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Abstract

Due to the emergence of staphylococcal biofilm infections, the need for advanced antibiotics is crucial. The aim of this investigation was to evaluate the potency and penetration of telavancin against staphylococcal biofilms using two different biofilm models. Multiple staphylococcal strains, including methicillin-sensitive *Staphylococcus aureus* ATCC 29213, vancomycin-intermediate *S. aureus* ATCC 700787, heterogeneously vancomycin-intermediate *S. aureus* ATCC 700698 and methicillin-sensitive *Staphylococcus epidermidis* ATCC 12228, were grown and treated in drip-flow reactors to determine log reductions due to telavancin treatment. After 3 days of biofilm growth and 24 h of treatment, mean log reductions for telavancin ranged from 1.65 to 2.17 depending on the bacterial strain tested. Penetration was evaluated qualitatively using confocal scanning laser microscopy to image the infiltration of fluorescently labelled antibiotic into a staphylococcal biofilm grown in a flow cell. Fluorescently labelled telavancin rapidly penetrated the biofilms with no alteration in the biofilm structure.

Keywords:

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Introduction

Multidrug-resistant staphylococcal infections have emerged as a major source both of hospital- and community-acquired infections. Healthcare-associated infections alone cost US hospitals an estimated \$45 billion annually in 2007 [1]. Many of these infections are associated with bacterial biofilms, which are difficult to eradicate [2]. Biofilm resistance to antibiotics is a result of several mechanisms, which may include failure of the agent to penetrate the full depth of the biofilm, inhibited diffusion within the biofilm, and phenotypic heterogeneity of bacteria within the biofilm [2]. As a result, the need for advanced antibiotics to treat multidrug-resistant staphylococci biofilms is critical.

Telavancin is a semisynthetic, bactericidal lipoglycopeptide. It has a core structure similar to vancomycin but is modified to include a lipophilic side chain [3]. Like vancomycin, telavancin inhibits cell wall synthesis; however, it has a second mechanism of action that disrupts bacterial cell membrane potential and permeability [4]. These two modes of action may explain why telavancin has greater bactericidal activity against staphylococci than vancomycin [4].

The effect of telavancin has been studied in a number of biofilm models. Using a Sorbarod model, telavancin exhibited substantial

antimicrobial activity against *Staphylococcus aureus* strains [5]. The Calgary Biofilm Device [6,7], 96-well flat-bottom plates, and biofilms grown on polystyrene disks inside a flow cell [7] have also been used. However, biofilm characteristics, such as cell density and antibiotic tolerance, can vary depending on the biofilm model used [8]. For example, *Staphylococcus epidermidis* ATCC 12228, used in this study, has been reported to be a non-biofilm forming strain in 96-well plate models using crystal violet assays, yet this strain readily formed biofilms in more robust biofilm culture systems such as flow cells [8]. Furthermore, despite the aforementioned telavancin studies, there are no direct experimental visualisations of the antibiotic penetrating into a biofilm, nor are there studies completed on robust biofilms grown at the solid-liquid-air interface. The aim of this project was to evaluate the potency and penetration of telavancin against staphylococcal biofilms using two different biofilm models. Quantitative analysis of potency was evaluated by plate count to determine log reductions of the antibiotic-treated biofilms relative to control biofilms grown in drip flow reactors (DFRs). Penetration was evaluated qualitatively using confocal scanning laser microscopy to image the penetration of fluorescently labelled telavancin into a biofilm grown in a flow cell.

2. Materials and methods

2.1. Quantitative analysis of telavancin potency

A quantitative analysis of antibiotic potency was performed using a Model DFR 110-4 drip flow reactor (BioSurface Technologies

