

Communication

Novel Antifungals and *Aspergillus Section Terrei* with Potpourri Susceptibility Profiles to Conventional Antifungals

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Abstract: The epidemiology of invasive fungal infections (IFIs) is currently changing, driven by aggressive immunosuppressive therapy, leading to an expanded spectrum of patients at risk of IFIs. Aspergillosis is a leading cause of IFIs, which usually affects immunocompromised patients. There is a limited number of antifungal medications available for treating IFIs and their effectiveness is often hindered by rising resistance rates and practical limitations. Consequently, new antifungals, especially those with novel mechanisms of action, have become increasingly required. This study assessed the activity of four novel antifungal agents with different mechanisms of activity: manogepix, rezafungin, ibrexafungerp, and olorofim against 100 isolates of *Aspergillus section Terrei*, containing amphotericin B (AmB) wildtypes/non-wildtypes and azole-susceptible/-resistant strains, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) method. In general, all tested agents showed potent and consistent activity against tested isolates, exhibiting a geometric mean (GM) and minimum effective concentration (MEC)/minimum inhibitory concentrations (MIC) range respectively as follows: manogepix (0.048 mg/L, 0.032-0.5 mg/L), rezafungin (0.020 mg/L, 0.016-0.5 mg/L), ibrexafungerp (0.071 mg/L, 0.032-2 mg/L), and olorofim (0.008 mg/L, 0.008-0.032 mg/L). In terms of MIC₉₀/MEC₉₀, olorofim had the lowest values (0.008 mg/L), followed by rezafungin (0.032 mg/L), manogepix (0.125 mg/L), and ibrexafungerp (0.25 mg/L). All antifungals tested demonstrated promising *in vitro* activity against *Aspergillus section Terrei*, including *A. terreus* and cryptic species that were azole-resistant and AmB non-wildtype.

Keywords: New Antifungals; *Aspergillus terreus*; aspergillosis; Antifungal Susceptibility Test; EUCAST; Ibrexafungerp; Manogepix; Olorofim; and Rezafungin; Resistance

1. Introduction

There is a growing trend of fungal infections affecting immuno- and medically compromised patients (1,2). The treatment of invasive fungal infections (IFIs), including invasive aspergillosis (IA), has remained challenging due to several factors, namely the limitations in currently available antifungal therapies and changing epidemiology (3,4). *A. terreus* is the third or fourth most common etiological agent of IA, depending on the geographical region (5). This species has a unique clinical position among the opportunistic pathogenic *Aspergillus* species due to the relatively high mortality rate and reduced susceptibility to amphotericin B (AmB), making treatment challenging (6–9). Currently, voriconazole remains the first therapeutic choice for aspergillosis, followed by other substituted agents, such as isavuconazole (ISA), liposomal AmB (L-AmB), and voriconazole (VRC) plus an echinocandin (10). In addition to the limited therapeutic options available, azole-resistant *A. terreus* and related species, along with the tolerance phenomenon, threaten the current pipeline of antifungals (11–14).

New generations of antifungals are needed to combat the rapidly rising levels of resistance and their associated clinical failures (15). The development of antifungal drugs has stagnated in the past two decades, with only ISA introduced (16). Although ISA has a broader spectrum than VRC and fewer drug-related side effects, it still displays cross-resistance with other azoles (17). Even though antifungal drug development is a lengthy process, it addresses the consequences of limited drug classes. Several antifungals are currently being developed in clinical trials and have substantial support from pharmaceutical companies (18).

