Madurella mycetomatis, the main causative agent of eumycetoma, is highly susceptible to olorofim

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Objectives: Eumycetoma is currently treated with a combination of itraconazole therapy and surgery, with limited success. Recently, olorofim, the lead candidate of the orotomides, a novel class of antifungal agents, entered a Phase II trial for the treatment of invasive fungal infections. Here we determined the activity of olorofim against *Madurella mycetomatis,* the main causative agent of eumycetoma.

Methods: Activity of olorofim against *M. mycetomatis* was determined by *in silico* comparison of the target gene, dihydroorotate dehydrogenase (DHODH), and *in vitro* susceptibility testing. We also investigated the *in vitro* interaction between olorofim and itraconazole against *M. mycetomatis.*

Results: *M. mycetomatis* and *Aspergillus fumigatus* share six out of seven predicted binding residues in their DHODH DNA sequence, predicting susceptibility to olorofim. Olorofim demonstrated excellent potency against *M. mycetomatis in vivo* with MICs ranging from 0.004 to 0.125 mg/L and an MIC_{90} of 0.063 mg/L. Olorofim MICs were mostly one dilution step lower than the itraconazole MICs. *In vitro* interaction studies demonstrated that olorofim and itraconazole work indifferently when combined.

Conclusions: We demonstrated olorofim has potent *in vitro* activity against *M. mycetomatis* and should be further evaluated *in vivo* as a treatment option for this disease.

Introduction

The poverty-associated disease mycetoma, which was added to the Neglected Tropical Disease List in 2016 by WHO, remains a major health problem in endemic areas.[1,2] Most cases occur in the mycetoma belt between latitudes 15° South and 30° North.[3,4] Mycetoma presents itself as a subcutaneous chronic granulomatous infectious and inflammatory disease characterized by the formation of grains in affected tissues.[3,5] In more than 80% of the cases, the foot and leg are affected.[4] This disease is divided into two groups: actinomycetoma (mycetoma caused by bacteria) and eumycetoma (mycetoma caused by fungi). Although many different fungal species are found to cause eumycetoma, *Madurella mycetomatis* dominates other fungal species and is present in more than 70% of all patients.[4,6]

Eumycetoma is recalcitrant in nature, which necessitates prolonged antifungal therapy combined with massive and repeated surgical debridement. In severe cases, amputation of the affected part may be the only remaining treatment option.[3,7,8] Previous reports determined that *M. mycetomatis* was most susceptible to the azole class of antifungal agents[9–11] and is currently treated with itraconazole.[12] Treatment with itraconazole may take years and, with an average monthly income of only $60/month, itraconazole at $330/month is considered to be too expensive for patients. Thus there is a dire need for another antifungal agent that is active against *M. mycetomatis.*[13]

Olorofim, formerly known as F901318 (F2G Ltd, Eccles, Manchester, UK), is the leading representative from a novel class of antifungal agents called the orotomides.[14] Olorofim inhibits the fungal enzyme dihydroorotate dehydrogenase (DHODH) leading to obstruction of the pyrimidine biosynthesis pathway.[14,15] Studies have demonstrated that olorofim is active against pathogenic and azole-resistant *Aspergillus* species,[16–19] *Scedosporium* species,[20] *Lomentospora prolificans,*[20] *Coccidioides immitis,*[21] *Fusarium proliferatum*[22] and other dimorphic fungi.[22] Oliver *et al.*[14] also demonstrated that olorofim exhibited much greater potency against *Aspergillus* spp. compared with other leading antifungal

classes. Given the potency and activity of olorofim, here we aim to evaluate its *in vitro* activity against *M. mycetomatis* and the *in vitro* interaction between olorofim and itraconazole as a first effort to determine whether olorofim shows potential as a new treatment for eumycetoma.

