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Targeting Multidrug-resistant Staphylococci with an anti-*rpoA* Peptide Nucleic Acid Conjugated to the HIV-1 TAT Cell Penetrating Peptide

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Staphylococcus aureus **infections present a serious challenge to healthcare practitioners due to the emergence of resistance to numerous conventional antibiotics. Due to their unique mode of action, peptide nucleic acids are novel alternatives to traditional antibiotics to tackle the issue of bacterial multidrug resistance. In this study, we designed a peptide nucleic acid covalently conjugated to the HIV-TAT cell penetrating peptide (GRKKKRRQRRRYK) in order to target the RNA polymerase α subunit gene (*rpoA*) required for bacterial genes transcription. We explored the antimicrobial activity of the anti-*rpoA* construct (peptide nucleic acid-TAT) against methicillin-resistant *S. aureus*, vancomycin-intermediate *S. aureus*, vancomycin-resistant *S. aureus*, linezolid-resistant *S. aureus,* and methicillin-resistant *S. epidermidis* in pure culture, infected mammalian cell culture, and in an *in vivo Caenorhabditis elegans* infection model. The anti-*rpoA* construct led to a concentration-dependent inhibition of bacterial growth (at micromolar concentrations) *in vitro* and in both infected cell culture and *in vivo* in *C. elegans*. Moreover, *rpo*A gene silencing resulted in suppression of its message as well as reduced expression of two important methicillin-resistant *S. aureus* USA300 toxins (α-hemolysin and Panton-Valentine leukocidin). This study confirms that *rpoA* gene is a potential target for development of novel antisense therapeutics to treat infections caused by methicillin-resistant *S. aureus*.**

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Introduction

One of the most pressing current medical issues facing healthcare practitioners and researchers is the rapid emergence of bacterial pathogens exhibiting resistance to traditional antibiotics. Of these pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA) has received considerable attention due to the diverse array of diseases it causes (ranging from superficial skin infections to invasive syndromes such as pneumonia and sepsis); MRSA was linked to nearly half of all fatalities in the USA attributed to infections caused by antibiotic-resistant bacteria in 2013.[1] The morbidity and mortality associated with MRSA infections is due in large part to the bacteria's ability to secrete a large number of virulence factors including toxins that induce pores in the plasma membrane of host cells leading to leakage of intracellular contents and lysis.[2,3] Though a large arsenal of traditional antibiotics was once capable of treating MRSA infections, resistance to these antibiotics (including agents of last resort such as vancomycin) has emerged indicating alternative therapeutic agents need to be explored in order to circumvent this challenge.[4,5] One such alternative that has received more attention in recent years is the use of peptide nucleic acids (PNAs) to target and suppress essential genes in bacterial pathogens.

PNAs are nucleic acid analogues that are capable of forming strong and stable complexes with RNA and DNA thus permitting targeted inhibition of specific genes.[6] One target of significant interest is bacterial RNA polymerase given it is highly conserved among bacterial species and is a key enzyme in gene transcription, directly interfering with its function is postulated to negatively impact bacterial viability.[7] Recently, RNA polymerase has emerged as a promising target for development of antisense agents, including PNAs, against both Gram-negative and Gram-positive pathogens.[8–13] However, one of the deficiencies of these specific PNAs is their limited uptake by host cells due to their high molecular weight and nonionic structure.[8] To overcome this limitation, researchers have examined the impact of conjugating PNAs to different cell penetrating peptides (CPPs), in order to significantly enhance PNA delivery to host tissues.[14]

In this study, we designed a peptide nucleic acid covalently conjugated to the TAT CPP in order to target a specific region of the *rpo*A gene in drug-resistant staphylococci. The HIV-1 TAT-based peptide has been previously shown to potentiate the antisense effect of anti-*gyrA* PNA against *Streptococcus pyogenes*.[15] We examined our anti-*rpoA* PNA-TAT construct's ability to disrupt growth of staphylococci (including MRSA) in pure culture, cell culture, and *in vivo* in a *Caenorhabditis elegans* model. Results garnered from this study confirm

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Table 1 Staphylococcal isolates used in this study

NARSA[a] ID	Alias strain	Origin	Molecular features			Phenotypic traits [d]
			SCC*mec* type	*mecA*	PVL[b]	
NRS382	USA100; 626	Ohio, USA	II	+	–	Resistant to methicillin, ciprofloxacin, clindamycin, and erythromycin
NRS71	Sanger 252 (MRSA 252)	United Kingdom	II	+	–	Resistant to tetracycline andmethicillin
NRS384	USA300-0114	Mississippi, USA	IV	+	+	Resistant to erythromycin, methicillin, and tetracycline
NRS385	USA500; 95938	Connecticut, USA	IV	+	–	Resistant to ciprofloxacin, clindamycin, erythromycin, gentamicin, methicillin, tetracycline, and trimethoprim
NRS386	USA700; 1078	Louisiana, USA	IV	+	–	Resistant to erythromycin and methicillin
NRS387	USA800;1045	Washington, USA	IV	+	–	Resistant to methicillin
NRS119	SA LinR #12	Massachusetts, USA	IV	+	NA[c]	Resistant to linezolid and methicillin
NRS19	HIP07256	Illinois, USA	IV	+	NA	Glycopeptide-intermediate *S. aureus*
VRS1	HIP11714	Michigan, USA	II	+	–	Resistant to vancomycin
VRS2	HIP11983	Pennsylvania, USA	II	+	–	Resistant to vancomycin, erythromycin, and spectinomycin
VRS4	HIP14300	Michigan, USA	II	+	–	Resistant to vancomycin, erythromycin, and spectinomycin
NRS101	RP62A (ATCC 35984)	Tennessee, USA	–	+	–	Prototype biofilm producer *S. epidermidis*; resistant to methicillin and gentamicin

[a]NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*. [b]PVL, Panton-Valentine leukocidin. [c]NA, No available information.[d]Phenotypic traits were reported from NARSA.

