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Subinhibitory concentrations of tedizolid potently inhibit extracellular toxin production by methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*

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Abstract

Purpose. Potent extracellular toxins including alpha-haemolysin, Panton–Valentine leukocidin (PVL) and toxic-shock syndrome toxin 1 (TSST-1) significantly contribute to *Staphylococcus aureus* pathogenesis, thus, toxin suppression is a primary focus in treatment of staphylococcal disease. *S. aureus* maintains complex strategies to regulate toxin expression and previous data have demonstrated that subinhibitory concentrations of beta-lactam antibiotics can adversely increase *S. aureus* exotoxin production. The current study evaluates the effects of subinhibitory concentrations of tedizolid, a second-generation oxazolidinone derivative, on expression of staphylococcal exotoxins in both methicillin-resistant and methicillin-sensitive *S. aureus*.

Methodology. *S. aureus* exotoxin expression levels were compared at 12 and 24 h following treatment with tedizolid, linezolid, nafcillin or vehicle control.

Results. Our findings show that the level of antibiotic required to alter toxin production was strain-dependent and corresponds with the quantity of toxin produced, but both tedizolid and linezolid could effectively reduce expression of alpha-haemolysin, PVL and TSST-1 toxin at subinhibitory concentrations. In contrast, nafcillin showed less attenuation and, in some *S. aureus* strains, led to an increase in toxin expression. Tedizolid consistently inhibited toxin production at a lower overall drug concentration than comparator agents.

Conclusion. Together, our data support that tedizolid has the potential to improve outcomes of infection due to its superior ability to inhibit *S. aureus* growth and attenuate exotoxin production.

INTRODUCTION

Staphylococcus aureus is a prevalent human pathogen capable of causing a wide range of diseases. The versatility of *S. aureus* as a pathogen is attributable, in part, to an assortment of virulence factors. Of these, *S. aureus* secreted toxins such as alpha-haemolysin, Panton–Valentine leukocidin (PVL) and toxic-shock syndrome toxin 1 (TSST-1) can contribute to infection in general and necrotizing fasciitis, haemorrhagic necrotizing pneumonia and toxic-shock syndrome, specifically [1–3]. Staphylococcal exotoxins can exert local effects, causing cellular injury and tissue necrosis, and systemically, causing life-threatening inflammatory disorders. Toxin neutralization studies, highlighting reduced cytopathic effects and improved outcomes [4–12], confirm the fundamental role that staphylococcal toxins play in pathogenesis of life-threatening infections.

Expression of staphylococcal exotoxins is controlled in response to organism growth, density and environmental cues [13–15]. During antibiotic therapy, pathogens encounter a range of antibiotic concentrations and may be exposed to subinhibitory levels dependent upon multiple factors including resistance by the pathogen or antibiotic penetration into the environmental niche (e.g. biofilm or host tissue). Our laboratory and others have demonstrated that subinhibitory doses of beta-lactam antibiotics can adversely increase and prolong *S. aureus* toxin production [16–18]. Additional studies have

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Keywords: *Staphylococcus aureus*; subinhibitory; antibiotic; bacterial; exotoxin.

Abbreviations: ABSSSI, acute bacterial skin and skin structure infections; AUC, area under the curve; HLA, alpha-haemolysin; MIC, minimal inhibitory concentration; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*; PVL, Panton-Valentine leucocidin; TSST-1, toxic-shock syndrome toxin 1.

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One supplementary table and three supplementary figures are available with the online version of this article.

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Table 1. Characteristics of *S. aureus* strains used in this study

S. aureus strain*	MIC (μg ml^{-1})†			Toxin profile‡		
	Nafcillin	Linezoild	Tedizolid	PVL	TSST-1	HLA
FPR3757 (MRSA)	6.3	2.0	0.5	+	−	+
Strain 1560 (MRSA)	12.5	4.0	0.5	+	−	+
CDC 368-04 (MRSA)	12.5	4.0	0.5	−	+	+
04-002 (MSSA)	0.8	2.0	0.5	−	+	+

**S. aureus* strains used in the present study.

†MIC for each antibiotic as determined by broth microdilution method (see Methods).

‡*S. aureus* toxins: PVL, Panton–Valentine leukocidin; TSST-1, toxic-shock syndrome toxin 1; HLA, alpha-haemolysin; −, negative; +, positive.

shown phenotypic and genotypic changes that result from exposure to subinhibitory concentrations of different classes of antibiotics [19]. Together, these findings demonstrate the significant ability of antibiotics to modify bacterial metabolism, biofilm formation and toxin production that influence bacterial virulence, underscoring a potentially detrimental impact on disease outcome.

