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


1 Institute of Medical Microbiology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, 2 Institute of Microbiology, University of Innsbruck, Innsbruck, Austria, 3 Institute of Medical Microbiology and Hospital Epidemiology, Hannover Medical School, Hannover, Germany, 4 Institute for Medical Microbiology, Immunology and Hygiene, University Hospital of Cologne, Cologne, Germany and 5 Twincore Center for Experimental and Clinical Infection Research, Department of Experimental Virology, Hannover, Germany

Summary

Because published reports indicate that the antibiotic colistin (COL) has antifungal properties, this study investigated the antifungal in vitro activity of COL as single agent and in combination with the antifungal compounds voriconazole (VRC), caspofungin (CAS) and amphotericin B (AMB) against Scedosporium/Pseudallescheria spp., Exophiala dermatitidis and Geosmithia argillacea. In total, susceptibility was determined for 77 Scedosporium/Pseudallescheria spp., 82 E. dermatitidis and 17 G. argillacea isolates. The minimal inhibitory concentrations (MICs) of COL 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 categorise the drugs’ interaction. The MIC50 value of COL was 12 μg ml⁻¹ for S. prolificans, 16 μg ml⁻¹ for P. apiosperma, 16 μg ml⁻¹ for P. boydii, 12 μg ml⁻¹ for E. dermatitidis and 6 μg ml⁻¹ for G. argillacea. VRC was the most active drug in combination without any antagonism with the exception of few P. boydii isolates. COL 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.

Key words: Colistin, susceptibility testing, Scedosporium/Pseudallescheria, Exophiala, Geosmithia, cystic fibrosis.

Introduction

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

The prevalence of filamentous fungi in the CF community shows great interstudy and geographical variations. 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.\(^{15}\) Nebulised COL is a frequently used agent for inhalation therapy in CF patients chronically colonised with *Pseudomonas aeruginosa* or other multi-drug-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 analyse the *in vitro* activity of COL as single agent and in combination with voriconazole (VRC), caspofungin (CAS) and amphotericin B (AMB) against *Pseudallescheria/Scedosporium* spp., *E. dermatitidis* and *G. argillacea*.

**Materials and methods**

**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, nine reference strains of international collections), 30 *P. apiosperma* (67% from CF, 16 reference strains) and 28 *P. boydii* isolates (71% from CF, 14 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 13 (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.

**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.

**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 Pfaffer *et al.* [22], the CLSI test method was modified from a broth dilution method to a minimal inhibitory concentration (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; Lioflichem s.r.l., Roseto degli, Italy).

Before susceptibility testing, isolates were grown on malt extract agar (Oxoid, Hampshire, UK) at 30 °C for 7 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 72–96 h as recommended by the manufacturer. The MIC reading in single testing was interpreted as the zone diameter of inhibition that intersected with the strip.\(^{22}\)

**Combination testing**

In accordance with the method described by 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 MIC\(_{50}\) 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 for 72–96 h. 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 = \( \frac{\text{MIC } A}{\text{MIC } A + \text{MIC } B(A + B)} + \frac{\text{MIC } B}{\text{MIC } B(A + B)} \) 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. All tests were performed twice.

**Results**

*Scedosporium/Pseudallescheria* spp.

The range, geometric mean, MIC50 and MIC90 of COL and the antifungal compounds against *Scedosporium/Pseudallescheria* spp. isolates (n = 77) are shown in Table 1. The MIC50 for COL was 12 µg ml\(^{-1}\) for *S. prolificans*, 16 µg ml\(^{-1}\) for *P. apiosperma* and 16 µg ml\(^{-1}\) for *P. boydii* (Table 1). *S. prolificans* exhibited high MICs (>32 µg ml\(^{-1}\)) 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\(^{-1}\)).

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* were lower than those for *S. prolificans*. CAS exhibited no activity against any tested isolate (>32 µg ml\(^{-1}\)).

The MIC50 of AMB in single testing against all tested isolates was >32 µg ml\(^{-1}\). 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 much as six times higher than the MIC50 of COL alone.