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Single-species staphylococcal biofilms were initiated by conditioning the slides with 10%-strength tryptic soy broth (TSB) (Becton Dickinson & Co., Sparks, MD) for 10 min and then inoculating each channel with 1 mL of an overnight culture (10^8 CFU/mL) of the test strain. The biofilm was allowed to grow at 37°C with 10%-strength TSB medium flowing at a rate of 10 mL/h/channel for a period of 3 days. After the growth period, flow to the reactor was halted, the reactor was set to a horizontal position and 20 mL of the treatment solution was added to the appropriate channel. The treatment solution consisted of 4 mg/mL telavancin (Theravance Inc., South San Francisco, CA) in 50% acidified dimethyl sulfoxide (DMSO), diluted to 80 µg/mL in sterile water. Sterile saline was used as a negative control. Following 24 h at 37°C, the treatments were drained and the chambers were rinsed with 20 mL of additional sterile saline. The slides were removed from the reactor. The biofilms were scraped into 10 mL of Dey–Engley neutralising broth (Becton Dickinson & Co.) and were then disaggregated by 30 s of vortexing, 2 min of sonication and an additional 30 s of vortexing. The resulting bacterial suspension was then serially diluted 10-fold with phosphate-buffered saline (PBS) and was plated on tryptic soy agar (Becton Dickinson & Co.). The plates were incubated at room temperature for 24–48 h and the number of CFU was counted. Based on the dilution and surface area of the slide, the number of CFU per unit area was calculated and logarithmically (base 10) transformed. The log reduction of each treatment was then calculated relative to the saline-treated control. Each drip flow experiment was repeated four times and the results are presented as the mean ± standard deviation. Statistical analysis was performed using Minitab v.16 software (Minitab Inc., State College, PA).

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2.3. Flow cell imaging

The penetration of fluorescently labelled telavancin into staphylococcal biofilms was assessed using capillary flow cells [12]. Capillary flow cells (Model FC91; BioSurface Technologies Corp.) were inoculated with 250 µL of a 10^8 CFU/mL suspension of *S. aureus* ATCC 29213 in TSB. Biofilms were grown for 24 h with perfusion of 10%-strength TSB medium at a flow rate of 1.0 mL/min at 37°C. Following biofilm growth, the flow cells were mounted on the stage of a Leica SP5 confocal scanning laser microscope

(Leica Microsystems, Inc., Buffalo Grove, IL). Using a 63× water immersion objective, an image plane was selected near the bottom of the biofilm cluster where it was attached to the glass surface, and 108 µg/mL telavancin was introduced into the flow cell at a flow rate of 1.0 mL/min at room temperature. Images were simultaneously collected at a wavelength of 500–550 nm (e.g. green fluorescence, labelled antibiotic) and transmitted light every 3 s for 12 min. This experiment was repeated three times.

Image analysis was conducted by comparing the mean fluorescence intensity of individual biofilm clusters with the mean fluorescence intensity of the bulk fluid using MetaMorph® 7.7.0.0 image analysis software (Molecular Devices, Downingtown, PA). The resulting data sets resulted in a mean fluorescence intensity versus time for multiple biofilm clusters for both treatments. However, the photomultiplier tube settings were optimised separately for each experimental run, resulting in fluorescence intensity values that could not be directly compared. To normalise these values, the peak mean intensity value for each data set was identified and the remaining data were calculated as a percentage of the peak value. Therefore, the percentage of peak mean fluorescence versus time was determined for multiple biofilm clusters for each labelled antibiotic treatment. The collected images were also used to create time-lapse videos using Imaris x64 7.6.4 software (Bitplane AG, South Windsor, CT) (Supplementary data).

3. Results

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After 3 days of biofilm growth and 24 h of saline treatment, control biofilms had mean log densities of 8.12 ± 0.40 , 8.12 ± 0.26 , 8.08 ± 0.31 and 7.93 ± 0.16 log₁₀ CFU/cm² for *S. aureus* 29213, *S. aureus* 700698, *S. aureus* 700787 and *S. epidermidis* 12228, respectively (Fig. 1). The respective mean log densities for telavancin-treated biofilms were 5.95 ± 1.54 , 6.05 ± 1.37 , 6.38 ± 1.39 and 6.28 ± 1.38 log₁₀ CFU/cm² (Fig. 1). The log density for each telavancin group was significantly lower than its control counterpart ($P < 0.05$) using Student's *t*-test assuming one-tail distribution and unequal variances.

Mean log reductions were then calculated by subtracting the mean log density of the treated biofilm from the mean log density of the untreated control. As a result, the mean log reductions for telavancin treatments were 2.17 ± 1.37 , 2.07 ± 1.26 , 1.70 ± 1.57 and 1.65 ± 1.43 log₁₀ CFU/cm² for *S. aureus* 29213, *S. aureus* 700698, *S. aureus* 700787 and *S. epidermidis* 12228, respectively. A general linear analysis of variance (ANOVA) model with a 95% confidence interval was then fitted to the log reduction data. 'Experiment' was included as a random effect, whilst 'strain' and 'treatment' were included as fixed effects with a potential interaction. Results of this statistical analysis indicated that there was no statistically significant effect of strain ($P = 0.943$) or interaction between strain and treatment ($P = 0.871$). Thus, the effect of telavancin treatment was not strain-dependent in these experiments.

