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F901318 represents a new 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 increasing numbers 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 named the orotomides, is a new 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* species, revealed minimal inhibitory concentrations of 0.06 μg/ml and below - 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 modelling of *A. 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 new class of agent with a novel mechanism of action.

Antifungal drug | Aspergillus fumigatus | Mechanism of action | Dihydroorotate dehydrogenase

Introduction.

A recent estimate puts the annual death toll from serious fungal infections at 1.5 million (1). As one of the four biggest killers, *Aspergillus* species are opportunistic human pathogens, particularly affecting the immunocompromised such as transplant recipients and those with haematological malignancies. Invasive aspergillosis has a high mortality (30-90%) and is estimated to affect more than 200,000 people a year. 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 for the treatment of systemic fungal infections arriving in the clinic, with the most recent being the echinocandins in 2001. Only three other classes of antifungal drug 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 analogue, converted to 5-fluorouracil within fungal cells, that disrupts DNA and RNA synthesis, however, due to rapid development of resistance, it is primarily used 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 worrying, as they are currently the only orally available antifungal for the treatment of aspergillosis (7). Azole-resistant clinical isolates of *Aspergillus fumigatus* have been observed and isolated from patients around the world including Europe, USA, Asia, Africa, Australia and the Middle East (8, 9). Apparently exacerbated by the environmental use of azole fungicides in agriculture (10), rates of azole-resistance have been observed approaching 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 *Aspergillus fumigatus* and multiple chemical series with antifungal activity were identified. The initial hits in one such series, originally named the 'F3-series', were developed by a medicinal chemistry programme that was driven by classical structure-activity relationships based on *in vitro* activity. This series was characterized by excellent *in vitro* potency against *Aspergillus* species but was devoid of activity against *Candida* species. This unusual pattern perhaps explains why similar chemicals have not been found before. Typically, antifungal screens have depended

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 new 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.

Reserved for Publication Footnotes

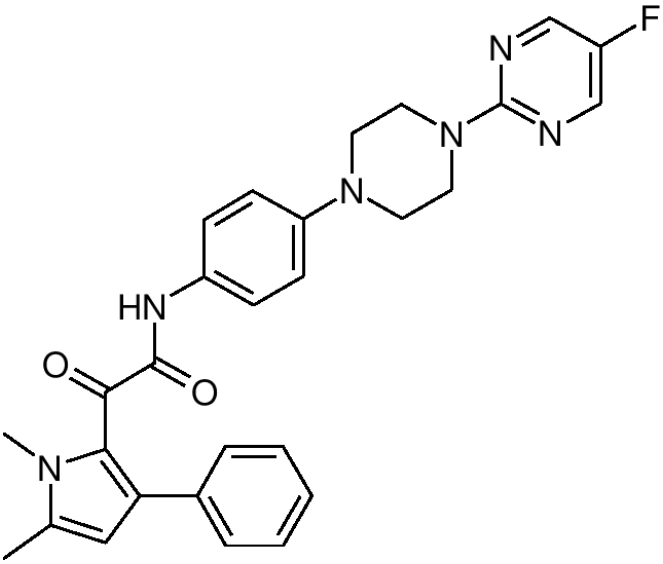

Fig. 1. Structure of F901318.

on first finding activity against *Candida*. Modifications to improve physico-chemical properties, antifungal potency, pharmacokinetics, ADMET properties, *in vivo* efficacy in infection models and toxicology have led to F901318 (Fig. 1). Antifungal susceptibility testing of F901318 using standardized techniques revealed it to have potent activity against clinical isolates of aspergilli, with sub 0.1 μg/ml minimal inhibitory concentrations observed against multiple strains of *A. fumigatus*, *A. terreus*, *A. niger* and *A. flavus* including isolates resistant to other antifungals (Table 1).

