Antisense Inhibition of RNase P
MECHANISTIC ASPECTS AND APPLICATION TO LIVE BACTERIA*§

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We explored bacterial RNase P as a drug target using antisense oligomers against the P15 loop region of Escherichia coli RNase P RNA. An RNA 14-mer, or locked nucleic acid (LNA) and peptide nucleic acid (PNA) versions thereof, disrupted local secondary structure in the catalytic core, forming hybrid duplexes over their entire length. Binding of the PNA and LNA 14-mers to RNase P RNA in vitro was essentially irreversible and even resisted denaturing PAGE. Association rates for the RNA, LNA, and PNA 14-mers were ~10^8 M^-1 s^-1 with a rate advantage for PNA and were thus rather fast despite the need to disrupt local structure. Conjugates in which the PNA 14-mer was coupled to an invasive peptide via a novel monolgycine linker showed RNase P RNA-specific growth inhibition of E. coli cells. Cell growth could be rescued when expressing a second bacterial RNase P RNA with an unrelated sequence in the target region. We report here for the first time specific and growth-inhibitory drug targeting of RNase P in live bacteria. This is also the first example of a duplex-forming oligomer that invades a structured catalytic RNA and inactivates the RNA by (i) trapping it in a state in which the catalytic core is partially unfolded, (ii) sterically interfering with substrate binding, and (iii) perturbing the coordination of catalytically relevant Mg^{2+} ions.

Also, its cellular abundance is low compared with ribosomal RNA or tRNA; for example, E. coli cells contain a 60–100-fold molar excess of ribosomes over RNase P RNA (2). Thus, compared with ribosomes, lower intracellular drug concentrations may be required to deplete cellular RNase P activity below a threshold essential for cell growth and survival. Because the enzyme contains a stable catalytic RNA subunit expected to turn over slowly relative to most mRNAs, de novo transcription rates of its RNA subunit are predicted to be relatively low, suggesting that its inactivation will result in a rather persistent phenotype.

Previously, the so-called P15 loop region of E. coli-type RNase P RNAs, known to interact with the 3′-CCA portion of precursor tRNA (ptRNA) substrates (3), was demonstrated to be a very effective target site for antisense-like inhibition strategies (4, 5). In a related but conceptually different approach termed oligonucleotide-directed misfolding of RNA, E. coli RNase P RNA was screened with consecutive DNA 12-mers for inhibition of ptRNA processing in a reaction mixture in which RNase P RNA was newly transcribed in the presence of its protein subunit (6). A 2′-O-methylated variant of one 12-mer complementary to nucleotides 289–300 in the P15 loop region turned out to be the most efficient inhibitor. This result supported our choice of the P15 loop region for the rational design of antisense agents.

Antisense strategies in general have to circumvent the problem of rapid DNA and RNA oligonucleotide degradation, and DNA additionally suffers from the relatively low stability of DNA:RNA hybrids. “Third generation” antisense agents, such as locked nucleic acid (LNA) and peptide nucleic acid (PNA), are highly resistant to degradation enzymes (7–9). In LNA, a methylene bridge connecting the 2′-oxygen and 4′-carbon atoms fixes the sugar pucker in an A-type helical conformation. PNA is composed of an uncharged peptide backbone with the nucleobases attached via methylene carbonyl linkages and is presented in a spatial arrangement mimicking natural nucleic acids. Because of its uncharged backbone, PNA was reported to be able to invade stable stem-loop structures that are not accessible to natural oligonucleotides, with helix stability being largely independent of salt concentration (10, 11). The melting temperatures of LNA:RNA duplexes are substantially increased relative to the corresponding RNA:RNA helices (12–14). The stability of LNA:RNA duplexes apparently exceeds that of PNA:RNA duplexes (15).

The most severe obstacle to inhibition of bacterial growth by antisense agents, however, is their uptake into bacterial cells.
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has previously been demonstrated that conjugates of PNA oligomers and invasive peptides derived from antimicrobial peptides of the innate immune system of eukaryotic organisms are able to enter Gram-negative and -positive bacteria and to interact with their specific target RNAs in vivo (7, 16). At present, such PNA-peptide conjugates appear to be the most efficient strategy for antisense-based inhibition of live bacteria.

