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A peptide-morpholino oligomer conjugate targeting Staphylococcus aureus gyrA mRNA improves healing in an infected mouse cutaneous wound model.

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Abstract

Management of skin wound infections presents a serious problem in the clinic, in the community, and in both civilian and military clinical treatment centers. Staphylococcus aureus is one of the most common microbial pathogens in cutaneous wounds. Peptide-morpholino oligomer (PMO) conjugates targeted to S. aureus gyrase A mRNA have shown the ability to reduce bacterial viability by direct site-specific mRNA cleavage via RNase P. As a treatment, these conjugates have the added advantages of not being susceptible to resistance due to genetic mutations and are effective against drug resistant strains. While this strategy has proven effective in liquid culture, it has yet to be evaluated in an animal model of infected surface wounds. In the present study, we combined PMO conjugates with a thermoresponsive gel delivery system to treat full-thickness mouse cutaneous wounds infected with S. aureus. Wounds treated with a single dose of PMO conjugate displayed improved healing that was associated with increased epithelialization, reduced bacterial load, and increased matrix deposition. Taken together, our findings demonstrate the efficacy and flexibility of the PMO conjugate drug delivery system and make it an attractive and novel topical antimicrobial agent.

Keywords

Staphylococcus aureus; RNase P; skin wounds; PMOs

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One of the most common combat wound pathogens is S. aureus, which also includes methicillin resistant strains (MRSA) (Murray, 2008; Murray et al., 2010). In the US, the rate of MRSA infections has increased steadily since the year 2000, increasing the cost of medical care and resulting in higher rates of morbidity and mortality (Klevens et al., 2007). In addition, genetic mutations leading to the development of antibiotic resistance have increased the difficulty in treating S. aureus infections (Daum, 2007).

New models and methods of treatment are currently being investigated to improve the efficacy of treatment of wounds infected by S. aureus and to combat drug resistance. These include adding antibacterial coatings to bandages, or replacing the standard antibiotics with therapeutic peptides or nanoparticulate drug delivery vehicles (Guthrie et al., 2012; Lee et al., 2011; Li et al., 2010; Martinez et al., 2009). Alternate avenues of therapy also include vaccines against S. aureus (Kim et al., 2010). However, the ability to directly target the genes that control replication, or drug resistance, could not only effectively reduce the bacterial load of S. aureus, but also limit the potential for off target effects.

RNase P is an essential enzyme that primarily cleaves transfer RNA (tRNA) (Lundblad and Altman, 2010). This conserved mechanism can be directed against specific mRNA by providing a complementary external guide sequence (EGS), which binds to the target mRNA and directs RNase P cleavage (Li et al., 1992). When given the proper EGS, RNase P is able to catalytically break down bacterial, viral, and eukaryotic mRNAs (Guerrier-Takada et al., 1995; Guerrier-Takada et al., 1997; Jiang et al., 2012; Trang et al., 2001; Yen et al., 2001). Currently, EGSs can be created using phosphorodiamidate morpholino oligonucleotides (MOs), conjugated with a cell-penetrating peptide (CPP) to improve intracellular transport, and then used to alter and kill both Gram positive and negative bacteria (Shen et al., 2009; Wesolowski et al., 2011). The improved conjugate system (PMO) has proven efficacy in inhibiting the development of Plasmodium falciparum, a parasite that causes malaria (Augagneur et al., 2012).

The effect of the conjugate on S. aureus growing in liquid culture was carried out as described previously for work on various bacterial species (Wesolowski et al., 2011). The conjugate was redesigned to target a S. aureus gyrA specific sequence (TGGCCAAGGT) (Table 1). In addition, a non-specific sequence targeting poly A sequences with significantly reduced efficacy against gyrA was used as a control conjugate. Specifically, the control conjugate had an eleven-mer poly dT (11-T) sequence and its effectiveness against S. aureus was 2-3 times less compared to the experimental conjugate.