Mechanism of Action Screen

Initially, due to the method of discovery, the mechanism of action of this series was unknown. A combination of microbiological, genetic and biochemical approaches were taken to discover the target of this drug series. A genetic screen, similar to a multi-copy suppressor screen, was carried out to identify genes that, when present in multiple copies, gave resistance to F901318. This approach has been validated previously with the antifungal drugs itraconazole and terbinafine, by demonstrating that the presence of additional copies of cytochrome P-450 C-14 lanosterol α-demethylase and squalene epoxidase, respectively, leads to resistance to these agents (11, 12). In this study, *A. 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 isolated and the genomic DNA insert sequenced. All resistant clones contained inserts that mapped to the same region of chromosome I (Fig. S1A). Although sequence data from 5 genes was retrieved, only one gene was intact in all 4 genomic fragments: gene ANIA_05909. This gene, named *pyrE* in *Aspergillus* spp., encodes the pyrimidine biosynthesis enzyme dihydroorotate dehydrogenase (DHODH, EC 1.3.5.2). In order 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 transformed into *A. nidulans*. Strains carrying the intact pAMA1_18.1 or the ANIA_05910 disruptant displayed resistance to F901318, however upon disruption of *pyrE* the strain returned to wild type levels of susceptibility to F901318 (Fig. S1B). This 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 catalyzing 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 following 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 approximately 15 μM (13), 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), disrupted in pyrimidine biosynthesis have attenuated virulence in animal models of infection indicating that targeting pyrimidine synthesis is a valid antifungal strategy.

Biochemical evidence confirming the target was gained from *in vitro* DHODH 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, +/- S.D.; Fig. 2). DHODH is also present in mammals, although there is a low overall identity to *Aspergillus* DHODH (approximately 30%; Fig S4). A known inhibitor of human DHODH, teriflunomide (18), used to treat multiple sclerosis in man, did not inhibit *A. fumigatus* DHODH *in vitro*. Species selectivity of F901318 was confirmed in an assay where little inhibition of human DHODH was observed, while as expected teriflunomide inhibited human DHODH. In fact the IC_{50} value for F901318 against human DHODH was not reached at 100 μM, the highest concentration in these experiments, indicating that F901318 was >2200-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. Upon elucidation of the mechanism of action, the F3-series was renamed the orotomides combining the mechanism (dihydro**orot**ate) with the chemistry (α-keto**amide**).

Further enzyme kinetic experiments revealed that F901318 is a reversible inhibitor of *A. fumigatus* DHODH (Fig. S5A) and is a competitive inhibitor with respect to the ubiquinone (coenzyme Q) co-factor that functions as an electron acceptor in the reaction (Fig. S5B). This latter point is perhaps not unexpected, as structural studies have revealed that known inhibitors of human DHODH (teriflunomide and brequinar, (19)) and the *Plasmodium falciparum* enzyme (DSM265, (20)) bind in a region of the protein that is predicted to be a channel where 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, the binding of F901318 to *A. fumigatus* DHODH was investigated with the creation of 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. 3A). Validation of the importance of two of these residues was obtained by mutagenesis of *Candida albicans* DHODH. The wild type *C. albicans* DHODH is 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, create a mutant *C. albicans* DHODH that is inhibited by F901318 (Fig. 3B). The IC_{50} of the mutant *C. albicans*_V_{162}_M_{171} was still approximately 40-fold higher than the IC_{50} of F901318 against the *A. fumigatus* enzyme, indicating further important differences between DHODH from the two species, but these two residues are clearly important for inhibi-

Table 1.

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

Antifungal potency of F901318 and other antifungal drugs against the major *Aspergillus* species. The minimal inhibitory concentrations (MIC) in µg/ml of F901318, amphotericin B and voriconazole were determined for the *Aspergillus spp.* indicated (n is the number of different strains tested). *For caspofungin, the minimal effective concentration (MEC) is displayed as growth is not completely inhibited with this drug. Data is displayed as the geometric mean of the MICs and the range of MIC from lowest to highest for the strains of a particular species.

