Inhibition of Growth and Gene Expression by PNA-peptide Conjugates in *Streptococcus pyogenes*

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While *Streptococcus pyogenes* is consistently susceptible toward penicillin, therapeutic failure of penicillin treatment has been reported repeatedly and a considerable number of patients exhibit allergic reactions to this substance. At the same time, streptococcal resistance to alternative antibiotics, e.g., macrolides, has increased. Taken together, these facts demand the development of novel therapeutic strategies. In this study, *S. pyogenes* growth was inhibited by application of peptide-conjugated antisense-peptide nucleic acids (PNAs) specific for the essential gyrase A gene (*gyrA*). Thereby, HIV-1 Tat peptide-coupled PNAs were more efficient inhibitors of streptococcal growth as compared with (KFF)3K-coupled PNAs. Peptide-anti-*gyrA* PNAs decreased the abundance of *gyrA* transcripts in *S. pyogenes*. Growth inhibition by antisense interference was enhanced by combination of peptide-coupled PNAs with protein-level inhibitors. Antimicrobial synergy could be detected with levofloxacin and novobiocin, targeting the gyrase enzyme, and with spectinomycin, impeding ribosomal function. The prospective application of carrier peptide-coupled antisense PNAs in *S. pyogenes* covers the use as an antimicrobial agent and the employment as a knock-down strategy for the investigation of virulence factor function.

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Introduction

*Streptococcus pyogenes* (group A streptococci (GAS)) is an exclusively human pathogen, which causes a wide spectrum of infectious diseases ranging from mild superficial infections of the skin and the mucosal membranes to invasive diseases like necrotizing fasciitis (flesh-eating disease) or streptococcal toxic shock syndrome. Typically, superficial infections are associated with spontaneous recovery. However, if mild infections remain untreated, severe invasive infections or autoimmune sequelae can develop as a consequence.1,2 Therefore, antibiotic therapy is strongly indicated upon streptococcal infections. Currently, penicillin is the standard treatment of streptococcal pharyngitis. Reasons are the continuing susceptibility of GAS toward penicillin, its efficiency, safety, and the comparably low costs of penicillin treatment.3–5 However, penicillin-related treatment failure has been reported repeatedly in cases of streptococcal pharyngitis.6,7 Factors that have been discussed to be responsible for this phenomenon include the coexistence of β-lactamase–producing bacteria,4 biofilm formation by GAS,5 and internalization of GAS into epithelial host cells.10–12 Another problem poses the rising occurrence of macrolide resistance in GAS,13,14 which limits the use of macrolides to patients with significant penicillin allergies.15 Consequently, the development of novel therapeutic strategies remains an imperative.

Among the innovative therapeutic approaches, antisense molecules gain increasing importance. One advantage of antisense interference is the specific effect on target molecules. Another is the lack of already established bacterial resistance mechanisms toward antisense agents. Peptide nucleic acids (PNAs) have been tested as antimicrobial agents in the past decade in a variety of bacterial species. Their chemical properties place PNAs between peptides and nucleic acids. Nucleobases, which are capable of sequence-specific base pairing, are present in PNAs. However, peptide bonds replace the nucleic acid-specific sugar-phosphate backbone.16 PNAs show a high stability in organic solutions as well as in water and their hybrid characteristics add to their stability in biological environments. So far, no nuclease or protease is known to be capable of hydrolyzing PNAs. Consequently, PNAs proved to be very stable in human serum and cellular extracts.17 PNA uptake is limited by the outer cell membrane in Gram-negative bacteria.18 Cell-penetrating peptides (CPPs) are naturally occurring or synthetic peptides containing positively charged residues that are able to enter eukaryotic cells and bacteria. They can be employed for the transduction of cargo into target cells.19,20 Transport of PNAs into Gram-negative bacteria could be facilitated by (KFF)3K CPPs coupled to the PNA molecules.21–24 The mRNA of several essential genes has been targeted by PNA antisense interference to achieve inhibition of bacterial growth, including the gene for an RNA polymerase primary sigma factor (*rpoD*), the gene for the gyrase A subunit (*gyrA*), the gene coding for the anticyl carrier protein (*acpP*), and the *ompA* gene, coding for an outer membrane protein.25–29 In a different approach, bacterial protein biosynthesis has been inhibited by targeting with PNAs specific for the 16S or the 23S RNA.30,31 In a limited number of studies, CPP-conjugated PNAs have been tested in Gram-positive bacteria. In general, the...
antisense effect was less pronounced in Gram-positive species than in *E. coli* and a higher PNA concentration was required to cause growth reduction.