In this study we have combined the targeted morpholino approach with a thermo-responsive gel delivery system in order to evaluate its efficacy in a murine cutaneous wound model, an established preclinical model for wound healing and treatment (Kyriakides and Bornstein, 2003; Kyriakides et al., 2009). GyrA and 11-T conjugates were evaluated in a mouse model of S. aureus infected skin wounds, in a procedure approved by Yale' Institutional Animal Care and Use Committee, previously described in (Kyriakides et al., 2009). Under isofluorane anesthesia (Baxter Healthcare) we created matching, 6mm, full-thickness, dermal wounds on the flanks of C57Bl/6 mice using a punch biopsy device (Acupunch), and then infected the wound with 1×10^8 colony forming units (cfu) of S. aureus, Newman strain RN4220. The following day the conjugates were added to the open wound at a dose of 10 μM in a 20% solution of Pluronic F-127, a low-toxicity polymer with reverse thermal gelation properties (Gilbert et al., 1986). Wounds were covered with a sterile, transparent, film dressing (Tegaderm). Treatment groups were either gel with conjugate targeting the gyrA mRNA (7, 10, and 14 days), or gel with a control, non-specific conjugate (11-T, 7 and 10 days), or gel only (14 days).

Based on superficial examination of wounds, mice that received the drug loaded gels showed accelerated closure when compared to the control mice (Figure 1). All wounds showed defined eschar formation under the bandage, which gradually sloughed off. Wounds that received the drug conjugate had a healthier appearance with smaller eschar formation and more rapid epithelialization.

To further examine the wound healing process, the wounds were removed from the mouse flanks, fixed overnight in neutral buffered formalin, and prepared for histology. Paraffin sections were generated by the Yale Research Histology Service and selected sections were stained for H&E, Trichrome, and Gram stain using standard protocols. Stained tissue sections were imaged using a Zeiss Axio Imager A.1 and an AxioCam HRC camera and analyzed using Metamorph software (Molecular Devices).

One of the final steps in the wound healing process is epithelialization, where epithelial cells divide and migrate from the edges of the wound towards the center (Singer and Clark, 1999). The migrating edge of the epithelial cell layer, which stretches from the wound surface to the basal surface, is called the epithelial tongue (indicated by blue line in Figure 2). Epithelial cell migration directly relates to the length of the epithelial tongue and subsequent wound closure. We measured the length of the epithelial tongue in H&E stained full-thickness wound tissue sections, from the center of the wound, and found that day 10 wounds treated with the peptide-morpholino conjugate showed increased epithelial tongue length, which correlates with the observations of improved wound closure.

Days after wounding, fibroblasts that have migrated to the wound site begin to replace the provisional matrix with a collagenous matrix (Singer and Clark, 1999). After 10 days, wounds treated with the gyrA PMO-CPP conjugate showed significantly increased collagen content (Figure 2), with collagen fibrils appropriately ordered in the wound bed. Untreated wounds showed a significant decrease in collagen content, which indicates delayed wound healing. Neither condition showed evidence of mature collagen at 7 days, indicating delayed healing as a consequence of S. aureus infection (Kyriakides et al., 1999).

We have previously demonstrated the efficacy of our conjugate system in treating S. aureus in liquid culture (Wesolowski et al., 2011); here we demonstrated its ability in reducing S. aureus presence in an animal system. Gram stains were used to detect the presence of Gram positive bacteria in the healing skin wounds (Figure 3). Conjugate-treated skin wounds show a reduced presence of bacteria, indicated by a decreased amount of deep purple stain, when compared to the wounds that received control treatment. In particular, a dense layer of bacteria is clearly visible at seven days in the control (11-T treated) wounds (Fig. 3c).

Our targeted conjugate and thermoresponsive gel delivery system seems to compare favorably to antibacterial wound dressings that are currently under investigation (Martinez et al., 2009; Zahedi et al., 2010). Because the gel is able to fill the wound bed before hardening it provides a physical barrier that retains moisture. More importantly, the gel serves as a reservoir for a highly potent antimicrobial drug. We believe that our ability to provide direct antimicrobial contact with the wound bed is advantageous, especially for chronic or complex wounds. It should also be noted that an important limitation of this study was the use of a single dose of the drug. There is no known dose limiting toxicities associated with the targeted conjugate, and we anticipate that by increasing the frequency of administration and/or the dose, we could further improve wound healing by more effective reduction of the bacterial load.

Drug resistant strains of S. aureus have become an increasing problem, from the emergence of methicillin resistant strains 50 years ago to the appearance of vancomycin resistant strains in the past decade (Daum, 2007; Hirsch et al., 2008). Inhibition of gene expression via

RNase P has been shown to target genes that confer drug resistance, and convert bacteria to a drug-sensitive phenotype (Guerrier-Takada et al., 1997). The PMO method has the added advantage of being resistant to single genetic mutation. The conjugate can recognize and direct the cleavage of mRNA with up to three nonconsecutive mutations with no decrease in antibacterial function (Shen et al., 2009). In the unlikely event that these mutations occur, an alternative 15 bp EGS can be used for targeting.

