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Tigecycline Induction of Phenol-Soluble Modulins by Invasive Methicillin-Resistant *Staphylococcus aureus* Strains

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We examined the effects of tigecycline on three types of exoproteins, α-type phenol-soluble modulins (PSMα$_1$ to PSMα$_4$), α-hemolysin, and protein A, in 13 methicillin-resistant *Staphylococcus aureus* isolates compared to those of clindamycin and linezolid. Paradoxical increases in PSMαs occurred in 77% of the isolates with tigecycline at 1/4 and 1/8 MICs and clindamycin at 1/8 MIC compared to only 23% of the isolates with linezolid at 1/8 MIC. Induction was specific to PSMα$_1$ to PSMα$_4$, as protein A and α-hemolysin production was decreased under the same conditions by all of the antibiotics used.

Methicillin-resistant *Staphylococcus aureus* (MRSA) virulence in pneumonia and bacteremia has been attributed to exoproteins, specifically, α-hemolysin (Hla) and α-type phenol-soluble modulins (PSMα$_1$ to PSMα$_4$), which are produced by nearly all *S. aureus* strains and in excess by community-acquired MRSA strains (1). These exoproteins not only cause direct damage to target host cells but also exacerbate the host inflammatory response, contributing to acute lung injury. Additionally, recent reports have demonstrated the importance of protein A (Spa) in invasive diseases such as pneumonia (2, 3).

In light of the impressive arsenal of virulence factors contributing to the success of MRSA as a pathogen, it is of keen interest to determine if anti-MRSA agents belonging to the antibiotic class of protein synthesis inhibitors provide the added antivirulence benefit of exoprotein inhibition. In the present study, we investigated the antivirulence potential of tigecycline, linezolid, and clindamycin which have been proven efficacious in the treatment of MRSA infections. Our goal was to determine whether antivirulence effects can be generalized across different clinical isolates, different agents that inhibit protein synthesis, and different exoproteins. Specifically, we tested the effects of the above three antibiotics at subinhibitory concentrations on formylated PSMα$_1$ to PSMα$_4$, Hla, and Spa production by invasive MRSA isolates.

Eleven invasive MRSA isolates were tested under the following conditions. A modified CLSI broth macrodilution assay was used to determine MICs after 24 h of incubation at 37°C and shaking at 250 rpm in tryptic soy broth. Supernatants were then analyzed by liquid chromatography-tandem mass spectrometry, and Hla and Spa were analyzed by Western blotting as previously described (4, 5). Measured exotoxin values were normalized to the cell optical density at 600 nm (OD$_{600}$) at the time the supernatant was harvested. PSMα concentrations under various test conditions were compared by analysis of variance with Dunn's correction using GraphPad Prism version 5.0 software (GraphPad, San Diego, CA).

Table 1 depicts the SCC*mec* type, PVL status, and baseline PSMα production characteristics of the 13 isolates studied (11 clinical isolates and two control strains). The PSMα$_1$ to PSMα$_4$ peptides have been shown to cause concentration-dependent neutrophil lysis (6, 7). A PSMα$_3$ concentration of 5 μg/ml has been shown to lyse nearly 50% of human polymorphonuclear neutrophils (PMNs), while 10 μg/ml of PSMα$_1$ and PSMα$_4$ can cause 60% and 10% PMN lysis, respectively (8, 9). Thus, isolates were grouped on the basis of the amount of the most potent peptide, PSMα$_3$, produced at the baseline as very low to low (≤5 μg/ml), medium (6 to 15 μg/ml), or high (>15 μg/ml) producers.