In the present study, the *in vitro* activity of some promising new drugs in development was analyzed, including ibrexafungerp, manogepix, olorofim, and rezafungin. Manogepix (formerly E1210) is the active component of fosmanogepix, a novel first-in-class broad-spectrum antifungal agent that inhibits the activity of Gwt1 enzyme, which is involved in the biosynthesis of glycosylphosphatidylinositol(GPI) anchors, an essential component of the fungal cell wall (19,20). This leads to defects in various steps of cell wall biosynthesis with accompanying inhibition of cell wall growth, hyphal elongation, and attachment of fungal cells to biological substrates (20). Manogepix has been shown to have broad-spectrum activity against various molds and yeasts (19). Ibrexafungerp (formerly SCY-078), a semisynthetic derivative of enfumafungin, is a potent inhibitor of fungal β -(1,3)-D-glucan synthases (21), with promising activity against *Aspergillus* and *Candida* species. Olorofim (formerly F901318), a new antifungal agent with a novel selective activity, inhibits fungal dihydroorotate dehydrogenase(DHODH), thus halting *de novo* pyrimidine biosynthesis and, ultimately DNA synthesis, cell growth, and division (22,23). The cyclic hexapeptide rezafungin (formerly CD101), which is structurally similar to anidulafungin, is an echinocandin highly active against *Aspergillus* (22). The current study aimed to evaluate the *in vitro* activity of the above-mentioned new antifungals against a collection of *Aspergillus* section *Terrei* isolates, including AmB wildtype/non-wildtype and azole-susceptible/-resistant *A. terreus* sensu stricto (s.s.) and related species, using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference method.

2. Materials and Methods

A total of 100 molecular identified *Aspergillus* section *Terrei* isolates, including *A. terreus* s.s. (n = 30), *A. citrinoterreus* (n = 9), *A. alabamensis* (n = 7), *A. hortae* (syn. *A. hortai*; n = 6), *A. carneus* (n = 6), *A. niveus* (n = 6), *A. aureoterreus* (n = 5), *A. neoindicus* (n = 5), *A. iranicus* (n = 5), *A. neoaffricanus* (n = 4), *A. pseudoterreus* (n = 4), *A. allahabadi* (n = 4), *A. floccosus* (n = 2), *A. barbosa* (n = 2), *A. bicephalus* (n = 1), *A. ambiguus* (n = 1), and *A. microcysticus* (n = 1) were analyzed. The isolate collection included strains previously obtained and included in the ISHAM-ECMM-EFISG TerrNet Study (www.isham.org/working-groups/aspergillus-terreus) (24) and those preserved in the CBS biobank housed at the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands. Strains were identified, as previously described (13,25). A selection of non-wildtype/wildtype and resistant/susceptible isolates was made based on the susceptibility profiles of tested conventional antifungals (AmB, ISA, VRC, posaconazole (PSC)) (data not shown) (Figure 1). In total, 10% of selected isolates showed cross-resistance to the tested conventional antifungals.

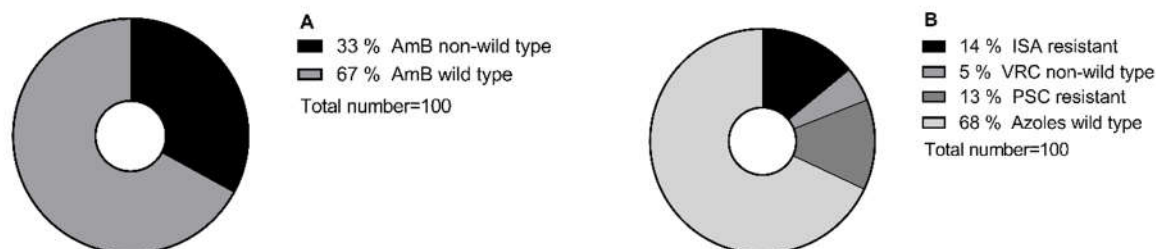


Figure 1. Pie chart illustrating the percentage of A. AmB-wild type/non-wild type B. ISA, PSC resistant/susceptible, and VRC wild type/non-wild type isolates, according to the clinical breakpoint and Epidemiological cutoff values defined by EUCAST

(https://www.eucast.org/mic_and_zone_distributions_and_ecoffs),(<https://www.eucast.org/astoffungi/clinicalbreakpointsforantifungals>). PSC; posaconazole, VRC; voriconazole, ISA; isavuconazole, AmB; amphotericin B.