Tedizolid, a novel second-generation oxazolidinone approved for treatment of acute bacterial skin and skin structure infections (ABSSSI), demonstrates potent antimicrobial activity against *S. aureus* with reduced selection for antibiotic resistance [20–22]. While previous studies support that protein synthesis inhibitors, such as oxazolidinones, can suppress production of alpha-haemolysin, PVL and TSST-1 [17, 18, 23, 24], others have shown that subinhibitory concentrations of protein synthesis inhibitors trigger a stress response and paradoxically induce bacterial virulence [25–27]. The study described herein examines the effects of tedizolid on four pathogenic *S. aureus* strains to determine how subinhibitory exposure influences *S. aureus* exotoxin production.

METHODS

Bacterial strains and culture

Strains of *S. aureus* used in the current study were as follows: *S. aureus* FPR3757 (an MRSA USA300 isolate [28]), *S. aureus* 1560 (an MRSA USA400 isolate [29]), *S. aureus* CDC 368–04 (an MRSA isolate that produces TSST-1 but not PVL), *S. aureus* 04–002 (an MSSA isolate from a patient with STSS [30]) and *S. aureus* ATCC 29213 (a reference strain for antibiotic susceptibility testing). All *S. aureus* strains were plated on blood agar medium (5 % sheep blood; Hardy Diagnostics, Santa Maria, CA) and cultured in cation-adjusted Mueller–Hinton II broth (Becton Dickinson, Franklin Lakes, NJ) for growth and analysis.

MIC determination

Antibiotics used in the current study were: tedizolid (Merck, Kenilworth, NJ), linezolid (Pfizer, New York, NY), and nafcillin (Bristol-Myers Squibb, New York, NY). Antibiotics were resuspended in Mueller–Hinton media (nafcillin, linezolid) or DMSO (tedizolid) prior to subsequent dilution in Mueller–Hinton media. DMSO (0.3 %) was included as a vehicle control for tedizolid. Susceptibility of *S. aureus* strains to individual antibiotics was determined using the broth microdilution method, in accordance with the Clinical and Laboratory Standards Institute guidelines. Briefly, isolated colonies were used to inoculate overnight cultures. The next day, *S. aureus* were sub-cultured and grown at 37 °C in 5 % CO_2 with shaking (200 r.p.m.) to mid-log phase. Bacteria were harvested by centrifugation, washed in sterile saline and seeded in 96-well flat-bottom plates at 5×10^5 c.f.u. ml^{-1} in the presence of antibiotic. Plates were incubated at 37 °C in 5 % CO_2 for 24 h at which point growth inhibition was determined.

Antibiotics' effects on *S. aureus* growth and toxin production

To monitor *S. aureus* growth, isolated colonies were used to inoculate overnight cultures. Log-phase cultures (0.3–0.4 AU) were diluted to a concentration of 5×10^5 c.f.u. ml^{-1}, at which time individual antibiotics were added (time=0 h). Cultures were maintained at 37 °C in 5 % CO_2 with shaking (200 r.p.m.). Viable bacterial counts were determined at 3, 6, 9, 12 and 24 h following the introduction of antibiotics by dilution plating on blood agar plates. Samples were collected at 12 and 24 h for quantitation and subsequent analysis of the effects of antibiotics on toxin production.

PVL and TSST-1 toxin quantification

Supernatants from individual 10 ml cultures were filter-sterilized (0.22 μm) and stored at −80 °C for toxin protein assays. PVL (LukS) and TSST-1 were quantified from
S. aureus culture supernatants by ELISA. For PVL, EIA/RIA plates were coated with 1 μg ml^{-1} anti-PVL mAb 1D9 (IBT Bioservices, Gaithersburg, MD) in 50 mM carbonate buffer (pH 9.6). Capture antibody was removed and plates were blocked with PBS+5 % skim milk overnight at 4 °C. LukS-PV standard (0.8–50 ng ml^{-1}; IBT Bioservices) and diluted culture supernatants (1 : 200 to 1 : 800) were applied to ELISA plates for 2 h at 37 °C. Plates were washed with PBS-Tween 20 (0.05 %) and successively incubated with 0.25 μg ml^{-1} rabbit polyclonal anti-PVL (LukS) (IBT Bioservices) and HRP-linked anti-rabbit IgG (H+L) (Cell Signaling, Danvers, MA) and assays were developed with 1-step Ultra TMB (Life

