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Inhaled tigecycline is effective against *Mycobacterium abscessus* *in vitro* and *in vivo*

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Background: *Mycobacterium abscessus* causes chronic pulmonary infections. Owing to its resistance to most classes of antibiotics, treatment is complex and cure rates are only 45%. Tigecycline is active against *M. abscessus*, but severe toxicity and the need for IV administration limit its use.

Objectives: To assess the potential of inhaled tigecycline as a treatment for *M. abscessus* pulmonary disease, by measuring its efficacy in a mouse model of chronic *M. abscessus* pulmonary disease, establishing the intracellular activity of tigecycline against *M. abscessus* in human macrophages and measuring the activity of tigecycline in the sputum of cystic fibrosis patients.

Methods: We infected GM-CSF knockout mice with *M. abscessus* by intrapulmonary aerosol. Infected mice were treated with tigecycline in 0.25, 1.25 and 2.5 mg doses, by inhalation, or untreated, for 28 days. Tigecycline was added to human peripheral blood-derived macrophages infected with *M. abscessus* to assess its intracellular activity. We performed a time–kill kinetics experiment of tigecycline against *M. abscessus* with and without sputum of cystic fibrosis patients.

Results: Inhaled tigecycline proved highly effective against *M. abscessus* in GM-CSF knockout mice. The effect was dose dependent. Tigecycline showed potent activity against *M. abscessus* in macrophages and retained most of its activity in the presence of sputum of cystic fibrosis patients.

Conclusions: Inhaled tigecycline may represent a viable treatment option for *M. abscessus* pulmonary disease, where treatment outcomes are currently very poor. A stable and safe formulation is required to proceed to further pharmacodynamic studies and ultimately clinical trials.

Introduction

Mycobacterium abscessus causes severe, chronic pulmonary infections in susceptible hosts. For its intrinsic resistance to most classes of antibiotics, *M. abscessus* has been rightfully dubbed an 'antibiotic nightmare'.[1] Treatment regimens for *M. abscessus* pulmonary disease typically involve an intensive phase first, with multiple IV antibiotics, which include imipenem, amikacin and tigecycline, with oral azithromycin. The second, continuation phase consists of multiple oral and inhaled agents whose selection is guided, in part, by susceptibility testing. These typically include azithromycin, clofazimine, moxifloxacin, minocycline, linezolid and inhaled amikacin.[2,3] For most of the oral drugs and inhaled amikacin, efficacy is unproven and *in vitro* data suggest limited to no activity.[4–6] Perhaps as a result, treatment outcomes in *M. abscessus* pulmonary disease are very poor, with treatment success rates of 45%.[7]

IV tigecycline became a drug of choice after a retrospective case series showed favourable responses to tigecycline-containing regimens in 16 of 26 patients (62%) with *M. abscessus* pulmonary disease who received >1 month of tigecycline treatment.[8] Yet adverse events were reported in 94% of patients, most commonly nausea and vomiting. Ten of the 36 patients (28%) had to stop tigecycline therapy early (within the first month), because of these adverse events and more than half of the patients treated for

>1 month received reduced doses of tigecycline (i.e. <100 mg/day) and anti-emetics to ameliorate nausea and vomiting.[8]

In vitro pharmacodynamic studies using the hollow-fibre model confirmed the efficacy of tigecycline against *M. abscessus*. Yet this study showed that increasing the daily dose to at least 200 mg would be required to optimize tigecycline's efficacy.[9]

Based on the toxicity profile and requirement of higher doses, an inhaled formulation of tigecycline could be attractive to deliver high doses at the site of infection, maximizing its activity while minimizing systemic toxicity. In the current study, we have tested our hypotheses that inhaled tigecycline is effective and well-tolerated in the treatment of *M. abscessus* pulmonary disease in a validated mouse model.

Materials and methods

Antibiotics and bacterial strains

Tigecycline (Tygacil®, powder for IV infusion; Pfizer) was obtained from the Department of Pharmacy at Radboud University Medical Center (Nijmegen, the Netherlands). We obtained the *M. abscessus* subspecies *abscessus* CIP104536 type strain (synonym ATCC 19977) from the Pasteur Institute (Paris, France) and *M. abscessus* subspecies *abscessus* #21, a clinical isolate with smooth colony morphology,[10] from the University of Colorado Hospital Clinical Microbiology Laboratory. Identification of both isolates was confirmed by WGS; the raw sequence files (FASTQ) were archived in the NCBI Sequence Read Archive (project number PRJNA566387).