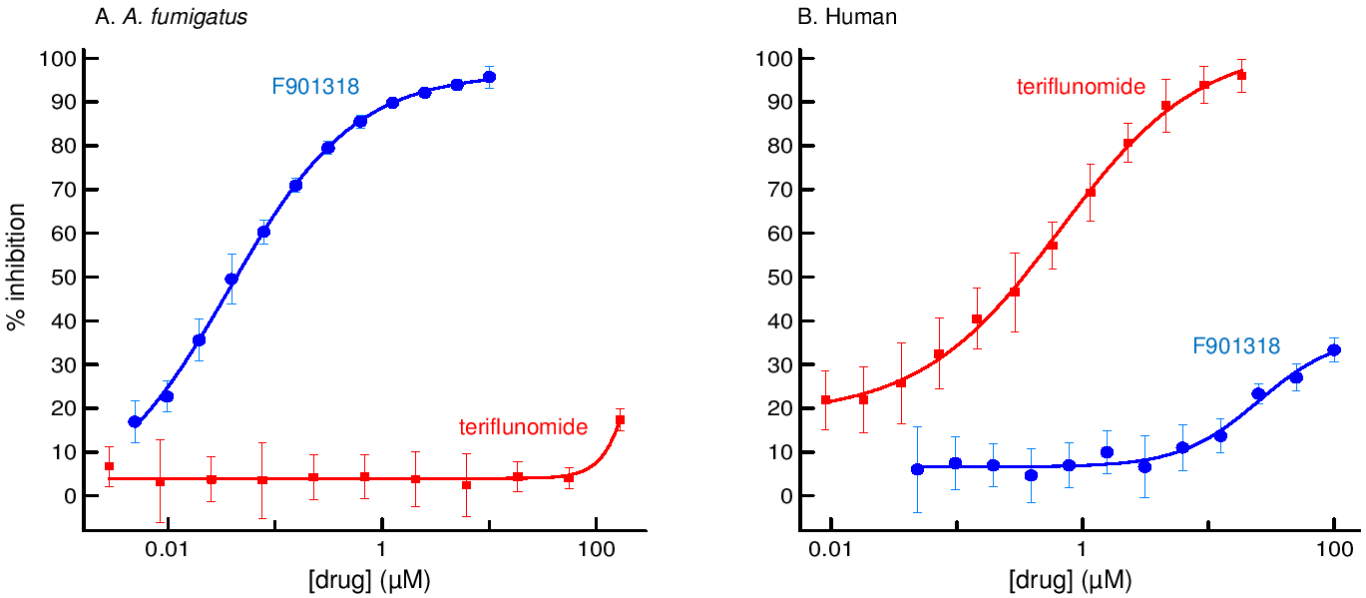

Fig. 2. F901318 inhibits *A. fumigatus* DHODH *in vitro*. Recombinant *A. fumigatus* DHODH (A) and human DHODH (B) were incubated in the presence and absence of varying concentrations of F901318 and teriflunomide. The activity of the enzymes was measured for each drug concentration and the percentage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

tion. These data, in addition to 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 the reoxidation of the $FMNH_2$ cofactor essential for the reaction to proceed (Fig. S2).

Spectrum, Resistance and *in vivo* efficacy of F901318

Although no activity against *Candida* spp. and the zygomycetes was observed in antifungal susceptibility testing, F901318 displays excellent potency against a broad range of pathogenic filamentous and dimorphic fungi including *Penicillium* spp., *Coccidiodes immitis*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Fusarium* spp. and the difficult to treat *Scedosporium* spp. This spectrum appears sequence-driven as the sensitive organisms grouped together in a phylogenetic analysis of DHODH (Fig. S7). This is consistent with observations that other DHODH inhibitors exhibit specificity of action due to interspecies variations in the architecture of the hydrophobic channel where 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.

