AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

SUSCEPTIBILITY

EUCAST Determination of Olorofim (F901318) Susceptibility of Mold Species, Method Validation, and MICs

Karin Meinike Jørgensen,[a] Karen M. T. Astvad,[a] Rasmus Krøger Hare,[a] Maiken Cavling Arendrup[a,b,c]

[a]Unit of Mycology, Statens Serum Institut, Copenhagen, Denmark
[b]Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark
[c]Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark

ABSTRACT Olorofim is a novel antifungal agent with *in vitro* activity against *Aspergillus* and some other molds. Here, we addressed technical aspects for EUCAST olorofim testing and generated contemporary MIC data. EUCAST E.Def 9.3.1 testing was performed comparing two plate preparation methods (serial dilution in medium [serial plates] versus predilution in DMSO [ISO plates]), two lots of olorofim, visual (visual-MIC) versus spectrophotometer (spec-MIC) reading, and four polystyrene plates using 34 to 53 *Aspergillus* isolates from five genera. Subsequently, olorofim MICs were compared to itraconazole, voriconazole, posaconazole, and amphotericin B MICs for 298 clinical mold isolates (2016 to 2017). Wild-type upper limits (WT-UL) were determined following EUCAST principles for epidemiologic cutoff value (ECOFF) setting. Olorofim median MICs comparing serial plates and ISO plates were identical (25/36 [69%]) or one dilution apart (11/36 [31%]). Interperson agreement for visual-MICs was 92% to 94%/100% for ≤1/≤2 dilutions, respectively. The visual-MIC values across tested microtiter plates and olorofim lots revealed only discrete differences (≤1 dilution lower for treated plates). No single spec-MIC criterion was applicable to all species. Olorofim MICs were low against 275 *Aspergillus* species isolates (modal MIC, 0.06 mg/liter; MIC range, < 0.004 to 0.25 mg/liter) and three dermatophytes (MICs 0.03 to 0.06 mg/liter). MICs against *Fusarium* were diverse, with full inhibition of *F. proliferatum* (MIC, 0.016), 50% growth inhibition of *Fusarium solani* at 1 to 2 mg/liter, and no inhibition of *F. dimerum*. Olorofim displayed potent *in vitro* activity against most mold isolates and was associated with limited variation in EUCAST susceptibility testing.

KEYWORDS olorofim, antifungal susceptibility, EUCAST, *Aspergillus*, AFST, F201318, *Fusarium*, amphotericin B, azoles, dermatophytes

Olorofim (formerly F901318) is a novel antifungal agent belonging to the new orotomide class of drugs which targets an important enzyme for pyrimidine biosynthesis, dihydroorotate dehydrogenase. It has *in vitro* activity against *Aspergillus* and other difficult-to-treat molds, including *Scedosporium*, *Lomentospora*, and *Fusarium* species, but no activity against *Candida*, *Cryptococcus*, or Mucorales species due to differences in the drug targets (1–3). For *Aspergillus* species specifically, *in vitro* activity has been demonstrated against azole-susceptible wild-type (WT) isolates as well as azole-resistant *cyp51A* mutant *Aspergillus fumigatus* isolates and also against other *Aspergillus* species, including *Aspergillus terreus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus flavus* complex, and the difficult-to-treat *Aspergillus calidoustus* and *Aspergillus tubingensis* species, using the standard EUCAST or CLSI reference methodologies and a limited number of isolates (1, 2). Interestingly, *in vivo* experimentation suggests potent efficacy against pulmonary aspergillosis in a mouse model involving WT *A. fumigatus* and azole-resistant TR_{34}/L98H *A. fumigatus*, as well as activity against *A. flavus* (2, 4), in a murine model of sinopulmonary aspergillosis. Olorofim has successfully

Received 13 March 2018 **Returned for modification** 9 April 2018 **Accepted** 11 May 2018

Accepted manuscript posted online 21 May 2018

Citation Jørgensen KM, Astvad KMT, Hare RK, Arendrup MC. 2018. EUCAST determination of olorofim (F901318) susceptibility of mold species, method validation, and MICs. Antimicrob Agents Chemother 62:e00487-18. https://doi.org/10.1128/AAC.00487-18.

Address correspondence to Maiken Cavling Arendrup, maca@ssi.dk.

progressed through 90-day toxicology studies in two species as well as phase I healthy volunteer trials. Initial patient studies are planned starting in 2018. Recently, olorofim has been granted orphan drug status for the treatment of invasive aspergillosis and rare mold infections caused by *Scedosporium* species by the European Medicines Agency (EMA) Committee for Orphan Medicinal Products.

Establishing a robust susceptibility testing procedure, associated endpoint reading method, and MICs for contemporary isolates are crucial parts of the clinical breakpoint setting process. CLSI and EUCAST have both provided methods for the licensed antifungal compounds (5, 6). Still, both organizations also recognize that despite standardized reference protocols, sources of variations still need to be thoroughly investigated and, if possible, controlled for and that not all compounds can be read with the same endpoint definition. For example, a morphological aberrant growth MEC (minimum effective concentration) endpoint is acknowledged for the echinocandins against *Aspergillus*, in contrast to a full visual inhibition MIC endpoint adopted for amphotericin B and azoles against *Aspergillus* and other molds. Moreover, some of the MICs obtained for some compounds have been found to vary with the type of microtiter plate used for microdilution, as exemplified by caspofungin and amphotericin B (7). This was recently also suggested for another investigational antifungal compound (CD101, rezafungin) and prohibited epidemiologic cutoff value (ECOFF) determination for the most susceptible *Candida* species (8). Finally, variations across different lot numbers have also been reported, particularly for caspofungin (7, 9).

