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In vitro activity of olorofim (F901318) against clinical isolates of cryptic species of *Aspergillus* by EUCAST and CLSI methodologies

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Objectives: To investigate the *in vitro* activity of olorofim (F901318), a novel broad-spectrum antifungal agent, against 150 strains belonging to 16 different cryptic species of *Aspergillus* by EUCAST and CLSI methodologies.

Methods: Olorofim, amphotericin B, micafungin, posaconazole and voriconazole were tested against cryptic species belonging to *Aspergillus fumigatus* complex (*n*=57), *Aspergillus ustus* complex (*n*=25), *Aspergillus niger* complex (*n*=20), *Aspergillus flavus* complex (*n*=20), *Aspergillus circumdati* complex (*n*=15) and *Aspergillus terreus* complex (*n*=13) using EUCAST and CLSI methodologies for broth microdilution susceptibility testing of antifungal agents.

Results: Olorofim was the only drug with activity against all cryptic species of *Aspergillus* tested, including the multiresistant species *Aspergillus lentulus, Aspergillus fumigatiaffinis* and *Aspergillus calidoustus*. Geometric means of MICs for olorofim were lower (0.017, 0.015 and 0.098 mg/L, respectively, for EUCAST; and 0.015, 0.015 and 0.048 mg/L, respectively, for CLSI) than for amphotericin B (4.438, 12.699 and 0.554 mg/L, respectively, for EUCAST; and 0.758, 1.320 and 0.447 mg/L, respectively, for CLSI), voriconazole (2.549, 2.297 and 5.856 mg/L, respectively, for EUCAST; and 2.071, 1.741 and 5.657 mg/L, respectively, for CLSI) and posaconazole (0.307, 0.308 and 12.996 mg/L, respectively, for EUCAST; and 0.391, 0.215 and 9.514 mg/L, respectively, for CLSI).

Conclusions: Olorofim shows encouraging *in vitro* activity against cryptic species of *Aspergillus* that can be hard to treat with current antifungal therapies. Further studies are warranted in order to assess its efficacy.

Introduction

Aspergillus spp. are some of the most common opportunistic human pathogens worldwide, especially in immunocompromised patients. They are the most frequent moulds isolated in clinical samples[1] and can produce a wide range of infections, such as invasive aspergillosis, chronic pulmonary aspergillosis or allergic bronchopulmonary aspergillosis. The leading cause of these life-threatening infections is *Aspergillus fumigatus* (85%), followed by *Aspergillus flavus* (5%–10%), *Aspergillus terreus* (2%–10%) and *Aspergillus niger* (2%–3%).[2]

However, the advances of molecular tools for identification during the last decade have led to the description of new species within the genus *Aspergillus* that are considered cryptic or sibling because they are difficult to differentiate by classical methods. Their prevalence in the clinical setting has been reported to be between 11% and 19% in three studies.[3–5] It has been recommended that *Aspergillus* isolates should be classified to the 'species complex' level,[6] as a way of gathering all these closely related cryptic species if appropriate speciation techniques are not performed. These species are important in the clinical setting because of their susceptibility profile, as azoles and amphotericin B frequently show poor activity against them, and some are considered to be MDR.[3,7] Therefore, new antifungal drugs that act via novel mechanisms are needed to overcome this ever-growing problem of resistance to current therapies.

The orotomides are a new chemical class of drugs whose most representative antifungal is olorofim (F901318). This synthetic small molecule inhibits dihydroorotate dehydrogenase (DHOH), which catalyses the conversion of dihydroorotate to orotate in the pyrimidine biosynthesis pathway.[8] Although it shows no activity against yeasts and Mucorales, olorofim has potent *in vitro* efficacy against a broad spectrum of pathogenic moulds such as *Coccidioides immitis*, *Coccidioides posadasii*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Penicillium* spp., *Scedosporium* spp., *Fusarium* spp. and *Aspergillus* spp., including azole-resistant *A. fumigatus*.[8–13] Murine models of invasive aspergillosis have confirmed the *in vivo* effect of this compound, indicating good tissue distribution.[8,14]

The aim of this study was to further evaluate the *in vitro* activity of olorofim and other comparators against a collection of clinical isolates of cryptic species of *Aspergillus* by comparing the results obtained by standard methodologies for broth microdilution susceptibility testing of antifungal agents (CLSI and EUCAST).