Here, we aimed at inhibiting growth of GAS M49, which is a generalist known to be responsible for skin and throat infections, 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. Growth reduction was achieved employing PNA sequences specific for *gyrA*, which were either coupled to (KFF)3K- or to Tat-peptides, respectively. Tat-conjugated anti-*gyrA* PNA sequences inhibited the growth of GAS M49 more efficiently than (KFF)3K-coupled anti-*gyrA* PNA sequences, while showing a lower unspecific CPP-related toxicity. Combination testing revealed antimicrobial synergy between antisense-PNA and conventional antibiotics.

**Results**

**GAS M49 growth reduction by carrier peptide-coupled anti-*gyrA* PNA**

Anti-*gyrA* PNA sequences were designed complementary to nucleotides covering the start codon of *gyrA* and three regions throughout the coding sequence of the gene (Table 1). We tested anti-*gyrA* PNA sequences with and without coupling to the synthetic (KFF)3K peptide, which had been used successfully before to support PNA uptake in a variety of bacterial species. In Figure 1a, a schematic of (KFF)3K-coupled anti-*gyrA* PNA sequences complementary to the start region is shown as a representative example. First, the four different target sequences within *gyrA* were compared. PNA sequences lacking the (KFF)3K-carrier peptide did not influence bacterial growth at all (data not shown). Also, (KFF)3K-coupled PNA sequences complementary to *gyrA* nucleotides 91-105, 867-881, and 1925-1939, respectively, did not interfere with GAS M49 growth (Table 1). Exclusively, (KFF)3K-coupled PNA sequences complementary to the start codon region of the *gyrA* transcript (nucleotides −5 to 5) led to a concentration-dependent reduction of GAS M49 growth (Table 1; Figure 2a). To control for specificity of the interaction, scrambled PNA sequences (scPNAs) were designed, which shared the same base composition with sequence-specific anti-*gyrA* PNA sequences but exhibited a randomized sequence (Figure 1b). Comparison with (KFF)3K-anti-*gyrA* scPNA revealed that growth reduction caused by treatment with (KFF)3K-anti-*gyrA* PNA was sequence specific (Figure 2b). No growth inhibition was achieved by addition of scPNA within a concentration range of 0.8–4.0 µmol/l. At PNA concentrations ≥5.6 µmol/l, unspecific toxic effects of the scPNAs were observed (Figure 2b). Application of the (KFF)3K peptide alone also resulted in the reduction of bacterial growth at concentrations ≥5.6 µmol/l (data not shown). It is likely that growth inhibition upon application of high concentrations of (KFF)3K peptide-coupled PNAS is mediated at least in part by toxic effects of the leader peptide. The minimal inhibitory concentration (MIC) of (KFF)3K-anti-*gyrA* PNA was 10 µmol/l. At this concentration, inhibition is caused by a combination of a sequence-specific action of the antisense molecule and an unspecific toxic effect of the leader peptide. Consequently, the following experiments were performed in the sub-MIC sequence-specific effective concentration range of (KFF)3K-anti-*gyrA* PNA. The observed effect upon treatment with (KFF)3K-anti-*gyrA* PNA was statistically relevant in the exponential (3 hours) as well as in the early stationary growth phase (6 hours) (Figure 2c–f). Dose dependency of GAS M49 growth inhibition became evident upon comparison of growth rates between samples (Supplementary Figure S1).

