Influence of Different Cell-Penetrating Peptides on the Antimicrobial Efficiency of PNAs in *Streptococcus pyogenes*

Gina Barkowsky,1 Anna-Lena Lemster,1 Roberto Pappesch,1 Anette Jacob,2,3 Selina Krüger,1 Anne Schröder,1 Bernd Kreikemeyer,1 and Nadja Patenge1

*Streptococcus pyogenes* is an exclusively human pathogen causing a wide range of clinical manifestations from mild superficial infections to severe, life-threatening, invasive diseases. *S. pyogenes* is consistently susceptible toward penicillin, but therapeutic failure of penicillin treatment has been reported frequently. At the same time, streptococcal resistance to alternative antibiotics, e.g., macrolides, is common. To reduce the application of antibiotics for treatment of *S. pyogenes* infections, it is mandatory to develop novel therapeutic strategies. Antisense peptide nucleic acids (PNAs) are synthetic DNA derivatives widely applied for hybridization-based microbial diagnostics. They have a high potential as therapeutic agents, because PNA antisense targeting of essential genes was shown to reduce growth of several pathogenic bacterial species. Spontaneous cellular uptake of PNAs is restricted in eukaryotes and in bacteria. To overcome this problem, PNAs can be coupled to cell-penetrating peptides (CPPs) that support PNA translocation over the cell membrane. In bacteria, the efficiency of CPP-mediated PNA uptake is species specific. Previously, HIV-1 transactivator of transcription (HIV-1 TAT) peptide-coupled anti-*gyrA* PNA was shown to inhibit growth of *S. pyogenes*. Here, we investigate the effect of 18 CPP-coupled anti-*gyrA* PNAs on *S. pyogenes* growth and virulence. HIV-1 TAT, oligolysine (K8), and (RXR)XB peptide-coupled anti-*gyrA* PNAs efficiently abolished bacterial growth in *vitro*. Consistently, treatment with these three CPP-PNAs increased survival of larvae in a *Galleria mellonella* infection model.

**INTRODUCTION**

*Streptococcus pyogenes* (group A streptococcus [GAS]) is a Gram-positive, exclusively human pathogen responsible for a variety of diseases ranging from mild self-limiting superficial infections of the throat or skin to life-threatening invasive diseases, including bacteremia and necrotizing fasciitis. The global burden of streptococcal infections is high, with 18 million invasive infections per year and 500,000 deaths.1 The impact of GAS diseases is especially high in resource-limited settings, and a rise of global invasive disease burden caused by GAS has been reported recently.2 Untreated superficial infections often lead to the development of severe invasive infections or autoimmune sequelae.1,3

To date, penicillin is the standard treatment of streptococcal pharyngitis, because GAS is invariably susceptible toward penicillin. Macrolides are recommended as alternate antibiotics for the treatment of *S. pyogenes* infections in patients who are allergic to β-lactams or in cases of penicillin failure.4 Resistance rates to macrolides in the United States have remained relatively low.5 In contrast, a rise of macrolide resistance in *S. pyogenes* has been observed in Europe, followed by a decrease in erythromycin resistance in several European countries.6 Today, a major goal of public health is to limit the application and distribution of antibiotics. One possible strategy is the application of antisense therapeutics targeting essential genes or antibiotic-resistance genes. Desired features of *S. pyogenes*-specific antimicrobials are a high specificity for the target gene, effective uptake into the bacterial cell, low unspecific toxicity, high stability, and—for the eradication of intracellular bacteria—import into eukaryotic cells. Antisense peptide nucleic acids (PNAs) potentially combine these properties and have been tested as antimicrobial agents in many bacterial species. PNAs are synthetic DNA derivatives, which bind sequence specific to DNA and RNA and are able to form stable duplexes and triplexes.7 The nucleic acid sugar-phosphate backbone is replaced by a pseudo-peptide backbone, resulting in a high chemical stability and resistance to nucleases and proteases.8,9 Cellular uptake of PNAs is limited by bacterial membranes and cell walls. Coupling of PNAs to cell-penetrating peptides (CPPs) may facilitate PNA translocation into bacteria and thereby enhance antimicrobial efficiency. CPPs are naturally occurring or designed peptides that are able to penetrate cell membranes and have been used for the...
introduction of different kinds of cargo into eukaryotic cells and bacteria.9,10 Typical examples of CPPs used in bacteria are the synthetic (KFF)3K and the HIV-1 transactivator of transcription (HIV-1 TAT)-derived peptides. (KFF)3K facilitated uptake of PNAs, among others, in Escherichia coli and Staphylococcus aureus.11,12 HIV-1 TAT was able to penetrate Listeria monocytogenes, S. aureus, and S. epidermidis.13,14 We observed previously that HIV-1 TAT-coupled anti-gyrA PNAs were able to inhibit growth in S. pyogenes.15 In this study, we tested anti-gyrA PNAs coupled to 18 different CPPs. We selected CPPs, which have been tested before as carrier molecules in eukaryotic cells and were known to exhibit low toxicity (Table 1). We found that HIV-1 TAT, oligolysine (K8), and (RXR)4XB-coupled anti-gyrA PNAs efficiently abolished growth of S. pyogenes in vitro. In a Galleria mellonella infection model, treatment of infected larvae with these CPP-PNAs increased survival.