Here we have characterized in vitro the efficiency, specificity, and kinetics of E. coli RNase P RNA inhibition by DNA, RNA, LNA, and PNA versions of a 14-mer complementary to the P15 loop. We further demonstrate, for the first time, the growth inhibition of live E. coli bacteria by an antisense oligomer specific to its highly structured and catalytic RNase P RNA. The duplex-forming oligomer inactivates the enzyme by sterically blocking substrate access, by arresting part of the catalytic core of the RNA in a non-functional conformation, and by disrupting the binding of catalytically relevant Mg$^{2+}$ in the P15 loop region.

EXPERIMENTAL PROCEDURES

RNA, LNA, PNA, and DNA Oligonucleotides—High pressure liquid chromatography-purified RNA oligonucleotides were purchased from IBA (Göttingen, Germany) or CureVac (Tübingen, Germany). DNA oligonucleotides were from Invitrogen. Further purification of oligonucleotides was performed on denaturing polyacrylamide gels. Oligonucleotides were localized by UV shadowing, excised from the gel, eluted overnight at 8 °C in NaOAc (1 M, pH 4.9), and recovered by ethanol precipitation. The following oligonucleotides were used in this study: 5′-CAAGCA-GCCUACCC (RNA E. coli 14-mer), 5′-CUAGCGCAUCCACC (RNA A. vinosum 14-mer), 5′-AAGCGGCCCAUCCC (RNA T. thermophiles 14-mer), 5′-CCUUUUUUUGGAAACAAUU-UUAGG (control RNA), 5′-CCAGCAUCCACC (DNA E. coli 14-mer). The LNA 14-mer, synthesized essentially as described previously (17), was identical in sequence to the RNA 14-mer (RNA 14-mer). The LNA 14-mer, synthesized essentially as described previously (4), was extended by RNase P RNA (Fig. 1) results in the disruption of the local structure and blocks the docking of the tRNA 3′-end to the active site of RNase P RNA (5). This inhibits RNase P activity, not only in the RNA alone but also in the holoenzyme reaction (4). We extended our inhibition analyses by including derivatives of the 14-mer containing a PNA or LNA backbone. As a first approach to assessing the relative binding affinities of the RNA, DNA, LNA, and PNA oligomer versions, we preincubated E. coli RNase P RNA with different oligomer concentrations for 40 min to allow the binding equilibrium to be

using a Bio-Imaging analyzer FLA 3000-2R (Fujifilm) and the analysis software PCBAS/AIDA (Raytest). Images were further edited by Corel Photopaint, version 11.

Cloning of Bacillus subtilis rnpB in Plasmid pSP64—B. subtilis rnpB, including its natural promoter and terminator, was amplified from genomic B. subtilis DNA (strain W168) using primers 5′-GGC AGC AAG CCT TTAT GAT TGA TCA C (including the native HindIII site upstream of rnpB) and 5′-CGC CCA AGC TTG TTG ATAT CTT CAT C GT ATC ACC CTG TC. The resulting PCR fragment was cloned in the HindIII site of pSP64 (Promega).

E. coli Growth Inhibition Experiments—PNA-peptide conjugates were tested for inhibition of E. coli cells (wild-type K12 and the lipopolysaccharide-defective E. coli strain AS19; Ref. 20) essentially as described in Ref. 7. 3 ml of LB (Luria-Bertani) medium were inoculated with a single colony, and cells were grown under aeration (180 revolutions/min in a thermostaker) at 37 °C to an A$_{578}$ of 2.4–3.7. Suspensions were diluted with double-distilled H$_2$O (strain K12) or 0.9% NaCl (strain AS19) to 10% LB and 5 × 10$^7$ cells/ml. To 100 μl (5 × 10$^4$ cells) of such a suspension, PNA-peptide inhibitor was added and 10 μl aliquots withdrawn after 0, 10, 30, 60, and 180 min; aliquots or appropriate dilutions (down to 1:10,000) were plated in 10 (for undiluted samples) or 100 μl portions (in 10% LB, including 0.9% NaCl for strain AS19), incubated on LB agar plates at 37 °C overnight, and the number of colonies counted.

