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Effect of ascorbic acid on *Mycobacterium tuberculosis* biofilms

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Planktonic cultures of *Mycobacterium tuberculosis,* the bacterium responsible for the lung disease tuberculosis (TB), are highly susceptible to killing by ascorbic acid (vitamin C). As planktonically grown *M. tuberculosis* are unlikely to be representative of the bacterium during infection, we set out to determine if ascorbic acid was also antibacterial against *M. tuberculosis* growing as a biofilm. We use biofilm growth as a model for the multiple phenotypic states *M. tuberculosis* can exist in during an infection. In our experiments we employed bioluminescent *M. tuberculosis* H37Rv (BSGTB1) in which light production is a non-destructive surrogate measure of bacterial viability. Light levels were monitored before and after treatment with 1mM to 256mM ascorbic acid. After 3 weeks of treatment, biofilms were disrupted, washed and inoculated into fresh media to look for sterilisation. Our findings show that ascorbic acid concentrations of 32mM or greater reduced bioluminescence produced by *M. tuberculosis* BSGTB1 growing in biofilms to background levels and resulted in the death of all cells within the biofilm. This indicates that *M. tuberculosis* biofilms are susceptible to inhibition and killing by ascorbic acid (vitamin C) and suggests that novel antibiotics with a mode of action similar to ascorbic acid could represent a useful avenue of investigation for TB treatment.

Effect of ascorbic acid on *Mycobacterium tuberculosis* biofilms

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Abstract

Planktonic cultures of *Mycobacterium tuberculosis,* the bacterium responsible for the lung disease tuberculosis (TB), are highly susceptible to killing by ascorbic acid (vitamin C). As planktonically grown *M. tuberculosis* are unlikely to be representative of the bacterium during infection, we set out to determine if ascorbic acid was also antibacterial against *M. tuberculosis* growing as a biofilm. We use biofilm growth as a model for the multiple phenotypic states *M. tuberculosis* can exist in during an infection. In our experiments we employed bioluminescent *M. tuberculosis* H37Rv (BSGTB1) in which light production is a non-destructive surrogate measure of bacterial viability. Light levels were monitored before and after treatment with 1mM to 256mM ascorbic

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Introduction

The bacterium *Mycobacterium tuberculosis* is responsible for the lung disease tuberculosis (TB). It is estimated that 1/3 of the world's population is infected with this deadly pathogen. While TB represents a huge burden on health care systems in its own right, it also complicates other serious illnesses such as HIV/AIDS. New compounds are desperately required to shorten the current TB treatment regimes, which routinely last longer than 6 months, and to combat the rise of resistant *M. tuberculosis* strains. Drug-resistant TB leads to extended hospital stays and treatment times and, in the worst case scenario, untreatable disease.[1, 2]

Planktonic *M. tuberculosis* has been shown to be particularly susceptible to killing via the products of ascorbic acid-induced Fenton chemistry. The observed killing was dependent on high levels of ferrous iron and the production of reactive oxygen species.[3] A concentration of 1mM ascorbic acid in the presence of excess ferrous iron was shown

to be enough to inhibit the growth of *M. tuberculosis*, with sterilisation occurring within 3 weeks in the presence of 4mM ascorbic acid.

Bacterial cells present in an infected host can display a range of phenotypes and occupy several divergent physiological niches.[4, 5] These are unlikely to be accurately reflected by planktonically-grown cultures. In contrast, many of these phenotypes and niches are replicated within a biofilm, a complex community of micro-organisms sticking together on a surface. Within a biofilm, bacterial cells are more resistant to disinfection and drug treatment and therefore represent a much harder target to sterilise.[6] *M. tuberculosis* can form a type of floating biofilm called a pellicle, which has similar properties[7] and correlates better to the disease environment. The population of cells present within the biofilm can be varied, with a mixture of replicating and non-replicating cells in various nutritional states which would be expected to respond differently to antimicrobial treatment. As such the biofilm represents a useful model for disease treatment. The purpose of this study was to determine if ascorbic acid has antibacterial activity against *M. tuberculosis* growing as a biofilm.

