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# *In vitro* activity of a novel compound, Mul-1867, against clinically significant fungi *Candida* spp. and *Aspergillus* spp.

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ABSTRACT

There is an urgent need for new antifungal compounds to treat various types of fungal infections, including pulmonary infections. This study was designed to investigate the potency of a novel compound (Mul-1867) against *Candida* spp. and *Aspergillus* spp. isolated from patients with fungal pneumonia, cystic fibrosis and chronic obstructive pulmonary disease. Mul-1867 was highly effective against susceptible control strains as well as resistant clinical isolates, with minimum fungicidal concentrations (MFCs) varying from 0.06 μg/mL to 0.5 μg/mL. It was also highly effective against pre-formed 48-h-old biofilms formed by yeasts and moulds. The half-minimal biofilm eradication concentration ($MBEC_{50}$) was equal to the MFC. The minimum biofilm eradication concentration to eliminate 90% of biofilms ($MBEC_{90}$) varied from 1 × to 4 × MFC. Scanning electron microscopy revealed morphological changes accompanied by the release of intracellular material from the fungal cells following exposure to Mul-1867. Furthermore, an increased concentration of nucleic acids was found in the medium after 5 min and 20 min of Mul-1867 treatment, indicating leakage of cytoplasmic contents. Overall, these data indicate that Mul-1867 may be a promising inhaled antifungal agent for the treatment and prevention of fungal respiratory infections.


## 1. Introduction

Fungal pathogens have become the leading cause of morbidity and mortality in people lacking protective mechanisms, and the lungs are the main site of opportunistic fungal infections [1,2]. Fungal spores are inhaled regularly and are normally eliminated by alveolar macrophages and mucociliary clearance [3]. It is well known that fungal pneumonia is often nosocomial. However, the non-hospital environment also contributes to a significant majority of fungal lung infections in immunocompromised hosts [2]. *Aspergillus* spp. are ubiquitous and widespread in outdoor (air, soil and water) and indoor environments; therefore, exposure to them and their spores is almost unavoidable [4].

If one of the protective mechanisms fails, inhalation of fungal spores or overgrowth of opportunistic filamentous fungal pathogens results in the development of fungal infection in the lungs that can be further disseminated to other body parts [5]. In particular, immunocompromised hosts, characterised by an impaired immune system and consequently lower protection against fungi, are susceptible to pulmonary fungal infections. Opportunistic invasive fungal infections caused by *Aspergillus* spp. and *Candida* spp. are more commonly observed in neutropenic patients, including those with blood malignancies as well as recipients of bone marrow, lung or heart transplants [6–8].

Among these immunocompromised hosts, 20% of fatal infections are caused by invasive fungal pneumonia. More than 200,000 cases of destructive pulmonary aspergillosis occur each year [1,9]. Another highly susceptible group to invasive fungal pneumonia comprises patients with ciliary activity impairment, such as those with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) [10,11]. COPD patients are at higher risk of developing invasive pulmonary aspergillosis owing to immunosuppression as well as the administration of steroids and broad-spectrum antibiotics [12,13].

Antibiotics used for the treatment of bacterial pulmonary exacerbations alter the microbial composition in the lungs. This may result in altered bacterial–fungal interactions and an increased fungal burden in the lungs. For example, CF patients treated with tobramycin for chronic pulmonary *Pseudomonas aeruginosa* infections showed an increase in the isolation both of *Candida* and *Aspergillus* spp. from the lungs as well as polymicrobial associations of bacteria with fungi [14,15]. In this patient population, fungi are associated with a rapid decline in lung function and the instigation of bronchopulmonary exacerbations [16,17].

During invasive fungal pneumonia, both yeasts and moulds have been shown to form biofilms, which are resistant to antifungal drugs

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[18–20]. In addition, surface films and extracellular polymeric substances were shown to retard the penetration and diffusion of antifungal agents through biofilms [21,22]. There are increasing reports of fungal isolates with reduced susceptibility to polyene and azole antimycotics, the most commonly used antifungal agents. Moreover, numerous studies have suggested that the widely used polyene antimycotics, including amphotericin B (AmB), are losing their efficacy, especially in immunocompromised hosts [23].

Therefore, development of novel antimicrobial agents effective both against yeasts and moulds is at the forefront of today's medicine. In this study, the fungicidal activity of the novel antimicrobial compound Mul-1867 [poly-N1-hydrazino(imino)methyl-1,6-hexanediamine], which is not related to any existing antifungal agents including polyenes or azoles, was investigated against planktonic and sessile *Candida* spp. and *Aspergillus* spp. isolated from patients with pulmonary infections and from environmental samples.

## 2. Materials and methods

### 2.1. Test substance and antimicrobials

Mul-1867 (Fig. 1) was synthesised by TGV-inhalonix Inc. (New York, NY). Polyene and azole antibiotics, namely AmB and voriconazole (VRZ), were purchased from Sigma-Aldrich (St Louis, MO).