Here, we investigated the influence of various technical issues related to EUCAST MIC determination for the novel compound olorofim, including inter-reader variability in visual endpoint determination, performance of spectrophotometric endpoint reading, endpoint variation across two lots of olorofim, and finally, the impact of various brands and types of polystyrene plates. Subsequently, the *in vitro* susceptibilities among prospectively collected contemporary clinical mold isolates to olorofim were investigated in comparison with that of the licensed antimold agents using the EUCAST E.Def 9.3.1 method.

RESULTS

Technical aspects. Olorofim susceptibility was first evaluated by comparing MICs generated using plates prepared by serial dilution (serial plates) and the ISO method (ISO plates) and by using visual (comparing two readers) and spectrophotometric endpoint reading. Median MICs and ranges were low and similar across the species and across *A. fumigatus* with or without Cyp51A alterations (Table 1). Species- and Cyp51A-specific median MICs for the two plate preparation methods were either identical (25/36 [69%]) or one dilution apart (11/36 [31%]). The closest agreement was seen for the visual reading for which 11/12 median MICs were identical using serial and ISO plates and the lowest for spectrophotometric reading using 50% endpoint for which 1/6 median MICs were identical using serial or ISO plates. If adopting MIC ranges spanning ≤3 dilutions as an indicator of low technical variability, this target was met for the visual endpoint method and for both plate preparations in all cases except for *A. niger* and *A. terreus*. Similarly, this target was met for the spectrophotometer reading method except (i) when 50% endpoint was adopted, (ii) for *A. niger* in general, and (iii) for the 95% endpoint reading of non-WT *A. fumigatus* (Table 1).

Interperson variability for visual endpoint determination was investigated by comparing individual endpoints across all species and Cyp51A types read by reader 1 with those of a second reader (reader 2, 3, or 4, with the second reader varied on different days). A 100% agreement was obtained within ±1 dilutions and ≤1 dilution apart in 92% to 94% of the cases independently if the serial dilution or the ISO method was adopted for plate production (see Table S1 in the supplemental material).

Spectrophotometric determinations of MIC endpoints (spec-MICs) using 95%, 90%, 85%, and 50% growth inhibition criteria are displayed in Table 1 and are the following compared to the visual MICs. Median spec-MICs using a 95% inhibition endpoint were ≤1 dilution apart from the visual endpoint on all occasions except for *A. niger* (using

TABLE 1 Comparison of olorofim MIC determined using plates prepared using serial dilution or ISO dilution and by adopting visual full inhibition or spectrophotometer endpoint criteria

	MIC (mg/liter)											
	Serial dilution plates						ISO plates					
	Full inhibition		Spectrophotometric inhibition (%)				Full inhibition		Spectrophotometric inhibition (%)			
Isolate (*n*)	Reader 1	Reader 2–4[a]	95	90	85	50	Reader 1	Reader 2–4[a]	95	90	85	50
A. flavus (10)												
Median	0.03	0.03	0.03	0.016	0.016	0.001	0.03	0.03	0.016	0.016	0.016	0.002
Range	0.03	0.03	0.016–0.03	0.016–0.03	0.008–0.03	0.0005–0.008	0.3–0.125	0.016–0.06	0.016–0.03	0.008–0.016	0.004–0.016	0.00024–0.008
No. 2-fold dilutions	1	1	2	2	3	5	3	3	2	2	3	6
A. fumigatus WT (5)												
Median	0.06	0.03	0.03	0.03	0.016	0.016	0.03	0.03	0.03	0.016	0.016	0.008
Range	0.06	0.016–0.06	0.03	0.016–0.03	0.016–0.03	0.004–0.016	0.03–0.06	0.016–0.06	0.016–0.03	0.016–0.03	0.008–0.016	0.001–0.008
No. 2-fold dilutions	1	3	1	2	2	3	2	3	2	2	2	4
A. fumigatus mutant (5)												
Median	0.06	0.03	0.03	0.03	0.03	0.004	0.06	0.03	0.03	0.03	0.03	0.004
Range	0.06–0.125	0.03–0.125	0.03–0.125	0.016–0.03	0.016–0.03	0.004–0.03	0.03–0.06	0.03–0.06	0.03 to >1	0.016–0.06	0.016–0.06	0.004–0.03
No. 2-fold dilutions	2	3	3	2	2	4	2	2	7	3	3	4
A. nidulans (10)												
Median	0.06	0.06	0.03	0.03	0.03	0.008	0.06	0.06	0.03	0.03	0.016	0.004
Range	0.03–0.125	0.03–0.125	0.03–0.06	0.016–0.03	0.016–0.03	0.004–0.016	0.03–0.125	0.3–0.125	0.016–0.06	0.016–0.03	0.016–0.03	0.002–0.016
No. 2-fold dilutions	3	3	2	2	2	3	3	3	3	2	2	4
A. niger (10)												
Median	0.06	0.06	0.5	0.06	0.03	0.016	0.06	0.06	0.5	0.06	0.03	0.008
Range	0.03–0.25	0.03–0.125	0.03 to >1	0.016 to >1	0.016–0.25	0.008–0.032	0.03–0.125	0.03–0.25	0.06 to >1	0.016 to >1	0.016–0.25	0.002–0.03
No. 2-fold dilutions	4	3	7	8	5	3	3	4	6	8	5	4
A. terreus (10)												
Median	0.03	0.016	0.016	0.016	0.008	0.004	0.03	0.016	0.008	0.008	0.008	0.002
Range	0.016–0.06	0.008–0.125	0.008–0.03	0.008–0.016	0.004–0.016	0.0005–0.008	0.008–0.06	0.008–0.03	0.004–0.016	0.004–0.008	0.004–0.008	0.00024–0.004
No. 2-fold dilutions	3	5	3	2	3	5	4	3	3	2	2	5

Species-specific MIC ranges broader than the optimal target (spanning ≤3 dilutions) are underlined.