Isolates were cultured from 10% glycerol frozen stocks (-80°C) on malt extract agar (Carl Roth, Karlsruhe, Germany) at 37°C for up to 5 days; spores were harvested by applying spore suspension buffer (0.9% NaCl, 0.01% Tween 20 [Sigma-P1379]). Antifungal susceptibility testing was performed according to the broth microdilution method of EUCAST (26). The antifungals used were ibrexafungerp (range 0.03–16 mg/L; Scynexis, Inc., Jersey City, NJ, USA), olorofim (range 0.008–4 mg/L; F2G Ltd., Manchester, UK), rezafungin (range 0.01–8 mg/L; MedChemExpress, Sollentuna, Sweden), and manogepix (range 0.03–16 mg/L; MedChemExpress, Sollentuna, Sweden). The minimum inhibitory concentrations (MICs), the concentration at which no hyphal growth was detected, was assessed for olorofim, and for the rest of the tested agents, the minimal effective concentrations (MECs), which markedly altered hyphal growth with blunted colonies, was assessed. A final reading of MIC results was performed with a stereoscope after 48

h. Geometric mean (GM), MIC₅₀/MEC₅₀ (MIC/MEC causing inhibition of 50% of the isolates tested) and MIC₉₀/MEC₉₀ (MIC/MEC causing inhibition of 90% of the isolates tested) were calculated.

3. Results

The MIC distribution and *in vitro* susceptibility testing results of manogepix, rezafungin, ibrexafungerp, and olorofim against 100 *Aspergillus* section *Terrei* isolates, including those with reduced susceptibility to AmB and resistance to azoles, are shown in Figure 2, Figure 3, and Table 1.

Manogepix demonstrated potent *in vitro* activity against all tested isolates, as shown in Figure 1, with MECs ranging from 0.032 to 0.5 mg/L, and the MEC₅₀ and MEC₉₀ values of 0.032 and 0.125 mg/L, respectively. Considering the species separately (Table 1), *A. citrinoterreus* and *A. bicephalus* demonstrated the highest MECs range (0.032-0.5 and 0.5 mg/L, respectively), and *A. carneus* and *A. niveus* the highest GM (both 0.086 mg/L). Furthermore, manogepix displayed potential activity at the lowest concentration (0.032 mg/L) against the majority of resistant/non-wildtype isolates (Figure 3A). The MEC range, MEC₅₀, and MEC₉₀ values of rezafungin were 0.016 to 0.5 mg/L, 0.016 mg/L, and 0.5 mg/L, respectively, against all tested *Aspergillus*. Among all tested species, *A. carneus* showed the highest MEC range and GM for rezafungin (0.016-0.5 and 0.026 mg/L, respectively). Rezafungin inhibited most isolates at the lowest concentration, 0.016 mg/L, when focusing on resistant/non-wildtype isolates (Figure 3B). Ibrexafungerp yielded MEC range, MEC₅₀, and MEC₉₀ values of 0.03 to 2 mg/L, 0.06 mg/L, and 0.25 mg/L, respectively. As compared to all other tested species, *A. citrinoterreus*, and *A. terreus* s.s, the most clinically isolated species, displayed the highest MEC range (both 0.032-2 mg/L), and *A. allahabadi* showed the highest GM (0.087 mg/L). According to the results, ibrexafungerp exhibited promising inhibitory activity at the lowest concentration range tested (0.032-0.06 mg/L) against most of the non-wildtype and resistant isolates (Figure 3C). Olorofim showed a high activity against all tested *Aspergillus* section *Terrei* isolates, exhibiting an MIC range, MEC₅₀, and MEC₉₀ values of 0.008–0.032 mg/L, 0.008 mg/L, and 0.008 mg/L, respectively. Comparatively, *A. neoindicus* had the highest MIC range for olorofim (0.008-0.032 mg/L), and *A. iranicus* showed the highest GM (0.012 mg/L). Considering non-wildtype/resistant isolates separately, olorofim showed a significant inhibitory effect at the lowest concentration tested (0.008-0.016 mg/L) (Figure 3D).