### 2.2. Fungal strains

Eleven fungal species were used in this study. Fungal cultures were prepared from clinical isolates obtained from the human respiratory tract of patients. Clinical isolates of *Candida albicans* CF-4, *C. albicans* CF-32, *Aspergillus flavus* CF-220f and *Aspergillus fumigatus* CF-135 were obtained from patients with CF. *Candida albicans* FP-16, *C. albicans* FP-22, *A. flavus* FP-8 and *A. fumigatus* FP-11 were isolated from patients with fungal pneumonia (all samples provided by Dr V. Tetz from a private collection). Control strains included *C. albicans* ATCC 14053, *A. fumigatus* ATCC 204305 and an environmental isolate of *A. flavus* UMC-3 (provided by the Clinical Microbiology Laboratory, Upstate Medical Center, Syracuse, NY). All fungal strains were subcultured from freezer stocks onto Sabouraud dextrose agar (SDA) (Oxoid Ltd., Basingstoke, UK) plates and were incubated at 37 °C for 48 h to obtain yeast and mould colonies for subsequent liquid subcultures in Sabouraud dextrose broth (SDB) (Oxoid Ltd.).

### 2.3. In vitro antifungal susceptibility testing

Minimum fungicidal concentrations (MFCs) for antimicrobials were determined by microtitre assays in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines [24,25]. A standard inoculum for yeast testing was $2.5 \times 10^3$ CFU/mL and the inoculum size for moulds was $5 \times 10^4$ CFU/mL. AmB and VRZ were dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St Louis, MO), whereas Mul-1867 was dissolved in sterile water. Antifungals were two-fold serially diluted. The MFC was defined as the lowest concentration of antimicrobial agent that completely inhibited visible fungal growth after 48 h at 37 °C. Fungal isolates were categorised as susceptible, intermediate or resistant according to susceptibility breakpoints for antifungals based on CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria [26–28].

### 2.4. Effect of Mul-1867 on pre-formed fungal biofilms

In each well of a 96-well round-bottom polystyrene tissue culture microtitre plate (Sarstedt, Nümbrecht, Germany), 200 μL of a standardised yeast or mould culture inoculum ($5 \times 10^5$ CFU/mL) in SDB was added [29]. Untreated biofilms were used as negative controls in this study. Following exposure, well contents were aspirated to prevent antimicrobial carryover and each well was washed three times with sterile deionised water.

The minimum biofilm eradication concentration (MBEC) of Mul-1867 was evaluated as the concentration that killed 50% ($MBEC_{50}$) and 90% ($MBEC_{90}$) of the fungi in pre-formed 48-h-old biofilms. The number of viable cells in the fungal biofilm was determined by estimating the CFU number in accordance with the 2008 CLSI guidelines for interpretation [24,25].

To estimate the CFU number, biofilms were scraped thoroughly, with particular attention to the well edges [21]. The well contents were aspirated and were placed in 2 mL of isotonic phosphate buffer (0.15 M, pH 7.2) and the total CFU number was determined by serial dilution and plating on SDA. Data were $\log_{10}$ transformed and were compared with untreated controls. All assays included three replicates and were repeated in three independent experiments.

### 2.5. Scanning electron microscopy (SEM)

Cultures of *C. albicans* ATCC 14053 and *A. flavus* UMC-3 were treated with a solution of Mul-1867 for 5 min and 20 min, respectively, and were compared with untreated control cultures from the

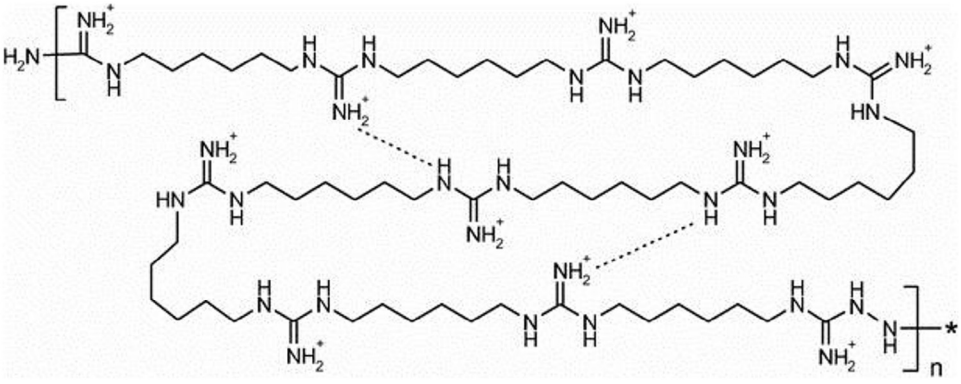

**Fig. 1.** Chemical structure of Mul-1867 ($n = 1–20$).