Reverse Transcription (RT)-PCR—To compare E. coli RNase P RNA levels in inhibited and uninhibited cells, AS19 cells harboring plasmid pSP64-Bsrnp were grown as described above and treated with either no inhibitor, 5 μM PNA-G-peptide, or 5 μM scPNA-G-peptide for 3 h. Cell amounts were verified by plating. Total RNA from each culture was prepared using the RNeasy Mini Kit (Qiagen) followed by DNase I treatment (Promega). RT-PCR was performed with the AccessQuick RT-PCR System (Promega) according to the manufacturer’s instructions. Primers specific for E. coli rnpB were 5′-CTC ACT GCC TCA AGC AGC CT (5′-end-labeled) and 5′-GAA GCT GAC CAG ACA GTC GC. Primers specific for rpsR (ribosomal protein S18) were 5′-TGG CAC GTT ATT TCC GTG C (5′-end-labeled) and 5′-TTA CTG ATG GCC ATC AGT GTA CCG. RT-PCR reactions contained the normal amounts of unlabeled primers and, in addition, trace amounts of the respective 5′-end-labeled primer.

RESULTS

Inhibition Efficiency and Specificity of DNA, RNA, LNA, and PNA 14-mer Versions—Hybrid formation of the 14-mer (E. coli 14-mer; either RNA or DNA) with its target site in the P15 loop region of E. coli RNase P RNA (Fig. 1) results in the disruption of the local structure and blocks the docking of the tRNA 3′-end to the active site of RNase P RNA (5). This inhibits RNase P activity, not only in the RNA alone but also in the holoenzyme reaction (4). We extended our inhibition analyses by including derivatives of the 14-mer containing a PNA or LNA backbone. As a first approach to assessing the relative binding affinities of the RNA, DNA, LNA, and PNA oligomer versions, we preincubated E. coli RNase P RNA with different oligomer concentrations for 40 min to allow the binding equilibrium to be
reached. Multiple turnover processing reactions containing 10 nM RNase P RNA were then started by adding 100 nM ptRNA substrate. Inhibition efficiencies (given as $K_i$ values, defined as the oligomer concentration resulting in a 2-fold reduced rate of ptRNA cleavage) (Table 1) provided a first measure for the relative inhibitor strength. $K_i$ values do not necessarily equal $K_D$ values but report the same trends in binding affinity differences (5). The RNA and LNA variants turned out to have the lowest and very similar $K_i$ values (2.2 for RNA, 3.9 for LNA) (Table 1) followed by PNA ($K_i = 12.5$) and DNA ($K_i = 25$). $K_i$ values of 2–4 nM are low for an enzyme concentration of 10 nM, as one would expect 5 nM to be the minimum inhibitor concentration for obtaining 50% inhibition. We attribute this to the finding that only $\sim 50$–60% of $E. coli$ RNase P RNA is in an active conformation after 40 min of preincubation at 37 °C in the standard buffer used here (21).

We then addressed the inhibition specificity of the RNA, LNA, and PNA versions of the 14-mer by testing their effects on the processing reaction catalyzed by three other bacterial RNase P RNAs with sequence variation in the target region, namely those from *Pseudomonas aeruginosa*, *A. vinosum*, and *Thermus thermophilus*. Complementarity of the $E. coli$ 14-mer was predicted to be the least compromised with *P. aeruginosa* RNase P RNA, of intermediate stability for the *A. vinosum* RNA, and most destabilized for *T. thermophilus* RNase P RNA (Fig. 2). As expected, inhibition efficiencies of RNA and PNA 14-mers decreased in the order $P. aeruginosa > A. vinosum > T. thermophilus$ (Table 1). Yet with the LNA 14-mer, there was little discrimination. This is attributable to the extraordinary stability of LNA:RNA hybrids (12, 13), which leads to increased mismatch tolerance, in line with a previous study (15).