Technologies, Grand Island, NY). Recombinant LukF-PV (IBT Bioservices) was undetectable by these methods, indicating that our PVL immunoassay was specific to LukS-PV. For TSST-1, EIA/RIA plates were coated with 5 μg ml^{-1} anti-TSST-1 affinity purified sheep antisera (Toxin Technology, Sarasota, FL). Capture antisera was removed and plates were blocked in PBS+5 % skim milk overnight at 4 °C. TSST-1 standard (0.15–20 ng ml^{-1}; Toxin Technology) and diluted culture supernatants (1 : 2000 to 1 : 8000) were applied to ELISA plates for 2 h at 37 °C. Plates were washed with PBS-Tween 20 (0.05 %) and incubated with a 1 : 1200 dilution of HRP-conjugated anti-TSST-1 sheep antisera (Toxin Technology) followed by development with 1-step Ultra TMB. Notably, *S. aureus* Protein A did not interfere with either immunoassay at concentrations <500 ng ml^{-1}.

Alpha-haemolysin analysis

Alpha-haemolysin activity was assayed by a standard rabbit erythrocyte lysis assay, as described previously [18]. In brief, sterile-filtered culture supernatants were diluted twofold in DPBS in a microtitre plate and an equal volume of washed rabbit erythrocytes (2 % in DPBS) was added. Sterile deionized water was included as a 100 % haemolysis control. After incubation for 1 h at 37 °C, plates were centrifuged, supernatants were transferred to a new microtitre plate, and the absorbance was read at 550 nm. Activity (haemolytic units ml^{-1}) was defined as the inverse of the dilution causing 50 % haemolysis, multiplied by 2.

Statistical analysis

For each experimental condition, a minimum of three independent biological replicates were collected and tested. The concentration of toxin was compared between antibiotic and control treatment for each antibiotic using a one-way ANOVA followed by Dunnett's multiple comparisons test (GraphPad Prism 7.03; GraphPad Software, La Jolla, CA).

RESULTS

S. aureus growth was monitored following the addition of sub-MIC concentrations of antibiotics at the mid-logarithmic stage of growth. The MICs for antibiotics used in this study are provided in Table 1.Notably, tedizolid inhibited growth at concentrations four to eightfold lower than that of linezolid in each *S. aureus* isolate. Dose-dependent effects on growth were observed for each antibiotic tested (Figs 1, S1–S3, available in the online version of this article). While 1/16× MIC and 1/8× MIC concentrations of antibiotics resulted in a lag in exponential bacterial growth, cultures still grew to densities comparable to that of vehicle control by 24 h. Higher antibiotic doses, particularly 1/2× MIC and 1× MIC, had a detrimental effect on bacterial growth and impaired their ability to reach densities detected in the control treatment at 24 h (Fig. 1 and Table S1). Due to the impact of higher antibiotic concentrations on bacterial density, the 1/16× MIC, 1/8× MIC, 1/4× MIC and vehicle control treatments were selected for analysis of subinhibitory antibiotic effects on *S. aureus* toxin production. *S. aureus* cultures treated with subinhibitory antibiotics, or vehicle control, were evaluated at late-log/early stationary (12 h) and stationary (24 h) phases of growth for effects on toxin expression.