TABLE 1 Characteristics of the 11 clinical isolates and two control strains used in this study

Isolate or strain	*mec* type	PVL[a]	Infection type	PSM[b] baseline	MIC (μg/ml)[c]		
					TYG	CL	LZ
1	IV	+	Blood	Low	0.125	0.188	3
2	IV	+	Blood	Medium	0.125	0.25	3
3	IV	+	Blood	Low	0.125	0.188	2
4	IV	+	Blood	Medium	0.125	0.25	2
5	IV	+	Blood	Medium	0.125	0.188	2
6	IV	+	Pneumonia	Low	0.188	0.125	2
7	IV	+	Pneumonia	Medium	0.125	0.188	3
8	IV	+	Necrotizing pneumonia	High	0.125	0.25	3
9	II	−	Pneumonia	Very low	0.125	>256	3
10	II	−	Pneumonia	Very low	0.125	>256	1
11	II	−	Pneumonia	Very low	0.125	0.188	2
Control strains							
USA300 (LAC)	IV	+	Wound	Medium	0.25	0.25	2
USA600	II	−	Blood	Low	0.125	>256	2

[a] A plus or minus sign denotes the presence or absence, respectively, of the *lukF/S* gene, which encodes the Panton-Valentine leukocidin.
[b] The PSMα$_3$ production level at the baseline was arbitrarily defined as very low (<1 μg/ml), low (1 to 5 μg/ml), medium (6 to 15 μg/ml), or high (>15 μg/ml).
[c] TYG, tigecycline; CL, clindamycin; LZ, linezolid. Resistance is defined as a MIC of >256 μg/ml (not tested for PSMα production at subinhibitory concentration).

Bacterial growth at 1/2 MICs of all three antibiotics was altered in half of the isolates tested by as much as 50% of the final OD compared to a no-antibiotic control, while no significant difference in the growth of any isolates was observed at 1/4 and 1/8 MICs. Thus, subsequent discussions will focus on the effect of antibiotics at the latter subinhibitory concentrations that did not affect growth. Measured PSMα values were normalized to the OD$_{600}$ at the time the supernatant was harvested.

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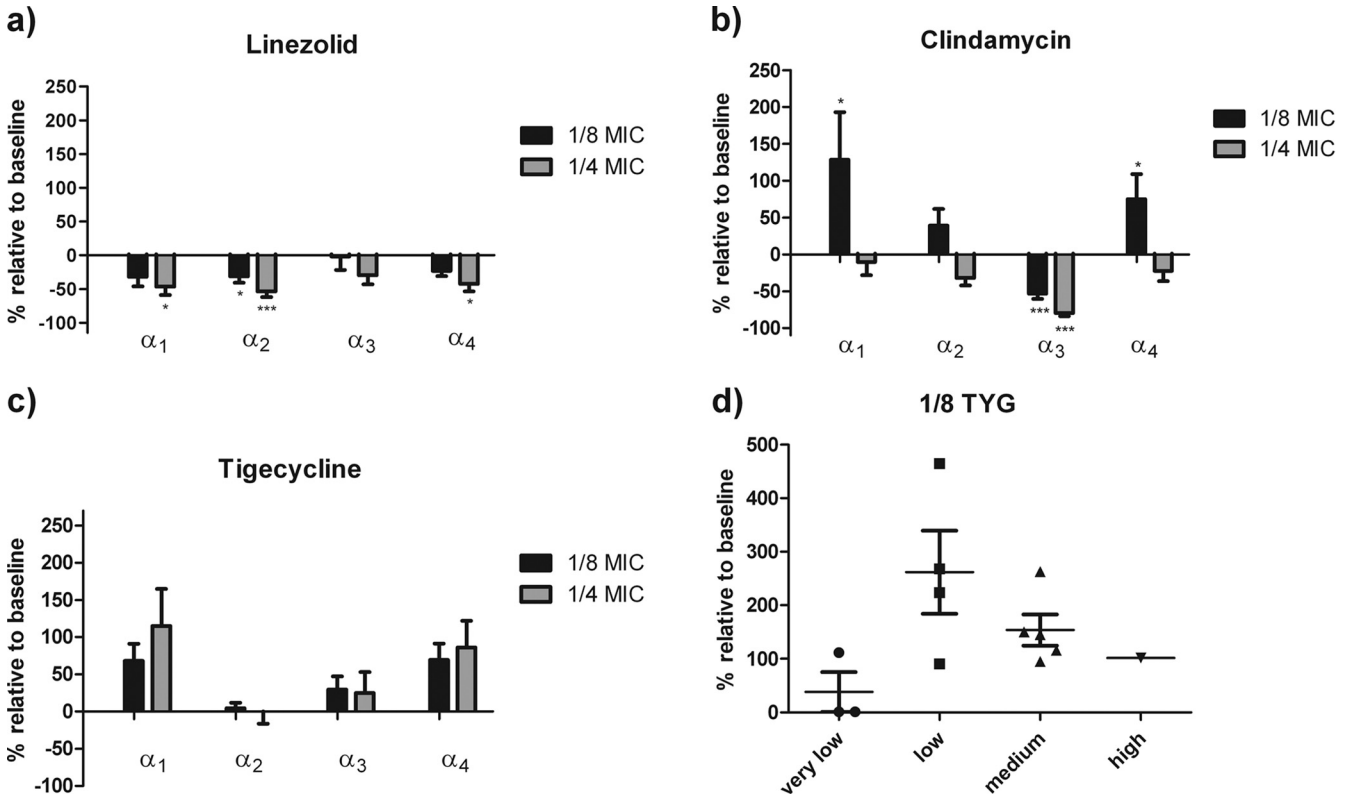