### Table 1. CPP-PNA Anti-gyrA Conjugates for Antisense Studies in S. pyogenes

| CPP Sequence | CPP-PNA Designation | Reference |
|--------------|---------------------|-----------|
| RQIKIWFQRMKK | Anti-gyrA PNA       | 16        |
| Dansyl-G-C-ELALEA | ELA-gyrA PNA | 17        |
| GRKKRRQRRRYK | TAT-gyrA PNA       | 18        |
| LGTYQDFNKFHTFPQTAIGVGAP | hCalcitonin-gyrA scPNA | 19        |
| KFFKKFKKKK | (KFF)3K-gyrA PNA | 20        |
| KKKKKKKK | K8-gyrA PNA       | 21        |
| LLLLL | L6-gyrA scPNA     | 22        |
| PDESTK | PDESTK-gyrA PNA | 23        |
| PLSSIFSRGDP | TLM-gyrA PNA | 24        |
| AGYLLGKINLKALA | TP10-gyrA PNA | 25        |
| GWTNLNSAGYLLGKINLKALAALKIK | Transportan-gyrA PNA | 26        |
| DPKDGPDKGVTVTFTVTVTGKDPKPD | VT5-gyrA PNA | 27        |
| RXRRXXRRXRXRB | (RXR)4XB-gyrA PNA | 28        |

www.moleculartherapy.org

Molecular Therapy: Nucleic Acids Vol. 18 December 2019 445
RESULTS

Design of CPP-Coupled Anti-gyrA PNAs Specific for S. pyogenes

In a previous study, we observed antimicrobial effects of peptide-coupled anti-gyrA antisense PNAs specific for S. pyogenes. Growth inhibition by this construct was caused by antisense targeting of the essential gene gyrA. Its gene product represents the subunit A of the DNA topoisomerase gyrase, which is involved in replication and is thus required for bacterial growth. Since carrier molecules show a species-specific influence on cargo uptake, we wanted to explore the effect of a variety of CPPs coupled to anti-gyrA antisense PNAs on S. pyogenes (Table 1). Peptides were coupled to PNAs via a flexible ethyleneglycol linker (8-amino-3, 6-dioxaoctanoic acid). The sequence of anti-gyrA antisense PNAs was tgtatcaag-NH₂, covering gyrA –5 to 5. The sequence of the corresponding control PNAs (scrambled PNAs [scPNAs]) was attagctgt-NH₂. scPNAs were composed of the same base pairs as the antisense PNAs in a randomized order.

Antimicrobial Effect of CPP-Coupled Anti-gyrA PNAs on S. pyogenes

To determine the impact of different CPPs on the efficacy of anti-sense PNAs targeting S. pyogenes, a prescreening approach was performed. S. pyogenes M49 strain 591 was incubated for 6 h with
10 μM CPP-anti-gyrA PNA conjugates. Reduction of bacterial counts caused by different CPP-coupled antisense PNAAs compared with an untreated control was determined. From 18 CPP-antisense PNA conjugates, three showed an antimicrobial effect in this assay: TAT-anti-gyrA PNA, K8-anti-gyrA PNA, and (RXR)₄XB-anti-gyrA PNA (Figure 1). Similar results were obtained with S. pyogenes M1 strain AP1, with the exception of K8-anti-gyrA PNAAs, which did not show any antimicrobial effect in AP1 (data not shown; Figure 3B).