**Table 1**

| Peptide nucleic acids (PNAs) and inhibitory concentrations |
|----------------------------------------------------------|
| **PNA** | **Target** | **Sequence** | **c (µmol/l)** |
| anti-*gyrA* PNA | *gyrA* -5 to 5 | Tgcattaag | — |
| anti-*gyrA* scPNA | *gyrA* -5 to 5 | Attagctgt | — |
| (KFF)3K-anti-*gyrA* PNA | *gyrA* -5 to 5 | (KFF)3K-eg*-tgcattaag | 1.6–4.0 |
| (KFF)3K-anti-*gyrA* scPNA | *gyrA* -5 to 5 | (KFF)3K-eg*-attagctgt | ≥5.6 |
| (KFF)3K-anti-*gyrA* _91 PNA | *gyrA* 91 to 105 | (KFF)3K-G-gcctggccagatg | — |
| (KFF)3K-anti-*gyrA* _867 PNA | *gyrA* 867 to 881 | (KFF)3K-G-tgcctggccagatg | — |
| (KFF)3K-anti-*gyrA* _1925 PNA | *gyrA* 1925 to 1939 | (KFF)3K-G-aagaggagatgc | — |
| (KFF)3K cell-penetrating peptides (CPP) | | (KFF)3K | ≥5.6 |
| Tat-anti-*gyrA* PNA | *gyrA* -5 to 5 | GRKKRKRQRQRQRQRQK-eg*-tgcattaag | 0.4–1.4 |
| Tat-anti-*gyrA* scPNA | *gyrA* -5 to 5 | GRKKRKRQRQRQRQRQK-eg*-attagctgt | — |
| Tat CPP | | GRKKRKRQRQRQRQRQK | ≥20.0 |

*ethyleneglycol linker: 8-amino-3,6-dioxaoctanoic acid.*
(KFF)3K-anti-gyrA PNA effects the abundance of gyrA transcripts in GAS M49

We asked whether antisense binding of (KFF)3K-anti-gyrA PNA to the gyrA mRNA was reflected by changes of the gyrA transcript level. The influence of the presence of sequence-specific PNAs on the amount of gyrA mRNA was tested by reverse transcription followed by quantitative reverse transcription polymerase chain reaction (Figure 3). Transcript abundance of the 5S RNA gene was used for normalization. The gyrA mRNA level in mock-treated GAS samples served as control. Upon treatment with 1.6 µmol/l (KFF)3K-anti-gyrA PNA, the amount of gyrA transcript was reduced to 50% of the amount detected in the untreated control sample. Addition of scPNA did not influence the relative gyrA mRNA level detected by quantitative reverse transcription polymerase chain reaction.

The HIV-1 Tat peptide supports the antimicrobial activity of anti-gyrA PNA more efficiently than the (KFF)3K-carrier

In GAS M49, PNA uptake and PNA-mediated growth inhibition were not achieved when PNAs lacking a leader peptide were employed. Fusion of the CPP (KFF)3K to gyrA-specific PNA enabled sequence-specific growth inhibition of GAS M49. The uptake efficiency of every given CPP varies between bacterial species. Similar to the cationic and hydrophobic (KFF)3K peptide, the HIV-1 Tat protein-derived arginine-rich recombinant peptide YGRKKRRQRRR (Tat) translocates by destabilizing phospholipid bilayers and thereby induces transient pores in the respective membrane.29 In contrast to (KFF)3K, Tat has not been used before for transduction into...
bacteria. To test whether the Tat peptide could be employed as a leader peptide for the uptake of peptide oligonucleotides into GAS M49, it was coupled to gyrA antisense PNA. The effect of Tat-anti-gyrA PNA on growth of GAS M49 was compared with the growth inhibition mediated by Tat-anti-gyrA scPNA. Significant growth reduction in the presence of Tat-anti-gyr PNA was observed in the concentration range of 0.4–1.4 µmol/l (Figure 4a,c,d). Addition of higher concentrations of Tat-PNA anti-gyrA did not lead to a further increase of the observed effects (data not shown). Application of Tat-anti-gyrA scPNA did not lead to growth inhibition of GAS M49 (Figure 4b), indicating a sequence-specific inhibitory effect. Addition of Tat peptide alone did not lead to GAS M49 growth inhibition up to a concentration of 10 µmol/l Tat peptide (data not shown). In comparison with anti-gyrA PNAs coupled to the (KFF)3K leader peptide, Tat-PNA anti-gyrA showed a much more efficient growth inhibition of GAS M49 (Table 1).