Tigecycline MIC measurements

Tigecycline MICs were determined by broth microdilution in CAMHB,[11] using SensiTitre RGMYCO plates with freeze-dried tigecycline at final concentrations ranging from 0.015 to 4 mg/L (Trek Diagnostics/ThermoFisher, Landsmeer, the Netherlands). For intracellular and time–kill assays, the MIC for *M. abscessus* CIP104536 was determined using an in-house broth microdilution assay in CAMHB.

Intracellular antimycobacterial activity

Peripheral blood mononuclear cells were obtained from three healthy volunteers (Sanquin Bloodbank, Nijmegen, the Netherlands). Informed consent was obtained for use of their blood for scientific purposes, as approved by the Ethics Committee of Radboud University Medical Center. The isolation and differentiation protocol was executed as previously described.[12] On the day of infection, *M. abscessus* CIP104536 was added at a ratio of 1:1 (cells:bacteria) for 1 h at 37°C to allow phagocytosis before the medium was changed to RPMI 1640 supplemented with 10% human pooled serum. After the 1 h incubation, tigecycline was added at 2 × MIC (8 mg/L) concentration; an untreated control was also included. After 1 day, 2 days and 3 days, the numbers of extracellular and intracellular bacteria were quantified as previously described.[12]

Time–kill kinetics in cystic fibrosis sputum

We performed in duplicate a time–kill kinetics experiment with tigecycline at 2 × MIC (8 mg/L) concentration against *M. abscessus* CIP104536 in CAMHB with and without 10% (v/v) sputum of cystic fibrosis patients, using previously published methodology.[6,13] Sputum of 18 cystic fibrosis patients was pooled and sterilized by overnight UV irradiation; sterility was checked by 24 h incubation on 5% Columbia sheep blood agar. At the start of the experiment and after 1, 2, 3, 4, 5 and 7 days of incubation at 30°C, samples were taken from the culture bottles and plated in triplicate on Middlebrook 7H10 agar for cfu counting.

Mouse model experiment

Mice

GM-CSF knockout mice[14] were bred at Colorado State University animal housing facilities. Prior to infection, mice were verified as genetic knockout through PCR genotyping. University facilities cared for the animals in line with both Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and NIH guidelines.

Bacteria

M. abscessus #21 was grown in Middlebrook 7H9 broth supplemented with OADC and 0.05% Tween 80 in a shaker incubator at 37°C. After ∼24 h of incubation, when the culture reached OD_{600} between 0.6 and 0.7 (∼1×10^7 cfu/mL), bacteria were aliquotted in preparation for infection. Aliquots were serially diluted into 1:5 dilutions and plated on Middlebrook 7H11 (M7H11) selective agar (BD Difco) supplemented with 10% OADC enrichment (BD Difco) and cfu were enumerated after 3–4 days of colony growth. All cfu values were converted to $\log_{10}$ cfu/mL.

Infection

GM-CSF knockout mice were infected with 1×10^6 cfu of *M. abscessus* #21 in a 50 μL saline solution, through the intrapulmonary aerosol route using an FMJ-250 high-pressure syringe device (PennCentury, Philadelphia, PA, USA) with an attached MicroSprayer (MicroSprayer, model IA-C; PennCentury) as previously described.[10] The mice were temporarily anaesthetized using a mixture of isoflurane and oxygen. The mouse was quickly placed abdomen-down with its teeth suspended up at a 45° angle, and its tongue gently extended out. The MicroSprayer tip was then inserted into the upper trachea and bacteria inoculum sprayed out in the 50 μL dose.[10] After the 50 μL administration, the mouse was placed back into the appropriate cage and monitored as it regained consciousness.