Table 2 Minimum inhibitory concentration (MIC) of conjugated PNA and TAT CPP against staphylococcal species

	MIC (μmol/l)											
	MRSA[a]						Lin[rb]	VISA[c]		VRSA[d]		MRSE[e]
Treatment	USA100	USA300	USA500	USA700	USA800	NRS71	NRS119	NRS19	VRS1	VRS2	VRS4	NRS101
PNA-TAT	32	16	32	32	8	8	16	16	32	8	16	16
Free PNA	>32	>32	>32	>32	>32	>32	32	>32	32	>32	>32	ND[f]
TAT CPP	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	ND

PNA, peptic nucleic acids, CPP, cell penetrating peptides.
[a]MRSA, Methicillin-resistant *Staphylococcus aureus*. [b]Lin[r], Linezolid-resistant *S. aureus*. [c]VISA, Vancomycin-intermediate *S. aureus*. [d]VRSA, Vancomycin-resistant *S. aureus*. [e]MRSE, Methicillin-resistant *S. epidermidis*. [f]ND, Not determined.

that the *rpoA* gene in MRSA is a promising antisense target and *C. elegans* AU37 is a suitable *in vivo* model to utilize for screening PNAs.

Results

PNA target site selection

The Basic Local Alignment Search Tool was utilized to perform sequence alignment of the *rpoA* 5′ terminal region across different *Staphylococcus* species (**Supplementary Table S1**). Based on the bioinformatics analysis, we set out to design a peptide nucleic acid covalently conjugated to HIV-1 TAT to target a specific conserved region with 100% identity to the *rpoA* gene's mRNA across different *Staphylococcus* species. The conserved region selected included the translation start codon and the 5′ terminal region, as this area is accessible for ribosome assembly and consistent success has been experienced targeting this particular region.[16,17] To confirm the efficiency of the designed PNA, the sequence was analyzed using the OligoWalk program.

Antimicrobial activity of PNA *in vitro*

Minimum inhibitory concentration. The minimum inhibitory concentrations (MICs) of PNA-TAT, free PNA, and TAT CPP were examined against a panel of *Staphylococcus* species (**Table 1**) in pure culture. The MIC of PNA-TAT, required to inhibit growth of different drug-resistant *Staphylococcus* clinical isolates, were found to be in the range of 8 to 32 μmol/l (**Table 2**). The PNA demonstrated good activity against multiple clinical isolates of MRSA, particularly MRSA USA300-0114, a community-associated strain responsible for outbreaks of staphylococcal skin and soft-tissue infections in the USA.[18] Similarly, bactericidal activity was observed in other important clinical MRSA isolates (USA100, USA500, USA700, and USA800) that exhibit resistance to various antibiotic classes including macrolides, aminoglycosides, lincosamides, and fluoroquinolones. In addition PNA demonstrated consistent activity against multidrug-resistant clinical isolates including vancomycin-intermediate *S. aureus*, vancomycin-resistant *S. aureus*, linezolid-resistant *S. aureus*, and methicillin-resistant *S. epidermidis*.

Bacterial reduction. Concentration-dependent bacterial reduction was determined against MRSA Sanger 252, MRSA USA300 and methicillin-resistant *S. epidermidis*. Against MRSA Sanger 252 cultures, the PNA-TAT cleared bacteria (produced an 8.86 $\log_{10}$ reduction) at the MIC (16 μmol/l); at 4 μmol/l and 8 μmol/l concentrations, the PNA-TAT produced a 1.67 and 3.21 $\log_{10}$ reduction, respectively (**Table 3**). With regards to MRSA USA300, significant bacterial reduction was achieved at the MIC value of 16

Table 3 Effect of PNA-TAT on pure culture of MRSA Sanger 252, MRSA USA300, and MRSE

	4 µmol/l		8 µmol/l		16 µmol/l		32 µmol/l	
Treatment	Log CFU[a] (±SD[b])	Log reduction	Log CFU (±SD)	Log reduction	Log CFU (±SD)	Log reduction	Log CFU (±SD)	Log reduction
MRSA Sanger 252								
PNA-TAT	7.19±0.43	1.67*[c]	5.65±0.79	3.21*	Cleared	8.86*	Cleared	8.86*
Control	8.86±0.03	0						
MRSA USA300								
PNA-TAT	ND		9.11±0.06	0.26	5.28±0.25	4.08*[c]	4.19±0.51	5.17*
Control			9.37±0.14	0				
MRSE								
PNA-TAT	8.59±0.19	0.23	8.53±0.17	0.29	5.48±0.26	3.34*[c]	ND	
Control	8.82±0.25	0						

MRSA, methicillin-resistant *S. aureus*; MRSE, methicillin-resistant *S. epidermidis*; PNA, peptide nucleic acid.
[a]CFU, colony forming units. [b]SD, standard deviation. [c]Asterisks (*) correspond to values found to be significantly different ($P< 0.05$) from water by statistical analysis (Student's *t*-test).
ND, Not determined.

µmol/l (observed a 4.08 $\log_{10}$ reduction) and at 32 µmol/l (observed a 5.17 $\log_{10}$ reduction), respectively (**Table 3**). Additionally, at the MIC (16 µmol/l), significant bacterial reduction (3.34 $\log_{10}$ reduction) was observed for the PNA-TAT against methicillin-resistant *S. epidermidis* (**Table 3**).

PNA-TAT displays rapid bactericidal activity against MRSA

After confirming the antimicrobial activity of the designed PNA-TAT construct against different staphylococcal species, we investigated how rapidly the construct kills MRSA in pure culture. MRSA Sanger 252 was exposed to 16 µmol/l of the PNA, vancomycin, linezolid, and a control (water) over a 24 hours period. Samples were collected every 2 hours and the number of viable colony forming units (CFU) was determined. As presented in **Figure 1**, the PNA-TAT construct exhibits a bactericidal effect and completely eradicates a high starting inoculum of MRSA (~2.75×10^5 CFU/ml) within 6 hours. Vancomycin requires 24 hours to achieve the same effect. Linezolid, in contrast to PNA-TAT and vancomycin, exhibits bacteriostatic activity.

PNA-TAT suppresses *rpoA* gene expression and expression of MRSA toxin genes

Real-time quantitative reverse-transcriptase polymerase chain reaction (PCR) was used to examine the impact of PNA on *rpoA* gene expression in MRSA USA300 and the subsequent suppression of gene expression for two important toxins, α-hemolysin (*hla*) and Panton-Valentine leukocidin (PVL). As depicted in **Figure 2a** PNA-TAT produced a 53.16% (at 8 µmol/l), 78.1% (at 16 µmol/l) and 86% (at 32 µmol/l) reduction in *rpoA* gene expression in MRSA USA300 indicating a concentration-dependent inhibition of gene expression. Interestingly, suppression of *rpoA* gene expression led to significant down regulation of the toxin genes *hla* and PVL in MRSA USA300 (**Figure 2b,c**). The suppression of toxins gene expression was found to be concentration-dependent (at 32 µmol/l, there was negligible expression of both *hla* and PVL).