In summary, we have shown the first use of EGS-mediated gene silencing in an infected skin wound. The S. aureus -targeted, PMO-conjugate was effective at reducing bacteria and improving the re-epithelialization and healing of S. aureus -infected skin wounds. These effects were realized with a single dose of our thermoresponsive gel drug delivery system. Moreover, this drug delivery system could be used to treat other types of wounds and to fight infections associated with injury or burns, including those involving drug-resistant strains.

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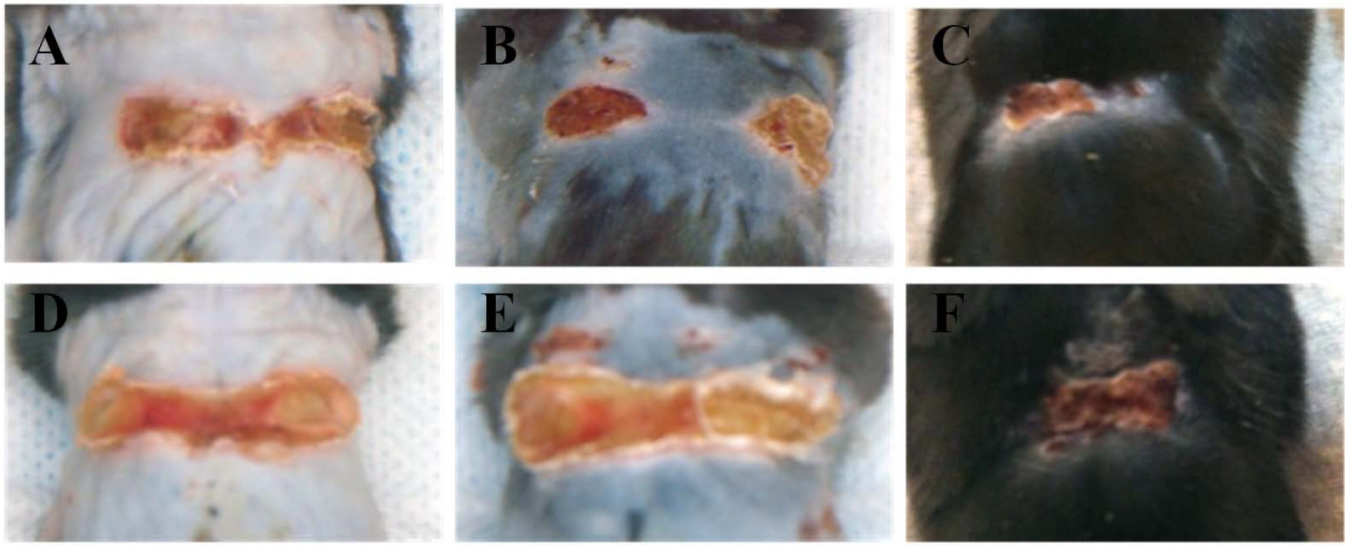

Figure 1.

Conjugate delivery produced improved wound closure in S. aureus infected skin wounds. Wounds in both treated (A, B, C) and untreated (D, E, F) animals improved from 7 (A, D) to 14 (C, F) days, a time period sufficient for near-complete healing in uninfected animals (B, E are 10 days). Scabs formed shortly after wounding and sloughed off as healing progress. Eschar formation was more severe in untreated animals.