[a]Reader 1 was constant throughout the study, whereas the second reader was any of 3 different individuals (Reader 2 to 4).

both serial dilution and ISO method prepared plates) and *A. terreus* (using the ISO method prepared plates). However, this strict endpoint resulted in a wide spec-MIC distribution for the *A. fumigatus* mutants (7 versus 2 dilutions for the visual MIC reading of ISO plates) and an artificially elevated median spec-MIC for *A. niger* (0.5 mg/liter versus 0.06 mg/liter) (Table 1). With a 90% inhibition endpoint, the overall median spec-MIC was one step below that for visual reading, but the spec-MIC ranges for all isolate groups, except *A. niger*, were acceptable (≤3 dilutions). For *A. niger*, the 85% inhibition endpoint was superior.

The impact of polystyrene plate type and variation between different lots of pure olorofim substance was next examined. Visual MICs were obtained using four different microtiter plates (treated and untreated Nunc and Greiner plates) prepared using serial as well as ISO methods, respectively, and with two different lots of olorofim pure substance. The MICs determined were overall similar across the different plate types (see Table S2). However, discrete differences of ≤1 dilution were noted, as the use of treated plates tended to lead to slightly lower median MICs, particularly when the plates were prepared using 2-fold serial dilutions. There were no differences in MICs obtained using two different batches of olorofim.

Olorofim EUCAST reference method MICs for contemporary mold isolates. In total, 298 mold isolates were referred for susceptibility testing March 2016 to October 2017 and thus tested for olorofim susceptibility. The isolates were derived from airway samples (239 [80%]), pleura (1), sinus (2), blood, brain, or cerebral spinal fluid (CSF) (1 each), ear (20 [7%]), eye (8 [3%]), skin, hair, or nail (5), other (11 [4%]), or unknown sites (9 [3%]). The referred isolates included 275 *Aspergillus* species, 10 *Fusarium* species, 10 Mucorales isolates, and 3 dermatophytes.

Olorofim displayed potent *in vitro* activity across the 275 isolates belonging to eight different *Aspergillus* spp., with an overall modal MIC of 0.06 mg/liter, geometric MIC (GM-MIC) of 0.037 mg/liter, and MIC range of <0.004 to 0.25 mg/liter (visual endpoint as per reference standard) (Table 2). For *A. fumigatus* specifically, the GM-MIC was 0.037 mg/liter and the wild-type upper limit (WT-UL) was 0.25 mg/liter unless the statistical method with the 99% endpoint was used (0.5 mg/liter) (Table 2). The olorofim *in vitro* activity against *A. fumigatus* was unaffected by concomitant azole resistance. Thus, olorofim susceptibility against the wild type was a modal MIC of 0.06 mg/liter, GM-MIC of 0.037 mg/liter, and MIC range of <0.004 to 0.25 mg/liter compared to a modal MIC of 0.06 mg/liter, GM-MIC of 0.042 mg/liter, and a range of 0.004 to 0.125 mg/liter against the non-wild type (Table 2). The MICs were similar for *A. flavus* and *A. niger* (modal MIC, 0.03 and 0.03 to 0.06 mg/liter, and GM-MICs, 0.050 and 0.052 mg/liter, respectively) and slightly lower for *A. terreus* (range, 0.008 to 0.03 mg/liter; GM-MIC, 0.022 mg/liter). A similar potent *in vitro* activity was observed against the three dermatophyte isolates (MICs, 0.03 to 0.06 mg/liter).

The *in vitro* activity for *Fusarium* spp. was more diverse and species specific. The most susceptible was *F. proliferatum* (olorofim MIC, 0.016 mg/liter) (Table 2). In contrast, the activity against the other *Fusarium* species was less potent on a mg/liter basis, with MICs of ≥1 mg/liter for all. Partial growth inhibition was observed, however, against *Fusarium solani* isolates. Repeated testing of *F. solani* and *F. dimerum* isolates using an extended concentration range and a 50% growth inhibition endpoint revealed activity against *F. solani* complex isolates (MIC_{50} = 1 mg/liter; range, 1 to 2 mg/liter) but no inhibition against *F. dimerum*. Finally, no inhibition was observed against Mucorales for which the drug target is known to be different.