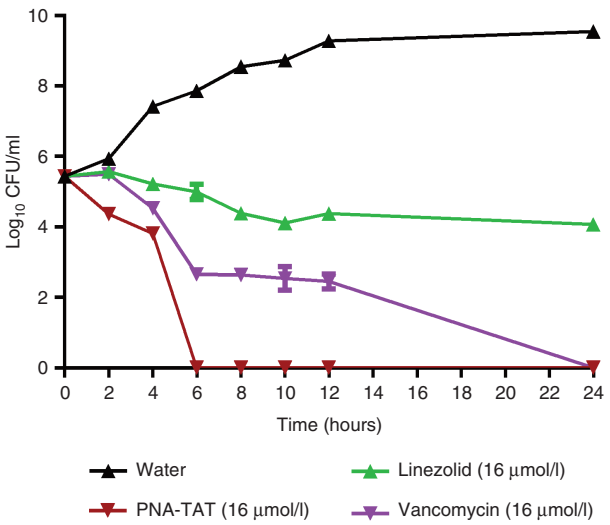

Figure 1 Time-kill kinetics of PNA-TAT against MRSA Sanger 252. Anti-*rpoA* PNA–TAT at 16 µmol/l (tested in triplicate) was incubated for 24 hours at 37°C with shaking and samples were collected for every 2 hours, serially diluted, and plated onto tryptic soy agar. Viable colony forming units per ml were determined after incubation at 37°C for 16 hours. Vancomycin and linezolid (both at 16 µmol/l) were used as positive controls. The results are presented as mean ± SD from two independent experiments. PNA, peptide nucleic acid; MRSA, methicillin-resistant *S. aureus.*

Anti-*rpoA* PNA-TAT constructs significantly reduces intracellular MRSA in infected cell culture

As PNA-TAT exhibited potent anti-MRSA activity against extracellular bacteria, we were interested to explore the ability of PNA-TAT to eliminate MRSA harboring inside macrophages. To examine the ability of anti-*rpoA* PNA-TAT to eradicate MRSA harboring inside host cells, murine macrophage J774 cells were initially infected with MRSA Sanger 252. Cells were subsequently exposed to either the PNA-TAT construct or vancomycin and the reduction in MRSA cells present inside the infected macrophages was determined. As presented in **Figure 3**, PNA-TAT outperformed vancomycin, eradicating 89% (at 8 µmol/l) and 93.1% (at 16 µmol/l) of

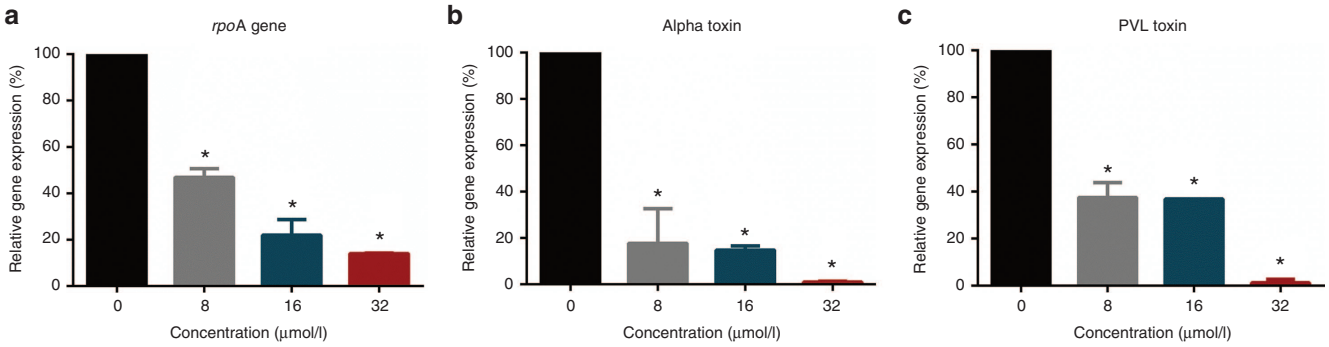

Figure 2 Dose-dependent down regulation of MRSA USA300 *rpo*A, *hla*, and *pvl* genes after incubation with anti-*rpoA* PNA-TAT. Bacterial cultures were maintained in tryptic soy broth with 8, 16, and 32 μmol/l PNA for 16 hours at 37°C with shaking. Total RNA was extracted from the treated and untreated cultures. The levels of transcripts were determined using RT-PCR. (**a**) The level of *rpoA* expression. (**b**) The level of *hla* (α-hemolysin) expression. (**c**) The level of *pvl* (Panton-Valentine leukocidin) expression. 16s rRNA was used as an internal control. **P* ≤ 0.05 using two-tailed Student's *t*-test was considered as significant. PNA, peptide nucleic acid; MRSA, methicillin-resistant *S. aureus*; RT-PCR, reverse transcriptase polymerase chain reaction.

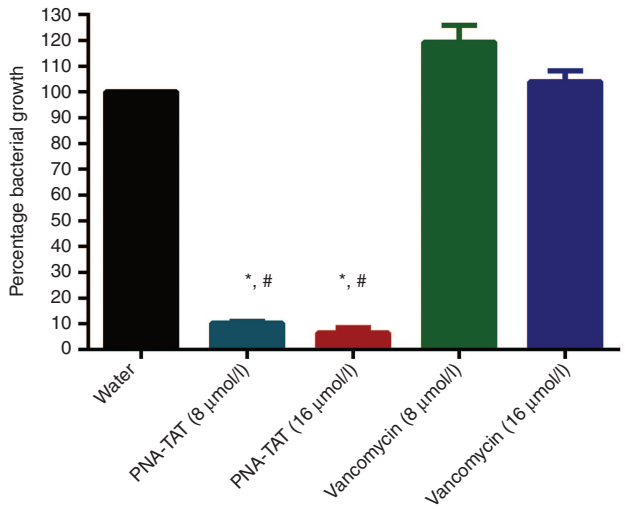

Figure 3 Examining PNA-TAT's ability to eradicate MRSA Sanger 252 present inside infected murine macrophages (J774). J774 cells were incubated with ~2×10^6 CFU/ml of bacteria for 45 minutes. After removal of the extracellular bacteria, the infected cells were treated with anti-*rpoA* PNA – TAT (at 8 and 16 μmol/l) for 4 hours with 5% CO_2. Vancomycin and sterile water were used as positive and negative controls. The results are presented as mean ± SD from two independent experiments. Data without error bars indicate that the SD is too small to be seen. **P* ≤ 0.05 using two-tailed Student's *t*-test are deemed significant. PNA, peptide nucleic acid; MRSA, methicillin-resistant *S. aureus*; CFU, colony forming units.