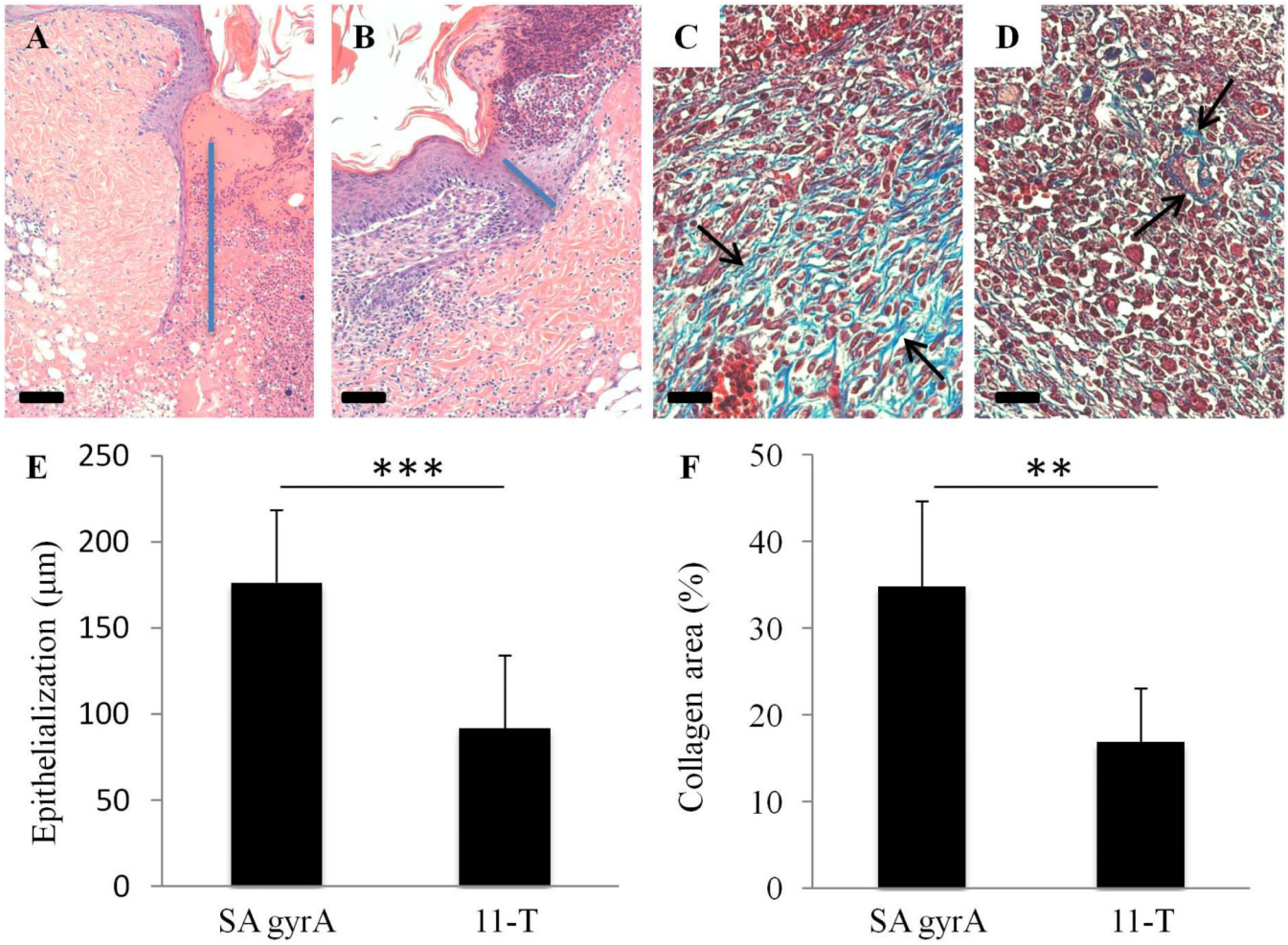

Figure 2.

Conjugate drug treated wounds show improved epithelialization and increased mature collagen. The increased epithelial tongue (blue bar) length at 10 days shows improved migration and wound closure in treated (A) wounds when compared to control (B) wounds. There is a statistically significant increase in epithelial tongue length in wounds that received the drug (E, $p < 0.001$, average + SD). Trichrome stain showed a significantly increased amount of mature collagen (highlighted by arrows), as indicated by a light blue stain, in conjugate treated tissue (C). S. aureus infected wounds that received inactive conjugate (D) showed minimal mature collagen at day 10. Quantification of collagen positive area per high power field (F) confirmed the increase in collagen ($p < 0.01$). Means compared using Student's t-test. Scale bar is 100 μm in A and B and 50 μm in C and D.