Resistance to F901318 in *A. fumigatus* was investigated 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 MIC observed for 40 passages and only a modest increase thereafter (Fig. S8). In contrast, voriconazole exhibited an uplift in MIC between 10 and 15 passages. From this study 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 kidney, liver and lung, with detection in the brain, albeit it at lower levels, suggesting that drug is getting to key sites of infection. Efficacy of F901318 was demonstrated in a persistently neutropenic murine model of invasive pulmonary aspergillosis. Following infection with a well-characterised *A. fumigatus* strain (NIH 4215), survival was significantly improved by F901318-treatment (Fig. 4A). Treatment with the triazole drug posaconazole also increased survival with this strain.

Mutations in the gene encoding the target molecule of the azole class of antifungal drugs, *Cyp51A*, have been identified that cause resistance. Several azole-resistant strains of *A. fumigatus* carry a combination of a tandem repeat in the promoter and a point mutation in the coding sequence. One such strain, *A. fumigatus* F16216, carrying the TR34/L98H mutation of *Cyp51A* has previously been shown to be resistant to multiple azole drugs

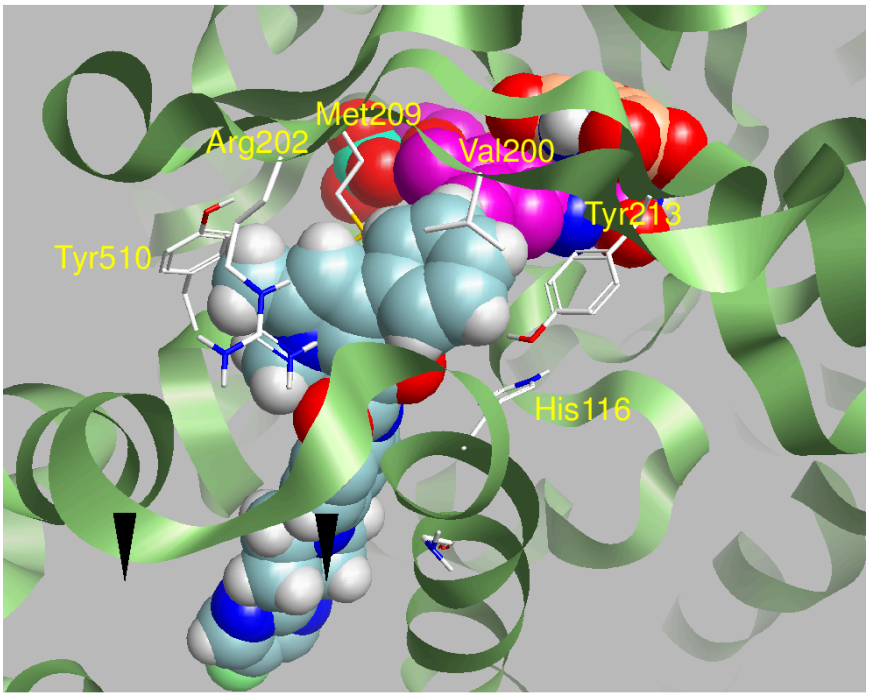

Fig. 3. (A) Binding of F901318 to *A. fumigatus* DHODH. A homology model of *A. fumigatus* DHODH was created and the binding mode of F901318 (cyan) estimated. The product orotate (orange) and the co-factor flavin mononucleotide (FMN, magenta) are also shown. Residues predicted to be close to the molecule are highlighted. (B) **F901318 inhibits a mutant version but not the wild type 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 wild type and mutant DHODH proteins is displayed in the right hand column. For the wild type *C. albicans* DHODH all 7 replicates had IC50 > 90 μM and for the *C. albicans*-V_{162}-M_{171} mutant DHODH: n=7; standard deviation = 0.91 μM.

including itraconazole, voriconazole and posaconazole (22). *In vitro*, *A. fumigatus* F16216 displayed no resistance to F901318, with an MIC of 0.03 μg/ml that is comparable to the data in Table 1. *In vivo*, in the pulmonary aspergillosis model, *A. fumigatus* F16216 causes an infection that cannot be treated with posaconazole (Fig. 4B). However, F901318 therapy leads 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 supported the progression and evaluation of this novel antifungal in Phase 1 oral and intravenous single and repeat 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 to existing classes 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 has mirrored the issues with target-based screening encountered in the anti-bacterial arena (24). In fact a review of new mechanism, first in class medicines approved by the FDA between 1999 and 2008 revealed that target-based screens were responsible for the discovery of only 3 out of 10 drugs for infectious disease, with the majority being discovered by phenotypic screening (ie '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 towards the clinic (26).