In a reverse setup, we designed 14-meric RNA oligomers that fully matched the P15 loop region of *A. vinosum* and *T. thermophilus* RNase P RNA, respectively. For the *A. vinosum* 14-mer, the 53-fold discrimination against *E. coli* RNase P RNA ($K_i = 3.0$ versus 160) (Table 1) exactly mirror-imaged what we had seen with the $E. coli$ 14-mer (Table 1). Surprisingly, discrimination against the *E. coli* RNase P RNA was only 6-fold for the *T. thermophilus* 14-mer (for details, see supplemental Fig. S1).

**Invasion of PNA and LNA 14-mers into the P15 Loop Region**—We next analyzed strand invasion of the 14-mers into the P15 loop region of *E. coli* RNase P RNA by lead probing. For the RNA and DNA 14-mers, we had shown earlier (5) that they anneal to the target region over their entire length, as inferred from the suppression of lead hydrolysis in the region spanning nucleotides 291–304 of RNase P RNA (Fig. 3, lanes 11 and 12). In addition, a novel lead hydrolysis product appears in the nucleotides 291–304 of RNase P RNA (Fig. 3, lanes 11 and 12). Prominent lead cleavage sites are marked by roman numerals.

**TABLE 1**

$k_i$ and $k_{on}$ values for the binding of antisense 14-mers to RNase P RNAs

| RNase P RNA | $E. coli$ 14-mer, RNA, $K_i$ | $E. coli$ 14-mer, LNA, $K_i$ | $E. coli$ 14-mer, PNA, $K_i$ | $E. coli$ 14-mer, DNA, $K_i$ | $A. vinosum$ 14-mer, RNA, $K_i$ | $T. thermophilus$ 14-mer, RNA, $K_i$ |
|-------------|-------------------|----------------|----------------|----------------|----------------|----------------|
| $E. coli$   | 2.2 ± 0.6         | 3.9 ± 1.8       | 12.5 ± 0.5     | 25 ± 5         | 160 ± 10       | 80 ± 20        |
| $P. aeruginosa$ (1 mismatch, 1 GU) | (6.8 ± 0.7) $\times 10^4$ | (8.6 ± 4.5) $\times 10^4$ | (2.2 ± 0.5) $\times 10^5$ | (2.3 ± 2.0) $\times 10^6$ | NA            | NA            |
| $A. vinosum$ (2 mismatches, 1 GU) | 19.0 ± 1.5        | 18.2 ± 0.8      | 580 ± 225      | NA             | NA            | NA            |
| $T. thermophilus$ (1 mismatch, 1 GU, 1 bulge) | 125 ± 53          | 25 ± 5          | 3200 ± 1500    | NA             | 3.0 ± 0.6      | 13.3 ± 2.5    |

$K_i$ values are based on at least three independent experiments; NA, not analyzed. In the first column, deviations from perfect Watson-Crick complementarity are indicated in parentheses for interaction of the *E. coli* 14-mer with the heterologous RNase P RNAs; GU, G-U wobble pair; $k_{on}$ values (in s$^{-1}$) are given in bold below $K_i$ values for *E. coli* RNase P RNA.

FIGURE 1. Secondary structure of *E. coli* RNase P RNA. The target region of the 14-mer (nucleotides 291–304) is indicated by the bold line. P, helical regions; J, joining segments named according to the helices they connect. The two guanosines (G292 and G293) that form Watson-Crick base pairs with C74 and C75 of tRNA 3'–ends are highlighted and boxed. Prominent lead cleavage sites are marked by roman numerals.
to imperfect complementarity and thus decreased affinity of this oligomer for E. coli RNase P RNA (Fig. 2). The lead cleavage pattern remained unchanged upon the addition of a control RNA lacking substantial sequence complementarity to E. coli RNase P RNA (Fig. 3, lanes 7 and 8). In the presence of the E. coli-specific LNA 14-mer (lanes 13 and 14), the same changes in the lead cleavage pattern as for the RNA analog were seen. However, all fragments that included the target site were shifted toward lower gel mobility, whereas the smaller ones (Fig. 3, bottom section of the gel) excluding this region showed unperturbed mobility. This result demonstrates that the LNA 14-mer remained bound to the RNase P RNA, even during electrophoresis in the presence of 8M urea. A very similar observation was made with the PNA 14-mer (Fig. 3, lanes 15 and 16), but there the upshifted fragments migrated more diffusely. One explanation is that the PNA 14-mer undergoes dissociation and reassociation cycles during electrophoresis. Alternatively, PNA is known to have a propensity to form RNA:PNA triplexes (22). Triplex formation is indeed conceivable based on the sequence of the PNA 14-mer (supplemental Fig. S2) and might have contributed to the diffuse gel mobility in lanes 15 and 16 (Fig. 3).