Overall, all agents demonstrated promising activity against tested isolates and considering GM of all species together, the lowest value was assigned to olorofim, followed by rezafungin, manogepix, and ibrexafungerp (0.008 mg/L, 0.020 mg/L, 0.048 mg/L, and 0.071 mg/L, respectively).

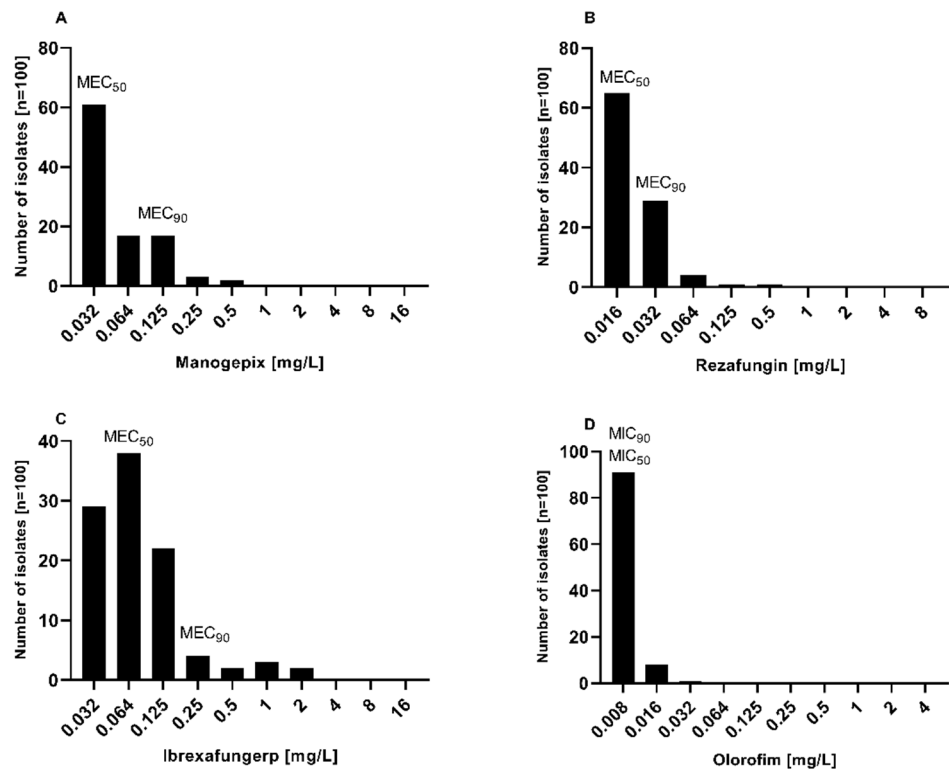


Figure 2. Distribution of minimum inhibitory concentrations (MICs) and minimum effective concentrations (MECs) of A. Manogepix, B. rezafungin, C. ibrexafungerp, and D. olorofim against *Aspergillus* section *Terrei* (n=100).