When the *in vitro* activity was compared to that of the mold-active azoles and amphotericin B (Table 3), olorofim was the most active agent on a mg/liter basis against all the mold species except the Mucorales and the group of other mold species. If applying the WT-UL/ECOFF values for *A. fumigatus* as an indicator of susceptibility (assuming that isolates with *in vitro* susceptibility comparable to that of *A. fumigatus* are likely susceptible), the proportion of isolates in this prospective Danish collection with MICs above the *A. fumigatus* WT-UL/ECOFFs was overall 7.3% for F901318 compared to

TABLE 2 EUCAST E.Def 9.3.1 MICs of olorofim against contemporary prospectively collected clinical mold isolates 2016 to 2017

Isolate	No.	No. of isolates at MIC (mg/liter) of:[a]										MIC (mg/liter)[b]					
		<0.004	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	≥1	Range	Modal	GM	WT-UL visual	WT-UL 97.5%	WT-UL 99%
Aspergillus spp.																	
A. flavus	12	1			1	**8**	2					<0.004–0.06	0.03	0.050	0.125	0.06	0.06
A. fumigatus	235	4	11	12	33	48	**90**	32	5			<0.004–0.25	0.06	0.037	0.25	0.25	0.5
WT for azoles	213	4	9	12	30	45	**81**	27	5			<0.004–0.25	0.06	0.037	0.25	0.25	0.5
Non-WT for azoles	22		2		3	3	**9**	5				0.004–0.125	0.06	0.042	0.25	2	4
A. giganteus	1				1												
A. nidulans complex	1					1											
A. niger	17			1		**6**	**6**	3	1			0.008–0.25	0.03/0.06	0.052	0.25	0.25	0.25
A. persii	1	1															
A. terreus	5			2	1	2						0.008–0.03		0.022			
A. tubingensis	2				1		1					0.016–0.06					
A. turcosus (A section *fumigati*)	1				1												
Fusarium spp.																	
F. dimerum	1										1						
F. solani complex	8										8	>0.5	>0.5				
F. proliferatum	1						1										
Dermatophytes[c]	3					2	1										
Aspergillus, *Fusarium*, and dermatophytes	288	6	11	15	38	67	**101**	35	6	0	9	<0.004 to >0.5	0.06	0.038	0.25	0.25	0.5
Mucorales[d]	10										10						
In total	298	6	11	15	38	67	101	35	6	0	19	<0.004 to >0.5	0.06	0.038	0.25	0.25	0.5

[a]Modal MICs are highlighted in bold. MICs were determined visually as per E.Def 9.3.1 protocol.
[b]WT-UL, wild type upper limit.
[c]One each of *Microsporum canis*, *Trichophyton interdigitale*, and *Trichophyton rubrum*.
[d]One each of *Absidia corymbifera*, *Circinella muscae*, *Lichtheimia ramose*, and *Rhizomucor miehei*, two *Rhizomucor pusillus*, and four *Rhizopus microsporus* isolates.

TABLE 3 *In vitro* susceptibility of contemporary mold isolates to olorofim and mold active azoles presented as MIC ranges, modal MICs, and the proportion of isolates with MICs above the *A. fumigatus* WT-UL (olorofim) and EUCAST ECOFF (licensed comparators), respectively[a]

	Olorofim			AMB			Itraconazole			Posaconazole			Voriconazole		
Isolate (*n*)	MIC range	Modal MIC	% or proportion >0.25 mg/liter	MIC range	Modal MIC	% or proportion >1 mg/liter	MIC Range	Modal MIC	% or proportion >1 mg/liter	MIC Range	Modal MIC	% or proportion >0.25 mg/liter	MIC Range	Modal MIC	% or proportion >1 mg/liter
Aspergillus spp.	<0.004–0.25	NA[b]	0	0.125–4	NA	2	<0.125 to >16	NA	11	<0.06 to >4	NA	8	0.25–16	NA	7
A. flavus (12)	<0.004–0.06	0.03	0	0.5–4	1	25	0.125–0.5	0.125	0	0.06–0.25	0.125	0	0.5–2	1	8
A. fumigatus (235)	<0.004–0.25	0.06	0	0.125–1	0.5	0	<0.125 to >16	0.25	8	<0.06 to >4	0.125	7	0.25–16	0.5	5
A. niger (17)	0.008–0.25	0.03/0.06	0	0.125–0.5	0.25	0	0.5 to >16	1	41	0.125–0.5	0.25	25	0.5–4	1	50
A. terreus (5)	0.008–0.03	NA	0	1–4	1	40	<0.125–0.5	0.125	0	<0.06–0.25	0.06	0	0.5–1	1	0
Other *Aspergillus* sp. (6)	0.004–0.6	NA	0	0.125 to >4	NA	1/6	0.125 to >16	NA	3/6	0.06–0.5	NA	2/6	0.25–2	NA	2/6
Fusarium spp. (10)	0.06 to >8	NA	90	1 to >4	NA	50	4 to >16	NA	100	>4	NA	100	8 to >16	NA	100
Dermatophytes (3)	0.03–0.06	NA	0	0.25–1	NA	0	<0.125–1	NA	0	<0.06–0.5	NA	1/3	0.125–1	NA	0
Mucorales[c] (10)	>0.5	NA	100	0.016–1	NA	0	<0.125 to >16	NA	60	<0.06–4	NA	50	4 to >16	NA	100

[a]MICs were determined visually as per E.Def 9.3.1 protocol.

[b]NA, not applicable.

[c]One each of *Absidia corymbifera*, *Circinella muscae*, *Lichtheimia ramose*, and *Rhizomucor miehei*, two *Rhizomucor pusillus*, and four *Rhizopus microsporus* isolates.

4% for amphotericin B, and 13% to 15% for itraconazole, posaconazole, and voriconazole.