intracellular MRSA, respectively. No reduction in MRSA CFU was observed with vancomycin at the same test concentrations. These findings suggest that PNA-TAT is a potential valuable treatment option for challenging MRSA infections (such as pneumonia) where MRSA resides inside host cells.

Anti-*rpoA* PNA-TAT construct did not *in vitro* cytotoxicity analysis of *rpo*A-TAT against J774 cells

Using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*tetrazolium) (MTS) assay with murine macrophage J774 cells, it was confirmed that

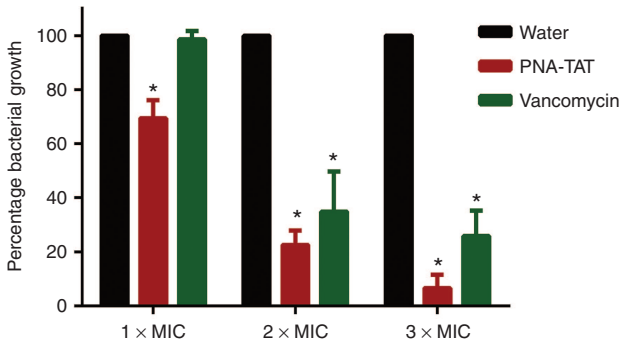

Figure 4 Efficiency of PNA-TAT in treatment of MRSA Sanger 252 infected *C. elegans*. L4-stage worms were grown on tryptic soy agar plates seeded with a lawn of bacteria for 2 hours. Worms were treated with anti-*rpoA* PNA-TAT at 1 × MIC, 2 × MIC or 3 × MIC for 18 hours using vancomycin and sterile water as controls. Worms were lysed, colony forming units (CFUs) were counted, and the percent bacterial reduction per worm in treated groups was calculated (relative to the untreated control groups). 10 worms (tested in triplicate) were used for each treatment. The results are presented as mean ± SD from two independent experiments. Data without error bars indicate that the SD is too small to be seen. **P* ≤ 0.05 using two-tailed Student's *t*-test are deemed significant. PNA, peptide nucleic acid; MRSA, methicillin-resistant *S. aureus.*

Anti-rpoA PNA-TAT construct was not toxic at a concentration of 16 μmol/l (**Supplementary Figure S1**).

Efficacy of PNA-TAT *in vivo* in a *C. elegans* animal model

In order to validate the *in vitro* results confirming PNA-TAT's anti-MRSA activity, the temperature-sensitive sterile mutant strain *C. elegans* AU37 (sek-1(km4); glp-4(bn2) I) was utilized as a whole animal model to assess the ability of the antisense construct to work in a living system. This strain is sterile at room temperature and capable of laying eggs only at 15°C. Additionally, this strain is more susceptible to infection due to a mutation in the *sek-1* gene of the p38 mitogen-activated protein kinase pathway.[19,20] Worms infected with MRSA Sanger 252 were treated with PNA-TAT at 1 × MIC, 2 × MIC, or 3 × MIC, vancomycin, or water for 18 hours. The reduction in MRSA CFU in infected worms was subsequently

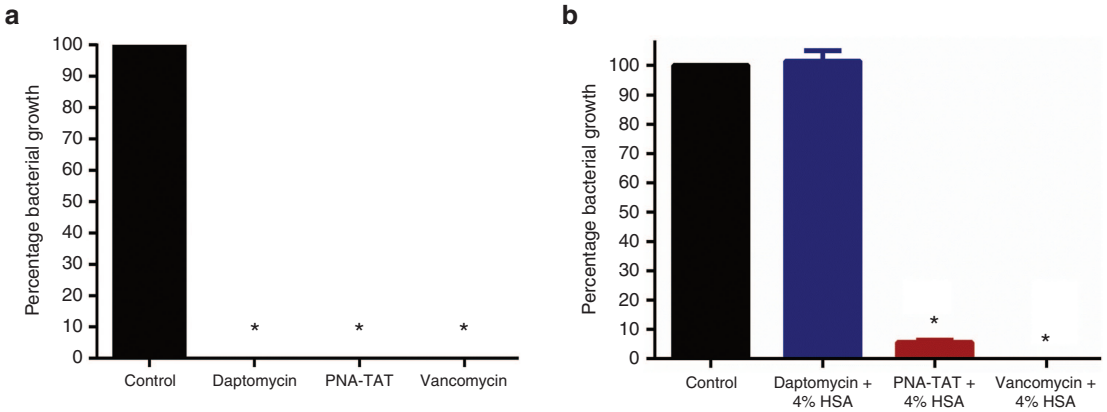

Figure 5 Impact of human serum albumin (HSA) on the efficacy of PNA-TAT inhibition of MRSA Sanger 252. Anti-*rpoA* PNA–TAT at 4 × MIC was incubated with ~5×10^5 CFU/ml in Mueller-Hinton broth II, in the presence and absence of 4% HSA. Daptomycin and vancomycin 4 × MIC served as positive and negative controls, respectively. The experiment was carried out in triplicate for 12 hours at 37 °C. (**a**) MRSA Sanger 252 inhibition in the absence of HSA. (**b**) MRSA Sanger 252 inhibition in the presence of 4% HSA. The results are presented as mean ± SD. *$P \leq 0.05$ using two-tailed Student's *t*-test are deemed significant. PNA, peptide nucleic acid; MRSA, methicillin-resistant *S. aureus*; MIC, minimum inhibitory concentration; CFU, colony forming units.

determined. In comparison to sterile water, PNA-TAT produced a concentration-dependent significant reduction in MRSA with no microscopically observed toxicity to the worms. A 30, 77.4, and 93.4% $\log_{10}$ MRSA CFU reduction was observed at 1 × MIC, 2 × MIC and 3 × MIC of PNA-TAT, respectively (**Figure 4**). A 65.1 and 74.2% reduction in MRSA CFU was observed in vancomycin-treated worms at concentrations = 2 × MIC and 3 × MIC, respectively.