Drug treatment

Tigecycline was aliquotted into separate labelled vials, which were wrapped in aluminium foil and stored in a temperature- and humidity-controlled room to maintain drug stability. The appropriate vial was reconstituted in 300 μL of 0.9% endotoxin-free saline solution (TekNova) immediately before administration to minimize drug degradation.[15] In two separate experiments, the mice received tigecycline via intrapulmonary aerosol delivery using the MicroSprayer device and methodology as explained above.[10] All treatment groups received one 50 μL dose 5 days a week for a total duration of 4 weeks. As control groups, we treated 8 infected mice with aerosolized endotoxin-free saline solution (0.9%; TekNova; six mice) and another group of 10 mice was infected but not treated.

Bacterial load determination

One day and 10 days after infection, as well as 4 weeks after initiation of therapy, mice were euthanized by CO_2 inhalation followed by cervical dislocation. During the necropsies, the lungs were examined for gross pathology and photographed. Thereafter, lungs were prepared for enumeration of bacterial burden and histopathology.

Mouse lung tissues were homogenized using a Next Advance Bullet Blender (Averill Park, NY, USA). Briefly, the left lobe of the lung was placed in a 1.5 mL sterile, safe-lock Eppendorf tube containing 0.5 mL of sterile 1 × PBS and 3×3.2 mm sterile stainless-steel beads and homogenized for 4 min at 8000 rpm in the Bullet Blender. After homogenization, each sample was used for bacterial enumeration. Briefly, serial dilutions of homogenized organs were prepared and cultured on the in-house-prepared M7H11 selective agar supplemented with 10% OADC and 10 μg/mL cycloheximide (Goldbio) and 50 μg/mL carbenicillin (Goldbio) to prevent fungal and bacterial contamination. Plates were incubated for 3–4 days at 37°C, after which

the cfu in each plate were enumerated, with the data expressed as mean ± SEM $\log_{10}$ cfu for each group.

To prevent a drug carry-over effect onto agar plates, the samples were concurrently plated on in-house-prepared M7H11 agar plates supplemented with 0.4% activated charcoal (Sigma–Aldrich). To further determine whether residual drug in tissue homogenate was inhibiting colony formation on agar plates, separate 100 μL lung tissue homogenates were spiked with 100 μL of 10^4 *M. abscessus* #21. After sample homogenizing, the 200 μL sample was used for dilution and plating. Plates were incubated at 37°C for 72 h and then cfu were counted.

Histology

The right lung lobe was immediately placed into 4% paraformaldehyde diluted in PBS during the necropsy. Tissues were then paraffin embedded and sectioned onto charged slides (6–10 μm) to be stained by haematoxylin and eosin (H&E).

Ethics

The Colorado State University Animal Users Committee (IACUC) approved all experimental protocols involving the animals.

Statistical analysis

For the macrophage infection, a factorial ANOVA with *post hoc* Tukey's honest significance test was used to assess differences in total mycobacterial load between treated and untreated macrophages. Calculations were performed in R, version 3.5.2. For the mouse model experiments, cfu data were analysed using Graph Pad Prism version 8.1.1; the statistical analysis was performed using Dunnett's multiple comparisons test as part of a one-way ANOVA test.

Results

In vitro and ex vivo assays

The tigecycline MIC for the *M. abscessus* #21 clinical isolate used for the mouse model experiments and that for the *M. abscessus* CIP104536 type strain was 0.5 mg/L using the SensiTitre RGMYCO assay (both isolates also showed inducible macrolide resistance); the tigecycline MIC for *M. abscessus* CIP104536 using the in-house method was 4 mg/L.

Tigecycline exhibited significant activity against *M. abscessus* CIP104536 in human macrophages, evidenced by a 3 $\log_{10}$ kill after 3 days (Figure 1). In the time–kill assay, after 1 day of unhindered activity, the sputum of cystic fibrosis patients temporarily inhibited tigecycline activity, causing a 1 $\log_{10}$ cfu difference in kill rate on Days 3–5 (Figure 2).

Mouse model experiments

Inhaled high-dose tigecycline proved effective *in vivo*, in a dose-dependent manner (Figure 3). In the 2.5 mg dose group, one animal had countable colonies of *M. abscessus*, owing to pulmonary abscess formation. The effect of both 1.25 mg and 2.50 mg tigecycline doses on lung cfu counts resulted in a significant reduction of 3.98 and 4.42 $\log_{10}$ cfu, respectively, when compared with the untreated control group ($P < 0.0001$). Charcoal-supplemented M7H11 plates and the spiked lung tissue samples did not yield evidence for significant carry-over or residual drug activity in the homogenized tissue (data not shown). There were no signs of distress or other drug toxicity in the tigecycline-treated mice.

