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SUSCEPTIBILITY

EUCAST-Obtained Olorofim MICs against *Aspergillus* and *Scedosporium* Species and *Lomentospora prolificans* Showed High Agreements between Visual Inspection and Spectrophotometric Readings

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ABSTRACT Previous studies show high agreement between MIC spectrophotometric readings and visual inspection of azoles and amphotericin B against *Aspergillus fumigatus* isolates. Here, we tested and compared the *in vitro* activity of a novel antifungal, olorofim, against *Aspergillus* spp., *Scedosporium* spp., and *Lomentospora prolificans* by visual inspection and spectrophotometric readings. Clinical isolates of *Aspergillus* (n = 686) and *Scedosporium* (n = 36) spp. and *L. prolificans* (n = 13) were tested. Olorofim MICs were evaluated—following the EUCAST E.Def 9.4 procedure—by visual inspection or spectrophotometric readings (combinations of either ≥90% or ≥95% fungal growth inhibition endpoints compared to drug-free control endpoints and different wavelengths [405 nm, 450 nm, 492 nm, 540 nm, and 620 nm]). We observed high *in vitro* activity of olorofim against all tested *Aspergillus* spp. (MICs up to 0.06 mg/L), except for *A. calidoustus*, and against *L. prolificans* and *Scedosporium* spp. (MICs up to 0.125 mg/L). The combination of ≥90% fungal growth inhibition endpoints at wavelengths of ≥492 nm resulted in high essential agreements with *A. fumigatus* and lesser agreement with non-*fumigatus Aspergillus*, *Scedosporium* spp., and *L. prolificans*, although the number of isolates studied was low. This single-center study shows high agreement among olorofim MICs against *A. fumigatus* by visual inspection and spectrophotometric readings (≥90% fungal growth inhibition endpoints and wavelengths of ≥492 nm) and encouraging results against non-*fumigatus Aspergillus* spp., *Scedosporium* spp., and *L. prolificans*.

KEYWORDS olorofim, *Aspergillus*, *Lomentospora prolificans*, *Scedosporium*, EUCAST, spectrophotometric reading

Recent reports have raised concern about the increasing rate of azole resistance in *Aspergillus fumigatus* isolates worldwide (1–3). Intrinsic resistance to amphotericin B is a trait of *Aspergillus* species such as *A. terreus*, *A. flavus*, *A. nidulans*, and *A. fumigatus* cryptic species (4–6). *Scedosporium* spp. and *Lomentospora prolificans*—rarely the cause of invasive fungal infections—frequently show resistance to multiple antifungal agents (7). The limited number of available antifungal agents greatly hampers the treatment of invasive fungal infections. The search for new antifungal agents is key in light of the emergence of multidrug-resistant fungal isolates.

Olorofim is the leading novel antifungal of the orotomide class of drugs. It inhibits the biosynthesis of pyrimidine by inhibiting dihydroorotate dehydrogenase. Because olorofim works by a new mechanism, it eliminates the presence of antifungal cross-resistance as shown by the full activity of olorofim against azole-resistant *A. fumigatus* isolates (8–11).

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Olorofim showed *in vitro* antibiofilm activity against early-stage *A. fumigatus* and *L. prolificans* biofilms (12, 13). Olorofim has shown *in vitro* antifungal activity against clinical mold isolates, including *Aspergillus* spp., *Scedosporium* spp., and *L. prolificans* following EUCAST procedures and MIC determination by visual inspection (14–17). However, visual inspection of MICs against mold isolates may be subjective, and spectrophotometric readings may help increase objectivity. Previous studies show high agreement between MIC spectrophotometric readings and visual inspection of azole and amphotericin B against *A. fumigatus* clinical isolates (18–21). These high agreements motivated an update of the EUCAST E.Def 9.4 document, which now includes MIC spectrophotometric readings of azoles and amphotericin B against *A. fumigatus* as an alternative to visual inspection (22).

To date there is only one study addressing olorofim MIC spectrophotometric readings against molds as an alternative to visual inspection (23). Thus, we tested the *in vitro* activity of olorofim against a collection of different clinical *Aspergillus* spp., *Scedosporium* spp., and *L. prolificans* isolates comparing visual inspection of the plates and spectrophotometric readings and using different endpoints and wavelengths.