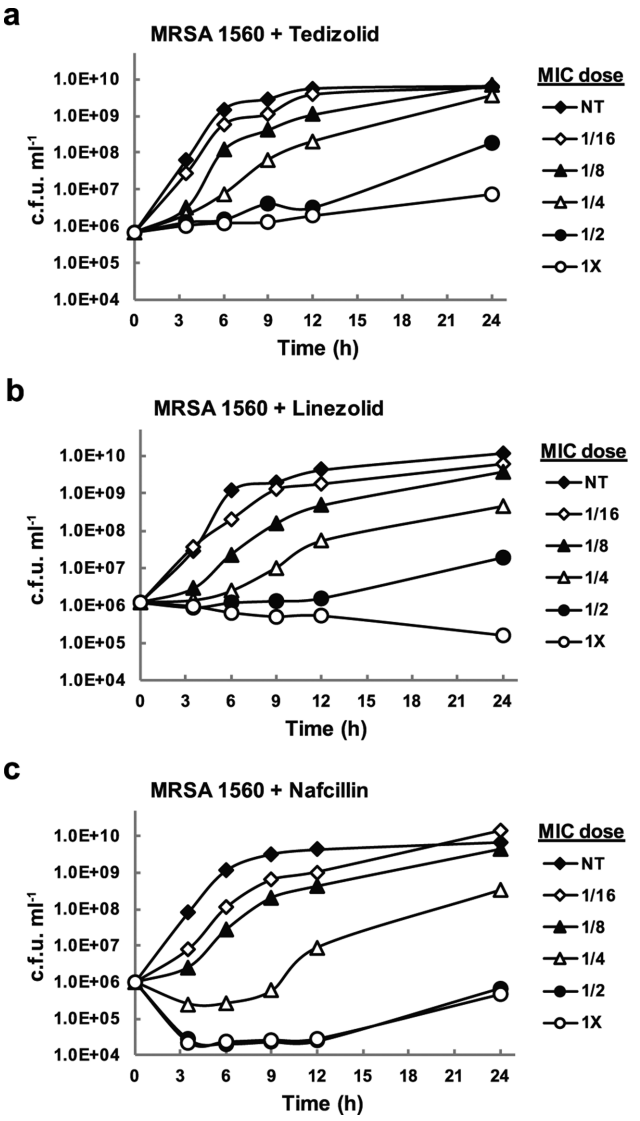

Fig. 1. Effect of sub-MIC antibiotics on *S. aureus* growth. The growth of methicillin-resistant *S. aureus* strain 1560 was monitored over time in the presence of varying concentrations of antibiotic. Bacteria were grown in Mueller–Hinton II broth containing (a) tedizolid, (b) linezolid or (c) nafcillin at 1/16X, 1/8×, 1/4×, 1/2× or 1× the MIC. Culture samples were collected at 0, 3, 6, 9, 12 and 24 h for bacterial quantitation. NT, no treatment control.

PVL, a bicomponent toxin encoded by staphylococcal bacteriophage, targets neutrophils, macrophages and monocytes [31, 32] and has been epidemiologically linked to severe staphylococcal infections [33, 34]. Recent studies have demonstrated the importance of PVL in the pathogenesis of *S. aureus* pneumonia and skin infection [35–37]. Consequently, we chose to assess levels of PVL toxin in response to subinhibitory antibiotic treatment. PVL expression was