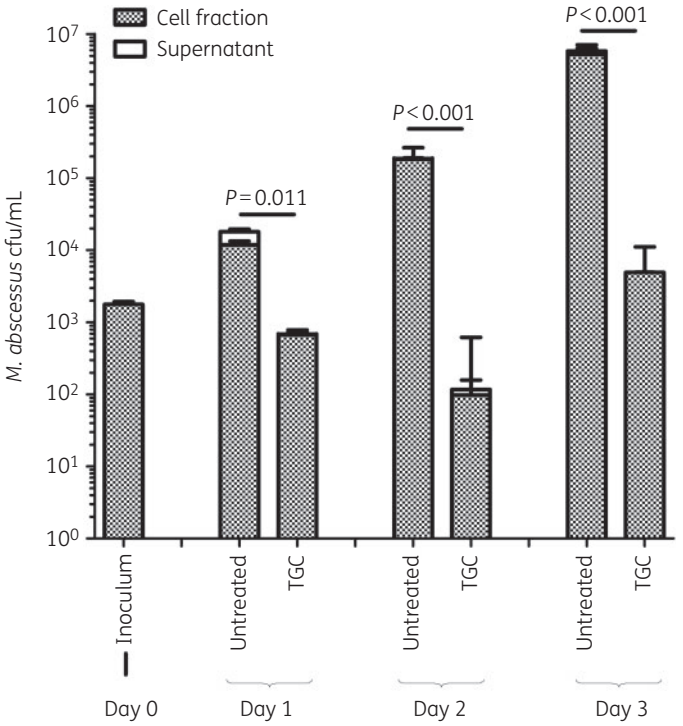

Figure 1. Intracellular activity of tigecycline against *M. abscessus* CIP104536. TGC, tigecycline.

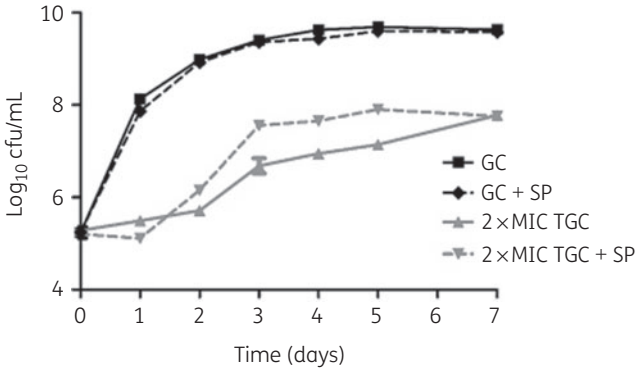

Figure 2. Time–kill kinetics of tigecycline against *M. abscessus* CIP104536 with and without cystic fibrosis sputum. GC, growth control; TGC, tigecycline; +SP, with 10% (v/v) cystic fibrosis sputum.

Histology

Histopathological analysis of the lung sections obtained from *M. abscessus*-infected GM-CSF knockout mice showed granuloma or pre-granuloma formations, influx of inflammatory cells, mainly plasma cells and neutrophils, in parenchyma and influx of lymphocytes in perivascular zones and an increase in foamy cells in granulomatous-like formations (Figure 4). Lung tissue of tigecycline-treated mice showed increased

