The effect of the orotomide antifungal olorofim on the growth and viability of Scedosporium and Lomentospora species.

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Introduction

- Olorofim (F901318) is the leading member of the new orotomide class of antifungals that act via inhibition of the pyrimidine biosynthetic enzyme dihydroorotate dehydrogenase (DHODH) (1).
- The spectrum of olorofim includes filamentous and dimorphic fungi including *Aspergillus*, *Taloromyces*, *Coccidioides*, *Histoplasma* and *Blastomyces.*
- *Scedosporium* spp. and the closely related *Lomentospora prolificans* are filamentous fungi that are frequently resistant to antifungal drugs. Olorofim has potent *in vitro* antifungal activity against *Scedosporium* spp. and *Lomentospora prolificans* (2, 3).
- Olorofim is in phase II clinical development for the treatment of serious systemic fungal infections including invasive aspergillosis, scedosporiosis, lomentosporiosis and other rare mould infections.

Methods

Strains used:
Scedosporium apiospermum strains 451 & 4883 (clinical strains)
Lomentospora prolificans 206 (clinical strain) & NCPF 7138 (type strain)

Minimal Inhibitory Concentration (MIC)
MICs for imaging were performed in 96-well µClear microplates (Greiner) in RPMI-1640 medium according to the CLSI M38-A2 protocol. The lowest drug concentration showing 100% inhibition of growth (detected visually) was recorded as the MIC.
XTT MIC assays – Metabolic activity in the MIC wells was measured by reduction of XTT to an orange formazan product. Following a 48h MIC, 0.3 mM XTT and 4 µM menadione were added to the wells, incubation was continued for 60 min at 35ºC and the absorbance of the supernatant was read at 492 nm.

Imaging & Cell Viability
Images were generated by Leica confocal SP8X microscope.
For fluorescent assays conidia were incubated for 8h in Vogel's minimal medium to prepare germlings (germinated spores) followed by 0-120 h incubation in the presence of 0.25 µg/ml olorofim. For assessment of cell viability 2 µg/ml bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC) was added, and after 5 min the fluorescent germlings were observed (excitation 488 nm; emission 500-590 nm) and counted.

Results - MICs

	MIC (µg/ml)	
	olorofim	voriconazole
S. apiospermum 451	0.25	1
S. apiospermum 4883	0.125	0.5
L. prolificans 206	0.25	>8
L. prolificans NCPF7138	0.5	>8

Table 1. MICs of strains used in this study.

As has been observed previously (2, 3) olorofim displays excellent *in vitro* potency against these species, including *L. prolificans* that is frequently resistant to all available antifungal agents as highlighted by the raised MICs to voriconazole.

Results – XTT assay

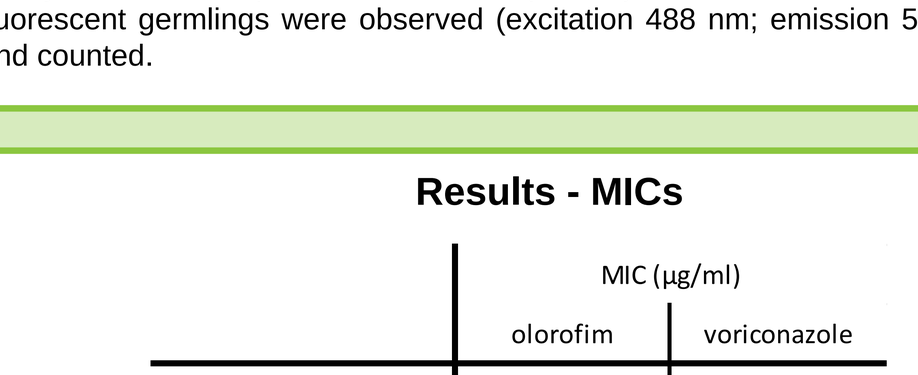

Figure 1. XTT evaluation of metabolic activity in MIC wells.
Following MIC evaluation the metabolic activity of the well contents were assessed by XTT assay and the values converted to a percentage of the activity in the absence of drug. **A.** *S. apiospermum* 451; **B.** *L. prolificans* 206. The visual MICs are indicated by the arrows.

There is an effect of olorofim on metabolic activity at concentrations below the visually observed MIC value (arrowed). Less than 25% of the no drug level of metabolic activity was observed at concentrations down to 0.06 µg/ml and 0.03 µg/ml in the *S. apiospermum* and the *L. prolificans* XTT assay respectively.

Results – Imaging of MIC wells

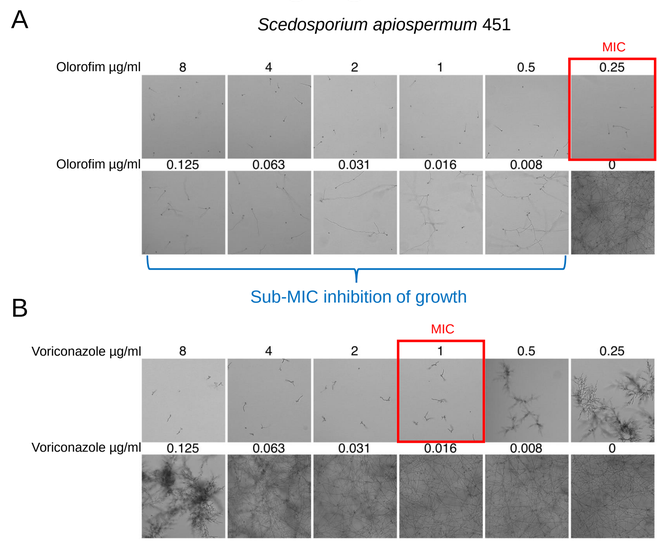

Figure 2. Imaging of *Scedosporium apiospermum* 451 MIC wells.
MICs were set up with a final drug concentration of 0 to 8 µg/ml olorofim (A) and voriconazole (B). The MIC estimated visually is highlighted in red. Microscopic images of each well were taken.