RESULTS AND DISCUSSION

Olorofim MICs obtained by visual inspection against *Aspergillus* spp., *Scedosporium* spp., and *L. prolificans*. MIC distributions against the species tested, with some exceptions, fit a Gaussian pattern (Table 1). Olorofim showed *in vitro* activity against all *Aspergillus* sp. isolates tested; all MICs reached 0.06 mg/L, except against *A. calidoustus* (highest MIC, 0.5 mg/L). Modal olorofim MICs were 0.008 mg/L (against *A. terreus*), 0.016 mg/L (*A. fumigatus*, *A. flavus*, and other *Aspergillus* spp.), 0.03 mg/L (*A. niger*), and 0.5 mg/L (*A. calidoustus*). Likewise, olorofim showed *in vitro* activity against the *Scedosporium* sp. and *L. prolificans* isolates tested; all MICs reached 0.125 mg/L. Modal olorofim MICs were 0.016 mg/L (the *S. apiospermum* complex) and 0.125 mg/L (*L. prolificans*). The obtained wild-type upper limit (wtUL) values (when possible) were 0.016 mg/L (*A. terreus*), 0.03 mg/L (*A. fumigatus*), 0.06 mg/L (*A. niger*), and 0.06 mg/L (*S. apiospermum* complex) (Table 1 and Table S1 in the supplemental material). Table 1 and Table S1 show olorofim MICs against quality control strains by visual inspection and spectrophotometric readings. Our study is in line with previous reports that demonstrate *in vitro* olorofim activity against *Aspergillus* spp. using EUCAST procedures (9, 24–27). *A. fumigatus* and *A. terreus* isolates were especially susceptible to the drug, whereas olorofim MICs against *A. calidoustus* were notably higher than those of the remaining *Aspergillus* spp., confirming previous observations (9, 24, 25). Further studies should be developed to assess the role of olorofim for treating patients infected by *A. calidoustus*.

Scedosporium spp. and *L. prolificans* cause difficult-to-treat infections due to the intrinsic low activity of azoles and amphotericin B against these fungi (17). Olorofim has shown *in vitro*

TABLE 1 MIC distributions by visual observation for olorofim against the isolates tested

	MIC distributions (no. of isolates at each MIC, mg/L)[a]														
Species (*n*)	**0.001**	**0.002**	**0.004**	**0.008**	**0.016**	**0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥ 16**
A. fumigatus sensu lato (566)[b]	0	2	6	86	**433**	37	2	0	–	–	–	–	–	–	–
A. flavus sensu lato (44)[b]	0	0	1	18	**25**	0	0	0	–	–	–	–	–	–	–
A. terreus sensu lato (33)[b]	0	0	4	**24**	5	0	0	0	–	–	–	–	–	–	–
A. niger sensu lato (23)[b]	0	0	0	0	9	**12**	2	0	–	–	–	–	–	–	–
A. calidoustus (7)	0	0	0	0	0	0	0	0	1	**6**	0	0	0	0	–
Aspergillus spp. (13)[b]	1	1	0	1	**8**	2	0	0	–	–	–	–	–	–	–
Scedosporium spp. (36)[c]	0	0	0	4	**12**	**11**	5	4	0	0	–	–	–	–	–
L. prolificans (13)	0	0	0	0	0	0	3	**10**	0	0	–	–	–	–	–
A. flavus ATCC 204304	0	0	0	0	**22**	2	0	0	–	–	–	–	–	–	–
A. fumigatus ATCC 204305	0	0	0	2	**19**	3	0	0	–	–	–	–	–	–	–