PNA-TAT does not bind to the major component of human serum

Several antibiotics have limited use systemically due to their proficiency in binding to components of human serum, in particular the protein albumin. In order to examine if the antimicrobial activity of the PNA-TAT construct would be impaired in the presence of serum, MRSA Sanger 252 was incubated with 4 × MIC of PNA-TAT, daptomycin, or vancomycin in the presence and absence of 4% human serum albumin (HSA). As presented in **Figure 5**, daptomycin, an antibiotic known to bind strongly to HSA, is unable to inhibit MRSA growth in the presence of 4% HSA. In contrast, vancomycin's antimicrobial activity is unaffected by the presence of HSA. The PNA-TAT construct mimics the behavior of vancomycin (a slight decrease in bacterial inhibition from 99.99 (in HSA-free media, **Figure 2a**) to 94.34% (in HSA-supplemented media, **Figure 2b**) is observed)). The result indicates the construct's antimicrobial activity is not impacted by the presence of HSA.

Discussion

MRSA infections continue to impose a significant problem both in the healthcare and community settings. MRSA has been associated with an array of different superficial and invasive diseases including skin infections, pneumonia, osteomyelitis, and sepsis.[21–24] These infections impact a diverse patient demographic ranging from individuals with weakened immune systems (namely neonates and geriatric patients) to healthcare workers to healthy athletes.[25–27] The challenge of treating patients infected with MRSA has been compounded in recent years with the rise in the pathogen's resistance to many conventional antibiotics, including agents of last resort such as vancomycin and linezolid.[28,29] This points to a critical need to find alternative antibacterial agents that can be used to address the burden of MRSA infections.

One alternative to conventional antibiotics that has been recently explored by our research group and others is developing antisense agents (such as PNAs) to silence expression of essential genes in bacterial pathogens.[8–12] This approach has been successfully employed in previous studies to target the RNA polymerase α subunit (*rpoA*) and RNA polymerase sigma 70 (*rpoD*) in both *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* indicating the viability of these genes as novel targets for antimicrobial agents. In addition, MRSA RNA polymerase sigma 70 encoded by *rpoD* gene has been shown as a promising target for PNA inhibition.[10,12] However, thus far, no published reports have examined the capability of direct inhibition of *rpoA* in MRSA using PNAs. One significant limitation of PNAs is their inability to cross cell membranes to attack their target inside the bacterial cytoplasm. This issue can be mitigated by conjugating the PNA to a suitable cell penetrating peptide. The aim of this study therefore was to examine the impact of conjugating the HIV-1 TAT cell penetrating peptide to a PNA designed to silence expression of the essential gene *rpoA* in MRSA.

We demonstrated that the presence of the TAT CPP did enhance the antimicrobial activity of the anti-*rpoA* PNA (the construct successfully inhibited MRSA growth at a concentration of 16 µmol/l). Additionally, using reverse transcriptase polymerase chain reaction, we confirmed that the PNA-TAT construct suppressed the expression of both *rpoA* and genes (*hla* and PVL) encoding for two key toxins, *hla* and PVL. These two toxins are responsible for promoting pathogenesis of MRSA infections and impairing the host immune response from clearing an infection. For example, PVL promotes lysis of leukocytes and is associated with more invasive forms of MRSA skin infections and community-acquired pneumonia.[30] We surmise that the ability of the PNA-TAT to suppress the

expression of *hla* and PVL will alleviate the morbidity associated with infections (such as necrotizing pneumonia) caused by PVL- and *hla* positive MRSA strains, such as MRSA USA300 and MRSA USA400. This would correlate with a previous report that demonstrated suppression of Hla and PVL toxins, by the antibiotic linezolid, improved the treatment outcome of rabbits infected with MRSA USA300.[31]

Confirmation of the anti-*rpoA* PNA-TAT construct's antimicrobial activity against MRSA in pure culture led us next to investigate if the presence of the CPP would permit the construct to clear an intracellular MRSA infection. Bacterial pathogens that harbor inside host cells pose a difficult challenge for both the host immune response and many traditional antibiotics to clear. For example, β-lactam antibiotics and aminoglycosides are not able to effectively penetrate host cells to clear intracellular pathogens.[32,33] While antibiotics from the macrolide and fluoroquinolone classes are capable of penetrating host cells, they are "poorly retained" and thus limited in their ability to clear an infection.[32,33] The inability of many traditional antibiotics to clear intracellular MRSA is postulated to contribute to the high rate of recurring infection present in the healthcare setting.[34–36] Accordingly, treatment with conventional drugs of choice such as vancomycin and aminoglycosides is often associated with high clinical failures that exceeded 40% in intracellular MRSA infections due to poor intracellular penetration of drugs.[37,38] Our investigation revealed the anti-*rpoA* PNA-TAT construct, at the same concentration as its MIC (16 µmol/l), was capable of eradicating more than 90% of intracellular MRSA present inside infected macrophages. This proved far superior to the antibiotic vancomycin (16 µmol/l), which was unable to kill MRSA harboring inside macrophages. The result indicates that the PNA-TAT construct possesses a selective advantage over antibiotics such as vancomycin in its ability to clear intracellular MRSA, thus potentially limiting the possibility of a recurring infection.