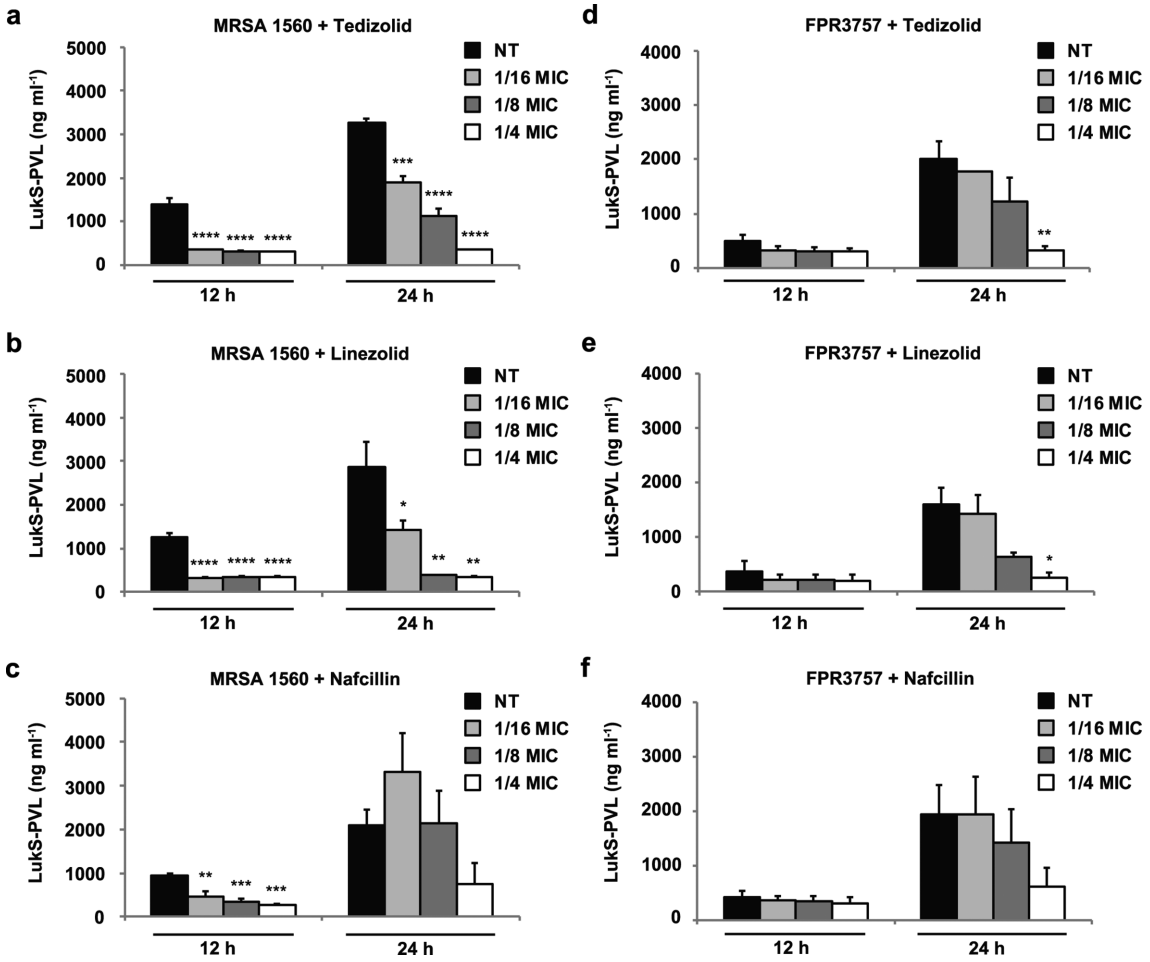

Fig. 2. Effects of tedizolid and comparator antibiotics on *S. aureus* PVL toxin expression. *S. aureus* strains were cultured in the presence of 1/16× MIC, 1/8× MIC, 1/4× MIC or vehicle control for 12 and 24 h. Levels of LukS-PVL were quantified from filter-sterilized culture supernatants by ELISA. Each panel shows LukS-PVL expression levels following treatment with the indicated antibiotic in (a–c) MRSA 1560 and in (d–f) *S. aureus* FPR3757. Bars represent average toxin expression±standard error from three biological replicates. Asterisks denote statistical significance of (*) $P \leq 0.05$, (**) $P \leq 0.01$, (***) $P \leq 0.001$ and (****) $P \leq 0.0001$ compared to the no treatment (NT) vehicle control at each corresponding timepoint.

monitored by indirect ELISA of the LukS-PVL subunit (Fig. 2). Both tedizolid and linezolid suppressed expression of LukS-PVL in MRSA 1560 by 12 h at 1/16× the MIC (Fig. 2a, b). While 1/8× MIC, 1/16× MIC and 1/4× MIC showed similar effects at 12 h, dose-dependent suppression of LukS-PVL was more evident at 24 h, when toxin expression was elevated. In contrast to treatments with tedizolid or linezolid, nafcillin failed to show consistent attenuation of toxin expression (Fig. 2c). To confirm these findings, PVL expression was evaluated in a second *S. aureus* strain following antibiotic exposure (Fig. 2d–f). While *S. aureus* FPR3757 expressed slightly less LukS-PVL, compared to MRSA 1560, higher doses of oxazolidinone antibiotics (i.e. 1/8v× MIC) were required for reduced toxin expression in *S. aureus* FPR3757. Together, these findings demonstrate that subinhibitory concentrations of tedizolid and linezolid effectively inhibit PVL expression in multiple strains of *S. aureus*. While strain-specific dosage effects were observed, no adverse induction of PVL toxin was detected in response to subinhibitory levels of oxazolidinone antibiotics.

Next, levels of TSST-1 toxin were evaluated from *S. aureus* clinical isolates following exposure to antibiotic treatment (Fig. 3). Encoded by a mobile pathogenicity island, TSST-1 is expressed during post-exponential growth and stimulates the release of large amounts of cytokines causing severe inflammation with fever, widespread effects on the vascular system and shock [1]. Subinhibitory levels of both tedizolid and linezolid suppressed expression of TSST-1 in *S. aureus* CDC 368–04 at 12 and 24 h (Fig. 3a, b). In MSSA 04–002, a strain that produces elevated levels of TSST-1, dose-dependent effects were also evident following treatment with subinhibitory concentrations of tedizolid and linezolid