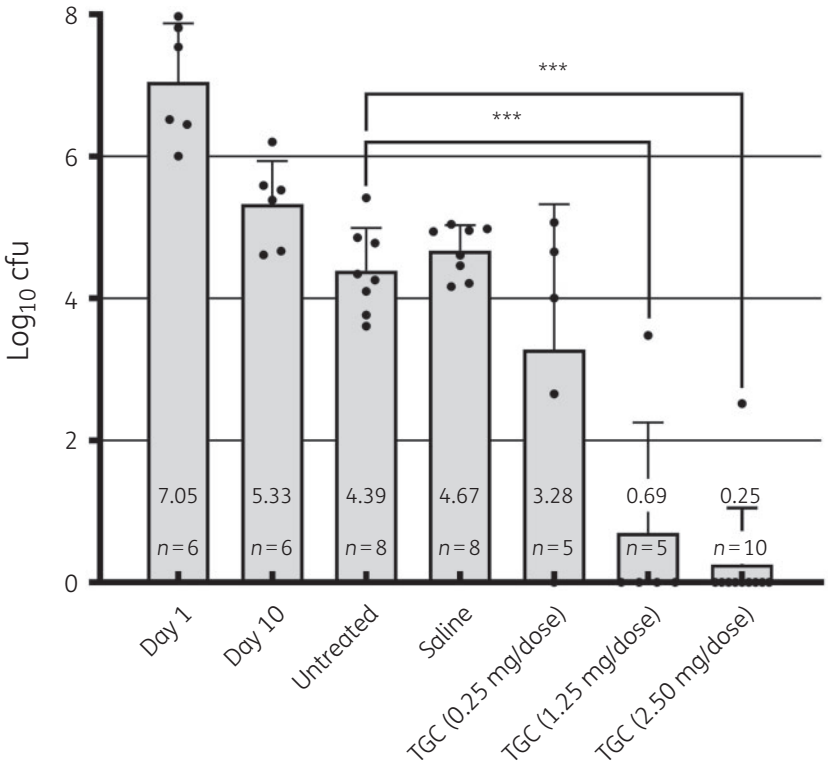

Figure 3. Bacterial load in the lungs of mice from the two experiments. Single data points in each group are represented by filled black circles. The *y*-axis represents the mean value and SEM of $\log_{10}$ cfu in the lungs. The *x*-axis represents each group and timepoint or group treatment in the study. Day 1 and Day 10 are timepoints included as infection control groups. All treatments were initiated on Day 10 and each group, excluding the untreated group, received an intrapulmonary aerosol dose of either saline or tigecycline at low/medium/high concentrations (0.25/1.25/2.50 mg/50 μL dose, respectively). Dunnett's multiple comparisons test: ***$P < 0.0001$. TGC, tigecycline.

necrotic debris in the alveolar lumen and proteinaceous oedema residue, and occasionally alveoli were lined by hypertrophied cells with vesicular nuclei, suggesting type II cell hyperplasia.

Discussion

Inhaled tigecycline proved highly efficacious in a mouse model of chronic pulmonary *M. abscessus* disease. This offers a new and interesting lead for an infectious disease where active antibiotics are few and treatment outcomes are dismal despite long-term complex multidrug regimens.[7] The possible clinical potential of inhaled tigecycline in pulmonary *M. abscessus* disease in humans is further supported by four other experimental findings. First, tigecycline shows antimycobacterial activity in human macrophages (Figure 1), which is a key asset in the treatment of intracellular pathogens like mycobacteria. Second, owing to the frequent occurrence of *M. abscessus* pulmonary disease in cystic fibrosis patients,[3,16] it is important to know that the activity of tigecycline is decreased but not annulled in the presence of cystic fibrosis sputum (Figure 2), although the clinical implications of this *in vitro* finding have not been studied.[13] Third, the tigecycline susceptibility of the strain used in the mouse model experiment was within the normal distribution reported by Wallace *et al.*[17] (≤0.06–1 mg/L); the higher MIC using the in-house assay likely results from tigecycline degradation in aqueous environments.[15] Last, we noted no signs of distress or inhalation-associated toxicity. Histology did not show airway damage in response to tigecycline inhalation. This is in line with previous observations in mice treated with inhaled doxycycline,[18] as well as recent clinical experience with topical tigecycline application.[19]

The effect of inhaled tigecycline was dose dependent, which echoes the previously recorded association between tigecycline AUC/MIC ratio and bactericidal effect in the hollow-fibre model.[9] This association is difficult to extract from the current experiments, as we have no pharmacokinetic data and only three dose groups, of which the two lowest dose groups are small in size. The effect of inhaled tigecycline seems not to result from carry-over, as cfu counts were similar on Middlebrook 7H11 with and without activated charcoal. In fact, the antibacterial effect of the inhaled tigecycline may have been underestimated in the mouse model experiment. Tigecycline rapidly degrades in aqueous environments,[15] so the dose applied in the mouse model experiments was likely lower than the calculated dose. An inhaled formulation of tigecycline should be designed to prevent drug degradation prior to and during administration; owing to its instability in aqueous environments,[15] a dry powder inhalation might be a preferred formulation.