Materials and methods

In silico modelling

The *M. mycetomatis* DHODH sequence was obtained by BLAST analysis using the *Aspergillus fumigatus* DHODH protein sequence as a guide (EC 1.3.5.2). *M. mycetomatis*, *A. fumigatus* and *Homo sapiens* DHODH sequences were aligned using Clustal Omega (EMBL-EBI, UK) and formatted using BOXSHADE (EMBnet node, Switzerland). Mitochondrial targeting sequences of *M. mycetomatis* and *A. fumigatus* DHODH were predicted by MitoFates[23] (Japan), while the transmembrane domains were predicted by Phobius[24] (Stockholm Bioinformatics Centre, Sweden).

Isolates

A total of 21 *M. mycetomatis* isolates with different genetic[25,26] and geographical backgrounds were used in this study. Among the isolates used, 14 isolates originated from Sudan, there was 1 isolate each from Algeria, Mali, India, Chad and the Netherlands and there were 2 isolates with unknown origin. Isolates were obtained from the Mycetoma Research Centre in Sudan, the Swiss Tropical Institute in Switzerland and the Westerdijk Fungal Biodiversity Institute and Erasmus Medical Centre mycetoma collection in the Netherlands. All isolates are maintained and preserved in the Erasmus Medical Centre's mycetoma collection. Isolates were identified to species level on the basis of morphology, PCR-based RFLP and sequencing of the internal transcribed spacer (ITS) regions.[27]

Fungal preparation

Fungal colonies were maintained on Sabouraud dextrose agar (BD Biosciences). After 3 weeks of growth at 37°C, colonies were scraped off, sonicated at maximum power for 5 s (Soniprep 150, Beun de Ronde, The Netherlands) and then inoculated into 50 mL Greiner tubes (Sigma–Aldrich) containing RPMI-1640 culture medium supplemented with 0.35 g/L L-glutamine and 1.94 mM MOPS. The isolates were then further incubated for 7 days at 37°C. After incubation, the mycelia within were washed once with RPMI-1640 culture medium. A fungal suspension of 69%–71% transmission was then prepared (Novaspec II spectrophotometer) for *in vitro* susceptibility testing.

In vitro susceptibility testing

Susceptibility testing was carried out according to the previously described and validated method developed for susceptibility testing using a standardized hyphal inoculum.[9,28] Antifungal activity of olorofim against *M. mycetomatis* was determined using the XTT assay. Efficacy of olorofim was compared with that of itraconazole. Olorofim was dissolved in DMSO and tested at a range of 0.004–2 mg/L at a 2-fold dilution rate. Itraconazole was also dissolved in DMSO and tested at a range of 0.008–16 mg/L at a 2-fold dilution rate. The assay was carried out in round-bottom microtitre plates (Greiner Bio-one, The Netherlands). Wells in the microtitre plates were filled with different concentrations of olorofim or itraconazole and 100 µL of fungal suspension. For each fungal isolate, a drug-free and a negative control were included. The microtitre plates were then sealed and placed at 37°C for 7 days. Endpoints were determined at Day 7 and supernatant was measured at 450 nm (Epoch 2, Biotek, USA). MICs of olorofim and itraconazole were determined. MIC was defined as the lowest

concentration with a minimum of 80% growth reduction. With the XTT assay, 100% reduction in viable fungal mass could not be used as an endpoint, since a number of strains had pigments that influenced the colour intensity.[9,28] MIC_{50} and MIC_{90} were defined as the MICs that inhibited growth of 50% and 90% of all isolates tested, respectively. All experiments were performed in triplicate.

Olorofim and itraconazole interaction

A chequerboard microdilution assay was used to evaluate the *in vitro* activity between olorofim and itraconazole. Olorofim was evaluated using a concentration ranging from 0.002 to 2 mg/L and itraconazole from 0.004 to 0.25 mg/L. The interaction between olorofim and itraconazole was analysed based on the FIC index and the interaction ratio (IR).[29,30] FIC index values were calculated as follows:

$$\text{FIC index} = \text{FIC}_A + \text{FIC}_B = (\text{MIC}_A{}^{comb}/\text{MIC}_A{}^{alone}) + (\text{MIC}_B{}^{comb}/\text{MIC}_B{}^{alone})$$