Materials and methods

Isolates

A total of 150 *Aspergillus* strains belonging to six different species complexes were tested in this study. All strains were obtained from clinical samples and identified to the species level by standard microscopic morphology and by sequencing the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) and part of the β-tubulin gene following methods previously reported.[1]

Susceptibility testing

The antifungal susceptibility testing was performed following EUCAST reference method 9.2[15] and CLSI M38-A2.[16] Antifungals used were olorofim (range 0.015–8 mg/L; F2G Limited, Manchester, UK), amphotericin B (range 0.03–16 mg/L; Sigma–Aldrich Quimica, Madrid, Spain), voriconazole (range 0.015–8 mg/L; Sigma–Aldrich Quimica), posaconazole (range 0.015–8 mg/L; Sigma–Aldrich Quimica) and micafungin (range 0.004–2 mg/L; Astellas Pharma Inc., Tokyo, Japan).

A. flavus ATCC 204304 and *A. fumigatus* ATCC 204305 were used as quality control strains in all tests performed for both methods. MICs of amphotericin B, voriconazole, posaconazole and olorofim, and minimum effective concentrations (MECs) of micafungin were visually read after 24 and 48 h of incubation at 35°C in a humid atmosphere. Geometric mean (GM), MIC/MEC_{50} (MIC/MEC causing inhibition of 50% of the isolates tested) and MIC/MEC_{90} (MIC/MEC causing inhibition of 90% of the isolates tested) were determined.

Results

Table 1 shows the GM, MIC_{50}, MIC_{90} and range for all the species tested at 48 h of incubation by EUCAST and CLSI methods with amphotericin B, voriconazole, posaconazole and olorofim. For micafungin, GM, MEC_{50}, MEC_{90} and range by species complex tested at 48 h of incubation by EUCAST and CLSI are shown. MIC/MEC_{50} and MIC/MEC_{90} were only calculated for species that had more than five isolates.

Overall, olorofim was active against all cryptic species of *Aspergillus*, showing more potent activity than the rest of the antifungals tested both by EUCAST and CLSI for all species complexes.

Regarding *A. fumigatus* complex, all five species tested showed different resistance patterns to amphotericin B and/or azoles as previously described.[3] Micafungin was active against these (MEC_{90}s ranged from 0.015 to 0.25 mg/L by EUCAST and from 0.007 to 0.06 mg/L by CLSI), and olorofim MIC_{90} was 0.015 mg/L for each of these (except for *Aspergillus lentulus* by EUCAST; MIC_{90}=0.03 mg/L). Specifically, *A. lentulus* and *Aspergillus fumigatiaffinis* have been reported to be highly resistant species, both to amphotericin B and azoles,[3,17] and olorofim was able to inhibit both species.

Species belonging to the *Aspergillus ustus* complex have also been described as MDR.[3,18] MICs of posaconazole and voriconazole ranged from 4 to 16 mg/L by EUCAST and from 2 to 16 mg/L by CLSI for the three species tested, whereas amphotericin B MICs ranged from 0.12 to 2 mg/L by both procedures. Micafungin showed moderate activity (GMs ranged from 0.063 to 0.311 mg/L by both methodologies), but olorofim was the most active compound against these species (GMs were lower than 0.196 mg/L by both methodologies).

The four species tested belonging to the *A. terreus* complex were inhibited by olorofim (GMs ranged from 0.015 to 0.019 mg/L by both methodologies), with micafungin showing high activity as well (GMs were $\leq$0.015 mg/L). Voriconazole and posaconazole showed moderate activity against these species, whereas amphotericin B showed high MICs against them (MIC values ranged from 0.12 to 8 mg/L by both methodologies).