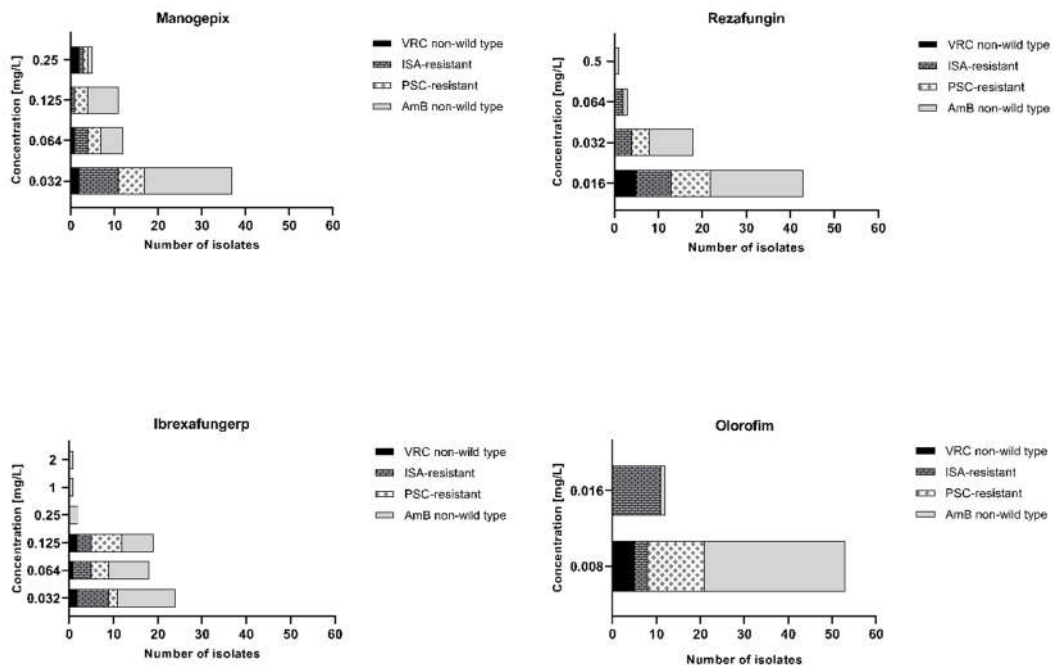


Figure 3. The activity of tested antifungals A. manogepix, B. rezafungin, C. ibrexafungerp, and D. olorofim with focus on AmB non-wild type (n=33), ISA-resistant (n=14), PSC-resistant (n=13), and VRC non-wild type (n=5) isolates of *Aspergillus* section *Terrei*. PSC; posaconazole, VRC; voriconazole, ISA; isavuconazole, AmB; amphotericin B.

Table 1. MIC values, ranges, and GMs for olorofim, and MEC values, ranges, and GMs for ibrexafungerp, manogepix, and rezafungin against azole-susceptible/resistant and AmB wildtype/non-wildtype *Aspergillus section Terrei* (n=100), as determined by EUCAST broth microdilution method. MIC50/MEC50 and MIC90/MEC90 stands for MICs/MECs inhibiting $\geq 50\%$ and $\geq 90\%$ of strains, respectively. The GM (geometric mean) is shown for species with at least four isolates or more.