FIG 1 Overall effects of linezolid, clindamycin, and tigecycline on PSMα$_1$ to PSMα$_4$ production. (a to c) Effects of antibiotics on the production of PSMα$_1$ to PSMα$_4$ (α1, α2, α3, and α4, respectively). All measured PSM values (μg/ml) were normalized to OD_{600} and then calculated as percentages relative to the baseline. Isolates ($n = 10$) included only those that produced measureable PSMα peptides at the baseline (***, $P < 0.0001$; **, $P < 0.001$; *, $P < 0.05$). (d) Strain-to-strain variability of PSMα$_1$ production at 1/8 MIC of tigecycline (TYG). Strains were grouped according to their baseline PSMα$_1$ production as very low (<1 μg/ml), low (1 to 5 μg/ml), medium (6 to 15 μg/ml), and high (>15 μg/ml) producers.

Tigecycline appears to have the least inhibitory potential overall, while linezolid has the greatest (Fig. 1a to c). Increased production of all four PSMα peptides was the primary response observed in the presence of tigecycline at 1/4 and 1/8 MICs, while with clindamycin this occurred only at 1/8 MIC (Fig. 1b and c). Notably, compared to clindamycin at 1/8 MIC, which significantly induced PSM production in seven isolates, linezolid at 1/8 MIC modestly induced PSM production in only three isolates (Fig. 1a).

Strain-specific responses to the presence of subinhibitory antibiotic concentrations in PSMα production were observed. Drug concentrations that induced PSMα production in some isolates did not do so in others, independently of the baseline production level. Specifically, with tigecycline at both 1/4 and 1/8 MICs (Fig. 1d), PSMα$_1$ was induced at least 1.5 times above the baseline in 77% (11/13) of the isolates, while induction did not occur in two isolates at any of the concentrations tested. Similar results were observed with clindamycin, where 54% (7/13) of the isolates were induced by greater than 150%; however, this was observed only at 1/8 MIC. In those isolates, PSMα$_1$ production was induced to greater than 10 μg/ml, which has been shown to cause significant PMN lysis (9). Linezolid at the same concentration resulted in increases in PSMα$_1$ in only 23% (3/13) of the isolates tested, was inhibitory in 5 isolates, and had no significant effects on the remaining isolates. Of additional interest is the observation that nonproducers at the baseline produced PSMα peptides in the presence of subinhibitory concentrations of both linezolid and tigecycline. However, it is noteworthy that PSMα production that was induced in those two isolates did not exceed 4 μg/ml and thus would not be expected to have a significant impact on host PMNs. In contrast to results observed with the PSMα$_1$ to PSMα$_4$ peptides, we found that the production of both Hla and Spa did not increase under any condition and was inhibited in a dose-dependent manner at the concentrations of all three antibiotics tested regardless of whether PSMα peptides were induced or suppressed in those isolates (Fig. 2).

