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F901318, a Novel Antifungal Agent from the Orotomide Class: Discovery and Mechanism of Action

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Abstract

Background: There is a need for new antifungal drugs acting via novel mechanisms to combat an increasing incidence of serious invasive fungal disease. The limited existing systemic therapies have liabilities such as toxicities, drug-drug interactions, variable kinetics, and increasing resistance. F901318 is the most advanced member of a new class of antifungal agents, the orotomides, which have a novel mechanism of action distinct from those of currently available clinical antifungal agents. **Methods & Results:** The orotomide class was discovered by whole cell screening of a small molecule chemical library against clinical fungal isolates. Initial hits were developed through iterative rounds of molecular design and whole cell susceptibility testing. F901318 is the most advanced analog from this program. The target of F901318 was identified as dihydroorotate dehydrogenase (DHODH) through a combination of microbiological, biochemical and molecular techniques. DHODH, an important enzyme in pyrimidine biosynthesis, was identified as the target of F901318 from a screen employing an *Aspergillus nidulans* genomic library carried on an AMA plasmid. AMA transformants overexpressing DHODH were shown to be resistant to F901318. Knockout of the DHODH gene on the AMA plasmid restored sensitivity to F901318. Attempts to obtain mutants resistant to F901318 by repeated passage in the presence of drug failed. The antifungal action of F901318 *in vitro* is reversed by the addition of high concentrations of pyrimidines (circa 5mM, compared to a human serum concentration of 15 µM) confirming that the pyrimidine biosynthesis pathway is targeted in the whole cell. Inhibition of DHODH causes rapid cessation of fungal growth, and F901318 is a potent, competitive, reversible inhibitor of the recombinant *Aspergillus fumigatus* protein *in vitro*: IC_{50} = 44 nM +/- 10 nM (+/- standard deviation; n=11). The enzyme is also present in mammalian cells but F901318 is a very poor inhibitor of the human form of the enzyme (IC_{50} > 90 µM) giving a >2000 fold degree of selectivity. **Conclusion:** F901318 belongs to a novel class of antifungal agents, the orotomides, which arrest pyrimidine biosynthesis via inhibition of DHODH. The discovery of the orotomides and F901318 represent an important step in the development of novel antifungal drugs to target systemic fungal infections in man.

Introduction

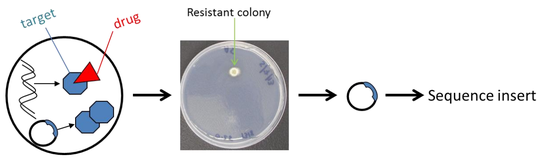
Only three classes of antifungal drugs are commonly used to treat serious systemic fungal infections: the azoles, the polyenes and the echinocandins. Issues such as toxicity, variable PK, drug:drug interactions and increasing resistance mean that there is an urgent need for the discovery of new agents acting via new mechanisms. However, fungi and humans share many genes and pathways making the discovery of new antifungal targets difficult (1).

F901318 is a novel agent and the most advanced representative of a class of antifungals for which the name “orotomides” was recently coined.

Methods

The orotomides were discovered by screening a 370,000 compound small molecule library for antifungal activity against *Aspergillus fumigatus*. The series was developed by standard SAR-driven iterative medicinal chemistry and antifungal susceptibility screening. Initially unknown, the mechanism of action was investigated in various ways:

1. Genetic screen – An *A. nidulans* genomic library carried on the AMA1 plasmid (2) was transformed into *A. nidulans* A767 and the resulting spores exposed to F901318. AMA DNA was recovered from resistant colonies and the genomic DNA insert sequenced.



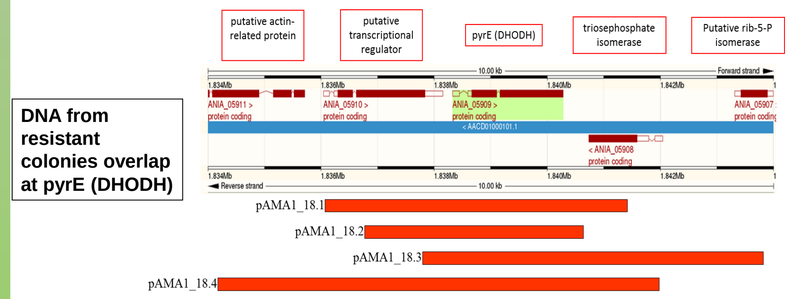
2. Reversal assays – Different concentrations of the pyrimidines uridine and uracil were added to antifungal susceptibility tests of F901318 against *A. fumigatus* Af293. Minimal inhibitory concentrations (CLSI M38-A2) were obtained after 48h at 35°C.
3. *In vitro* enzyme assays – Recombinant *A. fumigatus* and human DHODH were expressed in *E. coli* and purified by immobilised metal affinity chromatography. DHODH activity was determined by following the decrease in absorbance at 600 nm of a redox indicator dye, 2,6-dichloroindophenol, in the presence of the substrate dihydroorotate and coenzyme Q cofactor (3).