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Molar extinction coefficients determined for telavancin and FITC were determined to be 2808.1 mol⁻¹ cm⁻¹ and 72,833 mol⁻¹ cm⁻¹, respectively, and were used to determine the concentration of labelled antibiotic following purification. Optimum labelling was achieved by dissolving 5 µg in 1 mL of PBS and then adding 75 µL of a 10 mg/mL FITC solution. These solutions were prepared fresh for each experiment.

Fig. 1. Mean biofilm log densities for biofilms treated with saline (control) and telavancin (80 µg/mL). Results are the mean ± standard deviation of four separate experiments. *Significantly different from control ($P < 0.05$).

3.3. Flow cell imaging

Staphylococcus aureus 29213 formed extensive, heterogeneous biofilms in the glass capillary flow cell after 24 h of growth. Thick biofilms formed in the corners of the flow cell and also as large clusters in the middle of the tube walls. These isolated biofilms in the middle of the tube walls were used for the penetration experiments. Time-lapse images were taken of a single focal plane near the bottom of the biofilm cluster where it was attached to the glass surface every 3 s for 12 min, starting ca. 40 s prior to introducing the labelled antibiotic. The technique of capturing one image plane within the biofilm rather than an entire z-stack of the entire biofilm was selected because preliminary experiments indicated that labelled antibiotic penetration occurred rapidly. The rapid diffusion necessitated a scan rate that was not achievable if imaging the entire biofilm. Therefore, imaging one image plane was selected, using techniques similar to those previously published [12].

Using computer image analysis, a more densely packed region within the biofilm was selected to measure mean fluorescence intensity with time, and an identical region within the flow path was also identified to capture background fluorescence. Fluorescently labelled telavancin rapidly penetrated the biofilm clusters (Fig. 2) with a characteristic S-shaped curve (Fig. 3) [12,13], with the mean maximum fluorescence intensity plateauing at ca. 600 s. No alteration of biofilm structure was observed during the antibiotic exposure.

4. Discussion

Telavancin is approved in the USA and Canada for the treatment of adult patients with complicated skin and skin-structure infections due to susceptible Gram-positive pathogens. Telavancin is also approved in the USA and Europe for the treatment of hospital-acquired bacterial pneumonia and ventilator-associated bacterial pneumonia due to *S. aureus* [meticillin-resistant *S. aureus* (MRSA) only in Europe] when no other alternatives are suitable [14]. Hospital-acquired staphylococcal infections are often

associated with bacterial biofilms, which are difficult to eradicate [2]. An often-cited explanation for the failure of antibiotic biofilm treatment is the failure of the antibiotic to penetrate the biofilm [12]. The goal of this project was to visualise telavancin penetration into staphylococcal biofilms and to assess the efficacy of telavancin against robust staphylococcal biofilms.

In the DFR system, biofilms are grown on a solid surface that is continuously wetted with medium at a slow flow rate, providing low shear stress conditions at a solid-liquid-air interface [9]. Although in vitro model systems cannot replicate conditions within infected tissue, the DFR simulates slow flow of body fluids and nutrients that may flow through a biofilm-infected tissue. Furthermore, the substrate material used in this study (hydroxyapatite) is a model material for bone and certain orthopaedic implants. Previous biofilm studies have not examined the effects of telavancin on robust biofilms grown in this manner [5–7]. The robust nature of the DFR biofilm was further demonstrated by the *S. epidermidis* strain (12228) used in this study. This strain had been reported to be a ‘non-biofilm-former’ based on simple 96-well plate-based biofilm assays but readily formed biofilms in more complex systems [8] and the study described herein.

In a pharmacokinetic study, the peak plasma concentration for telavancin at the recommended daily therapeutic dosage (10 mg/kg/day) was 82.2 ± 27.3 µg/mL [15]. Therefore, 80 µg/mL telavancin was selected as the DFR treatment concentration because it aligned with the peak plasma concentration using the recommended daily therapeutic dosage. Using this concentration, the mean log reductions for telavancin treatments were 2.16 ± 1.37 , 2.07 ± 1.26 , 1.07 ± 1.57 and 1.65 ± 1.43 CFU/cm² for *S. aureus* 29213, *S. aureus* 700698, *S. aureus* 700787 and *S. epidermidis* 12228, respectively. The variability in these results are quite typical for a treatment using the DFR [10].