In vivo studies are critical to perform in order to validate *in vitro* results obtained for promising therapeutic agents. However, given the high cost of synthesizing and purifying a small quantity of PNA, it is very expensive to test PNAs in traditional animal models such as rodents. This requires the use of alternative animal models in order to examine the effect of PNAs on pathogens *in vivo*. *C. elegans* has been used as a whole animal model for more than four decades to examine critical questions in the field of biology; however, more recently, this model has gained traction as an alternative method to screen antimicrobial agents for suitable *in vivo* activity.[39,40] Thus we postulate that *C. elegans* is a good animal model to use as a proof-of-concept to confirm the antimicrobial activity of promising PNAs *in vivo* prior to examining these agents in more complex animal models such as rodents. Utilizing the *C. elegans* AU37 strain, which is more sensitive to the effects of pathogens, we confirmed that the anti-*rpoA* PNA-TAT construct does possess potent antimicrobial activity *in vivo*. At 2 × MIC, the antisense construct reduced the presence of MRSA in infected worms by >75% (compared to 64% for vancomycin). Interestingly, at 3 × MIC, the anti-*rpoA* PNA-TAT construct eradicated more than 93% of MRSA in infected worms in contrast to vancomycin (which produced a 74% reduction). These results further support the notion that *rpoA* is a good target for developing novel anti-MRSA agents and the anti-*rpoA* PNA-TAT construct is a promising antisense agent that warrants further investigation.

Several antibiotics used to treat staphylococcal infections, such as daptomycin, rifampicin, and clindamycin, bind strongly to components in human serum, thus limiting the amount of free/unbound drug available to treat an infection.[41–43] One limitation of using *C. elegans* as an animal model is the worm lacks plasma/blood (similar to vertebrate mammals) thus restricting a researcher's ability to examine the potential impact of PNA (or antimicrobial) binding to components of human serum.[41,42] This limitation can be addressed by examining the ability of PNAs to inhibit bacterial growth in the presence of major components of human serum (such as the protein albumin). Utilizing this method, we demonstrated there was no significant impairment in the PNA-TAT construct's ability to inhibit MRSA Sanger 252 growth in the presence of a physiological concentration of HSA. Thus, the PNA-TAT construct does not appear to bind to HSA in a manner similar to daptomycin.

RNA polymerase plays a critical role in DNA transcription. The enzyme is composed of multiple subunits, including α subunit (encoded by *rpoA*). The α subunit helps to stabilize the transcription complex, helps with promoter recognition, functions to assemble the core enzyme, and regulates the initiation of gene transcription.[44] Interfering with *rpoA* would be deleterious to the function of RNA polymerase as it would severely impair the functionality of the transcription complex. One of the negative consequences of this effect for bacteria would be decreasing the production of key virulence factors that promote MRSA pathogenesis (such as toxin production as demonstrated in this study). Furthermore, irreversible inhibition of RNA polymerase function (through inhibition of *rpoA*) in the target organism would ultimately lead to cell death.[45] In addition, suppression of *rpoA* leads to destabilization of the RNA polymerase complex. Destabilization of this complex will directly interfere with transcription of genes to mRNA and subsequently translation to the effector protein. One of the key systems involved in expression of virulence factors (including toxin production) in *S. aureus* is the accessory gene regulation (*agr*) quorum sensing system. High expression of *agr* is suspected to be a main contributor to the virulence of the MRSA USA300 strain.[46]

Interestingly, *agr* encodes two divergent promoters, one of which directs the synthesis of the small regulatory molecule RNAIII.[47] RNAIII is the effector for most genes of the *agr* regulon and RNAIII has been implicated in the increased production of many secreted *S. aureus* toxins.[46–48] RNAIII regulates the expression of many mRNAs both at the transcription and translation level.[49] For example, it has been shown that the 5′ domain of RNAIII is responsible for translation of *hla* mRNA to the effector protein.[50] Thus, decreased transcription of *hla* and *rnaIII* (due to downregulation of *rpo*A) limits RNAIII's ability to promote *hla* mRNA translation to the toxin. Decreased expression of *agr* leads to decreased expression of virulence gene expression, namely toxin genes like *hla*, *saeB* (enterotoxin B), and *tst* (toxic shock syndrome toxin-1).[51] This observation coincides with compounds isolated from *Photobacterium halotolerans* that inhibit the quorum sensing system in MRSA. Decreased expression of both *rnaIII* and *hla* were observed due to interference with

the *S. aureus agr* quorum sensing system.[46] We suspect that the decrease in expression of toxin genes observed from our reverse transcriptase PCR experiment is due directly to the suppression of *rpoA* gene expression. We postulate that suppression of *rpoA* expression leads to downregulation of key genes involved in virulence factor production including *rnaIII* and the *agr* quorum sensing system.

The multifaceted role of the RNA polymerase α subunit makes it a very good target for designing antisense agents for antibiotic-resistant pathogens. Few reports have discussed the promise of using PNAs as a novel therapeutic to target essential genes in MRSA. In addition to targeting *rpoA* in the RNA polymerase complex, the *rpoD* gene (encoding the RNA polymerase σ^{70} subunit) has been shown to be another viable target for designing antisense agents. Bai *et al.*, constructed PNAs (against a conserved region for *rpoD* mRNA) covalently conjugated to the $(KFF)_3K$ -cell penetrating peptide. The most promising antisense construct (anti-*rpoD* PPNA2332) was able to inhibit growth of four MRSA strains at a concentration of 12.5 µmol/l.[45] This is similar to the MIC obtained in our study with the anti-*rpoA*-TAT construct (MIC of 16 µmol/l). Substitution of the $(KFF)_3K$ CPP with an alternative peptide $((RXR)_4XB)$ resulted in an improvement in anti-MRSA activity (growth inhibition observed at 6.25 µmol/l); this demonstrates the impact altering the CPP can have on the antibacterial effect of the conjugated PNA. However, one drawback of PNAs conjugated to the $(RXR)_4XB$ peptide is that, these agents can permeabilize the bacterial membrane resulting in nonspecific inhibition of growth (as was observed in Bai *et al.*'s study).[45] The anti-*rpoD* PPNA2332 exhibited a concentration-dependent bactericidal activity against MRSA and inhibited *rpoD* expression in a similar concentration-dependent manner (with complete gene suppression observed at 40 µmol/l).