Aspergillus ochraceus showed an olorofim MIC_{90} of 0.03 mg/L by EUCAST and all isolates tested had an olorofim MIC of 0.015 mg/L by CLSI. Amphotericin B was inactive against this species (MIC_{90}=32 mg/L by both methodologies), azoles were moderately active (MIC_{90}=0.5 mg/L, except voriconazole MIC_{90}=1 mg/L by EUCAST) and micafungin showed MIC_{90}=0.06 mg/L by CLSI and MIC_{90}=0.25 mg/L by EUCAST. All *Aspergillus sclerotiorum* isolates tested displayed similar results to *A. ochraceus*.

Olorofim had increased activity against *Aspergillus alliaceus* strains tested, with low MICs by both methods (MICs ranged from 0.015 to 0.06 mg/L). Amphotericin B and micafungin showed high MIC/MEC values [up to 32 mg/L and 16/4 mg/L (EUCAST/CLSI, respectively)] and azoles had variable MICs (voriconazole MIC_{90} = 0.5 mg/L and posaconazole MIC_{90}=0.25 mg/L, by both procedures), as reported in previous studies.[3]

Finally, *Aspergillus tubingensis* strains were inhibited by olorofim (MICs ranging from 0.03 to 0.12 mg/L). Micafungin was the most active antifungal tested against this species (MEC_{90}=0.03 mg/L for both methodologies), whereas azoles and amphotericin B showed moderate effect (MIC_{90} values from 0.25 to 2 mg/L).

Discussion

Several studies have been carried out since the description of olorofim, demonstrating the *in vitro* activity of this compound. It has shown activity against *Lomentospora prolificans* isolates, being the only active compound described thus far against this species. When checked against *Scedosporium* species, olorofim yielded similar results to voriconazole but was more active than the rest of the drugs tested,[12] including isavuconazole.[9] Its effectiveness has also been assessed against *Coccidioides* isolates *in vitro* and *in vivo* with promising results.[13]

Olorofim has been proved to be active *in vitro* against azole-resistant *A. fumigatus* (harbouring *cyp51A*-associated point mutations and without known resistance mechanisms)[8,10] and has also been reported to be active *in vivo* in murine models of invasive pulmonary aspergillosis caused by an isolate carrying the TR34/L98H mutation in Cyp51A.[8] The pharmacodynamics of olorofim against *A. flavus* have also been recently described to be comparable with those from other azole drugs after studying *in vitro* and *in vivo* models of sinopulmonary invasive aspergillosis caused by this fungus.[14] This new drug has also displayed *in vitro* activity against other common *Aspergillus* species, such as *Aspergillus nidulans*, *A. terreus* and *A. niger*.[11]

Table 1. MIC values and ranges for amphotericin B, voriconazole, posaconazole and olorofim, and MEC values for micafungin for cryptic species of *Aspergillus* isolates, as determined by the CLSI and EUCAST broth microdilution methods