<i>Aspergillus section Terrei</i> (no.)	MEC range (mg/L)/(MEC GM)			MIC range (mg/L)/(MIC GM)
	Manogepix	Rezafungin	Ibrexafungerp (SCY-078)	Olorofim
<i>A. alabamensis</i> (n=7)	0.032/0.03	0.016-0.032/0.018	0.03-0.05/0.074	0.008/0.008
<i>A. allahabadii</i> (n=4)	0.032/0.03	0.016-0.032/0.017	0.06-0.125/0.087	0.008-0.016/0.009
<i>A. ambiguus</i> (n=1)	0.032/-	0.016/-	0.06/-	0.008/-
<i>A. aureoterreus</i> (n=5)	0.032-0.125/0.045	0.016-0.032/0.019	0.03-0.125/0.053	0.008/0.008
<i>A. barbosa</i> (n=2)	0.032/-	0.016/-	0.06-0.125/-	0.008/-
<i>A. bicephalus</i> (n=1)	0.5/-	0.016/-	0.03/-	0.008/-
<i>A. carneus</i> (n=6)	0.032-0.25/0.086	0.016-0.5/0.026	0.03-0.25/0.061	0.008-0.016/0.011
<i>A. citrinoterreus</i> (n=9)	0.032-0.5/0.070	0.016-0.032/0.018	0.03-2/0.076	0.008/0.008
<i>A. floccosus</i> (n=2)	0.064-0.125/-	0.016-0.032/-	0.03-0.25/-	0.008-0.016/-
<i>A. hortai</i> (n=6)	0.032-0.125/0.038	0.016-0.125/0.023	0.06-1/0.155	0.008/0.008
<i>A. iranicus</i> (n=5)	0.032-0.06/0.039	0.016-0.032/0.019	0.03-0.06/0.045	0.008-0.016/0.012
<i>A. micocysticus</i> (n=1)	0.032/-	0.016/-	0.03/-	0.008/-
<i>A. neoafricanus</i> (n=5)	0.032-0.125/0.039	0.016-0.06/0.025	0.03-1/0.173	0.008/0.008
<i>A. neoindicus</i> (n=5)	0.032-0.125/0.045	0.016-0.032/0.023	0.03-0.125/0.06	0.008-0.032/0.01
<i>A. niveus</i> (n=6)	0.032-0.25/0.086	0.016-0.06/0.023	0.3-0.125/0.061	0.008/0.008
<i>A. pseudoterreus</i> (n=4)	0.032-0.06/0.035	0.016-0.032/0.017	0.06/0.06	0.008/0.008
<i>A. recifensis</i> (n=2)	0.032-0.125/-	0.032/-	0.125/-	0.008/-
<i>A. terreus</i> s.s (n=30)	0.032-0.125/0.044	0.016-0.06/0.019	0.03-2/0.067	0.008/0.008
All isolates (n= 100)				
GM	0.048	0.020	0.071	0.008

Range	0.032-0.5	0.016-0.5	0.032-2	0.008-0.032
MEC 50/90	0.032/0.125	0.016/0.032	0.064/0.25	-
MIC50/90	-	-	-	0.008/0.008

4. Discussion

The mortality rate of aspergillosis infections remains high despite the improved diagnosis, and prophylaxis (27). There are currently four major classes of antifungal agents used to treat systemic mycoses: polyenes, azoles, echinocandins, and flucytosine (28). The effectiveness of present antifungals has been affected by toxicity, drug–drug interactions, variable pharmacokinetics, and reduced bioavailability (28). The emergence of drug resistance has introduced further limitations (29). For IA, VRC is the first line of treatment; alternatives include ISA, L-AmB, and VRC plus an echinocandin (30). Resistance to azoles, the first-line treatment, has grown alarmingly in the last decade, posing a serious challenge to the effective management of aspergillosis (29,31). The identification of antifungal resistance has relied on susceptibility testing, identifying MICs to define the susceptibility or resistance. Several factors further complicate treatment and lead to poor outcomes, such as method dependency of the susceptibility testing results and consequently discrepancies between *in vitro* and *in vivo* outcomes, as well as tolerance and persistence phenomena, which are not detectable by reference susceptibility testing methods (14,32,33). Therefore, the reduction in the currently limited antifungal arsenal leads to patient management complications and higher mortality due to resistant isolates, which calls for new antifungal agents and therapeutic approaches (3). Since *A. terreus* is naturally less susceptible to AmB, azole resistance in this species is of particular concern, which could lead to a loss of two primary lines of treatment (7,13). Furthermore, some less common species in section *Terrei*, exhibit high azole MICs, which, if not identified before antifungal therapy, may cause clinical failure (32). Thus, in this study, novel antifungals were tested against nearly all currently accepted species in section *Terrei*, including isolates with reduced susceptibility to conventional antifungals.