DISCUSSION

Technical aspects. Overall, the examined technical variables compared in this study had little impact on olorofim MICs. Interperson agreement was high for the visual MIC determinations, and MICs were comparable across the two methods for microtiter plate preparation, suggesting that the serial 2-fold dilution plates (where pipette tips were changed halfway through the dilution series) is equally appropriate for MIC determinations of olorofim against the tested *Aspergillus* isolates. Finally, spectrophotometric endpoint determination resulted in MIC endpoints comparable, although one dilution lower, to those obtained by visual reading if a 90% endpoint was adopted for *A. flavus*, *A. fumigatus*, *A. nidulans*, and *A. terreus*, whereas a less stringent 85% endpoint was required for *A. niger*. Altogether, these data suggest that the EUCAST E.Def 9.3.1 susceptibility testing is a robust method for MIC determinations of olorofim with both preparation types and reading methods.

Spectrophotometric endpoint reading was recently evaluated for *A. fumigatus* and mold-active azoles (10). In that study, the best correlation between visual and spec-MICs was found using a 95% growth inhibition and not 90% inhibition. Various factors may help explain the differences observed with the different endpoint methods. First, the spectrophotometric 95% endpoint is a very strict endpoint criterion. Not only is 5% growth very discrete, but the calculation of such an endpoint is normally done compared to a medium control, which does not take into account the contribution to the optical density (OD) provided by the inoculum itself. This is especially relevant for *A. niger*, which is colored, thus increasing the background OD further even before any growth has occurred. Second, a very strict endpoint will inevitably lead to notably elevated spec-MICs for compounds with static or "delayed-cidal" activity (the latter behavior is shown by F901318), because background levels will increase until growth arrests and thus, in such cases, may be elevated above a strict 95% endpoint calculated using medium as the background reference (11). We observed slight trailing growth and an occasional occurrence of tiny dots in the center surface of the wells in supra-MIC wells. Although this did not lead to significant variation in endpoint determination in this study, it may challenge spectrophotometer reading with a strict spectrophotometric endpoint and also accurate and reproducible visual endpoint determination between readers and laboratories, particularly when plates are read by people less familiar with antifungal susceptibility testing (AFST) in general and olorofim in particular.

Taken together, these data suggest 85% inhibition as an attractive endpoint, which could apply for hyaline as well as pigmented aspergilli. This would lead to a systematic but discrete MIC decrease of approximately 1 dilution step compared to those obtained by standard visual reading and in parallel serve to align EUCAST and CLSI olorofim MICs, as previous studies have shown that CLSI MICs may be lower than those obtained by the EUCAST method (using visual 100% growth inhibition) (12). However, a systematic MIC decrease might impact susceptibility classification unless separate EUCAST breakpoints for visual versus spectrophotometric reading were established.

A limitation associated with our evaluation of technical issues related to EUCAST testing of olorofim is that we have only evaluated the performance of visual and spectrophotometer endpoints for isolates with a WT phenotype for olorofim, because no resistant mutants have as yet been identified. Therefore, it is yet unknown how such isolates would behave in EUCAST AFST and if the individual endpoint criteria differentiate in their performance with respect to correctly identifying isolates with reduced clinical susceptibility. Moreover, a confirmation of our results in a multicenter study is warranted.

Olorofim *in vitro* activity against contemporary isolates. Olorofim displayed uniform and potent *in vitro* activity against all *A. fumigatus* isolates, including those with azole resistance. This aligns with recent mouse and rabbit model studies demonstrating *in vivo* efficacy of olorofim against wild-type *A. fumigatus* isolates as well as isolates

harboring TR_{34}/L98H or G138C Cyp51A alterations (13). The pharmacokinetic/pharmacodynamic (PK/PD) index that best linked drug exposure with observed effect in these studies was the ratio of the minimum total plasma concentration/MIC (C_{min}/MIC). On the basis of studies in murine and rabbit models of invasive pulmonary aspergillosis, an average olorofim C_{min} (mg/liter) and olorofim C_{min}/MIC of 0.1 to 0.3 and 3.3 to 9.1 were shown to have effects comparable to the effects of posaconazole and isavuconazole at efficacious human exposure levels (13).

A. fumigatus is the most prevalent mold causing invasive mold infections. This may suggest it is also the most pathogenic mold in human; thus, other molds against which olorofim MICs are equal to or lower than those against *A. fumigatus* are likely to be clinically susceptible as well. Such species include *A. niger*, which is less susceptible to azole compounds than *A. fumigatus*, and *A. terreus* and *A. flavus*, which are both less susceptible to amphotericin B. These findings are highly promising, with the limited armamentarium of antimold compounds in mind, as alternatives are few and currently no oral alternatives are available for isolates with decreased azole susceptibility (14).

Olorofim was active against the three dermatophyte isolates tested. In light of increasing terbinafine resistance in *Trichophyton rubrum* and *Trichophyton interdigitale*, further investigation of olorofim activity against dermatophytes is warranted (15–19). Although full growth inhibition was only obtained against the single *F. proliferatum* isolate among the *Fusarium* species included, partial growth inhibition was observed for *F. solani* in the 1- to 2-mg/liter concentration range. *Fusarium* spp. are, in general, much less susceptible than *A. fumigatus* to the antifungal compounds currently licensed, and infections are often difficult to treat. Voriconazole is the first-line therapy, but MICs above 16 mg/liter are not uncommon, and indeed, were also found in this study, where MICs were 8 to >16 mg/liter (20). From this perspective, partial inhibition seemed worthy of investigation as a basis for possible mono- or combination therapy scenarios. Thirteen isolates (4%) were *in vitro* resistant to olorofim and included 10 Mucorales isolates. This minority of isolates in our setting is thus unlikely an appropriate target for future therapy with olorofim.