[a]Modal MIC values are shown in bold. Dashes indicate nontested concentrations.
[b]*A. fumigatus sensu lato* (*A. fumigatus sensu lato* [*n* = 529], *A. lentulus* [*n* = 20], *Neosartorya udagawae* [*n* = 7], *A. fumigatiaffinis* [*n* = 4], *A. novofumigatus* [*n* = 2], *N. tsurutae* [*n* = 2], *A. felis* [*n* = 1], *A. thermomutatus* [*n* = 1]); *A. flavus sensu lato* (*A. flavus* [*n* = 42], *A. alliaceus* [*n* = 1], *A. tamarii* [*n* = 1]); *A. terreus sensu lato* (*A. terreus sensu stricto* [*n* = 23], *A. citrinoterreus* [*n* = 9], *A. hortai* [*n* = 1]); *A. niger sensu lato* (*A. tubingensis* [*n* = 10], *A. awamori* [*n* = 8], *A. niger sensu stricto* [*n* = 5]); other *Aspergillus* spp. (*Emericella nidulans* [*n* = 9], *A. sydowii* [*n* = 3], and *A. amoenus* [*n* = 1]).
[c]*S. apiospermum* complex (*S. apiospermum sensu stricto* [*n* = 29], *S. boydii* [*n* = 4], *S. ellipsoideum* [*n* = 1]), *S. auriantiacum* (*n* = 1), and *S. minutisporum* (*n* = 1).

TABLE 2 MIC distributions by visual observation and spectrophotometric readings against *A. fumigatus sensu lato* isolates[a,b]

Endpoints (%) (wavelength [nm]) used to obtain the MIC	MICs (no. of isolates at each MIC, mg/L)									Essential agreement (%)	Statistical wtUL at each percentage of modelled population				
	0.001	0.002	0.004	0.008	0.016	0.03	0.06	0.125	≥0.25		95%	97.5%	99%	99.5%	99.9%
Visual reading	0	2	6	86	**433**	37	2	0	0	**ND**	0.03	0.03	0.03	0.03	0.03
90 (405)	0	1	9	98	**374**	78	4	1	1	98.41	0.03	0.03	0.03	0.03	0.06
95 (405)	0	1	2	13	149	**163**	83	103	52	55.48	0.125	0.25	0.25	0.25	0.5
90 (450)	0	2	9	105	**346**	89	9	1	5	96.64	0.03	0.03	0.03	0.03	0.06
95 (450)	0	1	16	133	**149**	84	113	69	1	49.47	0.125	0.25	0.25	0.5	0.5
90 (492)	0	2	15	191	**332**	25	1	0	0	99.47	0.03	0.03	0.03	0.03	0.03
95 (492)	0	2	5	39	**322**	177	14	2	5	95.58	0.03	0.03	0.06	0.06	0.06
90 (540)	0	2	19	234	**296**	15	0	0	0	99.82	0.016	0.03	0.03	0.03	0.03
95 (540)	0	2	7	61	**397**	92	6	1	0	97.53	0.03	0.03	0.03	0.03	0.03
90 (620)	0	2	17	244	**287**	16	0	0	0	99.82	0.016	0.03	0.03	0.03	0.03
95 (620)	0	2	7	57	**404**	92	4	0	0	98.23	0.03	0.03	0.03	0.03	0.03

[a]Readings used two endpoints (≥90% and ≥95% inhibition of fungal growth versus a drug-free control) at different wavelengths (405 nm, 450 nm, 492 nm, 540 nm, and 620 nm) against *A. fumigatus sensu lato*. Essential agreements among visually obtained MICs and spectrophotometric MICs and wild-type upper limits were calculated.
[b]Underlined values indicate calculated non-wild-type isolates according to the statistical wtUL (99% of modeled population). Bold values indicate modal MIC. ND, not done. Cells in gray indicate the combination of fungal growth inhibition endpoint and wavelength values leading to an essential agreement value of >99% and wtULs similar to the one obtained by visual readings (using the 99% of modeled population).

activity against *Scedosporium* spp. and *L. prolificans* using the EUCAST procedure (17, 24). Moreover, a recent study proved the efficacy of olorofim to treat mice infected by *Scedosporium* spp. and *L. prolificans* (28). Our results confirm the remarkable *in vitro* activity of olorofim (higher against *Scedosporium* spp. than against *L. prolificans*) and its potential to become an effective antifungal agent for treating patients with *Scedosporium*/*Lomentospora* invasive infections.