Pyrimidines are essential to the cell, not just for the synthesis of DNA and RNA, but to form precursors for lipid and carbohydrate metabolism. For example, synthesis of the cell wall requires UDP-activated sugars at multiple stages including UDP-glucose for β-(1,3)-glucan synthesis. Pyrimidines are synthesized in the *de novo* pyrimidine biosynthesis pathway (Fig. S2), of which DHODH is a key enzyme, but they can also be scavenged by fungi from the environment via the salvage pathway. However, the pyrimidine salvage pathway appears to be inefficient for *A. fumigatus* (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 pyrim-

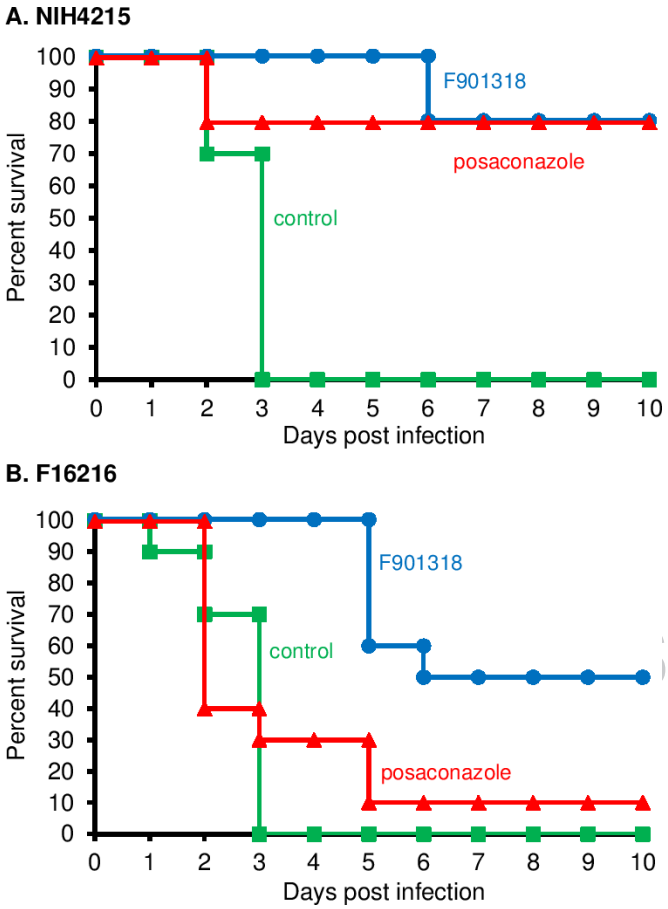

Fig. 4. ***In vivo*** **efficacy of F901318 in a mouse model of infection with a posaconazole resistant strain of** ***A. fumigatus*****.** Groups of 10 immunosuppressed mice were infected intranasally with (A) *A. fumigatus* NIH 4215 or (B) *A. fumigatus* F16216 conidia on day 0. Treatment with F901318 (15 mg/kg, three times daily, IV; blue circles), posaconazole (7.5 mg/kg, once daily, PO; red triangles) or control (green squares) began 6h post infection. Kaplin-Meier curves of surviving mice in each group were plotted. Following infection with NIH 4215, F901318-treatment significantly improved survival compared to controls ($p < 0.001$; Mantel-Haenszel test). Following infection with F16212, F901318-treatment significantly improved survival compared to controls ($p < 0.001$) and compared to posaconazole-treatment ($p < 0.005$).

idine biosynthesis was unable to survive *in vivo*, with a decrease in competitive index versus a wild type or a reconstituted strain observed after just 4h (27). In *Candida albicans*, *URA3* has been commonly used as a selectable marker, but concerns were raised that in some virulence studies the ectopic expression of *URA3*, leading to reduced OMP decarboxylase activity, had a greater effect on virulence than the disruption of the target gene of interest (28). Thus, the evidence from the literature supports the targeting of pyrimidine biosynthesis as a valid antifungal strategy.

