Resistance of planktonic and biofilm-grown Burkholderia cepacia complex isolates to the transition metal gallium

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Objectives: The Burkholderia cepacia complex is a group of pathogens that can cause severe pulmonary infections in cystic fibrosis (CF) patients. The aim of the present study was to investigate the in vitro activity of gallium against planktonic and biofilm-grown B. cepacia complex isolates.

Methods: Six B. cepacia complex isolates (belonging to three different species) as well as Pseudomonas aeruginosa PAO1 were included in the present study. MICs of Ga(NO$_3$)$_3$ for planktonic cells were determined using a broth microdilution method. Biofilms were formed in 96-well microtitre plates, and the fraction of surviving cells following Ga(NO$_3$)$_3$ treatment was determined using resazurin as a marker for cell viability. The antimicrobial effect of Ga(NO$_3$)$_3$ was assessed in the presence (50 µM) and absence of Fe$^{3+}$.

Results: When tested against planktonic cells, the MICs of Ga(NO$_3$)$_3$ in the absence of Fe$^{3+}$ were 64 mg/L for all B. cepacia complex strains investigated. However, the addition of 50 µM Fe$^{3+}$ in the presence of 64 mg/L Ga(NO$_3$)$_3$ resulted in increased growth for all B. cepacia complex strains investigated. In sessile cells, resistance to Ga(NO$_3$)$_3$ and the extent of the protective effect of 50 µM Fe$^{3+}$ against Ga(NO$_3$)$_3$ appear to be strain-dependent: the Burkholderia cenocepacia complex strains investigated are insensitive to Ga(NO$_3$)$_3$ in the presence of 50 µM Fe$^{3+}$, whereas the presence of Fe$^{3+}$ has no protective effect for both Burkholderia multivorans strains investigated.

Conclusions: As maximal tolerable Ga$^{3+}$ levels in plasma are estimated to be ~200 µM and considering the high levels of Fe$^{3+}$ in the lungs of people with CF, our data suggest that the added value of a Ga(NO$_3$)$_3$ treatment of B. cepacia complex-infected patients may be limited.

Keywords: iron metabolism, infection, cystic fibrosis, Pseudomonas aeruginosa

Introduction

Patients with cystic fibrosis (CF) are at particular risk for respiratory infections caused by members of the Burkholderia cepacia complex. Antibiotic resistance is widespread within the B. cepacia complex and is considered a key factor in the excessive mortality observed in CF patients, following B. cepacia complex infections.¹ Bacterial biofilms can play an important role in the pathogenesis of various human infections, and one of the striking properties of sessile (biofilm-grown) cells is their increased resistance to antimicrobial agents.² It has been previously shown that many B. cepacia complex bacteria readily form biofilms in vitro and that these sessile bacteria are more resistant to antibiotics than their planktonic counterparts.³ The use of the transition metal gallium was recently proposed to treat infections caused by another important CF pathogen, Pseudomonas aeruginosa.⁴ In vitro and in vivo studies showed that Ga$^{3+}$ inhibits P. aeruginosa growth and biofilm formation, and kills planktonic and sessile cells, in part by interfering with iron signalling through the transcriptional regulator pvdS.⁴

The aim of the present study was to investigate whether Ga$^{3+}$ could also be used to inhibit growth and biofilm formation of B. cepacia complex organisms.

Materials and methods

Strains and culture conditions

The following B. cepacia complex strains were used: Burkholderia cenocepacia LMG 16656 and LMG 18828, Burkholderia multivorans LMG 18822 and LMG 18825, and Burkholderia dolosa LMG 18941

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and LMG 18943. All strains were isolated from CF patients and were obtained from the BCCM/LMG Bacteria Collection (Gent, Belgium). For comparison, we also included \textit{P. aeruginosa} reference strain PAO1. All isolates were cultured on Nutrient Agar (Oxoid, Hampshire, UK) at 37°C.