	Antifungal test at 48 h									
	EUCAST (mg/L)					CLSI (mg/L)				
Species (no. tested)	AMB	VRC	POS	MCF	olorofim	AMB	VRC	POS	MCF	olorofim
A. fumigatus complex										
A. lentulus (20)										
GM	4.438	2.549	0.307	0.008	0.017	0.758	2.071	0.391	0.005	0.015
MIC_{50}/MEC_{50}	4	2	0.25	0.007	0.015	1	2	0.5	0.007	0.015
MIC_{90}/MEC_{90}	32	4	0.5	0.015	0.03	1	4	0.5	0.007	0.015
range	1–32	1–8	0.12–0.5	0.004–0.015	0.015–0.03	0.25–2	1–4	0.12–1	0.004–0.015	0.015–0.015
Aspergillus thermomutatus (10)										
GM	0.283	2.297	0.308	0.031	0.015	0.121	1.741	0.231	0.015	0.015
MIC_{50}/MEC_{50}	0.5	2	0.25	0.03	0.015	0.12	2	0.25	0.015	0.015
MIC_{90}/MEC_{90}	2	4	0.50	0.25	0.015	1	4	0.25	0.03	0.015
range	0.03–2	1–4	0.25–0.5	0.004–0.12	0.015–0.015	0.03–1	0.5–4	0.12–0.5	0.004–0.03	0.015–0.015
A. fumigatiaffinis (10)										
GM	12.699	2.297	0.308	0.012	0.015	1.320	1.741	0.215	0.005	0.015
MIC_{50}/MEC_{50}	16	2	0.25	0.015	0.015	2	2	0.25	0.004	0.015
MIC_{90}/MEC_{90}	32	4	0.5	0.03	0.015	2	2	0.25	0.007	0.015
range	2–32	2–8	0.25–0.5	0.004–0.03	0.015–0.015	0.25–4	1–4	0.12–0.5	0.004–0.015	0.015–0.015
Aspergillus udagawae (10)										
GM	2.297	1.866	0.231	0.009	0.015	0.660	1.149	0.186	0.005	0.015
MIC_{50}/MEC_{50}	2	2	0.25	0.007	0.015	0.5	1	0.25	0.004	0.015
MIC_{90}/MEC_{90}	4	4	0.5	0.03	0.015	1	2	0.5	0.007	0.015
range	1–4	1–4	0.12–0.5	0.004–0.03	0.015–0.015	0.5–1	1–2	0.12–0.5	0.004–0.015	0.015–0.015
Aspergillus hiratsukae (7)										
GM	0.500	2.000	0.247	0.009	0.015	0.163	1.486	0.224	0.011	0.015
MIC_{50}/MEC_{50}	0.5	2	0.25	0.007	0.015	0.12	1	0.25	0.007	0.015
MIC_{90}	2	8	1	0.015	0.015	0.5	4	1	0.06	0.015
range	0.25–2	0.5–8	0.03–1	0.004–0.015	0.015–0.015	0.12–0.5	1–4	0.03–1	0.004–0.015	0.015–0.015
A. ustus complex										
A. calidoustus (20)										
GM	0.554	5.856	12.996	0.085	0.098	0.447	5.657	9.514	0.063	0.048
MIC_{50}/MEC_{50}	0.5	6	16	0.12	0.12	0.5	4	16	0.06	0.03
MIC_{90}/MEC_{90}	2	8	16	4	0.25	1	16	16	0.25	0.12
range	0.12–2	4–16	4–16	0.004–4	0.015–0.5	0.12–2	2–16	2–16	0.03–0.25	0.0015–0.25
Aspergillus insuetus (3)										
GM	0.500	8.000	16.000	0.311	0.196	0.500	8.000	16.000	0.153	0.095
range	0.5	8	16	0.5	0.25	0.5	8	16	0.12	0.12
Aspergillus keveii (2)										
GM	0.354	16.000	16.000	0.085	0.085	0.173	16.000	16.000	0.120	0.030
range	0.5	16	16	0.12	0.12	0.25	16	16	0.12	0.06
A. terreus complex										
Aspergillus citrinoterreus (5)										
GM	6.964	0.758	0.069	0.013	0.015	1.320	0.250	0.040	0.007	0.015
range	4–8	0.5–1	0.03–0.25	0.007–0.03	0.015–0.015	1–2	0.25–0.25	0.03–0.06	0.004–0.015	0.015–0.015
Aspergillus aureoterreus (3)										
GM	8.000	1.000	0.060	0.009	0.015	2.000	0.500	0.048	0.009	0.015
range	8–8	1–1	0.03–0.12	0.007–0.015	0.015–0.015	2–2	0.5–0.5	0.06–0.06	0.007–0.015	0.015–0.015
Aspergillus hortai (2)										
GM	2.000	1.000	0.085	0.010	0.015	0.346	0.122	0.060	0.015	0.015
range	1–4	1–1	0.06–0.12	0.007–0.015	0.015–0.015	0.12–1	0.06–0.25	0.06–0.06	0.015–0.015	0.015–0.015