Comparisons of MICs obtained by visual inspection and spectrophotometric readings. Tables 2 and 3 and Table S1 summarize the MIC distributions of olorofim against *Aspergillus* and *Scedosporium* spp. and *L. prolificans* obtained by visual inspection and by spectrophotometric readings using different combinations of fungal growth inhibition endpoints and wavelengths. MIC distributions against *A. fumigatus sensu lato* are shown in Table 2. Overall, essential agreements between MICs by visual inspection and spectrophotometric readings were higher using ≥90% fungal inhibition endpoints; such agreement values increased to >99% when wavelengths were ≥492 nm. The combination of ≥90% fungal growth inhibition endpoints and wavelengths of ≥492 nm resulted in MIC distributions generated by either spectrophotometric readings or visual inspections with identical wtUL values (0.03 mg/L).

We sequenced the *pyrE* gene in *A. fumigatus sensu stricto* isolates showing an olorofim non-wild-type phenotype (MICs higher than the wtUL value derived from visual inspection MIC distributions and spectrophotometric readings using either ≥90% or ≥95% fungal growth inhibition endpoints and wavelengths of ≥492 nm). MIC distributions generated from visual inspection led to two non-wild-type isolates. As to wtUL values from MIC distributions obtained by spectrophotometric readings, only one isolate was classified as non-wild type (≥90% fungal growth inhibition endpoint and a wavelength of 492 nm; MIC, 0.06 mg/L). In contrast, ≥95% fungal growth inhibition endpoints led to several non-wild-type isolates (wavelengths of 492 nm [*n* = 7], 540 nm [*n* = 7], and 620 nm [*n* = 4]). The examined *pyrE* gene sequences did not have the G119 substitution previously linked to resistance to olorofim, thus supporting the use of ≥90% fungal growth inhibition endpoints, which resulted in the lowest number of isolates with a false non-wild-type phenotype.

Table S1 shows the MIC distributions obtained against non-*fumigatus Aspergillus* species. To harmonize the methodology against all *Aspergillus* spp., combinations of fungal inhibition endpoints of ≥90% and wavelengths of ≥492 nm were assessed. It led to 100% agreement values between MIC visual and spectrophotometric readings, although spectrophotometric versus visually obtained wtUL values did not match values for *A. terreus* (0.008 mg/L versus 0.016 mg/L) and *A. niger* when MICs were read at 540 nm (0.06 mg/L versus 0.125 mg/L) (Table S1). Unfortunately, wtUL values from MIC distributions by visual inspection could not be set against *A. flavus* because of a very narrow MIC range; if the wtUL value had been

TABLE 3 MIC distributions by visual observation and spectrophotometric readings[a,b]

Species	Endpoints (%) (wavelength [nm]) used to obtain the MIC	MICs (no. of isolates at each MIC, mg/L)											Essential agreement (%)	Statistical wtUL at each percentage of modelled population				
		0.001	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	≥1		95%	97.5%	99%	99.5%	99.9%
S. apiospermum complex (*n* = 34)	Visual	0	0	0	4	**12**	**11**	3	4	0	0	0	ND	0.06	0.06	0.06	0.125	0.125
	90 (405)	0	0	0	5	**14**	10	1	3	1	0	0	72.22	0.03	0.06	0.06	0.06	0.125
	95 (405)	0	0	0	1	3	7	5	**10**	7	1	0	50.00	0.5	1	1	2	4
	90 (450)	0	0	0	4	8	**14**	3	4	1	0	0	69.44	0.06	0.06	0.125	0.125	0.125
	95 (450)	0	0	0	0	2	5	3	9	**14**	1	0	33.33	1	1	2	2	4
	90 (492)	0	0	2	7	**16**	8	1	0	0	0	0	75.00	0.03	0.06	0.06	0.06	0.125
	95 (492)	0	0	0	2	7	**15**	7	2	0	1	0	83.33	0.06	0.125	0.125	0.125	0.25
	90 (540)	0	0	3	8	**14**	8	1	0	0	0	0	72.22	0.06	0.06	0.06	0.06	0.125
	95 (540)	0	0	0	4	9	**13**	6	1	1	0	0	88.89	0.06	0.125	0.125	0.125	0.25
	90 (620)	0	0	2	7	**16**	8	0	1	0	0	0	75.00	0.03	0.06	0.06	0.06	0.06
	95 (620)	0	0	0	4	11	**13**	4	1	1	0	0	86.11	0.06	0.06	0.125	0.125	0.125
L. prolificans (*n* = 13)	Visual	0	0	0	0	0	0	3	**10**	0	0	0	ND	ND	ND	ND	ND	ND
	90 (405)	0	0	0	0	1	**6**	**5**	1	0	0	0	69.23	ND	ND	ND	ND	ND
	95 (405)	0	0	0	0	0	1	2	**9**	1	0	0	92.31	ND	ND	ND	ND	ND
	90 (450)	0	0	0	0	1	**6**	**5**	1	0	0	0	69.23	ND	ND	ND	ND	ND
	95 (450)	0	0	0	0	0	1	2	**7**	3	0	0	92.31	ND	ND	ND	ND	ND
	90 (492)	0	0	0	0	1	**10**	2	0	0	0	0	38.46	ND	ND	ND	ND	ND
	95 (492)	0	0	0	0	0	1	**8**	4	0	0	0	92.31	ND	ND	ND	ND	ND
	90 (540)	0	0	0	0	1	**10**	2	0	0	0	0	38.46	ND	ND	ND	ND	ND
	95 (540)	0	0	0	0	0	2	**9**	2	0	0	0	84.62	ND	ND	ND	ND	ND
	90 (620)	0	0	0	0	1	**9**	3	0	0	0	0	46.15	ND	ND	ND	ND	ND
	95 (620)	0	0	0	0	0	3	**8**	2	0	0	0	76.92	ND	ND	ND	ND	ND

