosolic enzymes that occur in Gram-positive bacteria and the trypanosomatids that use alternative cofactors, such as fumarate.

DHODH has been suggested as a target for therapy in multiple diverse disease areas, including oncology, rheumatoid arthritis, multiple sclerosis, and infectious diseases caused by such agents as *Plasmodium*, bacteria, and viruses (21, 29). There are currently two marketed agents that have activity against human DHODH: leflunomide for rheumatoid arthritis and teriflunomide for multiple sclerosis. DSM265 is an antimalarial drug targeting plasmodial DHODH that is currently in Phase 2 clinical trials (20); however, to our knowledge, no other human antifungal therapies have progressed with DHODH as a target.

Although at first consideration, the breadth of therapeutic areas for which DHODH has been proposed as a drug target is surprising, in each case limiting the pool of pyrimidines prevents proliferation of a population of cells. In some cases, the host cells are targeted, such as lymphocytes in autoimmune diseases and proliferating cancerous cells in oncology. Alternatively, the DHODH of invading pathogens is targeted to selectively limit the pyrimidine pools of the infective agent. Between these two effects, antiviral action has been reported for human DHODH inhibitors, given that viruses require host pyrimidines for replication (29).

In conclusion, to combat the increasing problem of resistance to existing antifungal therapies, the discovery of new cellular targets for antifungals, along with viable chemistry against these new targets (23), is vitally important. F901318 is an antifungal drug, currently in both intravenous and oral Phase 1 clinical trials (ClinicalTrials.gov identifiers: NCT02142153, NCT02342574, NCT02394483, and NCT02737371), that acts via inhibition of the pyrimidine biosynthesis enzyme DHODH, validating an alternative target for antifungal drug discovery.

Materials and Methods

Primers. The sequences of the primers used in this work are listed in Table S1. Primers were supplied by Eurofins MWG.

Synthesis of F901318. The 2-(1,5-dimethyl-3-phenyl-1H-pyrro-2-yl)-*N*-(4-[4-(5-fluoro-pyrimidin-2-yl-piperazin-1yl]-phenyl)-2-oxo-acetamide (F901318) was prepared as described in *SI Materials and Methods*.

In Vitro Antifungal Susceptibility Testing. MICs of antifungal drugs were determined according to Clinical and Laboratory Standards Institute (CLSI) protocol M38-A2 in RPMI 1640 medium buffered to pH 7.0 with MOPS buffer at 35 °C. For caspofungin, the minimum effective concentration (MEC) was defined as the lowest drug concentration causing abnormal growth (i.e., short-branching hyphae).

Mechanism of Action Screen. An *A. nidulans* genomic library carried on the pRG3-AMA1-NotI vector was obtained from the Fungal Genetic Stock Center. Protoplasts from *pyrG*- strains of *A. nidulans* (A767) were transformed with the genomic library by PEG-mediated transformation. Transformants were exposed to lethal concentrations of F901318 on Vogel's minimal agar (30). Plasmid DNA was extracted from resistant colonies and sequenced.

DHODH Assays. These assays were carried out using recombinant DHODH prepared from *A. fumigatus* cDNA, *C. albicans* gDNA, or, for the human protein, IMAGE clone 6064723 (Geneservice Ltd.) cloned into the vector pET44 (Novagen) minus the N-terminal 88 (*A. fumigatus*), 56 (*C. albicans*), or 28 (human) amino acids. For *C. albicans*, CTG-encoded serines were mutated

to TCG. Further mutations altered Phe_{162} and Val_{171} to become Val and Met to create the mutant protein *C. albicans*_V_{162}_M_{171}. Primers are listed in Table S1, and further methodological details are provided in *SI Materials and Methods*. The assay was conducted as described elsewhere (31).

Homology Modeling of *A. fumigatus* DHODH. Human DHODH [Protein Data Bank (PDB) ID code 1D3G] served as the protein template for construction of the *A. fumigatus* DHODH model. Other DHODH structures from human (PDB ID codes 1PRH, 2PRL, 2PRM, 3G0X, 3KVM, 2WV8, and 2FQI), rat (PDB ID codes 1UUM and 1UUO), *Trypanosoma cruzi* (PDB ID code 2E68), *P. falciparum* (PDB ID codes 3I68, 1ITV, and 3O8A) *Leishmania major* (PDB ID code 3MJY), and *Escherichia coli* (PDB ID code 1F76) also informed the process. Coarse refinement of the structure with Discovery Studio 4.1 (Accelerys) was followed by fine refinement with XEDraw (Cresset). More details of the homology modeling process and ligand binding are provided in *SI Materials and Methods*.

Resistance Testing. *A. fumigatus* 210 conidia were inoculated onto Sabouraud agar (Oxoid) in a 9-cm Petri dish. An 8-mm-diameter circle of agar was removed from the center of the plate to create a well. 100 μL of 500 μg/mL drug was loaded into the well and allowed to diffuse into the agar, creating a concentration gradient. After 4 d of incubation at 35 °C, a zone of inhibition was observed, and conidia were collected from the margins of growth and then used to create the next plate. After every fifth passage, the MIC was determined as described above.

In Vivo Efficacy Testing. All experiments were conducted under U.K. Home Office project license 40/3630 and approved by the University of Liverpool's Animal Welfare Committee. Groups of 10 CD-1 mice were immunosuppressed with 200 mg/kg cyclophosphamide i.p. at 4 d before infection and with cyclophosphamide and 250 mg/kg cortisone acetate s.c. at 1 d before infection. *A. fumigatus* F16216 carries an L98H mutation of *cyp51A* and a 34-bp tandem repeat in the *cyp51A* promoter, leading to resistance to azole drugs (22). Conidia from this strain and from the WT *A. fumigatus* NIH 4215 were administered intranasally on day 0. Treatment with F901318 (15 mg/kg i.v. three times daily) or posaconazole (7.5 mg/kg/day orally) was initiated at 6 h postinfection.

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