In conclusion, this study suggests that *in vitro* testing is reproducible across various types of polystyrene microtiter plates, associated with low inter-reader variation in MIC determination using the traditional visual endpoint criteria and potentially also using spectrophotometric reading, although further likely requiring the adoption of an 85% endpoint criteria at least for *A. niger*. Moreover, the real-time testing in the routine setting suggested a broad activity, with more than 90% of recent mold isolates received by our reference laboratory being susceptible as wild-type *A. fumigatus*.

MATERIALS AND METHODS

Susceptibility testing. Stock solutions of antifungal compounds were prepared in dimethyl sulfoxide ([DMSO] 5,000 mg/liter; Sigma-Aldrich) and microtiter plates with 2-fold dilutions prepared in double-concentrated medium according to the EUCAST methodology (5). Cell culture-treated Nunc MicroWell 96-well microplates (cat. no. 167008; Thermo Fisher Scientific) were used throughout and frozen at −80°C prior to use, as is part of routine practice in most laboratories (see below regarding additional plates used for the plate comparison study).

Susceptibility testing methodological studies. (i) Plate preparation and endpoint reading. Plates were prepared using (i) a 2-fold serial dilution of olorofim (F2G; Manchester, UK) in microtiter plates in double-concentrated EUCAST medium supplemented with 1% DMSO (serial plates) (23) and (ii) the ISO method, performing predilution in DMSO and subsequently diluting 1:100 in double-concentrated growth medium for each drug dilution before transferring to the microtiter plates (ISO plates) (5, 6, 19). In total, 53 isolates were included: 50 clinical *Aspergillus* isolates, including 10 *A. fumigatus*, 5 of which harbored *cyp51A* alterations (one each of TR_{34}/L98H, TR_{46}/Y121F/T289A, G54W, M220I, and a double mutant [M220I+V101F]), 10 *A. flavus*, *10 A. nidulans*, 10 *A. niger*, and 10 *A. terreus*, and three EUCAST QC strains, including *A. fumigatus* ATCC 204305, *A. flavus* ATCC 204304, and *A. flavus* CNM-CM-1813. Serial and ISO plates were tested in parallel and read visually by two independent readers (the first reader was constant throughout the study, whereas the second reader was any of 3 different individuals) after 2 days of incubation (full inhibition endpoint) as well as spectrophotometrically using a single reading point per well followed by calculation of 95%, 90%, 85%, and 50% endpoints.

(ii) Impact of various polystyrene plates and comparison of two lots of olorofim pure substance. Four flat-bottomed polystyrene plate types produced by two different companies were compared: (i) Nunc MicroWell 96-well microplates, nontreated (cat. no. 243656; Thermo Fisher Scientific), (ii) Nunc MicroWell 96-well microplates, cell culture treated (cat. no. 167008; Thermo Fisher Scientific)

(SSI-standard plates), (iii) Greiner 96-well plates, nontreated (cat. no. 655161; Sigma-Aldrich), and (iv) Greiner Cellstar 96-well plates, tissue culture treated (cat. no. 655180; Sigma-Aldrich). Two lots of olorofim (lot 5 and lot 6) were tested against 34 *Aspergillus* isolates, including 5 *A. fumigatus*, 5 *A. flavus*, 5 *A. nidulans*, 6 *A. niger*, 5 *A. terreus*, and 5 *A. fumigatus* harboring *cyp51A* alterations (one each of TR_{34}/L98H, TR_{46}/Y121F/T289A, G54W, M220I, and a double mutant [M220I+V101F]), and three EUCAST mold control strains, including *Aspergillus fumigatus* ATCC 204305, *Aspergillus flavus* ATCC 204304, and *Aspergillus flavus* CNM-CM-1813.

(iii) Data management. For comparison of MICs obtained by different endpoints, plate preparation method, or drug lots, the percentage agreement between median MICs within 2-fold dilutions were calculated per species and Cyp51A genotype (WT or non-WT). The wideness of MIC ranges indicated as number of dilutions of MICs spanned for a given species and Cyp51A type were also compared using $\log_2$ transformation of individual MICs. MIC ranges spanning a maximum of 3 dilutions were regarded as a parameter for low technical variation.

Prospective evaluation of olorofim *in vitro* susceptibility of cont3emporary mold. (i) Isolates. MICs were determined prospectively in parallel with routine susceptibility testing following the E.Def 9.3.1 method (with visual endpoint reading as per protocol) for olorofim (final concentration range, 0.004 to 0.5 mg/liter), amphotericin B (0.016 to 4 mg/liter), itraconazole (0.125 to 16 mg/liter), posaconazole (0.06 to 4 mg/liter), and voriconazole (0.125 to 16 mg/liter). The exceptions were that azole EUCAST MICs were not determined for *A. fumigatus* isolates categorized as azole susceptible using the recently validated 4-well VIPcheck screening test (VIPcheck, Nijmegen, The Netherlands) (21). For *A. fumigatus*, olorofim activity was examined individually for azole wild-type and non-wild type organisms (non-wild type defined as itraconazole MIC of >1 mg/liter, posaconazole MIC of >0.25 mg/liter, voriconazole MIC of >1, or isavuconazole MIC of >2 mg/liter) (5, 22). The ECOFFfinder program (available upon request from the EUCAST secretary via http://www.eucast.org/organization/steering_committee/) was used for determining a statistical olorofim wild-type upper limit WT-UL for species represented with at ≥10 isolates. For *Fusarium* species isolates, the MIC_{50} was also determined using an extended concentration range (8 to 0.03 mg/liter), a spectrophotometer reading, and a 50% growth inhibition endpoint. Finally, the proportion of isolates with MICs above the WT-UL/ECOFF for *A. fumigatus* was calculated.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00487-18.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

We thank research technician Birgit Brandt for excellent technical assistance.