[a]Readings used two endpoints (≥90% and ≥95% inhibition of fungal growth versus a drug-free control) at different wavelengths (405 nm, 450 nm, 492 nm, 540 nm, and 620 nm) against *S. apiospermum* complex and *L. prolificans*. Essential agreements among visually obtained MICs, spectrophotometric MICs, and wild-type upper limits were calculated.

[b]Underlined values indicate calculated non-wild-type isolates according to the statistical wtUL (99% of modeled population). Bold values indicate modal MIC. ND, not done. Cells in gray indicate the combination of fungal growth inhibition endpoint with wavelength values leading to an essential agreement value of >70% and wtULs similar to the one obtained by visual readings (using the 99% of modeled population).

0.016 mg/L, it would have matched the wtUL values generated by spectrophotometric readings.

Previous studies have proved spectrophotometric readings to be an alternative to visual inspection for determining azole and amphotericin B MICs against *A. fumigatus sensu stricto* (18–21), and currently, it is included in the updated EUCAST E.Def 9.4 procedure in which fungal inhibition growth endpoints of ≥90% are recommended (22). Our observations extend olorofim spectrophotometric MIC readings against *A. fumigatus*—including cryptic species—using the same fungal growth inhibition endpoint (≥90%) if the wavelengths used are ≥492 nm. However, for non-*Aspergillus* spp., following the criteria of getting high essential agreements and identical wtUL values between MIC distributions generated by either visual inspection or spectrophotometric readings using ≥90% growth inhibition endpoints and wavelengths of ≥492 nm are not as clear as for *A. fumigatus*.

We also compared olorofim MICs by visual inspection and spectrophotometric readings against *S. apiospermum* complex (Table 3). The highest agreements were found when readings were done using wavelengths of ≥492 nm. Although the highest agreements with visual inspection were found with ≥95% fungal growth inhibition endpoints, the ≥90% endpoint led to wtUL values of MIC distributions matching those of visual inspection and to a lesser extent in non-wild-type isolates. The ≥95% fungal growth inhibition endpoint seemed to be more accurate against *L. prolificans*, although it was not possible to calculate wtUL values due to the small number of isolates.

Given that the E.Def 9.4 document recommends the ≥90% fungal growth inhibition endpoint when using spectrophotometric MIC readings of azoles and amphotericin B against *A. fumigatus*, the aim of the current study was to harmonize such endpoints when conducting olorofim antifungal susceptibility testing. This endpoint worked fine for *A. fumigatus sensu lato* isolates. Taking into account the small number of non-*fumigatus Aspergillus* sp. and *Scedosporium* sp./*L. prolificans* isolates, further studies with a larger number of isolates should be developed to determine which fungal growth inhibition endpoint is best for the species in question. Few works have been published to date on the activity of olorofim against non-*fumigatus* isolates and *Scedosporium* spp./*L. prolificans* and are almost nonexistent when it comes to MIC spectrophotometric readings.