Identifying DHODH as the target of the orotomides has helped us to explain the spectrum of antifungal activity observed. F901318 has activity against many pathogenic filamentous and dimorphic fungi including *Aspergillus spp.*, *Histoplasma capsulatum*, *Blastomyces dermatitides* and *Coccidiodes immitis*, together 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, Cryptococcus neoformans*, and the human and *Plasmodium* enzymes are more distantly related, whilst still being 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 trypanosmatids that utilize alternative co-factors 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 agents including *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 anti-malarial 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 therapy areas for which DHODH has been proposed to be 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 auto-immune 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 because viruses require host pyrimidines for replication (29).

In conclusion, to combat the increasing problem of resistance to existing antifungal therapies, it is vitally important that new cellular targets for antifungals are discovered, together with viable chemistry against these new targets (23). F901318 is a new antifungal drug, currently completing both IV and oral Phase 1 clinical trials*, that acts via inhibition of the pyrimidine biosynthesis enzyme dihydroorotate dehydrogenase, validating a new target for antifungal drug discovery.

* ClinicalTrials.gov identifiers of these studies are NCT02142153, NCT02342574, NCT02394483 and NCT02737371.

Materials and Methods

Primers

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

Synthesis of F901318

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 the supporting information.

In vitro **antifungal susceptibility testing.** Minimal inhibitory concentrations (MICs) of antifungal drugs were determined according to CLSI methodology (protocol M38-A2) in RPMI1640 medium buffered to pH7.0 with MOPS buffer at 35°C. For caspofungin the minimum effective concentration (MEC) was defined as the lowest drug concentration causing abnormal growth (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. Assays were carried out using recombinant DHODH prepared from *A. fumigatus* cDNA, *C. albicans* gDNA, or, for the human protein, from 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 (Table S1) and further method details are included in the Supplementary Information. The assay was carried out as described elsewhere (31).

Homology modelling of ***A. fumigatus*** **DHODH**

Human DHODH PDB 1D3G was used as the protein template for construction of the *A. fumigatus* DHODH model. Other DHODH structures from human (1PRH, 2PRL, 2PRM, 3G0X, 3KVM, 2WV8, 2FQI), rat (1UUM, 1UUO), *Trypanosoma cruzi* (2E68), *P. falciparum* (3I68, 1ITV, 3O8A) *Leishmania major* (3MJY) and *E. coli* (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 modelling process and ligand binding is given in the supporting information.

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

centre of the plate to create a well. Into the well 100 µl of a 500 µg/ml of drug was loaded into the well and allowed to diffuse into the agar creating a concentration gradient. Following 4 days incubation at 35 °C a zone of inhibition was observed and conidia collected from the margins of growth, that were then used to create the next plate. Every 5th passage the MIC was determined as described above.

***In vivo* efficacy testing**.

All experiments were conducted under UK Home Office project license (40/3630) and approved by the University of Liverpool Animal Welfare Committee. Groups of 10 CD-1 mice were immunosuppressed with 200mg/kg cyclophosphamide intraperitoneally 4 days before infection and with cyclophosphamide and 250mg/kg cortisone acetate subcutaneously 1 day before infection. *A. fumigatus* F16216 carries an L98H mutation of *cyp51A* and a 34 base pair tandem repeat in the *cyp51A* promoter leading to resistance to azole drugs (*22*). Conidia from this strain, and from the wild type *A. fumigatus* NIH 4215 were administered intranasally on day 0. Treatment with F901318 (15 mg/kg three times daily, IV) or posaconazole (7.5 mg/kg/day, orally) began 6h post-infection.

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