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***In vitro* activity of colistin as single agent and in combination with antifungals against filamentous fungi occurring in patients with cystic fibrosis**

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SUMMARY

Background and Objectives: Because published reports indicate that the antibiotic colistin has antifungal properties, this study investigated the antifungal *in vitro* activity of colistin as single agent and in combination with the antifungal compounds voriconazole, caspofungin, and amphotericin B against *Scedosporium/Pseudallescheria* spp., *Exophiala dermatitidis*, and *Geosmithia argillacea*.

Material and Methods. In total, susceptibility was determined for 77 *Scedosporium/Pseudallescheria* spp., 82 *E. dermatitidis*, and 17 *G. argillacea* isolates. The MICs of colistin and the antifungals as single compound and in combination were determined with MIC test strips. Drug interactions were detected by crossing the MIC test strips at a 90° angle. The fractional inhibitory concentration index was used to categorize the drugs' interaction.

Results. The MIC₅₀ value of colistin was 12 µg/mL for *S. prolificans*, 16 µg/mL for *P. apiosperma*, 16 µg/mL for *P. boydii*, 12 µg/mL for *E. dermatitidis*, and 6 µg/mL for *G. argillacea*. Voriconazole was the most active drug in combination without any antagonism with the exception of few *P. boydii* isolates.

Conclusions. Colistin as single agent and in most combinations with antifungals exhibits *in vitro* antifungal activity against filamentous ascomycetes occurring in cystic fibrosis patients and may offer a novel therapeutic option, especially for multidrug-resistant *S. prolificans*.

INTRODUCTION

Bacterial colonization or infection of the respiratory tract is one of the main reasons for increased morbidity and mortality rates among cystic fibrosis (CF) patients.¹ However, the respiratory tract secretions of CF patients often contain filamentous fungi, such as *Aspergillus* spp..² *Scedosporium/Pseudallescheria* spp. and *E. dermatitidis* are reported to be the most common non-*Aspergillus* species moulds, whereas *G. argillacea* was recently described as an emerging pathogen in this patient population.³⁻⁵

The prevalence of filamentous fungi in the CF community shows great interstudy and geographical variations.^{3,6} This is most likely due to differences in the procedures used for mycological examination of sputum samples (conventional vs. selective agar), the methods used for species identification (phenotypic vs. molecular) and environmental differences. In the context of CF, the exact clinical relevance of these fungal infections is still a matter of debate. However, invasive and fatal infections with *Scedosporium/ Pseudallescheria* spp., *E. dermatitidis*, and *G. argillacea*, especially in immunocompromised patients, have been repeatedly reported.⁷⁻¹⁴

Colistin (COL, polymyxin E) belongs to the antibiotic substance class of polymyxins, produced by *Bacillus polymyxa*. The mode of action of this antibiotic is the disruption of the outer membrane of gram-negative bacteria, which results in an increased cell permeability with subsequent leakage of cell content, cell lysis, and cell death.¹⁵ Nebulized COL is a frequently used agent for inhalation therapy in CF patients chronically colonized with *Pseudomonas aeruginosa* or other multidrug-resistant gram-negative rods.¹⁶ Other authors reported that the antibiotic COL shows reasonable antifungal activity against *Candida* spp., *Cryptococcus neoformans*, and *Mucorales* spp..^{17,18} The aim of this study was to analyze the *in vitro* activity of COL

as single agent and in combination with VRC, CAS, and AMB against *Pseudallescheria/Scedosporium* spp., *E. dermatitidis*, and *G. argillacea*.

MATERIAL & METHODS

1.1. Isolates

In total, we tested 77 *Scedosporium/Pseudallescheria spp.* isolates, 82 *E. dermatitidis* isolates, and 17 *G. argillacea* isolates, including 69 (39%) reference strains. The clinical isolates were obtained from the following institutions: Institute of Medical Microbiology, University Hospital Essen, Germany; Consultant Laboratory for Cystic Fibrosis-Bacteriology (Northern Germany), Institute of Medical Microbiology and Hospital Epidemiology, Hannover Medical School, Hannover, Germany; Institute for Medical Microbiology, Immunology and Hygiene, University Hospital of Cologne, Cologne, Germany; and the Collection of the ECMM-ISHAM Working Group on *Pseudallescheria/Scedosporium* infections.