Antimicrobial synergy between peptide-coupled anti-gyrA PNAs and peptide-level antibiotics

The bacterial gyrase protein is a well-known target of antibiotics including aminocoumarins and quinolones. Given the fact that antisense-PNA treatment is reducing bacterial growth effectively but not completely, we wanted to test whether the combined application of peptide-coupled anti-gyrA PNAs with conventional antibiotics leads to synergistic effects. Gyrase peptide-targeting antibiotics were compared with the antibiotics targeting proteins unrelated to DNA replication. A widely used test for the determination of antimicrobial synergy between different inhibitors is the chequerboard assay. Following this protocol, the MIC for each compound is determined independently. Following serial dilution of the individual inhibitors, each concentration of one agent is then combined with each concentration of the second agent. Bacterial growth is determined for the combinations after appropriate incubation of the samples. For analyses of the inhibitor interactions, the fractional inhibitory concentration indices (FICI) are calculated. Checkerboard assay data have been interpreted variably, leading to ambiguous results. Furthermore, a linear dose dependency, which cannot be assumed for all antimicrobial substances, is a prerequisite for correct synergy determination using this method. Moreover, as pointed out before, at the MIC determined for (KFF)3K-anti-gyrA PNA, the sequence-specific effect of PNA was superimposed by toxic effects of the carrier peptide. For these reasons, the checkerboard assay was not feasible for combination testing of PNAs with antibiotics in GAS M49. To be able to work within the sequence-specific effective concentration range, spectrophotometric assessment of dose–response curves for antimicrobial combinations was employed as examples for antibiotics interfering with the same pathway as anti-gyrA PNAs, inhibitors of gyrase A (levofloxacin) and gyrase B (novobiocin) were chosen. These were compared with spectinomycin, which binds to the ribosomal 30S subunit and thereby inhibits a pathway distinct from DNA replication. In Figure 5a–c, the spectrophotometric recordings for the interaction of (KFF)3K-anti-gyrA PNAs with levofloxacin are shown as one representative example. Reduced growth of GAS M49 in the presence of levofloxacin (0.5–10 µg/ml) could be detected (Figure 5a). At a concentration of 0.5 µg/ml levofloxacin, a slight growth repression was observed repeatedly. This concentration was chosen for combination testing. GAS M49 was incubated in the presence of 0.5 µg/ml levofloxacin and 0.8–4.0 µmol/l (KFF)3K-anti-gyrA PNAs, respectively. Upon addition of (KFF)3K-anti-gyrA PNAs to the culture, an increased inhibition of growth could be observed in comparison with samples containing levofloxacin alone (Figure 5b). Reduction of the growth constant \( q = \ln(\text{OD}_{tx}/\text{OD}_{o})/t \) in the presence of one inhibitor or following application of a combination of the two agents served as a measure of interaction of the two effectors. For the combination of levofloxacin with (KFF)3K-anti-gyrA PNA, synergy was observed over the entire

Figure 4 Effect of Tat-coupled peptide nucleic acids (PNAs) on GAS M49 growth. (a) Concentration-dependent growth inhibition of GAS M49 by Tat-anti-gyrA PNA. (b) No growth inhibition of GAS M49 by Tat-anti-gyrA scPNA. (c,d) Statistic evaluation of the growth inhibition effect of Tat-anti-gyrA PNA at 3 (c) and at 6 hours (d). Error bars represent the mean ± SD, \( n = 6 \).
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Table 2 Interpretation of the decreasing growth rate constant (6 hours) (KFF)K-anti-gyrA peptide nucleic acid (PNA)

| gPNA (µmol/l) | gantibiotic | g0 | gPNA+antibiotic | gPNA × gantibiotic | Interpretation |
|--------------|-------------|----|----------------|-------------------|----------------|
| 0.5 µg/ml levofloxacin | | | | | |
| 0 | 0.20 | 0.19 | 0.20 | 0.19 | n.a.* |
| 0.8 | 0.19 | 0.19 | 0.20 | 0.17 | Synergyc |
| 1.6 | 0.13 | 0.19 | 0.20 | 0.11 | 0.12 Synergyc |
| 3.2 | 0.13 | 0.19 | 0.20 | 0.10 | 0.12 Synergyc |
| 4.0 | 0.11 | 0.19 | 0.20 | 0.09 | 0.10 Synergyc |
| 0.2 µg/ml novobiocin | | | | | |
| 0 | 0.22 | 0.20 | 0.22 | 0.20 | n.a.* |
| 0.8 | 0.21 | 0.20 | 0.22 | 0.20 | 0.19 Autonomyc |
| 1.6 | 0.11 | 0.20 | 0.22 | 0.10 | 0.10 Autonomyc |
| 3.2 | 0.08 | 0.20 | 0.22 | 0.08 | 0.07 Autonomyc |
| 4.0 | 0.08 | 0.20 | 0.22 | 0.03 | 0.07 Synergyc |
| 5.0 µg/ml spectinomycin | | | | | |
| 0 | 0.20 | 0.20 | 0.20 | 0.20 | n.a.* |
| 0.8 | 0.19 | 0.20 | 0.20 | 0.17 | 0.19 Synergyc |
| 1.6 | 0.13 | 0.20 | 0.20 | 0.09 | 0.13 Synergyc |
| 3.2 | 0.13 | 0.20 | 0.20 | 0.06 | 0.13 Synergyc |
| 4.0 | 0.11 | 0.20 | 0.20 | 0.04 | 0.11 Synergyc |