Six CPP-anti-gyrA PNA conjugates were selected for further analyses: three constructs that showed antimicrobial activity in the pilot experiment and three constructs that did not show any effect. Concentration-dependent bactericidal activity was investigated by treatment of S. pyogenes in a CPP-anti-gyrA PNA conjugate concentration range from 1 to 10 μM (Figure 2). Reduction of bacterial counts was observed following incubation of S. pyogenes with TAT-anti-gyrA PNA (Figure 2A), K8-anti-gyrA PNA (Figure 2B), and (RXR)₄XB-anti-gyrA PNA (Figure 2C), respectively. Colony-forming units (CFU) per milliliter in treated samples were significantly reduced compared with the untreated control sample in a concentration range from 4 to 10 μM PNAa. TAT-anti-gyrA scPNA caused a significant reduction of CFU per milliliter following treatment with 5 and 10 μM scPNAa, hinting toward a toxic effect of TAT CPP at higher concentrations (Figure 2A). K8-anti-gyrA scPNA and (RXR)₄XB-anti-gyrA scPNA showed a significant reduction of bacterial counts following treatment with 10 μM scPNA (Figures 2B and 2C). In contrast, no reduction of CFU per milliliter was observed following incubation with an Antennapedia homeodomain-derived CPP (Anti-anti-gyrA) PNAa, ELA-anti-gyrA PNAa, mVE-cadherin-anti-gyrA PNAa, and the corresponding scrambled control CPP-PNAAs (Figures 2D–2F).

**Table 2. MIC of CPP-PNA Anti-gyrA Conjugates**

| CPP-PNA                     | MIC (μM) |
|-----------------------------|----------|
| K8-anti-gyrA PNA            | 15.6     |
| K8-anti-gyrA scPNA          | 62.5     |
| TAT-anti-gyrA PNA           | 15.6     |
| TAT-anti-gyrA scPNA         | 62.5     |
| (RXR)₄XB-anti-gyrA PNA      | 62.5     |
| (RXR)₄XB-anti-gyrA scPNA    | 125      |

**Minimum Inhibitory Concentration**

We determined the minimum inhibitory concentration (MIC) of the CPP-antisense PNAAs that showed antimicrobial activity in the kill assay (Table 2). K8-anti-gyrA PNA and TAT-anti-gyrA PNA showed the lowest MIC at 15.6 μM. (RXR)₄XB-anti-gyrA PNA was less effective with a MIC of 62.5 μM. All scPNA controls showed a lower antimicrobial activity than the corresponding antisense constructs.

**Bactericidal Kinetics of CPP-Coupled Anti-gyrA PNAAs**

To monitor reduction of bacterial counts over the course of the experiment, S. pyogenes was treated with 5 μM CPP-antisense PNA conjugates. Reduction of bacterial counts following treatment with 5 and 10 μM PNA. TAT-anti-gyrA PNA caused a significant reduction of CFU per milliliter following treatment with an untreated control in a concentration range from 1 to 10 μM (Figure 2A), K8-anti-gyrA PNA (Figure 2B), and (RXR)₄XB-anti-gyrA PNA (Figure 2C), respectively. Colony-forming units (CFU) per milliliter were determined by plating of serial dilutions. During the course of the experiment, no complete clearance was achieved.

**Susceptibility of Different S. pyogenes Isolates to CPP-Anti-gyrA PNAAs**

We determined the antimicrobial effect of CPP-PNA conjugates on different S. pyogenes isolates representing distinct M serotypes of epidemiological relevance. Bacterial strains were treated with 5 μM CPP-PNA constructs. Samples were collected after 6 h, and bacterial counts were analyzed (Figure 4). TAT-anti-gyrA PNA exhibited antimicrobial activity against all strains except MGAS8232 (M18). K8-anti-gyrA PNA was effective toward all strains with the exception of AP1 (M1). In contrast, (RXR)₄XB-anti-gyrA PNA was able to reduce bacterial counts of all strains tested in this experiment.

**Hyaluronic Acid Content in Different S. pyogenes Isolates**

To determine whether differential capsule production was correlated to sensitivity toward CPP-PNA conjugates, hyaluronic acid (HA) was measured.
extracted from *S. pyogenes* strains. HA content of MGAS8232 (M18) was significantly higher than in all other isolates tested (Figure 5).

Since TAT-anti- gyrA PNAs were not effective in MGAS8232 (M18) (Figure 4A), this result indicates that HA represents a barrier for TAT-antisense PNAs but neither for K8-antisense PNAs nor for (RXR)$_4$XB-antisense PNAs.

CPP-Anti- gyrA PNAs Affect the Abundance of Target Gene Transcripts in *S. pyogenes*

The influence of *S. pyogenes* treatment with CPP-anti- gyrA PNAs on the amount of gyrA mRNA was investigated by reverse transcription, followed by quantitative real-time PCR (Figure 6). Bacteria were treated with a sublethal dose of CPP-PNA conjugates.