We tested a total of 19 *S. prolificans* (52% isolated from CF patients, 9 reference strains of international collections), 30 *P. apiosperma* (67% from CF, 16 reference strains), and 28 *P. boydii* isolates (71% from CF, fourteen reference strains). Of the 82 tested *E. dermatitidis* isolates, 80% (n=66) were obtained from CF patients and 34% (n=28) were reference strains from the Centraalbureau voor Schimmelcultures (CBS), including those from CF patients. Of the tested *G. argillacea* strains, two isolates were CBS reference strains and thirteen (76%) were obtained from CF patients. In total, 131 of the 176 (74.4%) tested fungal species were obtained from the respiratory tract of CF patients.

1.2. Molecular species identification

All clinical isolates were identified by phenotypic characteristics to the genus level. Subsequent species identification was performed using molecular methods. All isolates from Essen, Cologne and Hannover were identified by sequencing the internally transcribed spacer (ITS1-5.8S-ITS2) region of the rDNA, as described

previously.¹⁹ Isolates from the Collection of the ECMM-ISHAM Working Group on *Pseudallescheria/Scedosporium* Infections were identified to the species level by amplified fragment length polymorphisms, as reported.²⁰ Species identification of reference strains were provided by the international collections.

1.3. Susceptibility testing

Susceptibility testing for all isolates was performed according to the guidelines of the Clinical Laboratory Standards Institute (CLSI) document M38-A2.²¹ However, in accordance with Pfaller *et al.* [22], the CLSI test method was modified from a broth dilution method to a MIC test strip method. The following drugs were tested as single compounds: COL, VRC, CAS, and AMB. In addition, COL was tested in combination with VRC, CAS, and AMB (MIC test strips, Liofilchem s.r.l., Roseto degli Abruzzi, Italy).

Before susceptibility testing isolates were grown on malt extract agar (Oxoid, Hampshire, UK) at 30°C for seven days. They were then sub-cultured on potato dextrose agar (Oxoid) and incubated at 35°C for 7 days so that adequate sporulation could be ensured.

Spore suspensions were prepared in sterile 0.9% NaCl solution, adjusted to a density of 0.5 McFarland standard and inoculated onto RPMI MOPS 2% Glucose Agar (Reactivos Para Diagnostico S.L., Barcelona, Spain). For single testing, the MIC test strip was placed in the middle of the plate. The plates were incubated at 30°C and read after 72 to 96 hours as recommended by the manufacturer. The MIC reading in single testing was interpreted as the zone diameter of inhibition that intersected with the strip.²²

1.4. Combination testing

In accordance with the method of Kiraz *et al.* [23] the MIC test strips were placed at a 90° angle to each other. The MIC strip was placed so that the crossing of the two strips was at the MIC₅₀ found in single testing for each substance. For combination testing, the COL strip was always placed first on the RPMI agar and the antifungal agent strip was placed above. The plates were incubated at 30°C and were read after 72 to 96 hours. For combination testing, the MIC was read as each drug concentration at which the inhibition zone between the test strips met.

We determined synergistic, indifferent, or antagonistic effects by using the following formula to calculate the fractional inhibitory concentration index (FICI): $FICI = [MIC A/MIC A(A+B)] + [MIC B/MIC B(A+B)]$ where MIC A is the MIC of drug A alone, MIC A(A+B) is the MIC of drug A in combination testing, MIC B is the MIC of drug B alone, and MIC B(A+B) is the MIC of drug B in companion testing. The FICI thus obtained was interpreted as follows: synergy, <0.5; indifference, 0.5 to 4.0; and antagonism, >4.0.²⁴ All tests were performed twice.

RESULTS

***Scedosporium/Pseudallescheria* spp.**

The range, geometric mean, MIC₅₀, and MIC₉₀ of COL and the antifungal compounds against *Scedosporium/Pseudoallescheria* spp. isolates (n=77) are shown in Table 1. The MIC₅₀ for COL was 12 µg/mL for *S. prolificans*, 16 µg/mL for *P. apiosperma*, and 16 µg/mL for *P. boydii* (Table 1). *S. prolificans* exhibited high MICs (>32 µg/mL) against all tested antifungals in single testing. The MIC values for VRC and AMB against *P. boydii* and *P. apiosperma* were lower than those for *S. prolificans*. CAS exhibited no activity against any tested isolate (>32 µg/mL).

The MICs and the FICIs determined by susceptibility testing for COL in combination with VRC, CAS, and AMB are shown in Tables 2 and 3. The MIC₅₀ of the combination of VRC and COL were decreased for both substances and the combination exerted no antagonistic effects against the tested species with the exception of some *P. boydii* isolates (11%). The interaction of CAS and COL against *P. boydii* and *P. apiosperma* increased the MIC₅₀ of COL up to 256 µg/mL, resulting in a high rate of antagonism (>50%).