In addition to Bai *et al.*'s study, investigators have examined the promise of PNAs targeting other essential genes in *S. aureus* including *fmhB* (involved in cell wall synthesis) and *gyrA* (involved in DNA synthesis).[52] As in Bai *et al.*'s study, the antisense constructs (which were also conjugated to the $(KFF)_3K$ CPP) designed against *fmhB* and *gyrA* exhibited a concentration-dependent bactericidal effect (with complete inhibition of bacterial growth observed at 10 µmol/l). A recent study conducted by Liang *et al.* further demonstrated the potential of using PNAs to target essential genes in MRSA.[53] In this study, the authors designed PNAs (conjugated to the $(RXR)_4XB$ CPP) to target a critical gene (*ftsZ*) involved in bacterial cell division. Two antisense constructs, designated as PPNA1 and PPNA2, were able to inhibit growth of MRSA CY11 at 30 µmol/l and 40 µmol/l, respectively. Though both constructs were bactericidal against MRSA, only PPNA1, at 40 µmol/l, was able to completely eradicate a high inoculum of MRSA CY11 (10^5 CFU/ml) (in 6 hours). Our anti-*rpoA*-TAT construct was able to achieve the same effect, however at a much lower concentration (16 µmol/l).

This study confirms *rpoA* is a viable drug target for developing novel antibacterial agents for treatment of MRSA infections. The PNA-TAT construct we designed successfully inhibits MRSA growth in pure culture, cell culture, and *in vivo* in a *C. elegans* whole animal model. The PNA-TAT construct does not bind to HSA indicating this agent has potential for use in treating systemic MRSA infections. Further work confirming the anti-*rpoA* PNA-TAT construct's activity in other animal models of MRSA infection (to examine toxicity to host tissues and identify an appropriate route of administration) is an important next step to further examine the potential of PNA-TAT as a novel antimicrobial agent.

Materials and methods

Chemicals, reagents, and kits. Mueller-Hinton broth II, Dulbecco's modified Eagle's medium, Dulbecco's phosphate buffered saline, chloroform, isopropanol, agarose, ethidium bromide, Tris-Borate-ethylenediaminetetraacetate buffer, free water and primers were purchased commercially from Sigma-Aldrich (St. Louis, MO). Trypticase soy broth (TSB) and trypticase soy agar (TSA) were purchased from BD/Difco (Sparks, Maryland) and mannitol salt agar was purchased from Hardy Diagnostics (Santa Maria, California). Vancomycin hydrochloride was purchased from Gold Biotechnology (St. Louis, MO). Fetal bovine serum was purchased from Life Technologies (Grand Island, NY). Recombinant lysostaphin was purchased from AMBI Products LLC (Lawrence, NY). *TRI*zol Max Bacterial RNA Isolation Kit, SYBR Green PCR Master Mix, SuperScript II Reverse Transcriptase and 1 kb plus DNA ladder were purchased from Invitrogen (Carlsbad, CA). Turbo DNA-free Kit and DEPC-treated water were purchased from Ambion (Foster city, CA). RNAprotect Bacteria Reagent (QIAGEN, Valencia, CA) and random hexamers (Applied Biosystems, Carlsbad, CA) were also purchased from commercial vendors.

Bacterial strains and C. elegans. Bacterial strains used in this study are presented in **Table 1**. The temperature-sensitive sterile mutant strain *C. elegans* AU37 (sek-1(km4); glp-4(bn2) I) was used as a whole animal model for testing the PNA's impact against MRSA infection *in vivo*. Worms were grown on nematode growth media plates cultivated with *Escherichia coli* OP50. For infection worms were maintained on TSA agar plates seeded with MRSA Sanger 252 as described earlier[54]

Cell penetrating peptide and PNA. The HIV-1 TAT CPP (GRKKKRRQRRRYK) was synthesized and purified by GenScript (Piscataway, NJ). A PNA was designed to be complementary to a conserved region of the *rpoA* gene (including the start codon) present in 12 different *Staphylococcus* species. The PNA-TAT (GRKKKRRQRRRYK-O-tttctatcattt-NH2) was synthesized and purified by PNA Bio (Thousand Oaks, CA) and was conjugated to the HIV-1 TAT using manual coupling chemistry.

In vitro *antimicrobial activity of CPP and PNA.* The MICs of the anti-*rpoA* PNA-TAT construct, free PNA and the TAT CPP were determined against multiple *Staphylococcus* species. Bacteria (ranging from 2×10^5 to 3.15×10^5 CFU/ml) were cultured in Mueller-Hinton broth II and a modified version of the broth microdilution method was used to determine the MIC.[55] Briefly, low-binding clear microcentrifuge tubes (USA Scientific, Ocala, FL) were used instead of 96-well plates. Bacteria were incubated with each agent for 16 hours at 37 °C before the numbers of viable bacteria were counted by serial dilution

and plating on TSA plates. The MIC was scored as the lowest concentration where no turbidity was observed in the microcentrifuge tubes.

Time-kill assay. MRSA Sanger 252 in late logarithmic growth phase was diluted to ~2.75×10^5 CFU/ml and incubated with 16 µmol/l (2 × MIC) of PNA-TAT (in triplicate) at 37°C for 24 hours and samples were collected every 2 hours.[56,57] Linezolid and vancomycin were used as controls at the same concentration (16 µmol/l). Samples were serially diluted and plated onto TSA plates. Plates were then incubated at 37°C for 16 hours before viable CFU were determined.

Effect of PNA on MRSA gene expression. To quantify the expression of *rpoA* and the subsequent effect on expression of toxin genes including, *hla* toxin and PVL, total RNA was extracted using the *TRIz*ol Max Bacterial RNA Isolation Kit, according to the manufacturer's protocol, with few modifications. Briefly, MRSA USA300 was cultured for 3 hours until reaching an optical density (OD_{600}) of 0.24. A small aliquot (20 µl) was then centrifuged at 6,000×*g* for 2 minutes. The supernatant was discarded and the pellet was resuspended in 50 µl fresh trypticase soy broth. PNA-TAT was subsequently added at the following concentrations 0.5 × MIC (8 µmol/l), 1 × MIC (16 µmol/l) and 2 × MIC (32 µmol/l). Water served as a negative control. The samples were incubated in a 37°C shaking incubator for 16 hours.

After immediate stabilization of RNA in all samples by RNAprotect Bacteria Reagent and subsequent lysis of bacteria using 100 µg/ml recombinant lysostaphin in Tris EDTA (TE) buffer (1mol/l Tris pH 8, 0.5 mol/l ethylenediaminetetraacetate pH 8, dH2O), total RNA was extracted from the treated and untreated samples using the *TRIz*ol Max Bacterial RNA Isolation Kit. The phase separation step, using chloroform, was repeated twice to minimize the carryover of phenol and guanidine isothiocyonate. To remove genomic DNA contamination, the RNA samples were treated with Turbo DNAse, according to the manufacturer's protocol. The absence of genomic DNA was confirmed using conventional PCR and running samples on a 1% agarose gel. RNA quantity and quality were determined using an Epoch Microplate Spectrophotometer (Bioteck Instruments, Winooski, Vermont).