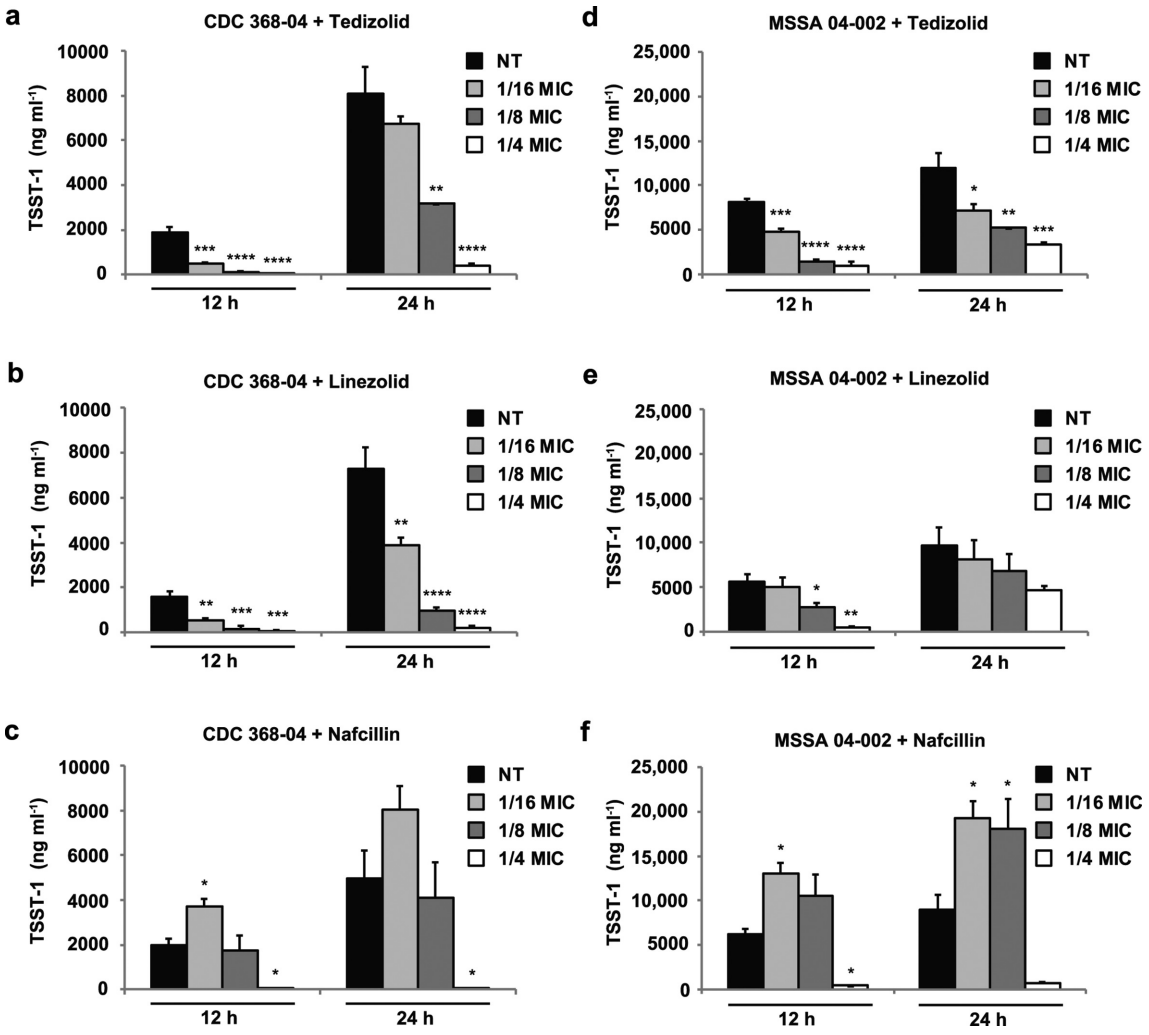

Fig. 3. Effects of tedizolid and comparator antibiotics on *S. aureus* TSST-1 toxin expression. Levels of TSST-1 were quantified from culture supernatants by ELISA. Each panel shows TSST-1 expression levels following treatment with the indicated antibiotic in (a–c) CDC 368–04 and in (d–f) MSSA 04–002. Bars represent average toxin expression±standard error from three biological replicates. Asterisks denote statistical significance of (*) $P \leq 0.05$, (**) $P \leq 0.01$, (***) $P \leq 0.001$ and (****) $P \leq 0.0001$ compared to the no treatment (NT) vehicle control at each corresponding timepoint.

(Fig. 3d, e). Interestingly, nafcillin exposure at 1/16× the MIC considerably increased TSST-1 toxin expression in both *S. aureus* strains (Fig. 3c, f). A 1/8× MIC nafcillin concentration also sustained increased toxin expression in MSSA 04–002, in comparison to vehicle control.

Lastly, alpha-haemolysin toxin activity was assayed from all four *S. aureus* strains in response to antibiotic treatment (Fig. 4). Part of the core genome, alpha-haemolysin encodes a pore-forming toxin that acts as one of the major virulence determinants in staphylococcal pathogenesis [38–40]. Consistent with the findings for PVL and TSST-1, alpha-haemolysin activity decreased in a dose-dependent manner upon treatment with subinhibitory levels of tedizolid and linezolid, particularly at 12 h (Fig. 4a–b, d–e). *S. aureus* FPR3757 represents a high alpha-haemolysin producer and antibiotic inhibition was most pronounced with lower toxin expression observed at 12 h. *S. aureus* 04–002 represents a low alpha-haemolysin producer and 1/16× MIC of tedizolid was sufficient to reduce alpha-haemolysin toxin activity at both 12 and 24 h. Notably, subinhibitory concentrations of nafcillin significantly increased haemolytic activity in MSSA 04–002 (Fig. 4f), but not *S. aureus* FPR3757 (Fig. 4c), MRSA 1560 or CDC 368–04 (data not shown). Taken together, our results provide evidence that subinhibitory concentrations of tedizolid and linezolid effectively reduce staphylococcal toxin expression while beta-lactam antibiotics, such as nafcillin, have less predictable effects and may adversely increase toxin expression.