This study was supported by an unrestricted grant from F2G.

The funder had no influence on the study design nor on the analysis of the results. Outside this work, the authors have the following potential conflicts to declare. M.C.A. has received personal speaker's honoraria for the past 5 years from Astellas, Basilea, Gilead, MSD, Pfizer, T2*Candida*, and Novartis. She has received research grants and contract work paid to the Statens Serum Institute from Astellas, Basilea, Gilead, MSD, NovaBiotics, Pfizer, T2Biosystems, F2G, Cidara, and Amplyx. K.M.J. has received meeting travel grants from Amplyx, MSD, and F2G. R.K.H. has received unrestricted research grant from Gilead and conference travel grants from Gilead, Pfizer, MSD, and Astellas. K.M.T.A. has received conference travel grant from Pfizer and Gilead.

REFERENCES

1. Buil JB, Rijs AJMM, Meis JF, Birch M, Law D, Melchers WJG, Verweij PE. 2017. *In vitro* activity of the novel antifungal compound F901318 against difficult-to-treat *Aspergillus* isolates. J Antimicrob Chemother 72:2548–2552. https://doi.org/10.1093/jac/dkx177.
2. Oliver JD, Sibley GEM, Beckmann N, Dobb KS, Slater MJ, McEntee L, du Pré S, Livermore J, Bromley MJ, Wiederhold NP, Hope WW, Kennedy AJ, Law D, Birch M. 2016. F901318 represents a novel class of antifungal drug that inhibits dihydroorotate dehydrogenase. Proc Natl Acad Sci U S A 113:12809–12814. https://doi.org/10.1073/pnas.1608304113.
3. Wiederhold NP, Law D, Birch M. 2017. Dihydroorotate dehydrogenase inhibitor F901318 has potent *in vitro* activity against *Scedosporium* species and *Lomentospora prolificans*. J Antimicrob Chemother 72:1977–1980. https://doi.org/10.1093/jac/dkx065.
4. Negri CE, Johnson A, McEntee L, Box H, Whalley S, Schwartz JA, Ramos-Martín V, Livermore J, Kolamunnage-Dona R, Colombo AL, Hope WW. 2017. Pharmacodynamics of the novel antifungal agent F901318 for acute sinopulmonary aspergillosis caused by *Aspergillus flavus*. J Infect Dis 217:1118–1127. https://doi.org/10.1093/infdis/jix479.
5. Arendrup MC, Meletiadis J, Mouton JW, Guinea J, Cuenca-Estrella M, Lagrou K, Howard SJ. 2016. EUCAST technical note on isavuconazole breakpoints for Aspergillus, itraconazole breakpoints for Candida and updates for the antifungal susceptibility testing method documents. Clin Microbiol Infect 22:571.e1–571.e4. https://doi.org/10.1016/j.cmi.2016.01.017.
6. Clinical and Laboratory Standards Institute. 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard—2nd ed. CLSI document M38-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
7. Fothergill AW, McCarthy DI, Albataineh MT, Sanders C, McElmeel M, Wiederhold NP. 2016. Effects of treated versus untreated polystyrene on caspofungin *in vitro* activity against *Candida* species. J Clin Microbiol 54:734–738. https://doi.org/10.1128/JCM.02659-15.
8. Arendrup MC, Meletiadis J, Zaragoza O, Jørgensen KM, Marcos-

Zambrano LJ, Kanioura L, Cuenca-Estrella M, Mouton JW, Guinea J. 2 March 2018. Multicentre determination of rezafungin (CD101) susceptibility of *Candida* species by the EUCAST method. Clin Microbiol Infect https://doi.org/10.1016/j.cmi.2018.02.021.