Inhibitor Association Rates—The lead probing experiments (Fig. 3) indicated that hybrid helices formed between the LNA 14-mer and bacterial RNase P RNAs from E. coli, P. aeruginosa, A. vinosum, and T. thermophilus. Duplex formations between the 14-mer specific to T. thermophilus RNase P RNA (T. th. 14-mer) and its cognate or E. coli RNase P RNA are depicted in the two sketches at the bottom. Individual P15 loop regions are shown in gray letters, and the two guanosines corresponding to G292 and G293 of E. coli RNase P RNA are highlighted in boxes. For more details, see Fig. 1.
or PNA 14-mer and the target region dissociate at very slow rates ($k_{off}$). Because the RNA 14-mer was previously shown to dissociate at a rate not exceeding 0.02 h$^{-1}$ under processing assay conditions (5), $k_{off}$ could be considered as practically zero for the RNA, LNA, and PNA 14-mers within the time frame of our inhibition analyses. We then addressed the question as to which extent these analogs may differ from their natural DNA and RNA counterparts in the rate of association ($k_{on}$). This was analyzed by an RNase P RNA activity assay performed under conditions where processing activity linearly depended on the amount of active enzyme. Thus, we determined the fraction of active (uninhibited) enzyme that remained after increasing enzyme-oligomer preincubation times, and the rate of enzyme inactivation equalled the rate of oligomer association (for details, see supplemental Fig. S3). The PNA 14-mer showed the fastest rate followed by the LNA and RNA versions and the DNA 14-mer with the slowest association rate (Table 1, values in bold lettering).

Inhibition by PNA-Peptide Conjugates in Vitro—Limited cellular uptake is a general problem of antisense agents, especially in bacteria. Because improved cell entry was demonstrated for PNA oligomers coupled to invasive peptides (7), we adopted this strategy for RNase P inhibition. Taking into account that the linker connecting the N-terminal peptide and the PNA oligomer may influence inhibition efficacy (7, 23), we utilized two different linkers. One is the commonly used 2-aminoethoxy-2-ethoxy acetic acid (AEEA) linker, (also termed 8-amino-3,6-di-oaoctanoic acid linker and abbreviated as eg1) (7, 24); and the other is the novel shorter monoglycine linker not analyzed so far (supplemental Fig. S4A). We first tested these PNA-peptide conjugates in our standard RNA-alone processing assay (see Table 1). At a concentration of 100 nM, both the PNA-AEEA peptide and PNA-G-peptide completely inhibited processing, in contrast to a control AEEA conjugate containing a scrambled PNA 14-mer (supplemental Fig. S4B). Similar results were obtained in the E. coli RNase P holoenzyme assay (supplemental Fig. S4C). There, specific inhibition was already evident at the lower concentration (12.5 nM) of PNA 14-mer or PNA-G-peptide.