Continued

Table 1. *Continued*

Species (no. tested)	EUCAST (mg/L) AMB	EUCAST VRC	EUCAST POS	EUCAST MCF	EUCAST olorofim	CLSI (mg/L) AMB	CLSI VRC	CLSI POS	CLSI MCF	CLSI olorofim
Aspergillus carneus (3)										
GM	1.587	1.260	0.196	0.015	0.019	0.500	1.260	0.153	0.009	0.015
range	1–2	1–2	0.12–0.25	0.007–0.03	0.015–0.03	0.25–1	1–2	0.12–0.25	0.007–0.015	0.015–0.015
Aspergillus circumdati complex										
A. ochraceus (10)										
GM	4.595	0.812	0.435	0.041	0.020	3.454	0.500	0.307	0.030	0.015
MIC_{50}/MEC_{50}	4	1	0.5	0.03	0.015	4	0.5	0.25	0.03	0.015
MIC_{90}/MEC_{90}	32	1	0.5	0.25	0.03	32	0.5	0.5	0.06	0.015
range	1–32	0.5–1	0.25–0.5	0.015–0.25	0.015–0.06	0.12–32	0.25–1	0.12–0.5	0.015–0.5	0.015–0.015
A. sclerotiorum (5)										
GM	6.063	1.149	0.660	0.017	0.017	1.741	0.660	0.214	0.015	0.015
range	2–8	0.5–4	0.5–1	0.007–0.06	0.015–0.03	2–8	0.5–2	0.06–0.5	0.007–0.03	0.015–0.015
A. flavus complex										
A. alliaceus (20)										
GM	27.858	0.342	0.077	0.086	0.024	12.996	0.193	0.067	0.017	0.015
MIC_{50}/MEC_{50}	32	0.25	0.06	0.03	0.03	32	0.25	0.06	0.007	0.015
MIC_{90}/MEC_{90}	32	0.5	0.25	2	0.03	32	0.5	0.25	0.5	0.015
range	4–32	0.25–1	0.03–0.25	0.015–16	0.015–0.06	1–32	0.12–0.25	0.03–0.5	0.004–4	0.015–0.015
A. niger complex										
A. tubingensis (20)										
GM	0.214	1.110	0.329	0.023	0.051	0.109	1.189	0.392	0.015	0.053
MIC_{50}/MEC_{50}	0.25	1	0.5	0.03	0.06	0.12	1	0.5	0.015	0.06
MIC_{90}/MEC_{90}	0.5	2	0.5	0.03	0.06	0.25	2	0.5	0.03	0.12
range	0.06–0.5	0.5–2	0.12–0.5	0.007–0.06	0.03–0.12	0.06–0.25	0.5–4	0.25–1	0.007–0.03	0.03–0.12

Antifungal test at 48 h (all columns).

AMB, amphotericin B; VRC, voriconazole; POS, posaconazole; MCF, micafungin.

In this work, the strong activity of olorofim has also been confirmed against cryptic species of *Aspergillus*, including those species that show intrinsic resistance to amphotericin B and/or azoles, such as *A. lentulus* or *Aspergillus calidoustus*, olorofim being the only drug of those tested with an inhibitory effect against all strains tested. Although micafungin had activity against most of the species tested, the routine use of echinocandins is not recommended as monotherapy for the treatment of invasive aspergillosis or other *Aspergillus* species because of their distinct mechanism of action, and their use is preferred in combination with other antifungals.[19] CLSI showed, in general, lower MICs (one or two dilutions) than EUCAST. The size of the inocula (10 times lower in CLSI) and the composition of the media (less glucose in CLSI) could explain these discrepancies in the results between the two methodologies used.[20,21]

Further development of this new antifungal is warranted, as well as a multicentre study to evaluate the reproducibility of olorofim activity against moulds and to establish breakpoints and epidemiological cut-off values (ECOFFs) for these species.

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