Following incubation, total RNA was extracted, and qRT-PCR was performed. Transcript abundance of the 5S RNA gene was used for normalization. The gyrA mRNA level in mock-treated *S. pyogenes* samples served as control. Treatment with 2 μM TAT-anti- gyrA PNA, K8-anti- gyrA PNA, and (RXR)$_4$XB-anti- gyrA PNA led to a significant reduction of gyrA transcript compared with the untreated control sample (Figure 6). The gyrA mRNA level decreased to 70%, 60%, and 56%, respectively, of the amount detected in the mock-treated bacteria.

Evaluation of CPP-Antisense PNA Conjugates in a *G. mellonella* Infection Model

Antimicrobial efficiency of CPP-antisense PNA conjugates was evaluated in vivo, using a *G. mellonella* infection model. Larvae were infected with *S. pyogenes* strain 591 (M49) and treated with 4 nmol CPP-PNAs. Survival of larvae was observed over 7 days. We compared survival of larvae treated with CPP-anti- gyrA PNAs with mock-treated larvae. Larvae treated with TAT-anti- gyrA PNA, K8-anti- gyrA PNA, or (RXR)$_4$XB-anti- gyrA PNA showed increased survival compared with mock-treated larvae (Figures 7A–7C). Ant-, mVE-cadherin-, and ELA-anti- gyrA PNAs that did not show antimicrobial effects in vitro did not affect survival of infected larvae (Figures 7D–7F).

One promising therapeutic strategy is a combination of antisense agents with conventional antibiotics to reduce the concentration needed for efficient antibiotics treatment.

Previously, we observed that a combination of TAT-anti- gyrA PNA with antibiotics targeting gyrase subunits resulted in synergistic or additive antimicrobial effects on *S. pyogenes* in vitro. To test whether TAT-anti- gyrA PNA treatment could enhance antibiotics efficiency in vivo, we first treated infected *G. mellonella* larvae with 1 μg levofloxacin, which increased survival of infected larvae from 20% to 46% (Figure 8A). In combination with 4 nmol TAT-anti- gyrA PNA, survival was increased to 63% (Figure 8B). A comparable survival of infected larvae was achieved by application of 15 μg levofloxacin (Figure 8A). Combination of PNA and levofloxacin also increased survival of the
infected larvae compared with TAT-anti-gyrA PNA treatment alone (33%) (Figure 8B).

DISCUSSION
PNAs are nucleic acid derivatives with a variety of properties rendering them suitable as antisense molecules, including chemical and thermal stability, strong binding to DNA and RNA, reasonable solubility, a lack of immunogenicity, and low intracellular toxicity.33 However, poor delivery into target cells hampers application of PNAs as antisense therapeutics. In bacteria, the cell membrane, the bacterial cell wall, and extracellular surface structures, such as the lipopolysaccharide layer or the capsule, represent barriers limiting cellular uptake of PNAs. Treatment of intracellular pathogens poses an additional challenge, because PNAs have to be delivered into the host cell, escape the endosomal pathway, and finally penetrate the bacteria. One possible strategy to improve cellular uptake is PNA coupling to CPPs. The efficiency of a given CPP to enhance delivery is species specific. In Gram-negative bacteria, among others, (KFF)3K and HIV-1-TAT have been identified as useful carriers.34,35 Growth of intracellular Salmonella enterica Serovar Typhimurium could be inhibited by (RXR)4XB-conjugated antisense peptide-phosphorodiimide morpholino oligomers.36 In Gram-positive bacteria, (KFF)3K was efficient in S. aureus.12 Intracellular L. monocytogenes could be targeted with HIV-1-TAT- and (RXR)4XB-conjugated PNAs.14 In our previous study, we found that HIV-1-TAT-coupled anti-gyrA PNAs showed antimicrobial activity in S. pyogenes.15

The aim of this study was to compare the efficiency of potential carrier molecules delivering antisense PNAs in S. pyogenes. We tested 18 CPPs belonging to different classes. Three CPPs were shown to support uptake of anti-gyrA PNAs: the cationic CPPs K8 and HIV-1-TAT and the arginine-rich, amphipathic peptide (RXR)4XB. In general, basic residues support internalization of CPPs into cells, because their positive charge initiates interaction with the negatively charged surface. Specifically, it has been shown that arginine residues were more effective than lysines and that the replacement of lysine residues with arginine improved cellular uptake.26,37 Here, we show that oligoarginine-coupled antisense PNAs were not able to inhibit S. pyogenes growth, whereas K8-conjugated anti-gyrA PNA showed an antimicrobial effect. In eukaryotic cells, insertion of 6-aminohexanoic acid (X) or β-alanine (B) residues into oligoarginine R8 decreased the cellular uptake but increased the splice-correction activity of the resulting compound.38 We observed that in contrast to R8, which did not function as a carrier in S. pyogenes, (RXR)4XB was able to mediate uptake of antisense PNAs. Penetratin (Ant) did not support antisense PNA uptake into S. pyogenes. This result is in accordance with an observation in L. monocytogenes. PNA uptake into L. monocytogenes was mediated by HIV-1-TAT and (RXR)4XB but not by Ant.14 PDSTK, a peptide derived from a PEST-like sequence from yeast, was able to support PNA antisense effects in S. aureus.12 We did not detect antimicrobial activity of PDSTK-conjugated antisense PNA in S. pyogenes.