To our knowledge, we are the first to investigate the effect of tigecycline on MRSA PSMα$_1$ to PSMα$_4$ peptide production. We also included clindamycin and linezolid for comparison and tested their antivirulence potential against two other key exotoxins, Hla and Spa. We found that at sub-MICs, agents in this class of antibiotics have pleiotropic effects on toxin production that are dependent on the drug, strain, and toxin tested.

All three of the antibiotics tested are known to have large volumes of distribution and would be expected to be present at high concentrations within tissues. However, the concentrations tested reflect clinical scenarios where sub-MICs might be achieved at sites of infection because of inadequate dosing, altered pharmacokinetic parameters of the patient, and differential drug distribution to various body sites. Specifically, compared to the concentrations achieved at other body sites, tigecycline achieves relatively low levels in serum (0.11 to 0.19 μg/ml) and lung epithelial lining fluid (0.11 to 0.31 μg/ml), which are relevant to bacteremia and

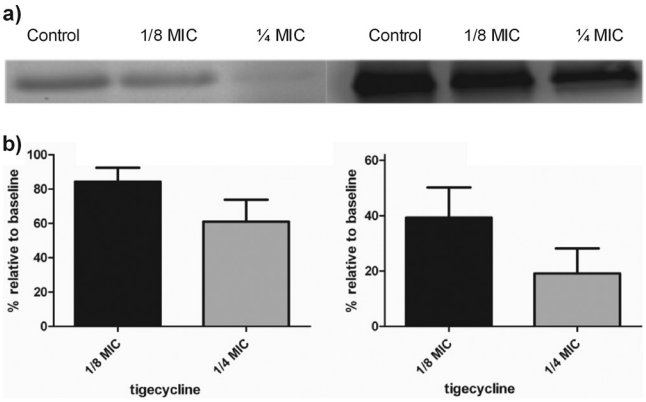

FIG 2 Effects of tigecycline on Hla and Spa production. (a) Representative images of Western blot assays of Hla and Spa, respectively. (b and c) Production of Hla and Spa in the presence of 1/8 and 1/4 MICs of tigecycline relative to the baseline. In no case was Hla or Spa production increased by subinhibitory concentrations of any of the antibiotics tested.

pneumonia, respectively, which are below the 0.5-μg/ml FDA MIC breakpoint of *S. aureus* susceptibility (10–12).

Low concentrations (1/4 and 1/8 MICs) of the antibiotics tested were more likely to induce PSMα production overall, with tigecycline having the greatest induction potential, clindamycin having less, and linezolid even less than that. Furthermore, the magnitudes of induction and inhibition differed among isolates, where the same drug could induce PSMα production in one isolate while inhibiting production in another. The observation of PSMα induction by protein synthesis-inhibiting antibiotics (e.g., linezolid and clindamycin) at sub-MICs is contrary to published literature on other exotoxins, where studies have consistently found toxin suppression under this class of antibiotics (13–16). The production of both Hla and Spa was suppressed by antibiotics tested at subinhibitory concentrations among the study isolates, independently of whether PSMα was induced in the same isolates. Hence, our results indicate that the induction effects may be specific for PSMα peptides. In contrast to our findings where significant inhibition was observed, a recent article showed tigecycline to be minimally inhibitory of Hla and Spa production (17); however, this difference may be due to the different strains and testing methods used.

Our study has several limitations. First, while we accounted for changes in cell counts by normalizing the amount of toxin measured to CFU counts, other factors, such as initial slowing of growth, could also affect toxin production. In addition, our observations are strictly *in vitro* and may not reflect how MRSA strains express virulence in the host environment, where the effects of toxin induction may be mitigated by host defenses that are present.

Further investigation is needed to examine if any other major toxins are induced, as PSMα peptides did when exposed to subinhibitory concentrations of different antibiotics. Elucidating the mechanism that underlies the induction of PSMα peptides is of great interest and has implications for the future development of new antibiotics. In light of the recent evidence implicating tigecycline in excess deaths and treatment failure in severe infections (18), the enhanced toxin production observed in this study may have clinical relevance and deserves further study. For now, our data caution against the general assumption by clinicians that all protein synthesis inhibitors possess inhibitory potential against all of the exotoxins produced by *S. aureus* and affirm the importance of adequate dosing.

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