Whilst the DFR provided quantitative data for the potency of telavancin, it did not provide for visualisation of the drug penetrating the biofilms. The flow-cell reactor was designed for microscope imaging [12] and allowed for the visualisation of FITC-labelled telavancin penetration into staphylococcal biofilms.

Fig. 2. Combined transmitted light and fluorescence images illustrating a cross-section of *Staphylococcus aureus* ATCC 29213 biofilm and the accumulation of 108 µg/mL of fluorescently labelled telavancin with time. Both the biofilm and the bulk fluid regions used to measure mean fluorescence intensity are outlined. Time zero corresponds to the first appearance of the labelled antibiotic in the focal plane.

Fig. 3. Image analysis results showing percentage of peak fluorescence intensity versus time for *Staphylococcus aureus* ATCC 29213 biofilms treated with fluorescently labelled telavancin. Results represent the mean $\pm$ standard deviation of three individual telavancin-treated biofilms. Time zero corresponds to the first appearance of the labelled antibiotic in the focal plane.

However, the telavancin concentration utilised had to be increased compared with the concentration used in the DFR experiments. At 80 µg/mL, FITC-labelled telavancin produced a weak fluorescent signal. Increasing the concentration to 108 µg/mL produced a

stronger fluorescent signal sufficient for visualisation. This concentration was still within the peak plasma concentration range [15]. The flow-cell experiments demonstrated that telavancin rapidly penetrated the *S. aureus* 29213 biofilms in a characteristic S-shaped curve (Fig. 3). Since the DFR results (Fig. 1) revealed that telavancin produced similar results regardless of the strain tested, the other staphylococcal strains were not examined in the flow-cell system.

Biofilm resistance to antibiotics is a result of several mechanisms, which may include failure of the agent to penetrate the full depth of the biofilm, inhibited diffusion within the biofilm, and phenotypic heterogeneity of bacteria within the biofilm [2]. The study described herein presents methods to evaluate antibiotic efficacy on robust biofilms and to visualise their penetration into such biofilms. The results indicated that telavancin rapidly penetrated staphylococcal biofilms. Thus, other mechanisms must be involved in the antibiotic tolerance of these biofilms. The techniques described may prove useful in further examining the efficacy of advanced antibiotics designed to treat multidrug-resistant biofilms.

Funding: Financial support for this work was provided by Theravance, Inc. (South San Francisco, CA). Telavancin was also supplied by Theravance, Inc.

Competing interests: None declared.

Ethical approval: Not required.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijantimicag.2015.05.022>

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Fig. 1. Mean biofilm log densities for biofilms treated with saline (control) and telavancin (80 µg/mL). Results are the mean ± standard deviation of four separate experiments. *Significantly different from control ($P < 0.05$).

3.3. Flow cell imaging

Staphylococcus aureus 29213 formed extensive, heterogeneous biofilms in the glass capillary flow cell after 24 h of growth. Thick biofilms formed in the corners of the flow cell and also as large clusters in the middle of the tube walls. These isolated biofilms in the middle of the tube walls were used for the penetration experiments. Time-lapse images were taken of a single focal plane near the bottom of the biofilm cluster where it was attached to the glass surface every 3 s for 12 min, starting ca. 40 s prior to introducing the labelled antibiotic. The technique of capturing one image plane within the biofilm rather than an entire z-stack of the entire biofilm was selected because preliminary experiments indicated that labelled antibiotic penetration occurred rapidly. The rapid diffusion necessitated a scan rate that was not achievable if imaging the entire biofilm. Therefore, imaging one image plane was selected, using techniques similar to those previously published [12].

Using computer image analysis, a more densely packed region within the biofilm was selected to measure mean fluorescence intensity with time, and an identical region within the flow path was also identified to capture background fluorescence. Fluorescently labelled telavancin rapidly penetrated the biofilm clusters (Fig. 2) with a characteristic S-shaped curve (Fig. 3) [12,13], with the mean maximum fluorescence intensity plateauing at ca. 600 s. No alteration of biofilm structure was observed during the antibiotic exposure.