Both (KFF)K-anti-gyrA PNA and Tat-anti-gyrA PNA showed synergy with all three antibiotics tested in this study. The effect was independent of the pathway targeted by the antimicrobial substance. Combination of peptide-coupled anti-gyrA PNAs with antibiotics achieved a stronger overall growth inhibition than application of PNA alone.

Discussion

In the era of increasing drug resistance, it is more important than ever to assure timely development of innovative antimicrobial agents. Among antisense molecules, PNAs are particularly promising candidates due to their specific structural features. They are known for their strong pairing to DNA as well as to RNA and their pseudopeptide backbone confers stability while allowing molecular modifications, e.g., the conjugation of CPPs. The potency of antisense PNAs has been studied most thoroughly in Escherichia coli. Few reports are available about the effectivity of PNAs in Gram-positive pathogens. We set out to test whether PNA antisense targeting of a gene coding for an essential enzyme in GAS M49 will pose an impediment to its growth. We choose gyrA, because the gyrase enzyme represents a well-characterized target of antibiotics, including aminocoumarins and quinolones, and because gyrA has been successfully used before for PNA antisense-studies in other species.24,28

We observed growth reduction in GAS M49 following the application of anti-gyrA PNAs. However, conjugation of anti-gyrA PNAs with CPPs was required for antimicrobial activity of the antisense-PNAs. This is in accordance with the previous studies. PNA uptake by the bacterial cell has repeatedly been described as a limiting factor in antimicrobial PNA action. To overcome this obstacle, a variety of
different strategies has been pursued in the past. The LPS
layer-defective E. coli-mutant AS1935 has been used fre-
quently for PNA studies, because of its increased perme-
ability. Another widely employed option is the conjugation
of PNAs with different CPPs, which proved to be effective in GAS M49. While no
toxicity is observed in GAS M49, a decrease of gyrA mRNA abundance to 50% could be detected upon treatment with (KFF)3K-anti-gyrA PNAs (Figure 3). Similar results have been obtained in E. coli, where application of (KFF)3K-anti-acpP-PNAs caused a decrease of acpP mRNA abundance to about 60% of the untreated control.36 Since ribosomal binding during translation acts as protective bar-
rier against cleavage and thereby stabilizes mRNA,41 a ham-
pered gyrA mRNA translation initiation due to PNA binding
might be responsible for a moderate destabilization of the
gyrA transcript in GAS M49.

The (KFF)3K-anti-gyrA PNA concentration required for the
implementation of growth inhibition in GAS M49 was much
higher than reported from E. coli inhibition studies. The MIC in
E. coli K12 varied between 2 and 6 µmol/l, depending on the
target,42 whereas the apparent MIC observed for GAS M49
was about 10 µmol/l, which is an approximate value, because
it is influenced by toxic effects of the (KFF)K leader. We
speculated that import of PNAs into M49 could be improved by
PNA coupling to a different CPP, preferentially to a peptide
exhibiting lower toxicity. Besides the synthetic (KFF)K pep-
tide, there are many naturally occurring CPPs known, which
represent short sequences of amino acids that are capable of
entering most mammalian cells.19 CPPs are often highly cat-
ionic and hydrophilic. Translocation of cargos across the cell
membrane seems to involve destabilization of the membrane
structure and formation of a pore by the cationic peptides.23 The HIV-1
Tat protein contains a small region corresponding to residues
YGRKKRRQRRR, which is required for membrane trans-
location by an apparently energy-independent mechanism.43
This fragment of the basic protein domain was shown to exhibit no cytotoxicity in HeLa cells at concentrations up to
100 µmol/l.44 However, translocation efficiency and toxicity
have not been tested in bacteria, yet.