We further analyzed the bactericidal effect of TAT-, K8-, and (RXR)4XB-anti-gyrA PNAs. All three CPP-anti-gyrA PNAs showed a dose-dependent antimicrobial effect in kill assays. Incubation of S. pyogenes with up to 5 μM K8- and (RXR)4XB-conjugated scPNAs did not lead to the reduction of bacterial counts. In contrast, TAT-anti-gyrA scPNA showed a sequence-independent
antimicrobial effect in this assay, indicating a toxic influence of the TAT peptide on *S. pyogenes* under these conditions. In a previous study, we tested the effect of the TAT peptide alone on *S. pyogenes* and did not observe any antibacterial activity up to 20 μM. Six hours following treatment with 5 μM or 10 μM TAT-, K8-, and (RXR)₄XB-anti-gyrA PNAs, a log CPU reduction of three or four, respectively, was observed, but no clearance was achieved. In contrast, *L. monocytogenes* could be cleared after 20 min incubation with 8 μM TAT- and (RXR)₄XB-antisense PNAs specific for the gene of RNA polymerase α subunit (*rpoA*). MIC determination revealed that TAT-anti-gyrA PNA and K8-anti-gyrA PNA were effective at the same MIC of 15.6 μM, whereas the respective scrambled controls showed a MIC of 62.5 μM. Here, no toxic effect of the TAT peptide was observed. The MICs of (RXR)₄XB-anti-gyrA PNA and its corresponding scrambled control were 62.5 μM and 125 μM, respectively. Compared with *L. monocytogenes*, these MIC values are rather high. TAT- and (RXR)₄XB-anti-*rpoA* PNAs exhibited a MIC of 1–4 μM, depending on the *L. monocytogenes* isolate tested.

To assess whether the bactericidal effect of the CPP-antisense PNAs is sufficient for treatment of a *S. pyogenes* infection in vivo, a *G. mellonella* infection model was used. TAT-, K8-, and (RXR)₄XB-anti-gyrA PNAs increased survival of infected *G. mellonella* larvae. In contrast, treatment of infected larvae with Ant-, mVE-cadherin-, and ELA-anti-gyrA PNAs, which did not show bactericidal activity in vitro, did not affect survival. A combination of anti-gyrA PNAs with antibiotics targeting gyrase subunit A was shown to result in synergistic or additive antimicrobial effects on *S. aureus* and *S. pyogenes* in vitro. Here, we demonstrate that combination therapy of infected larvae with TAT-anti-gyrA PNAs and levofloxacin led to increased survival rates compared with each treatment alone, supporting the idea that a combination of antisense PNAs with conventional antibiotics is a potent strategy to decrease the concentration of antibiotics during treatment of *S. pyogenes* infections.

Figure 7. Survival of *Galleria mellonella* Larvae Treated with 4 nmol CPP-PNAs following Infection with *S. pyogenes*

PNA conjugates are indicated by the name of the respective CPP: TAT (A), K8 (B), (RXR)₄XB (C), Ant (D), mVE-cadherin (E), and ELA (F). Scrambled PNA controls are indicated by sc. Statistical significance was determined using the log-rank test. Differences between curves were expressed as *p* ≤ 0.05; **** *p* ≤ 0.0001. Sample size: n = 60 larvae per group (A–C); n = 20 larvae per group (D–F).
We were surprised that from 18 CPP-PNA conjugates tested in this study, only three showed an efficient antimicrobial effect. For future experiments, different types of carriers should be investigated. One possible alternative to peptide carriers is vitamin B12, which has been successfully used in *E. coli* and *Salmonella Typhimurium.* The authors showed that vitamin B12 worked more efficiently in *E. coli* than (KFF)K, which is widely used in this organism.