**Antibacterial susceptibility testing**

MICs of Ga(NO\textsubscript{3})\textsubscript{3} (Sigma, Bornem, Belgium) were determined using a broth microdilution method based on EUCAST Discussion Document E.Dis 5.1.\textsuperscript{3} Isolates were cultured in flat-bottomed 96-well plates (TPP, Trasadingen, Switzerland) using a chemically defined minimal salt medium (CDM) without iron.\textsuperscript{6} The microtiter plates were incubated for 20 h at 37°C. Optical densities were measured using a Wallac Victor\textsuperscript{2} multilabel microtiter plate reader (Perkin Elmer LAS, Waltham, MA, USA) (\(\lambda = 535\) nm). MICs were also determined in CDM supplemented with Fe\textsuperscript{3+} (final concentration 50 \(\mu\)M) (added as FeCl\textsubscript{3} \(\cdot\) 6H\textsubscript{2}O) (designated as CDM-Fe). The MIC was recorded as the lowest concentration that completely inhibited growth.

**Biofilm experiments**

Biofilms were formed in 96-well microtiter plates and quantified using resazurin as a marker for cell viability. In brief, wells of a round-bottomed polystyrene 96-well microtiter plate (TPP) were inoculated with 100 \(\mu\)L of medium containing 10\textsuperscript{7} cfu. Following 4 h of adhesion, the supernatant with non-adherent cells was removed from each well and plates were rinsed using 100 \(\mu\)L of 0.9% (w/v) NaCl. In the resazurin assay, a colorimetric readout of the optical density was measured using a Wallac Victor\textsuperscript{2} multilabel microtiter plate reader (Perkin Elmer LAS, Waltham, MA, USA) (\(\lambda = 535\) nm). The MIC was recorded as the lowest concentration that completely inhibited growth.

**Results and discussion**

The MICs of Ga(NO\textsubscript{3})\textsubscript{3} in CDM were 64 mg/L for all \textit{B. cepacia} complex strains tested (Figure 1). This corresponds to \(\sim\)250 \(\mu\)M Ga\textsuperscript{3+}. The addition of 50 \(\mu\)M Fe\textsuperscript{3+} (a concentration similar to the total Fe\textsuperscript{3+} concentration found in lungs of CF patients\textsuperscript{1}) in the presence of 64 mg/L Ga(NO\textsubscript{3})\textsubscript{3} resulted in increased growth for all \textit{B. cepacia} complex strains investigated, and no clear MIC of Ga(NO\textsubscript{3})\textsubscript{3} in CDM-Fe could be determined (Figure 1). The MICs observed for the \textit{B. cepacia} complex strains are higher than the concentrations required to obtain a 100-fold reduction in \textit{Rhodococcus equi} cell numbers (50 \(\mu\)M)\textsuperscript{8} and similar to the concentrations required to inhibit the growth of \textit{Mycobacterium tuberculosis} and \textit{Mycobacterium avium} complex strains in broth culture.\textsuperscript{9} For control purposes, we also included \textit{P. aeruginosa} PAO1 in our experiments. It has previously been shown that the use of low Ga\textsuperscript{3+} concentrations (2–10 \(\mu\)M) results in growth inhibition and significant killing of planktonic \textit{P. aeruginosa} PAO1 cells.\textsuperscript{7} We observed that in the absence of Fe\textsuperscript{3+}, complete inhibition of planktonic growth required at least 16 mg/L (62.5 \(\mu\)M) of Ga(NO\textsubscript{3})\textsubscript{3}. In CDM-Fe, partial growth inhibition (i.e. the optical density measured in the treated wells is less than half of the optical density in the control wells) was observed starting from a Ga(NO\textsubscript{3})\textsubscript{3} concentration of 2 mg/L (7.8 \(\mu\)M), confirming previous results\textsuperscript{4} describing that a molar ratio Fe\textsuperscript{3+}:Ga\textsuperscript{3+} of at least 5:1 is necessary to overcome the inhibiting effects of Ga\textsuperscript{3+} in \textit{P. aeruginosa} PAO1.

In order to determine whether Ga(NO\textsubscript{3})\textsubscript{3} had any activity against \textit{B. cepacia} complex biofilms, we treated these biofilms with two concentrations of Ga(NO\textsubscript{3})\textsubscript{3} (32 and 64 mg/L) in CDM or CDM-Fe for 24 h. Treatment was initiated after 4 or 28 h of biofilm formation.

**Statistical analyses**

Analysis of variance with the Scheffe post hoc test was performed using SPSS 15.0 software (SPSS, Chicago, IL, USA).