Inhibition by PNA-Peptide Conjugates in Vivo—The conjugates were then tested for in vivo inhibition of E. coli wild-type K12 and a more permeable mutant strain (AS19) defective in lipopolysaccharide synthesis (20). To this end, cell suspensions were incubated with the corresponding conjugate for up to 180 min followed by a plating assay to determine the number of surviving colony-forming cells. Incubation of AS19 cells at 10 μM PNA-G-peptide for 180 min did not leave any survivors in all experiments, whereas inhibition by 5 μM was, on average, substantial but incomplete (Fig. 4A). Surprisingly, inhibitor effects were less pronounced with the PNA-AEEA peptide (Fig. 4B) containing the linker used in a related previous study (7). 10 μM PNA-AEEA-peptide had a much weaker inhibitory effect than the PNA-G-peptide at the same concentration. Scrambled versions of the two conjugate types showed a very mild inhibitory effect, suggesting that 10 μM is at the threshold of concentrations at which PNA-peptide conjugates start to inhibit E. coli cells in an antisense-independent manner. The minimal inhibitory concentration of the decamerpeptide (KFF)$_3$K itself was reported as 30 μg/ml (~20 μM) for E. coli K12 strains (25). Thus, the carrier peptide moiety may well be
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the cause of the weak nonspecific inhibition effects seen here. Inhibition effects were not markedly different for the K12 compared with the AS19 strain but somewhat attenuated (Fig. 4 C). This is consistent with the more permeable cell barrier of the AS19 mutant strain. The PNA 14-mer alone also showed some inhibition at a concentration of 10 μM (more pronounced with the AS19 relative to the K12 strain) (Fig. 4, B and D).

To demonstrate intracellular PNA-peptide association with RNase P RNA, we performed a genetic complementation experiment. The B. subtilis RNase P RNA gene (rnpB) was previously shown to be able to functionally replace the native E. coli rnpB gene in vivo (26, 27). We also did not observe inhibition of B. subtilis RNase P RNA by oligonucleotides targeted to the P15 loop region of E. coli RNase P RNA (4). AS19 and K12 bacteria transformed with a plasmid expressing B. subtilis rnpB (pSP64-BSrnpB) indeed rescued inhibition caused by the presence of the PNA-G-peptide (Fig. 4, E and F), further supporting the specificity of inhibition. Transformation with the multicopy plasmid pSP64 and growth in the presence of the antibiotic ampicillin made the AS19 and K12 bacteria somewhat more sensitive to inhibitors, which explains why we observed a more pronounced inhibition effect at 5 μM PNA-G-peptide compared with the data in Fig. 4, A and C.

In addition, RT-PCR was applied to analyze the fate of E. coli RNase P RNA upon inhibition by the PNA-G-peptide. AS19 bacteria harboring the complementation plasmid pSP64-BSrnpB were used, which had the advantage that cell growth was nearly the same in the presence and absence of PNA-G-peptide due to the expression of B. subtilis RNase P RNA (Fig. 4, E and F). The mRNA encoding ribosomal protein S18 (rpsR) was used to control for fluctuations in RNA template amounts used for RT-PCR reactions. Levels of E. coli RNase P RNA were clearly decreased in cells incubated with the PNA-G-peptide but not in those treated with the scrambled control conjugate (Fig. 5), suggesting that complexation with the inhibitor induces RNase P RNA degradation.

DISCUSSION

Our starting point was an RNA 14-mer that had the strongest inhibition effect on in vitro processing by E. coli RNase P RNA among several related oligoribonucleotides (5). To study stabilizing oligomer modifications in a systematic manner and for comparability reasons, we analyzed the PNA and LNA versions of the identical sequence and (in the case of the LNA 14-mer) carrying this ribose modification at each nucleotide position. We were aware that oligonucleotides with LNA modifications at only a few positions might be preferable for antisense strategies, as the gain in duplex stabilization (ΔTm/C◦C) per LNA modification is maximal if only, for example, every third nucleoside is replaced with the analog (12). Our in vitro inhibition experiments have shown that the RNA, DNA, PNA, and LNA versions of the 14-mer disrupt the structured P15 loop region of type A RNase P RNAs, forming a hybrid duplex over the entire oligomer length (Fig. 3) (5). Beyond disruption of the catalytic core structure, oligomer invasion prevents anchoring of tRNA 3′-NCCA-ends to the P15 loop and, thereby, perturbs coordination of catalytically relevant Mg2+ (28). The rate of dissociation was negligible for RNA, LNA, and PNA 14-mers under the applied conditions. The extraordinary stability of the target RNA-LNA duplex was associated with reduced discrimination against mismatched target sequences (Table 1) consistent with previous observations (15). The PNA 14-mer combined excellent specificity and the fastest association rate among the inhibitors tested (Table 1). Its relatively high Ki value (12.5 nM) (Table 1) can be explained by a certain propensity of hydrophobic PNA to self-aggregate (29), which likely reduced the effective concentration of PNA single strands, although we tried to minimize this problem by preparing, handling, and quantifying PNA solutions at 80 °C. At explicitly smaller oligomer sizes, however, LNA may become advantageous over PNA. For example, an LNA 8-mer directed against the telomerase RNA subunit inhibited telomerase in human prostate cancer cells with an IC50 of 25 nM, whereas a corresponding PNA 8-mer had a 200-fold lower potency; an LNA 8-mer with a single mismatch showed an at least 40-fold lower efficacy (15). One may argue that LNA octamers might elicit substantial off-target effects because of the multiple occurrence of identical octanucleotide sequences in bacterial and eukaryotic genomes. However, it is rather unlikely that oligomer annealing to sequence-identical non-target sites, if accessible to hybridization at all, entails the similarly severe functional consequences as duplex formation with the specific target site. Indeed, transfection of a human cell line with the aforementioned telomerase RNA subunit-specific LNA 8-mer did not reveal any signs of toxicity (15). These results, although not conclusive yet, are encouraging in the exploration of whether LNA-modified oligomers of <10 nucleotides may substantially
improve entry into bacteria without a substantial decrease in target selectivity or increase in toxicity.