The MIC₅₀ of AMB in single testing against all tested isolates was >32 µg/mL. When AMB was tested in combination with COL against *S. prolificans*, no antagonistic effects were detected. This combination resulted in fewer than 25% antagonistic effects against *P. apiosperma*, but in 54% antagonism against *P. boydii* isolates. For these last two species, the MIC₅₀ of COL in combination with AMB was as much as six times higher than the MIC₅₀ of COL alone.

Exophiala dermatitidis

The range, geometric mean, MIC₅₀, and MIC₉₀ of COL against all tested *E. dermatitidis* isolates (n=82) are shown in Table 1. The combination of COL and VRC was synergistic against 20% of isolates, and no antagonism was observed (Table 3).

In single testing, *E. dermatitidis* exhibited high MIC values against CAS (MIC₅₀, >32 µg/mL). Interestingly, the MIC₅₀ of CAS decreased from >32 µg/mL to 7 µg/mL when COL and CAS were applied in combination; this decrease resulted in a rate of synergy against 7.3% of the strains. AMB alone exhibited low MICs against all isolates (MIC₅₀, 0.25 µg/mL). When AMB was tested in combination with COL, the MIC₅₀ of both drugs decreased and showed indifference to 95% of the isolates.

Geosmithia argillacea

The range, geometric mean, MIC₅₀, and MIC₉₀ of COL against all tested *G. argillacea* isolates (n=17) are shown in Table 1. VRC alone and AMB alone did not exert any antifungal activity (MIC₅₀, >32 µg/mL for both substances). The combination of COL and CAS was antagonistic against 88% of *G. argillacea* isolates. When COL was combined with either AMB or VRC, only indifferent effects were observed, but the MIC₅₀ of AMB and VRC decreased from >32 µg/mL to 2 µg/mL and to 0.5 µg/mL, respectively.

Discussion

In this study, we could demonstrate that the antibiotic COL has besides its bactericidal activity also antifungal activity against *Scedosporium/Pseudallescheria* spp., *E. dermatitidis* and *G. argillaceae*. In addition, we showed that combining COL with antifungal substances could reduce the MIC values of antifungal agents' species specific. Based on the *in vitro* data, COL might be considered as a novel agent for the antifungal therapy in combination with an antifungal compound.

Infections in CF patients with the emerging fungal pathogen *S. prolificans* can be life-threatening due to the therapy-refractory nature of this fungus.¹² As shown in this study and by others, *S. prolificans* is resistant to nearly all antifungals tested as

single agent.^{20,25-27} One novelty of our study is the finding that COL exerts *in vitro* antifungal activity against *S. prolificans* (MIC₉₀=12 µg/mL). To the best of our knowledge, no other single agent that is approved for clinical use has to date demonstrated such low MIC value and this fact suggests that the efficacy of COL may be superior to that of any other antifungal agent against *S. prolificans*.

The combination of either ravuconazole and CAS or terbinafine and miconazole or VRC and micafungin has been reported to be synergistic against *S. prolificans*.^{25,28,29} We could also show that the combination of COL and VRC at low concentrations inhibits the growth of *S. prolificans*. In addition, no antagonism against *S. prolificans* was observed when COL was combined with VRC, CAS, or AMB.

In general clinical practice, COL is administered via inhalation as a common prophylaxis and also as treatment for chronic *P. aeruginosa* colonization in CF patients. In a pharmacokinetic study, Ratjen and colleagues demonstrated that inhalation of 2 million units of COL produces a sputum peak above 30 µg/mL after 90 min and reaches sputum levels higher than 12 µg/mL for 8 hours after administration.³⁰ Thus, inhalation twice daily is recommended for maintaining sufficient sputum concentrations of COL.³⁰ However, one must consider whether a peak COL concentration >30 µg/mL can actually exhibit fungicidal effects in the lungs of CF patients. Nonetheless, our *in vitro* data suggest that COL may exert antifungal therapeutic effects alone or in combination with antifungal agents.