$MIC_A{}^{comb}$ and $MIC_B{}^{comb}$ represent the concentration of drugs A and B, respectively, when tested in combination and $MIC_A{}^{alone}$ and $MIC_B{}^{alone}$ represent the concentration of drugs A and B, respectively, when acting individually. An FIC index value of ≤ 0.5 is considered synergistic, a value of >0.5 to 4 is considered indifferent and a value of >4 is considered antagonistic.[29,30] The IRs were calculated using the formula:

$$\text{IR} = \text{Io}/\text{Ie}$$

Io and Ie represent the observed and expected percentage of inhibition for a given interaction, respectively. Ie is calculated as follows:

$$\text{Ie} = A + B - (AB/100)$$

A and *B* represent the percentage of inhibition observed for each compound when acting alone. The interaction was considered synergistic when IR was >1.5, indifferent when IR was between 0.5 and 1.5, and antagonistic when IR was <0.5.[30,31] The chequerboard assay was performed twice using *M. mycetomatis* genome isolate MM55. Using the XTT endpoint read, MIC was defined as the lowest concentration with a minimum of 80% growth reduction.

Statistical analysis

MICs of olorofim and itraconazole were statistically compared using a Mann–Whitney test. A *P* value of <0.05 was deemed statically significant.

Results

In silico modelling predicts that M. mycetomatis is susceptible to olorofim