We used a synthetic HIV-1 Tat peptide derivative (Table
1) for conjugation to anti-gyrA PNA. Growth reduction of
GAS M49 in a dose-dependent manner was achieved with
0.4–1.4 µmol/l Tat-anti-gyrA PNA (Figure 4a–d). Thereby,
Tat-conjugated anti-gyrA PNA showed an enhanced anti-
microbial activity compared with PNA coupled to (KFF)K.
Growth inhibition of GAS M49 was already detectable at
Tat-PNA concentrations below 1 µmol/l. Upon application of the Tat-peptide alone, no toxicity was observed in GAS M49 cultures up to a concentration of 10 µmol/l Tat-peptide. Improved antisense effects in combination with low general toxicity are very desirable properties. It has been speculated that treatment failure and recurrent infections are caused by the internalization of S. pyogenes into host cells.17 Anti-
sense agents coupled to Tat, which allows import into host
cells in order to target intracellular bacteria and at the same
time exhibits low cytotoxicity, might help to circumvent these
therapeutic obstacles. Recently, growth of Brucella suis in

![Table 3](https://example.com/table3.png)

**Table 3** Interpretation of the decreasing growth rate constant (6 hours)
Tat-anti-gyrA peptide nucleic acid (PNA)

| c_{PNA} (µmol/l) | g_{PNA} | g_{antibiotic} | g_{0} | g_{PNA} | g_{PNA} \times | g_{antibiotic} | Interpretation |
|-----------------|---------|-----------------|------|---------|-----------------|-----------------|---------------|
| 0.5 µg/ml levofloxacin | 0 | 0.21 | 0.20 | 0.21 | 0.20 | 0.20 | n.a. |
| 0.2 | 0.21 | 0.20 | 0.21 | 0.20 | 0.20 | 0.20 | Autonomy |
| 0.4 | 0.20 | 0.19 | 0.20 | 0.19 | 0.19 | 0.19 | Synergy |
| 0.6 | 0.15 | 0.20 | 0.19 | 0.11 | 0.14 | 0.14 | Synergy |
| 0.8 | 0.12 | 0.20 | 0.20 | 0.11 | 0.14 | 0.14 | Synergy |
| 1.0 | 0.09 | 0.20 | 0.20 | 0.09 | 0.09 | 0.09 | Additivity |
| 1.2 | 0.06 | 0.20 | 0.20 | 0.07 | 0.07 | 0.07 | Additivity |

**Figure 3**

**Figure 4**

**Figure 5**
infected macrophages was shown to be inhibited by 30 µmol/l (KFF). K peptide-coupled PNAs. Application of Tat-coupled PNAs might reduce the required PNA concentration for the targeting of intracellular bacteria. In the near future, we plan to use Tat-conjugated antisense-PNAs in GAS for the directed knock down of putative virulence genes. Experimental regulation of gene expression by antisense technology will help to study the function of virulence factors, including regulatory small RNAs, and will allow straight-forward screening for virulence specific phenotypes. Antisense-mediated downregulation of virulence-related streptococcal genes may provide also a therapeutic advantage. GAS with attenuated virulence could be targeted by the host immune system leading to a subsequent clearance of infection without the requirement of further antimicrobial treatment. Combination of antimicrobial drugs is an interesting option for the improvement of therapy. The development of resistance to the respective drugs can be delayed or prevented by combinatorial administration. Another advantage is that the killing rate of bacteriostatic agents can be potentially increased in combination with a second drug. Moreover, in some cases, antimicrobial synergy can be observed upon combined treatment. Combination therapy may also be advantageous when resistance to a single agent develops rapidly. Combined in vitro application of antisense PNAs and peptide-targeting antibiotics have been tested in E. coli and in S. aureus. The authors described synergistic antimicrobial effects for combinations of drugs sharing the same genetic targets. By contrast, we found synergistic effects between peptide-coupled anti-gyrA PNA and three peptide level antibiotics, independent of the pathway targeted by the antibiotics (Tables 2 and 3). We speculate that during growth, inhibition of translation by ribosomal deficiency will interfere with DNA replication, because the required proteins are not replenished. Enhanced growth inhibition by (KFF)3K-anti-gyrA PNAs as well as Tat-anti-gyrA PNAs upon coapplication with conventional antibiotics suggests that antisense PNAs are promising candidates for combination therapy.