A limitation to this study is the small number of isolates of non-*fumigatus Aspergillus* spp. and *Scedosporium* spp./*L. prolificans*. Moreover, this is a single-center study, and broadening our observations to multiple centers may support and validate the findings of this work.

In conclusion, our study proves *in vitro* activity of olorofim against clinical mold isolates, including different *Aspergillus* spp. and the highly antifungal-resistant *Scedosporium* spp. and *L. prolificans*. This work also shows a high agreement among MIC values of olorofim against *A. fumigatus* by visual inspection or spectrophotometric readings (≥90% fungal growth inhibition endpoint and wavelengths of ≥492 nm) and presents encouraging results against non-*fumigatus Aspergillus* spp., *Scedosporium* spp., and *L. prolificans*.

MATERIALS AND METHODS

Isolates studied and molecular identification. A total of 735 clinical isolates—one isolate per patient and species—collected from the lower respiratory tract (91%) and belonging to *Aspergillus* (n = 686) or *Scedosporium* (n = 36) spp. and *L. prolificans* (n = 13) were tested. *Scedosporium* spp., *L. prolificans*, non-*fumigatus Aspergillus*, and some *A. fumigatus sensu lato* isolates (n = 402) were from patients admitted to Gregorio Marañón hospital (Madrid, Spain) between 1999 and 2021; the remaining *A. fumigatus sensu lato* isolates (n = 284) were collected as part of an multicenter azole resistance surveillance study conducted in Spain in 2019 (1).

Isolates were identified by amplifying and sequencing the β-tubulin gene using btub-2a and btub-2b primers; calmodulin was used to identify *A. niger* isolates (Table 1) (29).

Olorofim antifungal susceptibility testing. Antifungal susceptibility to olorofim (F2G, Inc., Manchester, UK) was studied using the EUCAST E.Def 9.4 procedure; MICs were obtained after plates were incubated for 48 h (tissue-treated trays, CELLSTAR reference [ref.] 655 180; Greiner Bio-One, Frickenhausen, Germany) at 35°C without shaking (22). Tested olorofim concentrations ranged from 0.001 to 0.125 mg/L; higher concentrations were used against *Scedosporium* spp./*L. polificans* (0.5 mg/L) and *A. ustus* (8 mg/L). MICs were obtained by visual inspection (defined as the concentration that completely inhibits visible fungal growth) or by spectrophotometric readings (combinations of either ≥90% or ≥95% inhibition of fungal growth compared to drug-free control endpoints and different wavelengths [405 nm, 450 nm, 492 nm, 540 nm, and 620 nm]). Quality control was ensured by testing the *A. flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 strains.

Underlying mechanism of olorofim resistance in *A. fumigatus sensu stricto* isolates. The *pyrE* gene was amplified and sequenced in *A. fumigatus sensu stricto* isolates showing a non-wild-type olorofim phenotype an MIC above the statistical wild-type upper limit [wtUL], calculated either from the MIC distribution obtained by visual inspection or by spectrophotometric readings using ≥90% fungal growth inhibition endpoints and wavelengths of ≥492 nm (27).

Data analysis. MIC visual inspection was used as the gold standard and compared against spectrophotometric MIC readings; MICs (percentage) within ±1 2-fold dilutions were considered to be in essential agreement (19). In species-specific olorofim MIC distributions with a minimum number of 15 isolates (for *A. ustus* and *L. prolificans* there were fewer than 15 isolates) wtULs were set following the principles applied for epidemiological cutoff (ECOFF) calculation using the ECOFFinder program (30). The best combination of fungal growth inhibition endpoint/wavelength to obtain MIC spectrophotometric reading (against each species complex tested) was determined when essential agreement between MICs obtained by visual inspection and spectrophotometric readings were high and wtULs (99% of the modeled population included) were identical.

Ethical consideration. Given the *in vitro* nature of this study, approval of the ethics committee was not required.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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