The analysis of DHODH sequences showed that the *M. mycetomatis* DHODH homologue (accession number: KXX79707) shares 58.7% homology with that of *A. fumigatus* and 40.1% homology with that of *H. sapiens.* When comparing the amino acid residues that are predicted to be important in olorofim binding[14] in both *A. fumigatus* and *M. mycetomatis* DHODH, we observed a similarity of 86% between the two species. *M. mycetomatis* DHODH shares six out of seven predicted binding residues with *A. fumigatus* DHODH. The amino acid that differed was Leu[195], which corresponded to the Met[209] position in *A. fumigatus* (Figure 1). This is a conservative replacement, with both amino acids having
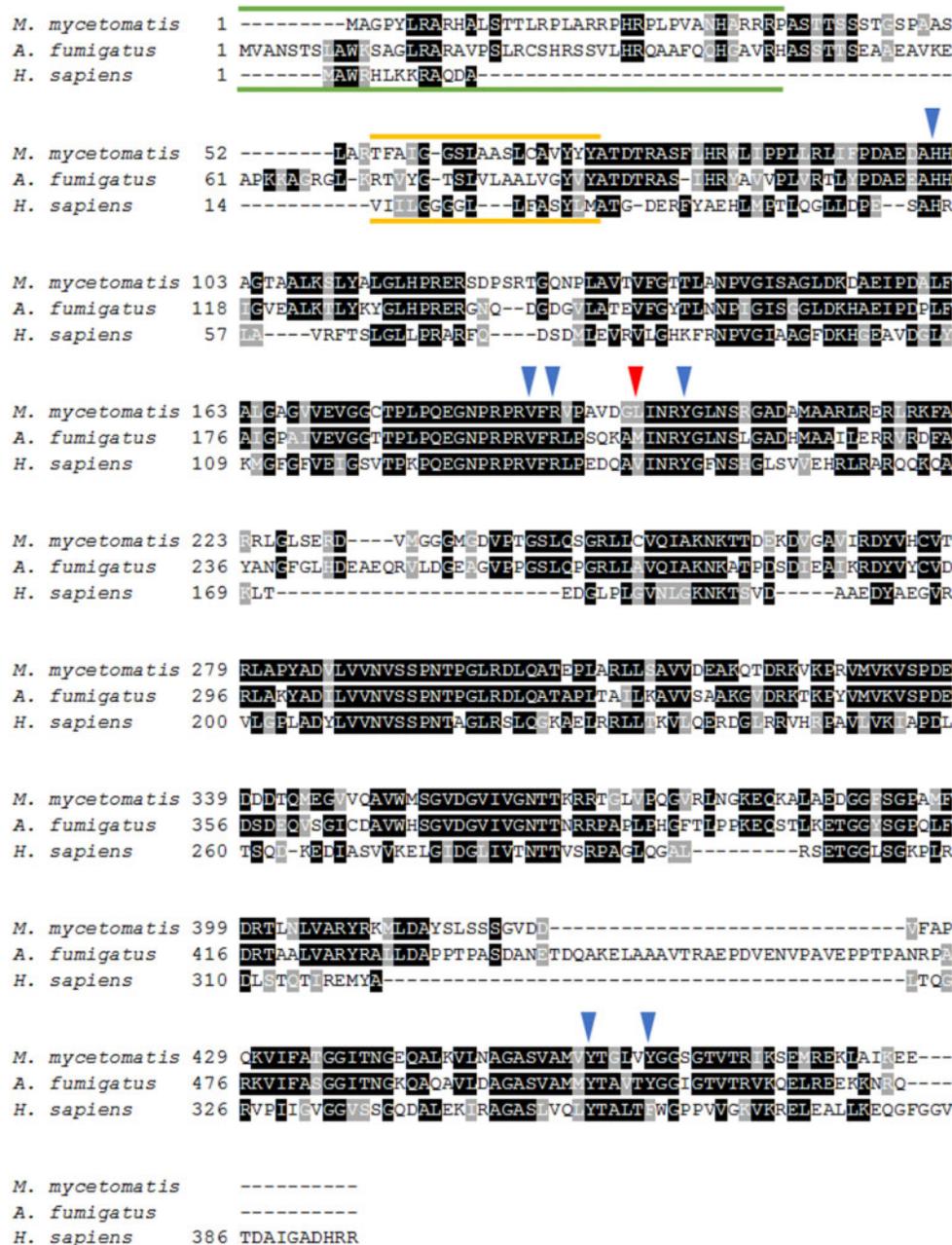
Figure 1. Alignment of *M. mycetomatis*, *A. fumigatus* and human (*H. sapiens*) DHODH amino acid sequences. Conserved residues are highlighted in black and similar residues are highlighted in grey. The predicted mitochondrial targeting sequences are indicated by the green lines and the predicted transmembrane domains are indicated by the yellow lines. The blue arrows depict the amino acid residues predicted to be important for olorofim binding in *A. fumigatus* DHODH[15] that are identical in *M. mycetomatis*. The red arrow depicts the amino acid residue predicted to be important for olorofim binding in *A. fumigatus* DHODH that deviates in *M. mycetomatis*. In *A. fumigatus* this amino acid is Met[209], while in *M. mycetomatis* the amino acid is Leu[195]. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

hydrophobic side chains, indicating that *M. mycetomatis* might be susceptible to olorofim.

