Molecular mechanism and frequency of olorofim resistance in *Aspergillus fumigatus*

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Introduction

- Olorofim (OLO) is a **new antifungal agent** with a novel mechanism of action.
- It is active against *Aspergillus fumigatus* both in vitro and in animal models including azole-resistant strains.
- OLO targets dihydroorotate dehydrogenase (**DHODH**) in the de novo pyrimidine biosynthesis pathway.
- **No intrinsic OLO resistance** has been detected in surveys to date of >2000 isolates of *A. fumigatus*. However, we observed that resistance could be selected in vitro using a very high inoculum of spores.

Objective
In this study, we analysed the frequency of resistance mutation induction of OLO in *A. fumigatus* using two experimental induction methods and using itraconazole (ITZ) as control. Resistant isolates were analysed and the resistance mechanism of OLO in *A. fumigatus* was investigated.

Results

- Method 1: The **resistance rate** of OLO was 1 in 5 x 10^7 (frequency of 2x10^{-8}) for strain AZN 8196 compared to 1 in 5 x 10^6 (2 x 10^{-7}) for ITZ. For strain V139-36 the resistance rate of OLO was 1 in 9 x 10^7 (1.1 x 10^{-8}) while the **rate for ITZ** was 1 in 8 x 10^6 (1.3 x 10^{-7})

- Method 2: Resistance to OLO in Af293 occurred at a **rate** of 1 in 6 x 10^8 (frequency of 1.7 x 10^{-9}).

- The phenotype and growth characteristics of OLO-resistant progeny were similar to that of the parent strains for 5 of 6 resistant mutants.

- Sequencing of *pyrE* revealed a hotspot for resistance mutations in the *pyrE*-gene for OLO resistance in *A. fumigatus* at locus Gly119. Four amino acid substitutions were found; G119V, G119C, G119S and G119A. A single OLO-resistant mutant was obtained that carried the wild type DHODH sequence, but this mutant was slow growing compared with the parent strain.

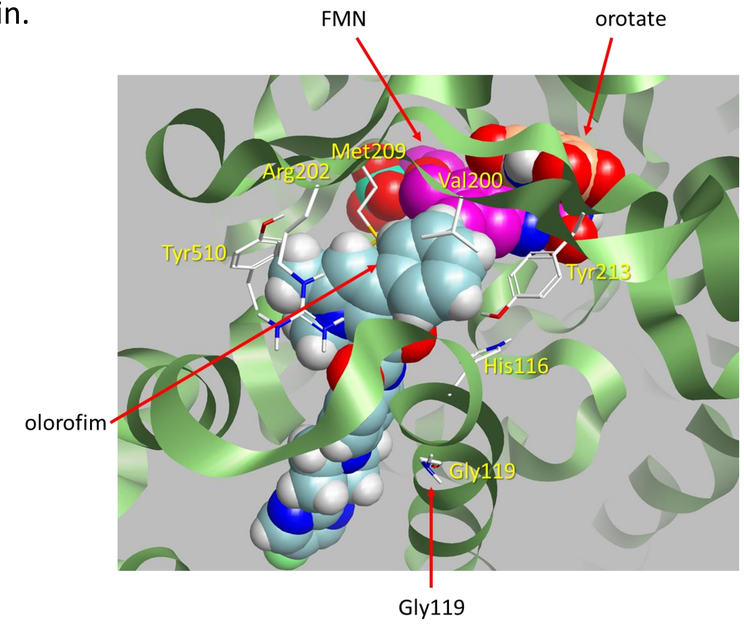

Figure 1: Predicted binding of olorofim to *A. fumigatus* DHODH. Olorofim was docked into a homology model of *A. fumigatus* DHODH. The position of Gly119 is indicated.

Materials and Methods

The mean rate of OLO resistance induction was calculated for selected isolates and compared with that of ITZ. The *pyrE*-gene, which codes for DHODH, was sequenced for OLO-susceptible parent strains and OLO-resistant progeny isolates and analyzed for mutations.

- Method 1: From two *A. fumigatus* isolates, AZN 8196 and V139-36, 10 single colonies were **separately inoculated** onto Sabouraud agar and incubated at 37°C for 96h. From each culture 1 x 10^8 conidia were applied to 6 RPMI agar plates containing either 0.5 mg/L OLO or 8 mg/L ITZ. Plates were incubated at 37°C for up to 7 days. Colonies growing on **drug-containing plates** were subcultured on RPMI agar containing 0.5 mg/L OLO or 8 mg/L ITZ to confirm resistance.

- Method 2: Spore stocks of *A. fumigatus* **strain Af293** were prepared and inoculated onto yeast nitrogen base with glucose agar (YNBG) containing 0.25 mg/L OLO. A total of 8 x 10^9 spores were inoculated into 12 x 100 ml YNBG-OLO agar plates that were subsequently incubated for 5 days at 35°C. Colonies growing on drug-containing plates were subcultured on YNBG-OLO **to confirm resistance**.

Discussion and Conclusion

- The OLO resistance rate was **5 to 10-fold lower** compared to ITZ, indicating that resistance selection might be **less frequent** in patients with aspergillosis treated with OLO.
- A hotspot for OLO resistance mutations was identified in the ***pyrE*-gene** at locus G119.
- Modelling predicts that **Gly119** is on a helix close to the olorofim molecule. Changing the residue at this position potentially affects olorofim binding to DHODH.

Conclusion
To date there is a very low frequency of resistance to olorofim seen in clinical isolates of *Aspergillus* spp., and no resistance has been seen in a collection of over 2000 isolates of *A. fumigatus*.

- *In vitro* we demonstrate that OLO resistance can be selected in *A. fumigatus* and can be mediated by mutations in the *pyrE*-gene at locus G119.
- The frequency of resistance varied from approximately 2 x 10^{-8} to 1.7 x 10^{-9} for spontaneous mutations.

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