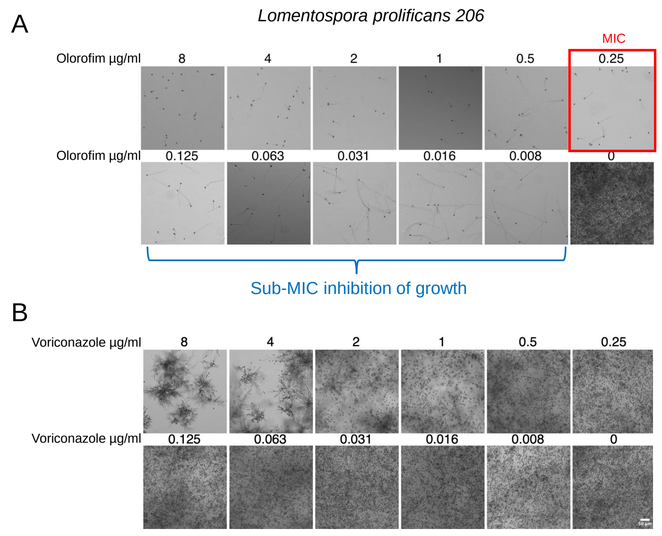

Figure 3. Imaging of *Lomentospora prolificans 206* MIC wells.
MICs were set up and analysed as described in the legend to Figure 2.

Microscopic examination of the fungal growth in the wells of MIC plates reveals inhibition of growth in multiple wells below the MIC value for olorofim (Figure 2A and 3A). This sub-MIC inhibition of growth is apparent from 0.008 – 0.125 µg/ml olorofim against both *S. apiospermum* 451 and *L. prolificans* 206 and was also observed in the other strains tested (4883 & NCPF7138; data not shown).
Sub-MIC inhibition of growth was also observed for voriconazole (Figure 2B and 3B) but the effect appears to be less dramatic.

Results – Viability staining

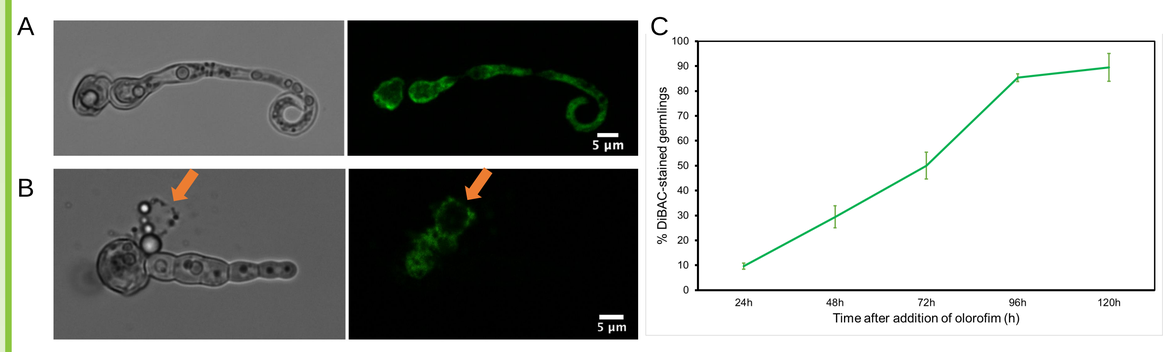

Figure 4. Olorofim causes time-dependent killing of *L. prolificans.*
L. prolificans 206 germlings were exposed to 0.25 µg/ml olorofim for 0-120 h and cell viability was assessed using the fluorescent dye DiBAC.

Figure 4A shows an *L. prolificans* germling where the hypha has taken up DiBAC and is no longer viable. Figure 4B shows a germling that has lysed and released some cellular contents that have become labelled by DiBAC (arrowed).
Figure 4C is a graph showing that the number of DiBAC-labelled germlings increases with time of exposure to olorofim. After 120h 90% of germlings are non-viable.
Hyphal lysis and time-dependent killing was previously observed in *A. fumigatus* exposed to olorofim (4).

Conclusions

- Olorofim has potent *in vitro* antifungal activity against *Scedosporium apiospermum* and *Lomentospora prolificans*.
- Microscopic examination of the wells of MIC plates reveals that olorofim has profound effects on growth at concentrations below the MIC, even down to 0.008 µg/ml.
- XTT-assay estimations of metabolic activity confirm inhibition below the visual MIC.
- *L. prolificans* germlings lose viability when exposed to olorofim as revealed by DiBAC staining. Cell contents were observed outside the hyphae indicating hyphal lysis had occurred. Time-dependent killing of the germlings occurred following addition of olorofim.

References: (1) Oliver, JD et al. 2016. PNAS 113 (45), 12809-14. (2) Wiederhold, NP et al. 2017. J Antimicrob Chemother 72: 1977–1980. (3) Biswas, C et al. 2018. Med Mycol 56: 1050–1054. (4) du Pré, S et al. 2018. Antimicrob Agents Chemother. 62(8). pii: e00231-18.