M. mycetomatis is highly susceptible to olorofim in vitro

As shown in Figure 2, MICs of olorofim ranged from 0.004 to 0.125 mg/L and MICs of itraconazole ranged from 0.008 to 0.25 mg/L. *M. mycetomatis* is more susceptible to olorofim compared with itraconazole. Significantly lower MICs were obtained for olorofim (median = 0.016 mg/L) than for itraconazole (median = 0.031 mg/L) (*P* = 0.047) (Table 1). For olorofim, a concentration of 0.016 mg/L was needed to inhibit 50% of isolates and a concentration of 0.063 mg/L was needed to inhibit 90% of *M. mycetomatis* isolates. For itraconazole, 0.031 and 0.125 mg/L was needed to inhibit 50% and 90% of isolates, respectively.
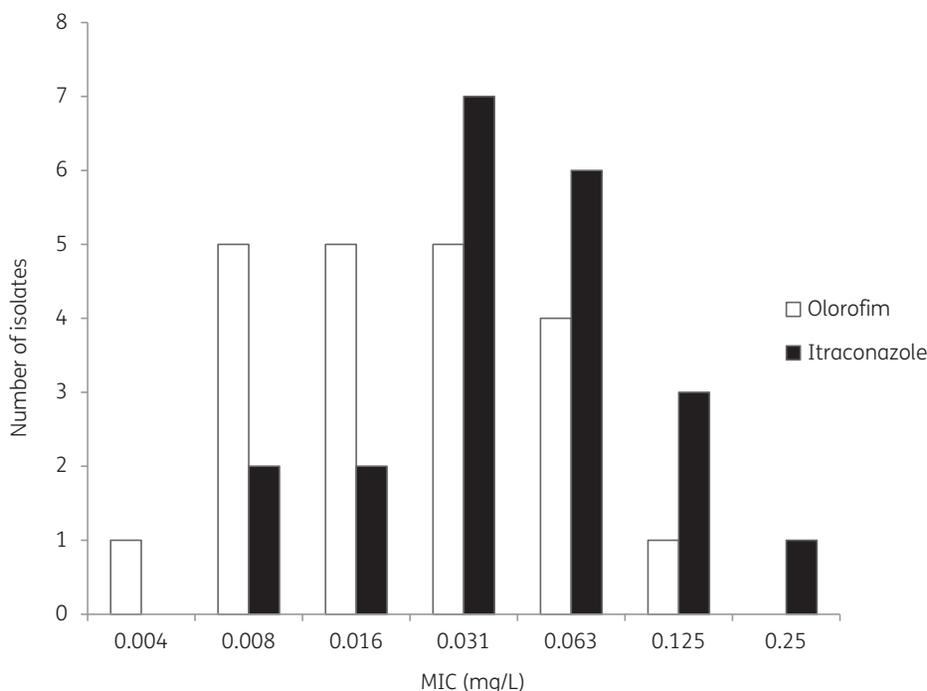
Figure 2. *In vitro* activities of olorofim and itraconazole against 21 *M. mycetomatis* isolates, represented by MICs.

Indifferent interaction between olorofim and itraconazole

To determine whether itraconazole and olorofim could potentially be combined in a therapy, a chequerboard assay for olorofim and itraconazole was performed on *M. mycetomatis* genome isolate MM55. As shown in Table 1, an FIC index of 3.2 and an IR of 0.92 were obtained. Both these values indicate that olorofim and itraconazole are indifferent when combined.

Discussion

Despite treatment with itraconazole at 200–400 mg daily, only 25.9% of eumycetoma patients are cured and 2.8% end up with amputation. This in turn leads to a high morbidity and dependency on family members. Therefore, there is an urgent need to identify novel drugs with activity against the causative agents of eumycetoma.

Since the discovery of olorofim, several studies have been carried out on the antifungal properties of olorofim, demonstrating its effectiveness against several fungal species, notably *Aspergillus* spp.[19] Olorofim showed a lower MIC compared with other drugs tested.[16,18–20] To evaluate whether olorofim would be active against *M. mycetomatis*, we first analysed and compared the amino acid sequence of *M. mycetomatis* DHODH with that of *A. fumigatus*. A homology of 58.7% was determined between the two DHODH sequences. Furthermore, six out of seven amino acids predicted by Oliver *et al.*[14] to be important in olorofim binding were shared. The single remaining amino acid, Met[209] in the *A. fumigatus* DHODH amino acid sequence, was replaced by Leu[195] in *M. mycetomatis*. Apparently this substitution did not affect the susceptibility to olorofim as we demonstrated that *M. mycetomatis* is indeed susceptible to olorofim with MICs ranging from 0.004

Table 1. *In vitro* susceptibility to olorofim and itraconazole, and the interaction of the combined drugs

	Olorofim	Itraconazole	Combined
MIC, median (mg/L)	0.016	0.031	—
MIC, range (mg/L)	0.004–0.125	0.008–0.25	—
MIC$_{50}$ (mg/L)	0.016	0.031	—
MIC$_{90}$ (mg/L)	0.063	0.125	—
MIC for *M. mycetomatis* isolate MM55 (mg/L)	0.063	0.063	—
FIC index for *M. mycetomatis* isolate MM55	—	—	3.2 (indifferent)
IR for *M. mycetomatis* isolate MM55	—	—	0.91 (indifferent)