<table>
<thead>
<tr>
<th>Species</th>
<th>COL [µg ml(^{-1})]</th>
<th>VRC [µg ml(^{-1})]</th>
<th>CAS [µg ml(^{-1})]</th>
<th>AMB [µg ml(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>GM</td>
<td>MIC50</td>
<td>MIC90</td>
</tr>
<tr>
<td><em>S. prolificans</em></td>
<td>3–16</td>
<td>8.1</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td><em>P. apiosperma</em></td>
<td>6–32</td>
<td>14.7</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td><em>P. boydii</em></td>
<td>4–36.8</td>
<td>18.5</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td><em>E. dermatitidis</em></td>
<td>1.5–64</td>
<td>11.7</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td><em>G. argillacea</em></td>
<td>4–8</td>
<td>6.2</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

MIC: minimum inhibitory concentration; GM: geometric mean; MIC50: minimal inhibitory concentrations of 50% of the isolates; MIC90: minimal inhibitory concentrations of 90% of the isolates.
**Exophiala dermatitidis**

The range, geometric mean, MIC$_{50}$ and MIC$_{90}$ 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 (MIC$_{50}$, 32 μg ml$^{-1}$). Interestingly, the MIC$_{50}$ of CAS decreased from >32 μg ml$^{-1}$ to 7 μg ml$^{-1}$ 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 (MIC$_{50}$, 0.25 μg ml$^{-1}$). When AMB was tested in combination with COL, the MIC$_{50}$ of both drugs decreased and showed indifference to 95% of the isolates.

**Geosmithia argillacea**

The range, geometric mean, MIC$_{50}$ and MIC$_{90}$ 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 (MIC$_{50}$, 32 μg ml$^{-1}$ 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 MIC$_{50}$ of AMB and VRC decreased from >32 to 2 μg ml$^{-1}$ and to 0.5 μg ml$^{-1}$ 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. argillacea*. 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. As shown in this study and by others, *S. prolificans* is resistant to nearly all antifungals tested as single agent. One novelty of our study is the finding that COL exerts in vitro antifungal activity against *S. prolificans* (MIC$_{90}$ = 12 μg ml$^{-1}$). 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*. 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* colonisation 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$^{-1}$ after 90 min and reaches sputum levels higher than 12 μg ml$^{-1}$ for 8 h after administration. Thus, inhalation twice daily is recommended for maintaining sufficient sputum concentrations of COL. However, one must consider whether a peak COL concentration >30 μg ml$^{-1}$ can actually exhibit fungicidal effects in...
Table 3: Combination testing of colistin (COL) with voriconazole (VRC), caspofungin (CAS) and amphotericin B (AMB) against Scedosporium prolificans, Pseudallescheria apiospermum, P. boydii, Exophiala dermaitididis and Geosmithia argillacea by MIC test strips.

<table>
<thead>
<tr>
<th>Species</th>
<th>COL + VRC</th>
<th>COL + CAS</th>
<th>COL + AMB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Sigma$FICI (median)</td>
<td>Synergy$^3$ (n/%)</td>
<td>Indifference$^3$ (n/%)</td>
</tr>
<tr>
<td>S. prolificans (n = 19)</td>
<td>0.8</td>
<td>1/5.3</td>
<td>18/94.7</td>
</tr>
<tr>
<td>P. apiosperma (n = 30)</td>
<td>0.7</td>
<td>4/13.3</td>
<td>26/86.7</td>
</tr>
<tr>
<td>P. boydii (n = 28)</td>
<td>1.3</td>
<td>2/7.1</td>
<td>23/82.1</td>
</tr>
<tr>
<td>E. dermatididis (n = 82)</td>
<td>0.7</td>
<td>16/19.5</td>
<td>66/80.5</td>
</tr>
<tr>
<td>G. argillacea (n = 17)</td>
<td>0.9</td>
<td>0/0</td>
<td>17/100</td>
</tr>
</tbody>
</table>

MIC, Minimal inhibitory concentration; n, Number of isolates.

$^1$SigmaFICI, fractional inhibitory concentration index, resulting from the addition of single-drug FICI.

$^2$Synergy; FICI ≤ 0.5.

$^3$Indifference; FICI > 0.5–4.0.

$^4$Antagonism; FICI > 4.0.
combination of COL with the less-effective drug CAS resulted in a fourfold MIC$_{50}$ 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. The MICs of AMB were more heterogeneous (range 0.75 to >32 µg ml$^{-1}$), as previously reported. COL exerted antifungal activity against *G. argillacea* with only indiffrent 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 analysing 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. A previous study used Etest to test *E. dermatitidis* and found that isolates were susceptible to CAS. Other studies using BDM found results similar to ours. 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. 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. Although the mode of action and cellular target of COL in fungi remains 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 COL in humans.

**Acknowledgment**

We thank the staff members of the Laboratory of Molecular Microbiology for excellent technical assistance.

**Conflict of interest**

The study was financially supported by the company Grünenthal.

**References**

Activity of colistin against filamentous fungi