Similarly to previous studies (34,35), manogepix exhibited encouraging activity against all tested *Aspergillus* spp., including AmB non-wildtype and azole-resistant isolates. Manogepix inhibited all tested isolates at 0.5 mg/L (MEC₅₀, 0.032 mg/L; MEC₉₀, 0.125 mg/L) (Figures 1A, 2A, and Table 1). Despite the similar MEC₅₀ and MEC₉₀ of *A. terreus* s.s. and *A. terreus* non-s.s. when compared separately, all *A. terreus* s.s. were inhibited at 0.125 mg/L, while all *A. terreus* non-s.s. were suppressed at 0.5 mg/L. As we found, a study of clinical isolates from Spanish patients found manogepix effective against cryptic *Aspergillus* species, including those resistant to PSC and AmB (36). Furthermore, according to a recent study, *in vivo* combining manogepix and L-AmB showed a synergistic effect in reducing invasive pulmonary aspergillosis fungal burden and improving survival (37). Synergistic effects with L-AmB may have greater utility in cases where azole resistance is suspected.

Rezafungin demonstrated significant *in vitro* activity against all tested isolates, by 0.5 mg/L (MEC₅₀, 0.016 mg/L; MEC₉₀, 0.032 mg/L) (Figures 1B, 2B, and Table 1). Rezafungin MECs were higher for *A. terreus* non-s.s. than *A. terreus* s.s., 0.5 mg/L (MEC₅₀, 0.016 mg/L; MEC₉₀, 0.032 mg/L) and 0.06 mg/L (MEC₅₀, 0.016 mg/L; MEC₉₀, 0.032 mg/L), respectively. The prolonged half-life of rezafungin *in vivo* (38), along with its potent *in vitro* activity against *Aspergillus* spp. (39), suggest that it may be beneficial in treating patients with infections caused by azole-resistant *Aspergillus*. However, it should be noted that monotherapy with an echinocandin is not currently recommended as a primary treatment for IA. To determine whether this potent *in vitro* activity would accelerate with combination therapy, and whether it would translate into *in vivo* efficacy against infections caused by resistant *Aspergillus* isolates, additional studies are warranted.

In vitro, ibrexafungerp, the new beta-glucan synthase inhibitor, showed promising antifungal activity against tested *Aspergillus* section *Terrei*, by MEC of 2 mg/L (MEC₅₀, 0.06 mg/L; MEC₉₀, 0.25 mg/L) (Figures 1C, 2C, and Table 1). There were no significant differences between MECs of *A. terreus* s.s. 2 mg/L (MEC₅₀, 0.064 mg/L; MEC₉₀, 0.125 mg/L) and *A. terreus* non-s.s. 2 mg/L (MEC₅₀, 0.064 mg/L; MEC₉₀, 0.25 mg/L). Ibrexafungerp has previously been shown to have *in vitro* and *in vivo* activity against *Aspergillus* species, including azole-resistant and caspofungin-resistant strains, which is consistent with this study (Figure 2, and 3) (40,41). Furthermore, the synergistic effect of ibrexafungerp in combination with ISA, VRC, and AmB was shown (42). These results are likely to

increase the appeal of using ibrexafungerp in combination with other agents for infections that are difficult to treat.

The strong activity of olorofim has been confirmed against the tested *Aspergillus* section *Terrei*, including those species that showed reduced susceptibility to AmB and/or azoles (Figures 2 D, 3D, and Table 1). Olorofim had the lowest MICs by 0.032 mg/L (MEC50 and MEC90, both 0.008 mg/L), with no differences between *A. terreus* s.s., and *A. terreus* non-s.s. Beside the present study, other studies have also shown that olorofim is effective against azole-resistant *A. fumigatus* *in vitro* and *in vivo* in murine models of invasive pulmonary aspergillosis (22). Additionally this new drug has shown activity against other common *Aspergillus* species, including *A. terreus* (43–45). Olorofim's activity has been retained against isolates showing resistance to azoles and/or AmB, and given its entirely different target to the azoles, cross-resistance would not be expected.

In conclusion, a set of novel antifungals (manogepix, rezafungin, ibrexafungerp, and olorofim) has been demonstrated to have promising and consistent *in vitro* activity against nearly all currently accepted species of *Aspergillus* section *Terrei* regardless of azole and AmB resistance. The development of novel agents could play a pivotal role in treating multi-resistant mold infections, including azole-resistant aspergillosis.

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