9. Arendrup MC, Rodriguez-Tudela J-L, Park S, Garcia-Effron G, Delmas G, Cuenca-Estrella M, Gomez-Lopez A, Perlin DS. 2011. Echinocandin susceptibility testing of *Candida* spp. Using EUCAST EDef 7.1 and CLSI M27-A3 standard procedures: analysis of the influence of bovine serum albumin supplementation, storage time, and drug lots. Antimicrob Agents Chemother 55:1580–1587. https://doi.org/10.1128/AAC.01364-10.
10. Meletiadis J, Mortensen KL, Verweij PE, Mouton JWW, Arendrup MCC, Leth Mortensen K, Verweij PE, Mouton JWW, Arendrup MCC. 2016. Spectrophotometric reading of EUCAST antifungal susceptibility testing of *Aspergillus fumigatus*. Clin Microbiol Infect 23:98–103. https://doi.org/10.1016/j.cmi.2016.10.017.
11. du Pre S, Beckman N, Sibley GEM, Law D, Bromley M, Read N, Birch M, Oliver J. 2016. Impact of the novel orotomide antifungal F901318 on viability of *Aspergillus fumigatus*, p 14. Abstr 7th Advances Against Aspergillosis, Manchester, UK.
12. Binder U, Lackner M, Grässle D, Naschberger V, Beckmann N, Warn P, Gould J, Law D, Birch M, Lass-Flörl C. 2018. The novel antifungal compound F901318 has potent *in vitro* and *in vivo* activity against *Aspergillus terreus*, including cryptic species, p 14. Abstr 8th Advances against Aspergillosis, Lissbon, Portugal.
13. Hope WW, McEntee L, Livermore J, Whalley S, Johnson A, Farrington N, Kolamunnage-Dona R, Schwartz J, Kennedy A, Law D, Birch M, Rex JH. 2017. Pharmacodynamics of the orotomides against *Aspergillus fumigatus*: new opportunities for treatment of multidrug-resistant fungal disease. mBio 8:e0115-17. https://doi.org/10.1128/mBio.01157-17.
14. Verweij PE, Ananda-Rajah M, Andes D, Arendrup MC, Bruggemann RJ, Chowdhary A, Cornely OA, Denning DW, Groll AH, Izumikawa K, Kullberg BJ, Lagrou K, Maertens J, Meis JF, Newton P, Page I, Seyedmousavi S, Sheppard DC, Viscoli C, Warris A, Donnelly JP. 2015. International expert opinion on the management of infection caused by azole-resistant *Aspergillus fumigatus*. Drug Resist Updat 21–22:30–40. https://doi.org/10.1016/j.drup.2015.08.001.
15. Digby S, Hald M, Arendrup M, Hjort S, Kofoed K. 2017. Darier disease complicated by terbinafine-resistant *Trichophyton rubrum*: a case report. Acta Derm Venereol 97:139–140. https://doi.org/10.2340/00015555-2455.
16. Yamada T, Maeda M, Alshahni MM, Tanaka R, Yaguchi T, Bontems O, Salamin K, Fratti M, Monod M. 2017. Terbinafine resistance of *Trichophyton* clinical isolates caused by specific point mutations in the squalene epoxidase gene. Antimicrob Agents Chemother 61:e00115-17. https://doi.org/10.1128/AAC.00115-17.
17. Osborne CS, Leitner I, Favre B, Ryder NS. 2005. Amino acid substitution in *Trichophyton rubrum* squalene epoxidase associated with resistance to terbinafine. Antimicrob Agents Chemother 49:2840–2844. https://doi.org/10.1128/AAC.49.7.2840-2844.2005.
18. Singh A, Masih A, Khurana A, Singh PK, Gupta M, Hagen F, Meis JF, Chowdhary A. 25 March 2018. High terbinafine resistance in *Trichophyton* interdigitale isolates in Delhi, India harbouring mutations in the squalene epoxidase (SQLE) gene. Mycoses https://doi.org/10.1111/myc.12772.
19. Rudramurthy SM, Shankarnarayan SA, Dogra S, Shaw D, Mushtaq K, Paul RA, Narang T, Chakrabarti A. 2018. Mutation in the squalene epoxidase gene of *Trichophyton interdigitale* and *Trichophyton rubrum* associated with allylamine resistance. Antimicrob Agents Chemother 62:e02522-17. https://doi.org/10.1128/AAC.02522-17.
20. Tortorano AM, Richardson M, Roilides E, van Diepeningen A, Caira M, Munoz P, Johnson E, Meletiadis J, Pana Z-D, Lackner M, Verweij P, Freiberger T, Cornely OA, Arikan-Akdagli S, Dannaoui E, Groll AH, Lagrou K, Chakrabarti A, Lanternier F, Pagano L, Skiada A, Akova M, Arendrup MC, Boekhout T, Chowdhary A, Cuenca-Estrella M, Guinea J, Guarro J, de Hoog S, Hope W, Kathuria S, Lortholary O, Meis JF, Ullmann AJ, Petrikkos G, Lass-Flörl C. 2014. ESCMID and ECMM joint guidelines on diagnosis and management of hyalohyphomycosis: *Fusarium* spp., *Scedosporium* spp. and others. Clin Microbiol Infect 20(Suppl 3):S27–S46. https://doi.org/10.1111/1469-0691.12465.
21. Arendrup MC, Verweij PE, Mouton JW, Lagrou K, Meletiadis J. 2017. Multicentre validation of 4-well azole agar plates as a screening method for detection of clinically relevant azole-resistant *Aspergillus fumigatus*. J Antimicrob Chemother 72:3325–3333. https://doi.org/10.1093/jac/dkx319.
22. Arendrup MC, Cuenca-Estrella M, Lass-Flörl C, Hope WW. 2013. Breakpoints for antifungal agents: an update from EUCAST focusing on echinocandins against *Candida* spp. and triazoles against *Aspergillus* spp. Drug Resist Updat 16:81–95. https://doi.org/10.1016/j.drup.2014.01.001.
23. Gomez-Lopez A, Arendrup MC, Lass-Floerl C, Rodriguez-Tudela J-L, Cuenca-Estrella M. 2010. Multicenter comparison of the ISO standard 20776-1 and the serial 2-fold dilution procedures to dilute hydrophilic and hydrophobic antifungal agents for susceptibility testing. J Clin Microbiol 48:1918–1920. https://doi.org/10.1128/JCM.00123-10.