The observation that anatomical changes like abscess formation impede the activity of inhaled tigecycline in the mouse model is relevant; *M. abscessus* pulmonary disease affects patients with underlying structural abnormalities of the lungs or may itself lead

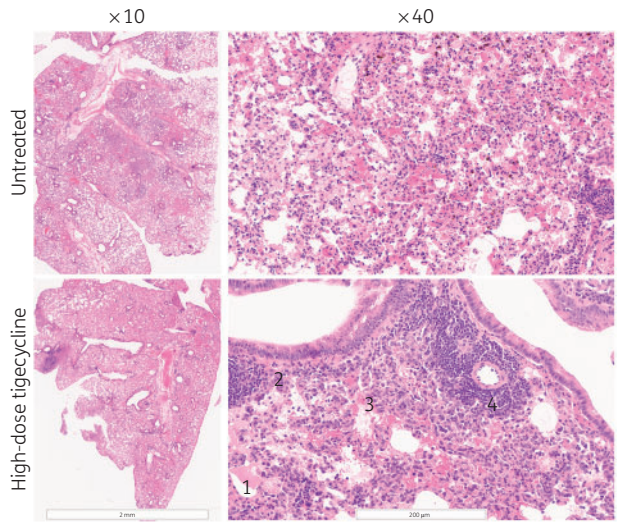

Figure 4. Representative images of H&E-stained lung-tissue sections from *M. abscessus*-infected GM-CSF knockout mice not receiving treatment (top row) and receiving high-dose tigecycline (bottom row) at ×10 and ×40 magnification. Histology shows granuloma-like formations and perivascular lymphocyte infiltration. Representative histological features are shown in the bottom right-hand photograph: 1, oedema and granulomatous formation with numerous foamy cells; 2, type II cell hyperplasia; 3, necrotic debris; and 4, perivascular lymphocyte cuffs.

to such abnormalities, particularly fibro-cavitary lesions.[20] If the activity or deposition of inhaled tigecycline is limited within such lesions, that might affect its use. Similarly, the efficacy may be different in the altered chemical environment of the lungs of cystic fibrosis patients. Mouse models mimicking cystic fibrosis have recently been applied to model *M. abscessus* lung infections and treated with inhaled antibiotics;[21] this may be an interesting model for further preclinical development of inhaled tigecycline.

One of the major limitations of the current study is the lack of pharmacokinetic analyses in the mouse model experiments. Measuring plasma concentrations could have helped to establish which fraction of the inhaled dose of tigecycline makes it into the bloodstream, which could have helped in estimating the likelihood of systemic adverse events as well as the likelihood of causing tigecycline resistance in commensal flora. Second, we used different *M. abscessus* isolates for *in vivo* and *in vitro* and *ex vivo* studies. The *M. abscessus* #21 isolate was previously shown to yield a stable chronic infection in GM-CSF knockout mice.[10,22] For *in vitro* and *ex vivo* work we used the *M. abscessus* CIP104536 type strain, to align with previous studies[6,9] and for reproducibility. Also, the inherent limitations of *in vitro* studies and mouse models apply here; for example, the applied doses cannot easily be translated into a dose suitable for use in clinical trials.

The clinical relevance of the observations on safety and efficacy of inhaled tigecycline against *M. abscessus* can only be proven in clinical trials. These will have to start with creating a stable formulation coupled with an effective inhalation device, followed by Phase 1 trials of safety and tolerability of this novel formulation, before moving into actual efficacy studies.

In summary, we show that inhaled tigecycline yields a very potent effect in GM-CSF knockout mice with chronic *M. abscessus* pulmonary disease. In an infection where treatment outcomes are so very poor, inhaled tigecycline may represent a viable treatment option. A stable and safe formulation is required to proceed to further pharmacodynamic studies and ultimately clinical trials.

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Transparency declarations

None to declare.

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