DISCUSSION

Antibiotics are some of the most well-studied natural and synthetic compounds. With their prevalent usage, concerns have been raised regarding "off-target" bacterial responses. At high concentrations, antimicrobial agents lead to bactericidal or bacteriostatic effects, whereas exposure to lower concentrations of antibiotics can result in genetic and phenotypic variations that enable adaptation and alter bacterial pathogenicity [19, 41]. Previous studies documenting the influence of subinhibitory antibiotic exposure on biofilm formation, the bacterial stress response and toxin production in *S. aureus* [16–18, 26, 42] highlight adverse effects of antibiotics that underscore a potentially detrimental impact on disease outcome.

The contributions of staphylococcal toxins and other secreted factors to human disease are well-recognized. As such, protein-synthesis inhibitors are expected to have a distinct advantage in treating toxigenic *S. aureus* infections by attenuating toxin production and virulence factor expression. Studies in our laboratory have shown that clindamycin and linezolid inhibit *S. aureus* toxin production *in vitro* and improve the clinical response to staphylococcal toxic shock syndrome [30]. Intriguingly, others have shown that subinhibitory concentrations of protein synthesis inhibitors can induce bacterial virulence [25–27]. The current study demonstrates that tedizolid effectively inhibits *S. aureus* exotoxin production, thus, reducing the likelihood of an adverse *S. aureus* toxigenic response. While, we would expect expression of other virulence factors