Results

1. Genetic screen

AMA DNA from four F901318-resistant colonies was sequenced and mapped to the *A. nidulans* genome as indicated below. All inserts mapped to the same region of chromosome I, with only one gene intact in all inserts – pyrE, coding for dihydroorotate dehydrogenase (DHODH). This result was verified by re-transforming *A. nidulans* with the pAMA1_18.1 plasmid and a version with pyrE knocked out. The intact plasmid gave rise to F901318-resistant colonies, whereas the pyrE KO version produced F901318-sensitive spores.

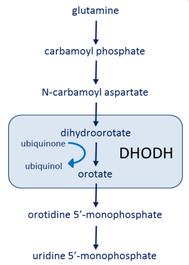
Thus, extra copies of pyrE lead to F901318-resistance.



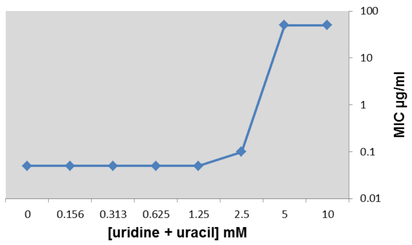
DNA from resistant colonies overlap at pyrE (DHODH)

2. Reversal assays

DHODH is an enzyme of the *de novo* pyrimidine biosynthesis pathway. This pathway has been shown to be important for virulence in several pathogenic fungi (4,5). Addition of pyrimidines, the end products of this pathway, can reverse the effect of F901318 *in vitro*, confirming that the pyrimidine pathway is affected by the drug in the whole cell. However, 5 mM pyrimidines were required for reversal, a high concentration when compared to the 15 µM pyrimidines present in human serum.



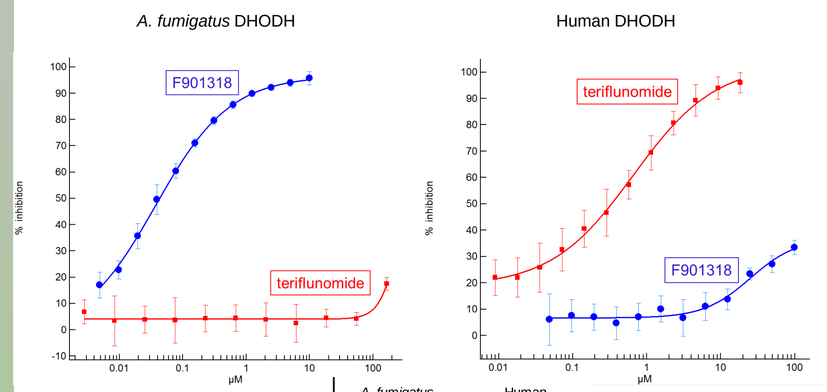
Pyrimidine biosynthesis pathway



F901318 MIC +/- addition of pyrimidines

3. *In vitro* enzyme assays

F901318 was compared to a known inhibitor of human DHODH, teriflunomide:



	A. fumigatus	Human
F901318	0.044 +/- 0.01	> 100
teriflunomide	> 166	0.36 +/- 0.095

DHODH IC50s (µM)

F901318 is a dose-dependent inhibitor of *A. fumigatus* DHODH, but a very poor inhibitor of human DHODH.

Conclusions

- ❑ F901318 is the most advanced in a new class of antifungal drug – the orotomides
- ❑ The orotomides were discovered in a whole-cell antifungal screen
- ❑ F901318 acts via a new mechanism of action for human antifungal therapy:

Inhibition of dihydroorotate dehydrogenase (DHODH)

- ❑ F901318 has >2000-fold selectivity for *Aspergillus* DHODH over human DHODH

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