4. Discussion

Telavancin is approved in the USA and Canada for the treatment of adult patients with complicated skin and skin-structure infections due to susceptible Gram-positive pathogens. Telavancin is also approved in the USA and Europe for the treatment of hospital-acquired bacterial pneumonia and ventilator-associated bacterial pneumonia due to *S. aureus* [meticillin-resistant *S. aureus* (MRSA) only in Europe] when no other alternatives are suitable [14]. Hospital-acquired staphylococcal infections are often

associated with bacterial biofilms, which are difficult to eradicate [2]. An often-cited explanation for the failure of antibiotic biofilm treatment is the failure of the antibiotic to penetrate the biofilm [12]. The goal of this project was to visualise telavancin penetration into staphylococcal biofilms and to assess the efficacy of telavancin against robust staphylococcal biofilms.

In the DFR system, biofilms are grown on a solid surface that is continuously wetted with medium at a slow flow rate, providing low shear stress conditions at a solid-liquid-air interface [9]. Although in vitro model systems cannot replicate conditions within infected tissue, the DFR simulates slow flow of body fluids and nutrients that may flow through a biofilm-infected tissue. Furthermore, the substrate material used in this study (hydroxyapatite) is a model material for bone and certain orthopaedic implants. Previous biofilm studies have not examined the effects of telavancin on robust biofilms grown in this manner [5–7]. The robust nature of the DFR biofilm was further demonstrated by the *S. epidermidis* strain (12228) used in this study. This strain had been reported to be a 'non-biofilm-former' based on simple 96-well plate-based biofilm assays but readily formed biofilms in more complex systems [8] and the study described herein.

In a pharmacokinetic study, the peak plasma concentration for telavancin at the recommended daily therapeutic dosage (10 mg/kg/day) was 82.2 ± 27.3 µg/mL [15]. Therefore, 80 µg/mL telavancin was selected as the DFR treatment concentration because it aligned with the peak plasma concentration using the recommended daily therapeutic dosage. Using this concentration, the mean log reductions for telavancin treatments were 2.16 ± 1.37 , 2.07 ± 1.26 , 1.07 ± 1.57 and 1.65 ± 1.43 CFU/cm² for *S. aureus* 29213, *S. aureus* 700698, *S. aureus* 700787 and *S. epidermidis* 12228, respectively. The variability in these results are quite typical for a treatment using the DFR [10].

Whilst the DFR provided quantitative data for the potency of telavancin, it did not provide for visualisation of the drug penetrating the biofilms. The flow-cell reactor was designed for microscope imaging [12] and allowed for the visualisation of FITC-labelled telavancin penetration into staphylococcal biofilms.

Fig. 2. Combined transmitted light and fluorescence images illustrating a cross-section of *Staphylococcus aureus* ATCC 29213 biofilm and the accumulation of 108 µg/mL of fluorescently labelled telavancin with time. Both the biofilm and the bulk fluid regions used to measure mean fluorescence intensity are outlined. Time zero corresponds to the first appearance of the labelled antibiotic in the focal plane.

Fig. 3. Image analysis results showing percentage of peak fluorescence intensity versus time for *Staphylococcus aureus* ATCC 29213 biofilms treated with fluorescently labelled telavancin. Results represent the mean $\pm$ standard deviation of three individual telavancin-treated biofilms. Time zero corresponds to the first appearance of the labelled antibiotic in the focal plane.

However, the telavancin concentration utilised had to be increased compared with the concentration used in the DFR experiments. At 80 µg/mL, FITC-labelled telavancin produced a weak fluorescent signal. Increasing the concentration to 108 µg/mL produced a

stronger fluorescent signal sufficient for visualisation. This concentration was still within the peak plasma concentration range [15]. The flow-cell experiments demonstrated that telavancin rapidly penetrated the *S. aureus* 29213 biofilms in a characteristic S-shaped curve (Fig. 3). Since the DFR results (Fig. 1) revealed that telavancin produced similar results regardless of the strain tested, the other staphylococcal strains were not examined in the flow-cell system.

Biofilm resistance to antibiotics is a result of several mechanisms, which may include failure of the agent to penetrate the full depth of the biofilm, inhibited diffusion within the biofilm, and phenotypic heterogeneity of bacteria within the biofilm [2]. The study described herein presents methods to evaluate antibiotic efficacy on robust biofilms and to visualise their penetration into such biofilms. The results indicated that telavancin rapidly penetrated staphylococcal biofilms. Thus, other mechanisms must be involved in the antibiotic tolerance of these biofilms. The techniques described may prove useful in further examining the efficacy of advanced antibiotics designed to treat multidrug-resistant biofilms.

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Ethical approval: Not required.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijantimicag.2015.05.022>

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