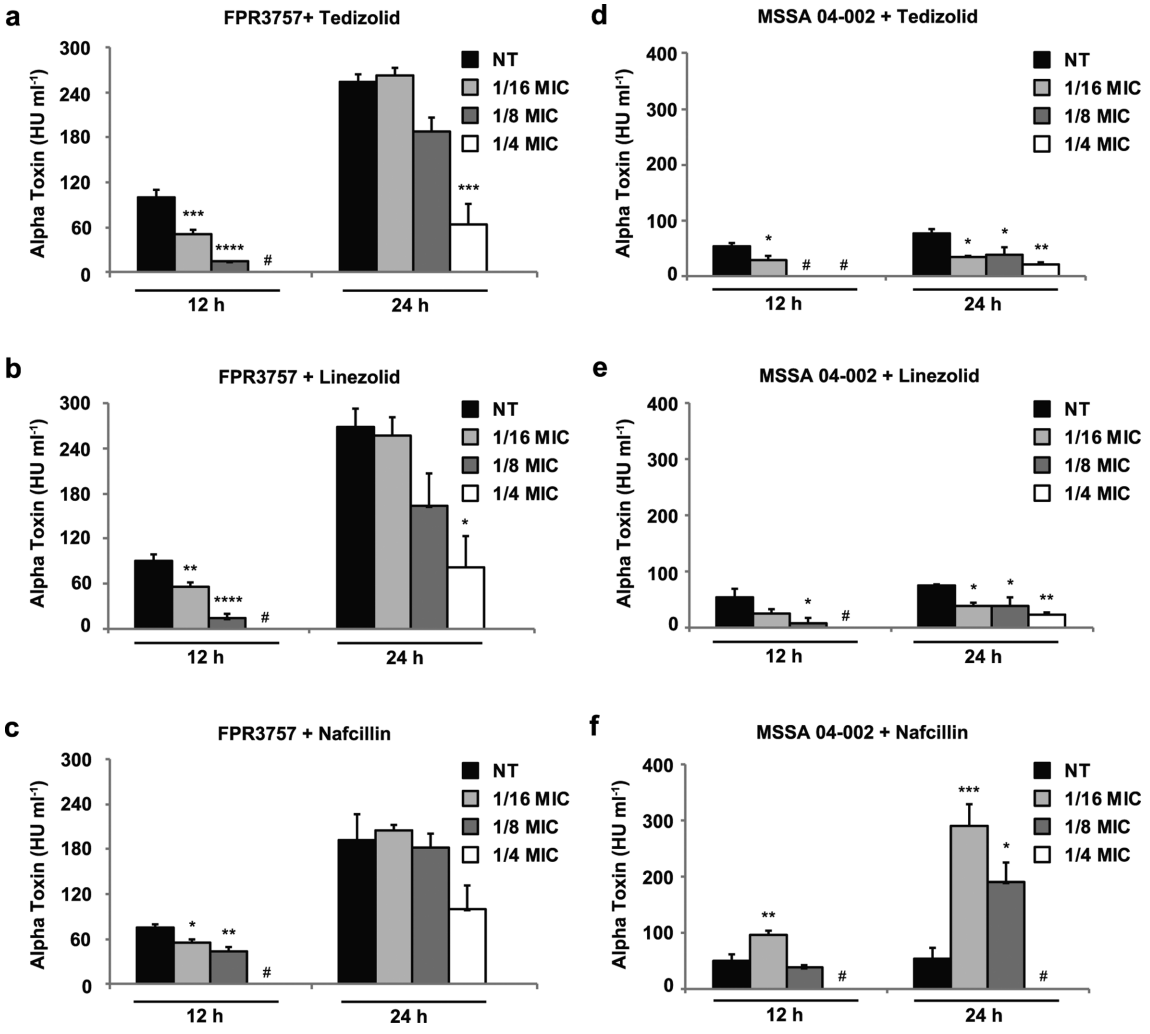

Fig. 4. Effects of tedizolid and comparator antibiotics on *S. aureus* alpha-haemolysin activity. Alpha-haemolysin activity was quantified from culture supernatants by rabbit erythrocyte lysis assay. Each panel shows alpha-haemolysin activity following treatment with the indicated antibiotic in (a–c) *S. aureus* FPR3757 and in (d–f) MSSA 04–002. Bars represent average toxin expression±standard error from three biological replicates. Asterisks denote statistical significance of (*) $P \leq 0.05$, (**) $P \leq 0.01$, (***) $P \leq 0.001$ and (****) $P \leq 0.0001$ compared to the no treatment (NT) vehicle control at each corresponding timepoint. A hash mark (#) indicates that no toxin was detected.

to be inhibited by subinhibitory concentrations of tedizolid as well, further analysis is required to assess the global impact of tedizolid on *S. aureus*.

Notably, toxin expression levels varied among individual *S. aureus* isolates and our findings demonstrate that subinhibitory concentrations of antibiotics affected toxin expression in a strain-dependent manner. For example, suppression of alpha-haemolysin in *S. aureus* FPR3757 requires higher levels of tedizolid than *S. aureus* 04–002. Similarly, low-dose nafcillin did not induce expression of alpha-haemolysin in *S. aureus* FPR3757, likely due to already high endogenous expression. Overall, the evaluation of a larger collection of *S. aureus* strains might better establish effects of tedizolid on toxin expression. While our understanding of mechanisms governing variable endogenous toxin expression among *S. aureus* isolates remains incomplete, subinhibitory concentrations of tedizolid and linezolid consistently suppressed toxin expression in different isolates and throughout different stages of *S. aureus* growth.

The current study demonstrates effective toxin suppression by tedizolid *in vitro*. While the effects of sub-MIC oxazolidinone, or beta-lactam, antibiotics have not been directly evaluated during experimental *S. aureus* infection, evidence from other infection models support that low-dose antibiotic effects are relevant during bacterial infection. Low-dose ciprofloxacin has been demonstrated to prime uropathogens for adherence and invasion and exacerbate chronic infection [43]. Similarly, chloramphenicol and erythromycin have been shown to modulate *Acinetobacter baumannii* capsule production providing increased resistance to killing during systemic disease [44]. Thus, conducting such *in vivo* studies for *S. aureus* in the future would validate the ability of tedizolid to inhibit toxin expression over a range of antibiotic concentrations.

Overall, the mechanism of action of antibiotics remains a prime factor in treating *S. aureus* and other toxin-related infections. Namely, protein synthesis inhibitors have proven superior to cell wall-active antibiotics for treatment of clostridial gas gangrene and group A streptococcus soft tissue infections using conventional antibiotic doses [45–47]. Linezolid has been shown to significantly reduce staphylococcal toxin expression *in vivo* [30, 48] and Le *et al.* recently demonstrated that inhibitory concentrations of tedizolid ($AUC_{0\text{-}24}$; $14.9\pm1.6\ \mu g{\cdot}h\ ml^{-1}$) suppressed alpha-haemolysin and PVL production in a necrotizing pneumonia model, resulting in reduced bacterial burden and increased survival [49]. Our findings add convincing evidence that tedizolid can potently reduce staphylococcal toxin expression at subinhibitory concentrations, contrasting effects observed with beta-lactam antibiotics. As concerns grow regarding the emergence of clindamycin and linezolid resistance, tedizolid offers a compelling treatment alternative for ABSSSI to improve outcomes for *S. aureus* and other toxin-related infections.

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Conflicts of interest
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