—, not applicable.

to 0.125 mg/L. Oliver *et al.*[14] successfully created a mutant *Candida albicans* DHODH that became susceptible to olorofim by replacing Phe[162] and Val[171] (equivalent to Val[200] and Met[209] in *A. fumigatus*) with Val[162] and Met[171]. This indicated that these two residues at their respective positions in each species were important for olorofim binding and subsequent inhibition of DHODH. However, as for the two residues in *M. mycetomatis*, the presence of Leu[195] (Met[209] in *A. fumigatus*) at the latter position apparently did not impair the binding of olorofim to DHODH. Since the difference in the latter amino acid residue between *M. mycetomatis* and *A. fumigatus* did not affect susceptibility to olorofim, taking these data together, the resistance of *C. albicans* to olorofim is most likely due to the difference in the former of the two residues (position

162 in *C. albicans*, 200 in *A. fumigatus* and 186 in *M. mycetomatis*). As indicated by Oliver *et al.*,[14] there must also be other important differences in DHODH between these species and more studies are needed to understand the importance of the amino acids involved in olorofim binding.

Olorofim is currently in a Phase II study (ClinicalTrials.gov identifier NCT03583164) for treatment of invasive fungal infections caused by *Scedosporium* spp., *Aspergillus* spp. and other resistant fungi in patients lacking suitable alternative treatment options. This study will provide a good understanding of the dosage and the efficacy of olorofim in patients, which might also be applicable for mycetoma patients. However, for actinomycetoma, the bacterial form of mycetoma, it was discovered that patients were more responsive to combination therapy than to a single drug alone. The combination therapy differs between countries; in Mexico and Sudan the Welsh regimen is used and in India the Raman regimen is used.[3,32–35] Until now, combination therapy for eumycetoma has not extensively been explored in animal models and clinical trials. This is because the results of *in vitro* combination studies may differ according to the methodologies used and thus cannot be relied upon to predict the clinical effect that may be obtained. For *M. mycetomatis*, hyphal fragments are exposed to the antifungal agents *in vitro*, while *in vivo* it structures itself as grains. Therefore, the efficacy of combination therapy should always be determined both *in vitro* and *in vivo*.[36] In the past, combination therapy for eumycetoma did not seem feasible since all antifungal agents with activity against the causative agents had the same mode of action, which could lead to antagonism instead of synergy.[37] When azoles were combined with terbinafine *in vitro* by Ahmed *et al.*[30] (2015), indifference and antagonism were noted. The *in vivo* study by Eadie *et al.*[37] (2017) demonstrated that combining the drugs resulted in antagonism and treatment significantly decreased larvae survival. Eadie *et al.*[37] confirmed the discovery of Scheven and Schwegler[38] that antagonism occurs when ergosterol is inhibited via two different pathways. In this study, the combination of olorofim and itraconazole, two drugs with different modes of action, was studied. The components of the *in vitro* combination of olorofim and itraconazole against *M. mycetomatis* acted indifferently to each other, as no antagonism or synergy was noted. Since olorofim and itraconazole inhibit fungal growth via different mechanisms, this highlights the room for further evaluation *in vivo* and in a clinical setting where they could be potentially combined to treat eumycetoma.

In conclusion, we showed that olorofim inhibits growth of *M. mycetomatis* and, although olorofim and itraconazole inhibit fungal growth by different mechanisms, when combined they show no antagonism or synergism. The next step will be to study the efficacy of olorofim against *M. mycetomatis* in an *in vivo* model.[39]

Transparency declarations

Olorofim was obtained from F2G Ltd. Jason D. Oliver and Mike Birch are employees and shareholders of F2G Ltd. Bart Rijnders is an investigator of a Phase II study on olorofim. His employer receives patient fees for the inclusion of patients in this study. All other authors: none to declare.

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