Furthermore, we will aim at the identification of additional antisense target genes specific for *S. pyogenes*. Beside other essential genes, antisense targeting of virulence factor genes is a promising strategy. For instance, antisense PNAs directed against *ska*, the gene coding for streptokinase, could potentially diminish *S. pyogenes* virulence. Streptokinase is involved in the lysis of fibrin clots and thereby supports bacterial spreading. It has been shown that a small compound inhibiting *ska* expression was able to improve survival in a murine infection model.*

In this study, we were able to confirm that *gyrA* is a suitable target for PNA-mediated antisense inhibition of gene expression in *S. pyogenes*. We found that TAT-, K8-, and (RXR)XB-antis*gyrA* PNAs showed antibacterial activity *in vitro* and *in vivo* with comparable characteristics. TAT-conjugated scPNAs showed *in vitro* an unspecific effect, probably caused by TAT toxicity, which was not apparent *in vivo*. K8-coupled anti-*gyrA* PNA showed high antimicrobial efficiency *in vitro* and was effective on all *S. pyogenes* serotypes tested except AP1. In contrast, (RXR)XB-coupled anti-*gyrA* PNA showed high bactericidal efficiency in the kill assay but exhibited higher MICs than TAT and K8-PNA conjugates. Additionally, the effect of (RXR)XB-coupled anti-*gyrA* PNA on larvae survival in the *G. mellonella* infection model was lower compared with the other conjugates. Overall, our results underline the importance of suitable vectors for PNA delivery to achieve optimal antimicrobial function and identified efficient CPPs for testing of additional *S. pyogenes* target genes.

**MATERIALS AND METHODS**

**PNA Synthesis**

CPP-PNAs were synthesized and purified by high-performance liquid chromatography (HPLC) (Peps4LS, Heidelberg, Germany). Sequences of all CPP-PNAs used in this work are listed in Table 1.

**Bacterial Strains and Culture Conditions**

*S. pyogenes* strains were cultured in Todd-Hewitt broth, supplemented with 0.5% yeast extract (THY; Oxoid, Thermo Fisher Scientific, Darmstadt, Germany), at 37°C under a 5% CO2/20% O2 atmosphere. All strains used in this study are listed in Table 3.

**Bacterial Kill Assay**

Overnight cultures of the respective *S. pyogenes* strain were diluted in PBS/brain heart infusion (BHI) (7/2) to approximately 2 × 10⁵ CFU/mL. 450 μL bacterial suspension, containing ~10⁵ CFU, was transferred to a 2-mL reaction tube. 50 μL PNA was added to a bacterial suspension, containing 10⁵ CFU/mL. The initial CFU count corresponded to 1–3 × 10⁵ CFU/mL. The initial CPP screen has been performed in two biological replicates. Each subsequent experiment has been performed in at least three independent biological replicates, as indicated in the figure legends.

**Extraction of Total RNA**

For RNA isolation, 450 μL bacterial suspension (10⁷ CFU/mL in THY) was prepared for each experimental condition, treated with 2 μM CPP-PNA conjugates, and incubated in a 2-mL
reaction tube for 6 h at 37°C and 7 rpm (Rotor SB3; Stuart, Staffordshire, UK). Subsequently, five samples per condition were pooled. Bacteria were pelleted immediately, shock frozen in liquid nitrogen, and stored at −80°C until use. Bacterial cells were disrupted in a homogenizer (Peqlab Biotechnologie, Erlangen, Germany). Total RNA was extracted, according to the protocol, supplied with the Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA). After extraction, RNA was treated with acid phenol–chloroform:isoamyl alcohol (125:24:1; pH 4.5; Thermo Fisher Scientific), and TURBO DNase (Thermo Fisher Scientific), according to the manufacturer’s instructions. RNA was stored at −80°C until further use.

Reverse Transcription Followed by quantitative real-time PCR
cDNA synthesis was performed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Thermo Fisher Scientific). Quantitative real-time PCR amplification was conducted with SYBR Green (Thermo Fisher Scientific) using the ViiA 7 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). The 5S rRNA gene served as internal control. Relative expression was calculated using the 2−ΔΔCt method.43 Primers were designed based on the full genome sequence of *S. pyogenes* M49 strain NZ131 (NCBI: NC011375): *gyrA*-specific primers: 5’-TGAAGTGTCTATTGGCAAGAGC-3’ and 5’-AGAGAATACGACGATGCACAGG-3’; 5S-specific primers: 5’-AGCGACTACCTTATCTCACAG-3’ and 5’-AGAGAATACGACGATGCACAGG-3’.

Determination of the MIC
MIC determination was performed following the protocol of the Clinical and Laboratory Standards Institute (CLSI).44 In MIC assays containing CPP-PNAs, lysed horse blood (LHB)/cation-adjusted Mueller-Hinton broth (CAMHB) medium was supplemented with 0.02% acetic acid and 0.4% BSA. MICs were recorded as the lowest concentration where no turbidity was observed in the wells.