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**Figure 1.** Antibacterial activity of Ga(NO\textsubscript{3})\textsubscript{3} against planktonic cultures of \textit{B. multivorans} LMG 18822 in CDM (open squares) and Fe-CDM (filled squares), \textit{B. cenocepacia} LMG 16656 in CDM (open diamonds) and Fe-CDM (filled diamonds), and \textit{B. dolosa} LMG 18941 in CDM (open triangles) and Fe-CDM (filled triangles).
with high doses of Ga(NO$_3$)$_3$ (32 and 64 mg/L) for 24 h and determined the fraction of surviving (i.e. metabolically active) cells in the biofilm (Table 1). Treating young (4 h old) B. cepacia complex biofilms with Ga(NO$_3$)$_3$, in the absence of Fe$^{3+}$ resulted in statistically significant reductions of the number of viable cells in most cases, although there were considerable differences between strains. Except for B. multivorans LMG 18825, reductions were at least 45.2% and 45.4% when Ga(NO$_3$)$_3$ concentrations of 32 or 64 mg/L were used, respectively. Although reductions were higher when using the higher Ga(NO$_3$)$_3$ concentration for five of the six strains tested, the differences in reductions between both concentrations were only statistically significant for both B. multivorans isolates and for B. dolosa LMG 18943 (64 mg/L). It resulted in statistically significant reductions of the number of viable cells, with the highest reductions observed for B. multivorans LMG 18822 (78.3% and 80.9% for 32 and 64 mg/L, respectively) and the lowest reductions for B. multivorans LMG 18825 (31.1% and 38.1%). Similarly, as observed for the young biofilms, reductions were higher when using the higher Ga(NO$_3$)$_3$ concentration (except for B. cenocepacia LMG 18828), but this difference was only statistically significant (P < 0.001) for both B. dolosa strains. The presence of 50 μM Fe$^{3+}$ resulted in a statistically significant (P < 0.01) loss of susceptibility to Ga(NO$_3$)$_3$ (i.e. lower reductions) for both B. cenocepacia strains investigated. This protective effect was not observed in the B. multivorans and B. dolosa isolates investigated (except for B. dolosa LMG 18943, 64 mg/L). It remains to be seen whether the lack of protection against the action of Ga(NO$_3$)$_3$ by Fe$^{3+}$ is common to all B. multivorans isolates. Similarly, it is at present unclear whether the loss of susceptibility to Ga(NO$_3$)$_3$ in the presence of Fe$^{3+}$ is common to all B. cenocepacia isolates or whether this is a strain-dependent characteristic. Kaneko et al.$^4$ reported that low (0.5 μM) Ga(NO$_3$)$_3$ concentrations were sufficient to completely inhibit P. aeruginosa biofilm formation, whereas mature biofilms could be killed by treating them with higher Ga(NO$_3$)$_3$ concentrations (100 and 1000 μM). Although there was no quantification of the anti-biofilm effect (biofilm killing was assessed using propidium iodide to visualize dead cells), a close inspection of the pictures presented by Kaneko et al. suggests that at least half of the cells were dead after 24 h of treatment with 1000 μM Ga(NO$_3$)$_3$. This is roughly in agreement with the reductions observed in the present study (Table 1). The addition of iron (50 μM) to the medium resulted in a partial but significant (P < 0.01) reduction of the anti-biofilm effect of Ga(NO$_3$)$_3$ in P. aeruginosa PAO1; higher concentrations are probably required to completely restore biofilm biomass to control levels. Overall, our data confirm the results obtained by Kaneko et al.$^4$ and the minor differences observed between both studies probably relate to differences in methodology (growth medium, incubation temperature and biofilm model system).

Our data clearly indicate that biofilm-grown B. cepacia complex organisms are resistant to high levels of Ga(NO$_3$)$_3$. The exact level of resistance and the extent of the protective effect of 50 μM Fe$^{3+}$ against Ga(NO$_3$)$_3$ appear to be strain- or species-dependent. As maximal tolerable Ga$^{3+}$ levels in plasma are estimated to be ~200 μM$^{10}$ and considering the high levels of Fe$^{3+}$ in the lungs of patients with CF$^2$, our data suggest that the added value of a Ga(NO$_3$)$_3$ treatment of B. cepacia complex-infected patients may be limited, although further research is required.

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Gallium resistance in *B. cepacia* complex

Transparency declarations

None to declare.

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