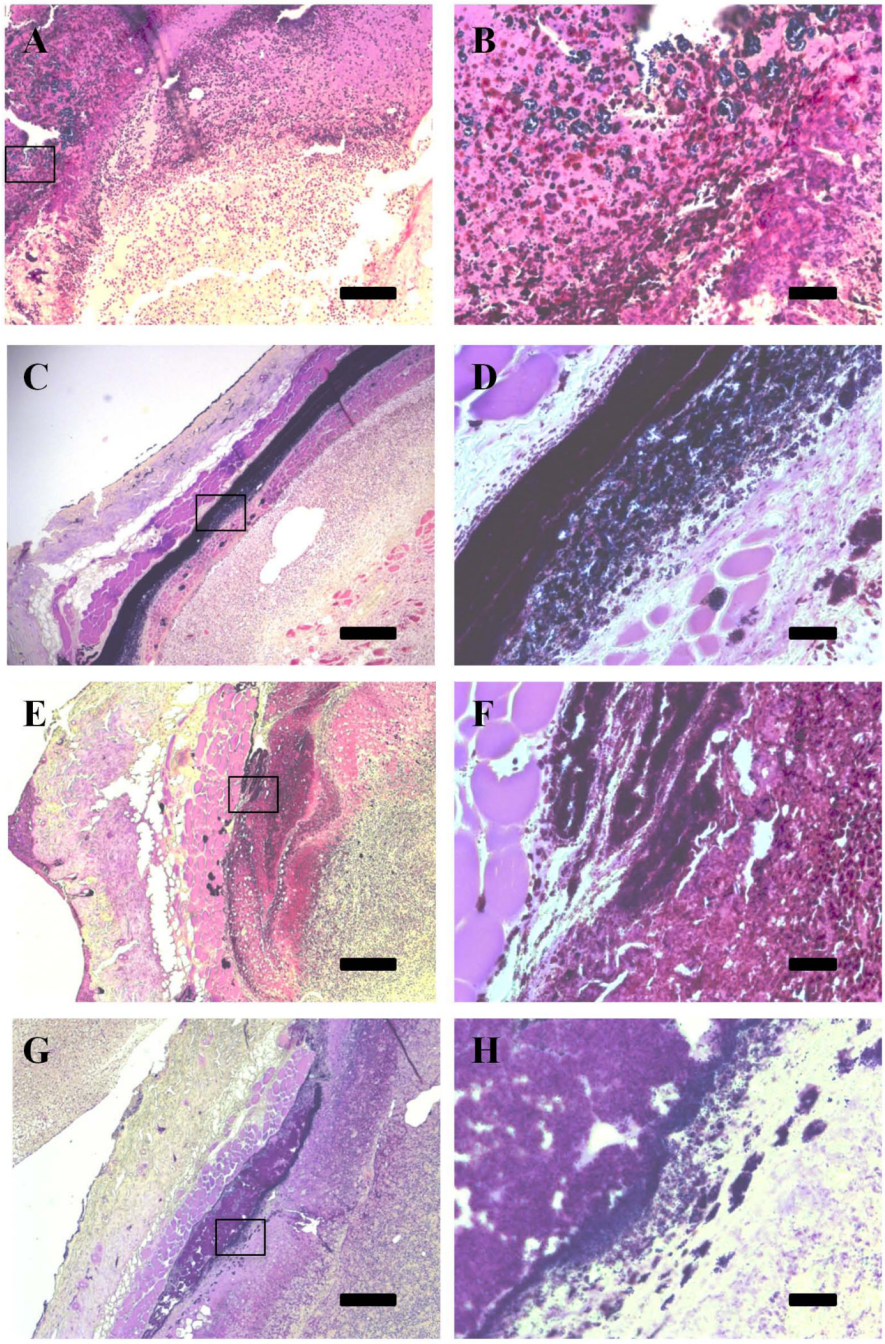

Figure 3.
Gram stain shows reduced bacterial load in wounds receiving active conjugate. Treated wounds at 7 (A,B) and 10 days (E, F) show decreased gram positive staining when compared to control wounds at 7 (C, D) and 10 (G, H) days. Inset images (B, D, F, and H) highlight areas in the tissue where staphylococcal colonies are visible in the tissue. Scale is 200 μm for A, C, E, and G, and is 25 μm for B, D, F, and H.

Table 1

Viability with different conjugates

Strain	Drug resistance	Viability (2 μM)	MIC_{50}
S. aureus Newman RN4220	Wild type	9×10^{-3}	2 μM
S. aureus ATCC43300 *	Methicillin, oxacillin	2×10^{-4}	< 2 μM

Bacteria viabilities were assayed as described in (18). MIC_{50} were assayed by standard methods.

* MRSA strain