**G. mellonella Infection Model**
Larvae of the greater wax moth *G. mellonella* were obtained from Reptilienkosmos (Niederkrüchten, Germany). For infection experiments, *S. pyogenes* strains were grown overnight in THY, washed twice in a 0.9% NaCl solution, and suspended in 0.9% NaCl to a final concentration of 1–3 × 10⁵ CFU/mL. Larvae with a weight of 150–200 mg were infected with 1–3 × 10⁶ CFU/larva. Bacteria were injected into the hemocoel of the larvae between the last two pairs of pro-legs using a microapplicator (World Precisions Instruments, Sarasota, FL) and a fine dosage syringe (Omnican F; B. Braun, Melsungen, Germany; 0.01–1 mL, 0.30 × 12 mm). As mock control, 0.9% NaCl was injected. For CPP-PNA treatment, larvae were injected 30 min postinfection with 4 nmol CPP-PNA/larva. Larvae were incubated for 7 days, and survival was monitored daily.46

### Table 3. *S. pyogenes* Strains

| Strain | M Type | Isolation                  | Reference                                      |
|--------|--------|----------------------------|------------------------------------------------|
| 5448   | MT1    | STSS                       | Dr. Nikolai Siemens, Karolinska Institut, Lund, Sweden |
| API    | M1     | sepsis                     | Centre for Reference and Research on Streptococci, Prague, Czech Republic |
| M3 8003| M3     | necrotizing fasciitis       | Dr. Nikolai Siemens, Karolinska Institut, Lund, Sweden |
| HERO-K-035 | M4 | throat infection      | clinical isolate, University Medicine Rostock, Germany |
| MGAS8232 | M18   | ARF                        |                                               |
| 591    | M49    | skin                       | R. Lütticken, Aachen, Germany                  |

ARF, acute rheumatic fever; STSS, streptococcal toxic-shock syndrome.

Statistical Analyses
All experiments were performed at least three times or as indicated by sample size (n). Statistical significance was determined using the tests indicated in the respective figure legends. Statistical analyses were performed using GraphPad Prism 7 software.

### ACKNOWLEDGMENTS
Support for the work of G.B. and B.K. was provided by a grant from the Ministerium für Bildung, Wissenschaft und Kultur, Mecklenburg-Vorpommern (ESF/14-BM-A55-0010/16). Support for the work of N.P. was provided by the University Medicine Rostock (FORUM 889008).