Materials and Methods

Strains and growth conditions

The integrating plasmid pMV306hspLuxABG13CDE[8] was used to transform *M. tuberculosis* H37Rv to create a stable bioluminescent strain (BSGTB1) as previously described.[9] This strain was used to grow biofilms in sterile, black 96 well plates (Nunc) using a previously described method.[10] Briefly, *M. tuberculosis* BSGTB1 was grown for 3 weeks at 37°C with gentle shaking (100rpm) in Middlebrook 7H9 broth (Fort Richard)

supplemented with Middlebrook ADC enrichment media (Fort Richard) and glycerol. The optical density (OD) at 600nm was measured and the culture adjusted to give an OD_{600nm} of 1.0. This culture was then diluted 1:100 in modified Sauton's media (0.5g L^{-1} KH_2PO_4, 0.5g L^{-1} $MgSO_4$, 4g L^{-1} L-Asparagine, 2g L^{-1} Citric acid, 0.05g L^{-1} Ferric Ammonium Citrate, 60mL L^{-1} glycerol, 0.1% $ZnSO_4$, pH to 7.0 [chemicals were supplied by Sigma-Aldrich]) and 100µL of culture was added to each inner well of a 96 well plate. The outer wells were filled with 200 µL of sterile water to reduce evaporation from the *M. tuberculosis* containing wells. Plates were incubated for 8 weeks at 37°C.

Determination of Ascorbic acid activity

After incubation, we quantified light levels from the bioluminescent biofilms (given as Relative Light Units [RLU]) using a Victor X-3 luminometer (Perkin Elmer) and determined how much media remained in the wells; due to the long incubation time some evaporation occurs and this has to be accounted for when calculating final concentrations. We added ascorbic acid dissolved in Sauton's media in a two-fold dilution gradient at a range of concentrations (1mM- 256mM) to the BSGTB1 biofilms to determine if the ascorbic acid was able to reduce light production. Each concentration was done in triplicate, using three independent BSGTB1 cultures. To determine if changes in light levels were a result of changes in pH, biofilms were also treated with acidified Sauton's media (pH3) to correlate with the lowest pH of ascorbic acid cells were treated with. Biofilms were incubated for a further 21 days at 37°C with no shaking and light levels were measured at 7 day intervals.

To determine if the addition of ascorbic acid had sterilised the biofilms, after 3 weeks incubation the biofilms were re-suspended and washed 3 times in Sauton's media

before they were re-suspended in fresh 7H9 broth (5ml) supplemented as described above. We monitored the broths for growth for a further 8 weeks.

Results

We investigated the impact of ascorbic acid on the viability of a bioluminescent strain of *M. tuberculosis* (BSGTB1) growing as a biofilm. Luminometry demonstrated that light levels from the BSGTB1 biofilms were reduced to background levels when treated with 32mM of ascorbic acid, or greater (Fig. 1). The reduction took place within the first week of treatment and light levels remained consistently low from this point onwards. We observed no reduction in light when the acidified control media was added in place of ascorbic acid, indicating that any effect was unrelated to changes in pH. Below the critical concentration of 32mM ascorbic acid, light production from the treated biofilms slightly increased compared to the untreated control (Fig. 1).

To determine whether viable bacteria remained within the biofilms after ascorbic acid treatment, we washed the treated BSGTB1 biofilms, re-inoculated them into fresh media and monitored for growth and light production over a further 8 weeks. We observed no growth or light production for ascorbic acid concentrations of 32mM and above (Fig. 2). In contrast, we did observe growth and light production in those treated with concentrations below 32mM and the non-treated controls.

Discussion

Many infectious bacteria form biofilms within their host. Bacteria living within a biofilm are notoriously difficult to treat and can persist for extended periods of time, as they have the ability to resist the immune system,[11] display increased virulence [12, 13] and can become phenotypically more resistant to antibiotics. It is common for antibiotic concentrations required to control bacteria within biofilms to be 100 to 1000 fold greater than those needed to treat planktonic forms.[6] In contrast, we observed that *M. tuberculosis* growing as a floating biofilm, known as a pellicle, is susceptible to a concentration of ascorbic acid similar to that reported for planktonic forms. This concentration was also sufficient to cause sterilisation of the *M. tuberculosis* pellicle within 3 weeks of treatment. It is possible that the comparative ease in which test compounds can access bacteria within a pellicle, as compared to a biofilm attached to a surface, make this form of biofilm easier to kill. However we have seen with other drugs that this is not the case (unpublished data). While it is still unknown if *M. tuberculosis* forms biofilms *in vivo*, many mycobacterial species do form complex, secondary structures such as pellicles *in vitro*.[14] Researchers have also reported histological evidence for the presence of multicellular structures involving *M. tuberculosis* outside of the macrophage.[15] The biphasic response of *M. tuberculosis* infections, in which a large kill is seen early on in drug treatment followed by a marked reduction in the bactericidal activity of therapeutic agents due to phenotypic rather than genetic resistance, could also be evidence that *M. tuberculosis* is able to form biofilms *in vivo*. Such structures could act as a reservoir for drug tolerant bacilli which are responsible for the increased duration of drug treatment required in cases of TB. Regardless, the biofilm is a useful multi-phenotypic environment in which a novel compound can be tested against cells with a range of susceptibilities. The susceptibility of *M. tuberculosis*

within this model indicates that drugs based on the activity of ascorbic acid could be utilised in TB treatment and their design should be researched.