COL was found to have *in vitro* activity against non-*Aspergillus* molds from the genus *Mucorales* and in combination with AMB also exerts synergistic activity against *Rhizopus oryzae*.¹⁸ More importantly, the results of *in vivo* experiments have demonstrated that the prophylactic intranasal administration of colistimethate to intranasally infected immunosuppressed mice harboring *R. oryzae* spores resulted in

a higher survival rate and a lower fungal burden than in untreated control mice.¹⁸ The authors suggested that preclinical and clinical studies evaluating the use of COL for the treatment of mucormycosis should be conducted promptly.

The activity of polymyxin B in combination with fluconazole against *C. neoformans*, *C. albicans*, and non-albicans *Candida* species has been previously assessed.¹⁷ This study showed that polymyxin B plus fluconazole was potent at low concentrations against *C. neoformans* isolates. The authors postulated that this drug combination could potentially be an effective treatment option for cryptococcal meningitis.

Because *A. fumigatus* is the mold most frequently isolated in samples from CF patients, we also tested several isolates of this species. In agreement with the findings of a previous study,¹⁸ we found that COL as single agent exerted no antifungal activity (MIC >32 µg/ml) against *A. fumigatus* isolates (n=10) from CF patients (data not shown).

As shown by Gilgado *et al.* [31] and by Lackner *et al.* [20], *P. boydii* exhibits high MIC values for AMB, but VRC shows good antifungal activity *in vitro*. In our study, the highest MICs for COL were also recorded among *P. boydii* isolates (MIC₉₀=36.8 µg/mL). Furthermore, the combination of COL with either CAS or AMB exerted antagonistic effects of more than 50% against *P. boydii*. These findings underline the importance of correct identification and determination of the clade origin of the isolate, because the *Scedosporium/Pseudallescheria* spp. are known to have heterogenous antifungal susceptibilities.^{20,31} As pointed out recently, susceptibility testing for targeted therapy against *Scedosporium/Pseudallescheria* species is recommended because of a wide MIC distribution for most substances.²⁰ Our findings confirm these observations: the activity of COL alone and in combination greatly differs among the various species of this genus.

Single testing of COL against *E. dermatitidis* yielded results that were not superior to those of VRC or AMB, for which low MIC values were recorded. However, the combination of COL with the less-effective drug CAS resulted in a 4-fold MIC₅₀ of CAS. Moreover, the combination of COL and VRC exhibited synergistic effects in up to 20% of isolates. Consequently, COL seems to have no major disadvantages in combination with antifungal agents against *E. dermatitidis*.

Like in previous studies, we also found high MICs for VRC and low MICs for CAS in *G. argillacea* isolates from CF patients.^{4,5} The MICs of AMB were more heterogenous (range 0.75 to >32 µg/mL), as previously reported.⁵ COL exerted antifungal activity against *G. argillacea* with only indifferent effects when it was combined with VRC or AMB. However, COL plus CAS exhibited nearly 90% antagonistic effects. These *in vitro* findings may indicate that concomitant administration of COL and CAS is less effective in treating *G. argillacea* infections.

Most studies analyzing antifungal susceptibility of these fungal species have used broth dilution method (BDM), as recommended by CLSI. However, our MIC strip test results for MIC determination of *Scedosporium/Pseudallescheria* spp. are mostly in agreement with the results of a recent study that used BDM.²⁰ A previous study used Etest to test *E. dermatitidis* and found that isolates were susceptible to CAS.³² Other studies using BDM found results similar to ours.^{33,34} In agreement with Pfaller *et al.* [24], we observed that in case of discrepancies the MIC values for VRC tend to be lower with a strip test than with BDM. In our study, we used MIC test strips to determine the activity of combinations of agents against filamentous fungi. A previous study demonstrated that Etest could be used successfully to test antifungal interactions for *Candida* spp..²⁵ However, one limitation of our study is that we did not compare the results of MIC strip interaction testing with the results achieved by BDM.

To the best of our knowledge, no previous studies have tested the activity of COL as single agent or in combination with other antifungal agents against fungi of the genera *Scedosporium/Pseudallescheria*, *Exophiala*, and *Geosmithia*. COL is the first agent to demonstrate activity against multidrug-resistant *S. prolificans*. Interaction testing with three antifungal agents demonstrated that VRC appears to be the best combination partner for COL, because almost no antagonism was observed for most species.

In conclusion, we have provided new insights into the antifungal *in vitro* activity of COL. Even though the mode of action and cellular target of COL in fungi is unclear, this substance shows antifungal activity against different filamentous ascomycetes. As our evidence is based on *in vitro* data only, our future perspective is to verify the results by *in vivo* animal models. Furthermore, clinical trials are needed to study the antifungal activity of colistin in humans.

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