### REFERENCES
1. Carapetis, J.R., Steer, A.C., Mulholland, E.K., and Weber, M. (2005). The global burden of group A streptococcal diseases. Lancet Infect. Dis. 5, 685–694.
2. Sims Sanyahumbi, A., Colquhoun, S., Wyber, R., and Carapetis, J.R. (2016). Global disease burden of group A streptococcus. In *Streptococcus pyogenes: Basic Biology to Clinical Manifestations*, J.J. Ferretti, D.L. Stevens, and V.A. Fischetti, eds. (University of Oklahoma Health Sciences Center).
3. Cunningham, M.W. (2008). Pathogenesis of group A streptococcal infections and their sequelae. Adv. Exp. Med. Biol. 609, 29–42.
4. Bisno, A.L., Gerber, M.A., Gwatney, J.M., Jr., Kaplan, E.L., and Schwartz, R.H.; Infectious Diseases Society of America (2002). Practice guidelines for the diag-
nosis and management of group A streptococcal pharyngitis. Clin. Infect. Dis. 35, 113–125.
5. Richter, S.S., Heidmann, K.P., Beekmann, S.E., Miller, N.J., Miller, A.L., Rice, C.L.,
Doern, C.D., Reid, S.D., and Doern, G.V. (2005). Macrolide-resistant Streptococcus
pyogenes in the United States. 2002-2003. Clin. Infect. Dis. 41, 599–608.
6. Cattoir, V. (2016). Mechanisms of antibiotic resistance. In Streptococcus pyogenes: Basic Biology to Clinical Manifestations, J.I. Ferretti, D.I. Stevens, and V.A. Fischetti, eds. (University of Oklahoma Health Sciences Center).
7. Nielsen, P.E., and Egholm, M. (1999). An introduction to peptide nucleic acid. Curr.
Issues Mol. Biol. 1, 89–104.
8. Demidov, V.V., Potaman, V.N., Frank-Kamenetski, M.D., Egholm, M., Buchard, O.,
Sonnichsen, S.H., and Nielsen, P.E. (1999). Stability of peptide nucleic acids in human
serum and cellular extracts. Biochem. Pharmacol. 48, 1310–1313.
9. Gupta, B., Levecchenko, T.S., and Torchilin, V.P. (2005). Intracellular delivery of large
molecules and small particles by cell-penetrating peptides and peptides. Adv. Drug
Deliv. Rev. 57, 637–651.
10. Lehto, T., Erzat, K., Wood, M.J.A., and El Andaloussi, S. (2016). Peptides for nucleic
acid delivery. Adv. Drug Deliv. Rev. 106 (Pt A), 172–182.
11. Eriksson, M., Nielsen, P.E., and Good, L. (2002). Cell permeabilization and uptake of
antisense peptide-peptide nucleic acid (PNA) into Escherichia coli. J. Biol. Chem. 277, 7144–7147.
12. Nekhotiaeva, N., Awasthi, S.K., Nielsen, P.E., and Good, L. (2004). Inhibition of
Staphylococcus aureus gene expression and growth using antisense peptide nucleic acids.
Mol. Ther. 10, 652–659.
13. Abushabha, M.F., Mohammad, H., and Seleem, M.N. (2016). Targeting multidrug-
resistant Staphylococci with an anti-rpoA peptide nucleic acid conjugated to the
HIV-1 Tat cell penetrating peptide. Mol. Ther. Nucleic Acids 5, e339.
14. Abushabha, M.F., Mohammad, H., Thangamani, S., Hussein, A.A., and Seleem, M.N.
(2016). Impact of different cell penetrating peptides on the efficacy of antisense ther-
apoetics for targeting intracellular pathogens. Sci. Rep. 6, 20832.
15. Patenge, N., Pappesch, R., Krawack, F., Walda, C., Mraheil, M.A., Jacob, A.,
Hain, T., and Kreikemeyer, B. (2013). Inhibition of growth and gene expression by
PNA-peptide conjugates in Streptococcus pyogenes. Mol. Ther. Nucleic Acids 2, e132.
16. Derossi, D., Joliot, A.H., Chassaing, G., and Prochiantz, A. (1994). The third helix of
the Antennapedia homeodomain translocates through biological membranes. J. Biol.
Chem. 269, 10444–10450.
17. Turner, Y., Wallukat, G., Saálik, P., Wiesner, B., Pritz, S., and Oehlke, J. (2010).
Cellular uptake and biological activity of peptide nucleic acid conjugates with pep-
tides with and without cell-penetrating ability. J. Pept. Sci. 16, 71–80.
18. Vivès, E., Brodin, P., and Lebleu, B. (1997). A truncated HIV-1 Tat protein basic
domain rapidly translocates through the plasma membrane and accumulates in the
cell nucleus. J. Biol. Chem. 272, 16010–16017.
19. Treinh, R., Krauss, U., Beck-Sickinger, A.G., Merkle, H.P., and Nielsen, H.M. (2004).
Cellular uptake but low permeation of human calcitonin-derived cell penetrating
peptides and Tat(47-57) through well-differentiated epithelial models. Pharm. Res.
21, 1248–1256.
20. Vaaza, M., and Porro, M. (1996). Group of peptides that act synergistically with
hydrophobic antibiotics against gram-negative enteric bacteria. Antimicrob. Agents
Chemother. 40, 1801–1805.
21. Mueller, J., Kretzschmar, I., Voelker, R., and Boisguerin, P. (2008). Comparison of
cellular uptake using 22 CPPs in 4 different cell lines. Bioconjug. Chem. 19, 2363–
2374.
43. Smoot, J.C., Barbian, K.D., Van Gompel, J.J., Smoot, L.M., Chaussee, M.S., Sylva, G.L., Sturdevant, D.E., Ricklefs, S.M., Porcella, S.F., Parkins, L.D., et al. (2002). Genome sequence and comparative microarray analysis of serotype M18 group A streptococcus strains associated with acute rheumatic fever outbreaks. Proc. Natl. Acad. Sci. USA 99, 4668–4673.

44. Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(−ΔΔC(T)) method. Methods 25, 402–408.

45. Weinstein, M.P., Patel, J.B., Burnham, C.A., Campeau, S., Convile, P.S., Doern, C., Eliopoulos, G.M., Galas, M.F., Humphries, R.M., Jenkins, S.G., et al. (2018). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 11th Edition (Clinical and Laboratory Standards Institute).

46. Mukherjee, K., Altincicek, B., Hain, T., Domann, E., Vilcinskas, A., and Chakraborty, T. (2010). Galleria mellonella as a model system for studying Listeria pathogenesis. Appl. Environ. Microbiol. 76, 310–317.