Acknowledgements

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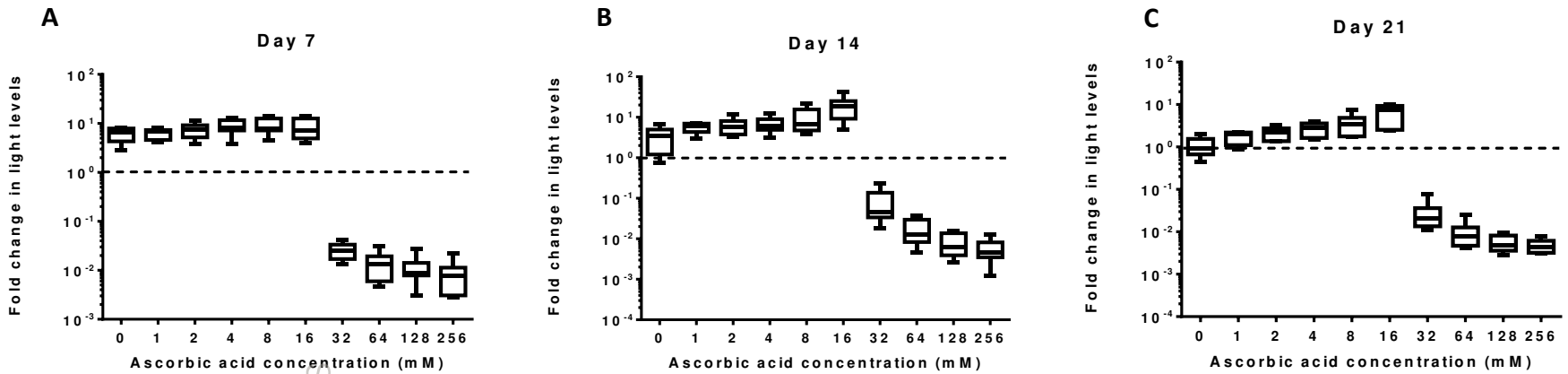

Figure 1: Treatment of mature (8 week old) *M. tuberculosis* BSGTB1 pellicles with 32mM ascorbic acid and greater results in decreased light levels (given as fold change) at days 7 (A), 14 (B) and 21 (C) post-treatment. Results are given as box whisker plots with the box representing values from the lower to upper quartile and the whiskers representing the range. Values above the dotted line indicate an increase in light levels from day 0, values below indicate a reduction.

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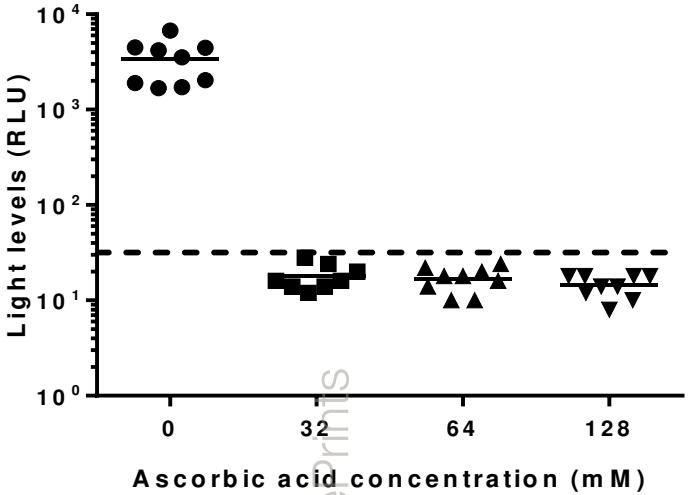

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152 **Figure 2**: Treatment of mature (8 week old) *M. tuberculosis* BSGTB1 pellicles with 32mM ascorbic acid and greater results in
153 sterilisation as indicated by a lack of bioluminescence after incubation of treated pellicles in fresh media. Values plotted here represent
154 4 weeks incubation. Cultures were monitored for 12 weeks post treatment and no light was observed from treated samples. Dotted line
155 indicates highest background level recorded.

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