Materials and Methods

PNA synthesis. PNAs and PNAs with carrier peptide conjugates were synthesized and purified by HPLC at the DKFZ (Heidelberg, Germany). All PNAs used in this work are listed in Table 1.

Bacterial strains and growth conditions. GAS serotype M49, strain 591, a clinical isolate from a skin infection, was kindly provided from R. Lütticken (Aachen, Germany). GAS M49 was cultured in Todd–Hewitt broth (Invitrogen, Life Technologies GmbH, Darmstadt, Germany) supplemented with 0.5% yeast extract (THY; Invitrogen) at 37 °C under a 5% CO2 atmosphere. All PNAs used in this work are listed following the protocol provided by the manufacturer. The purified total RNA was extracted with acidic phenol and digested with DNaseI (Ambion, Life Technologies GmbH, Darmstadt, Germany) to remove remaining traces of chromosomal DNA. The RNA preparation was treated with 10 U of DNaseI for 30 minutes at 37 °C. The enzyme was subsequently heat inactivated at 72 °C for 5 minutes. Quantitative reverse transcription polymerase chain reaction. Fifty nanogram of acidic phenol-extracted and DNaseI-treated total RNA was reverse transcribed to generate cDNA using the First-Strand cDNA Synthesis Kit from Invitrogen following the protocol provided by the manufacturer. For reverse transcription, two reactions were performed using random hexamer primers provided by the kit. One reaction contained reverse transcriptase, a second control reaction was performed without enzyme to exclude product formation from residual genomic DNA templates in the following gene-specific polymerase chain reaction. All reactions were performed in triplicates. The real-time polymerase chain reaction amplification was performed with SYBR Green (Fermentas, Fisher Scientific-Germany GmbH, Schwerte, Germany) using an ABI PRISM 7000 Sequence Detection system (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany). The level of 5S RNA gene transcription was determined by visual inspection. At time point 0, the viable cell count corresponded to 1–3 × 106 CFU/well. Each sample was prepared in triplicate; each experiment has been performed in at least four independent biological replicates. Growth rates were determined in the exponential growth phase: μ = log2x1/log(e) × (t2 – t1); t1 = 3 hours; t2 = 3.5 hours; x1 = OD600 at t1, x2 = OD600 at t2. The interaction of antimicrobial substances was determined in combination testing by calculation of the growth constant q = ln (ODtx/ODt0)/tx in the presence of one inhibitor or following application of a combination of the two agents. Thereby, autonomy was defined as q(A+B) = QA or gB and synergy as g(A + B) < gA × gB/g0.

RNA isolation. For RNA isolation, five wells were prepared for each experimental condition, treated as indicated, and pooled after 6 hours of incubation. Total bacterial RNA was then isolated using the FastRNAProBlue Kit from MP Biomedicals, Illkirch, France as outlined in the protocol provided by the manufacturer. The purified total RNA was extracted with acidic phenol and digested with DNasel (Ambion, Life Technologies GmbH, Darmstadt, Germany) to remove remaining traces of chromosomal DNA. The RNA preparation was treated with 10 U of DNaseI for 30 minutes at 37 °C. The enzyme was subsequently heat inactivated at 72 °C for 5 minutes.
Supplementary material

Figure S1. Dose-dependent inhibition of the GAS M49 growth rate (μ) by (KFF)3K-anti-gyrA PNA.

Figure S2. Combination testing of novobiocin with (KFF)3K-anti-gyrA PNA.

Figure S3. Combination testing of spectinomycin with (KFF)3K-anti-gyrA PNA.

Figure S4. Combination testing of levofloxacin with Tat-anti-gyrA PNA.

Figure S5. Combination testing of novobiocin with Tat-anti-gyrA PNA.

Figure S6. Combination testing of spectinomycin with Tat-anti-gyrA PNA.

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