For first strand cDNA synthesis, 150 nanograms of Turbo DNAse treated RNA was reverse transcribed using random hexamers and SuperScript II Reverse Transcriptase (according to the manufacturer's protocol). Specific primers for MRSA300 16S rRNA*, rpoA, hla*, and PVL genes were manually designed from GenBank sequences of *S. aureus subsp. aureus* USA300_FPR3757 (GenBank: CP000255.1) and purchased from Sigma-Aldrich (**Supplementary Table S2**). The specificity of the primers was confirmed using Basic Local Alignment Search Tool and conventional PCR using the genomic DNA of MRSA USA300.

Relative quantification for all cDNA samples was carried out, in triplicate, using an ABi 7300 Real-Time PCR System (Applied Biosystems) with the following conditions; 95 ºC for 10 minutes as an initial step for DNA polymerase activation (1 cycle) and 95ºC for 15 seconds, 58ºC for 30 seconds and 72ºC for 45 seconds for melting, annealing, and extension, respectively (40 cycles for each). 16S rRNA was used as the internal reference gene.[12] Real-Time quantitative reverse-transcriptase PCR results were analyzed by using the 2(-Delta Delta C(T)) method.[58]

Cell culture infection assay. To determine the ability of PNA-TAT to inhibit growth of MRSA Sanger 252 present inside macrophages, a modified version of a cell culture infection assay was performed, as described elsewhere.[9,59,60] Briefly, J774 cells in 96-well plates were infected with MRSA Sanger 252 at a 1:10 multiplicity of infection (MOI) for 45 minutes and treated subsequently with recombinant lysostaphin to kill extracellular bacteria. PNA-TAT at a final concentration of 8 or 16 µmol/l was added (in triplicate) to the infected J774 cells (maintained with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum) and the cells were incubated for 4 hours at 37°C with 5% CO_2. Vancomycin was used as a control. After incubation the cells were washed three times with phosphate buffered saline, lysed using 0.1% Triton X-100 and the intracellular bacteria were serially diluted and plated on TSA plates. Plates were incubated at 37°C for 16 hours before viable CFU were counted.

In vitro *cytotoxicity analysis of rpoA-TAT against J774 cells. rpo*A-TAT PNA was assayed (at concentrations of 4, 8, and 16 µmol/l) against murine macrophage (J774) cells to determine the potential toxic effect to mammalian cells *in vitro.*[61] Briefly, cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum at 37 °C with CO_2 (5%). Control cells received sterile water alone at a concentration equal to that in drug-treated cell samples. The cells were incubated with the PNA (in triplicate) in a 96-well plate at 37 ºC with CO_2 (5%) for 4 hours prior to addition of the assay reagent MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) (Promega, Madison, WI, USA) for 4 hours. Absorbance readings (at OD_{490}) were taken using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA). The quantity of viable cells after treatment was expressed as a percentage of the viability of water-treated control cells (average of triplicate wells ± SD). The toxicity data was analyzed via Student *t*-test, ($P < 0.05$), utilizing GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

PNA efficacy in infected C. elegans animal model. To assess the efficacy of PNA-TAT *in vivo*, *C. elegans* were infected with MRSA Sanger 252. Briefly, worms were grown for 5 days at 15°C (permitting worms to lay eggs) on nematode growth media agar plates seeded with a lawn of *E. coli* OP50. The eggs were harvested by bleaching[62] and maintained for 24 hours at room temperature with gentle agitation for hatching. Hatched larvae were transferred to a new nematode growth media plate seeded with *E. coli* OP50 and were kept at room temperature for 5 days until worms reached the adult stage of growth. Adult worms were collected and washed three times with M9 media in a 1:10 ratio to get rid of *E. coli* before transfer to TSA agar plates seeded with a lawn of MRSA Sanger 252 for infection.[63] After 2 hours of infection, worms were collected and washed with M9 buffer five times before incubation with PNA-TAT. Worms were transferred to low-binding microcentrifuge tubes (10 worms

per tube). PNA-TAT and vancomycin were added to the tubes (in triplicate) to achieve a final concentration equivalent to 1 × MIC, 2 × MIC, or 3 × MIC. Sterile water served as a negative control. After treatment for 18 hours, worms were washed five times with M9 buffer. Worms were examined microscopically to examine morphological changes and viability. Worms were lysed in microcentrifuge tubes containing 200 mg of 1.0-mm silicon carbide particles (Biospec Products, Bartlesville, OK) that were vortexed for one minute. Samples were serially diluted and plated onto mannitol salt agar to select for MRSA. Plates were incubated at 37°C for 18–20 hours before viable CFU was determined.

Assessment of PNA-TAT binding to HAS. The impact of protein binding on the antimicrobial effect of PNA-TAT was evaluated using HSA (Sigma-Aldrich). Briefly, PNA-TAT, at 4 × MIC (32 µmol/l), was added to diluted overnight cultures (~5×10^5 CFU/ml) of MRSA Sanger 252, in triplicate, using Mueller-Hinton broth II in the presence or absence of 4% HSA (in low adhesion tubes). Vancomycin (4 × MIC) and daptomycin (4 × MIC) served as negative and positive controls, respectively.[64] The media for testing daptomycin was supplemented with 50 µg/ml $CaCl_2$. Sterile water and 4% HSA were used as negative controls. After 12 hours incubation of the tubes with shaking at 37ºC, the samples were serially diluted in sterile water and 5 µl drops were plated onto TSA plates. The agar plates were incubated at 37 ºC for 24 hours before visual scoring of the bacteria.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). Statistical significance was determined using the two-tailed Student's *t*-test. $P \leq 0.05$ was considered significant. Data are presented as mean ± SD.

Supplementary material

Figure S1. *In vitro* cytotoxicity analysis of *rpo*A-TAT against J774 cells.
Table S1. Sequence alignment of *rpoA* gene among *Staphylococcus* species.
Table S2. Primers used in this study.

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