Measured association rates \( (k_{\text{on}}) \) for the RNA, LNA, and PNA 14-mers were in the range of \( 10^7 \text{ M}^{-1} \text{ s}^{-1} \) (Table 1). The value for the RNA 14-mer was previously calculated to be close to \( 10^6 \text{ M}^{-1} \text{ s}^{-1} \) based on experimentally determined values for \( k_{\text{on}} \) and \( K_d \) (5). We consider the direct experimental determination of \( k_{\text{on}} \) reported here to provide the more accurate values. The value of \( 10^7 \text{ M}^{-1} \text{ s}^{-1} \) is only a factor of \( \sim 10 \) below the values observed for natural antisense systems and exceeds the association rates of many artificial antisense interactions by a similar factor (30, 31).

We conclude that the oligomer annealing to the P15 loop region is rather fast despite the need to disrupt the helix P15 and possibly additional tertiary interaction in the catalytic core of RNase P RNA. This finding may be related to the fact that the P15 loop has been optimized during evolution as a binding site for the single-stranded 3’-overhangs of tRNA molecules. Mechanistically, oligomers may initially interact with the fully accessible CCA-binding loop and, during transient thermal opening of helix P15, snap into the P15 target sequence to extend base pairing in a zipper-like manner. When base pairing is established over the entire oligomer length, the RNase P RNA is trapped in the partially unfolded conformation, which appears to trigger RNA degradation \textit{in vivo} (Fig. 5).

Based on our results, it should be worthwhile to explore the loop of helix 80 of 23 S rRNA by a very similar antisense strategy, as G2251 and G2252 in this loop form Watson-Crick pairs with C74 and C75 of tRNA bound to the P site (32).

The PNA oligomer was coupled to an artificial carrier peptide that was shown to facilitate bacterial uptake in a previous study (7). Here, we could further improve efficacy of such conjugates by introducing a novel monoglycine linker connecting PNA and the carrier peptide. The monoglycine linker seems to improve bacterial uptake, as conjugates with the monoglycine and AEEA linker inhibited processing by RNase P RNA \textit{in vitro} to very similar extents (supplemental Fig. S4). Invasive peptides of the type used here, rich in basic and hydrophobic residues, form amphipathic secondary structures and interact preferentially with bacterial membranes whose outer layers expose anionic groups for electrostatic interactions; the presence of cholesterol in eukaryotic membranes contributes to the preference of these peptides for bacterial membranes (33). The preference of invasive peptides for bacterial membranes may reduce (but not necessarily eliminate) their potential toxicity in therapeutic applications. Indeed, the (KFF)_3K-type peptides are able to induce histamine release in some mammals (16) and hemolysis of human erythrocytes (25). Several other peptides were recently coupled to PNA antisense oligomers and tested for target inhibition in live \textit{Staphylococcus aureus} cells. Hexa- to decapeptides chemically unrelated to (KFF)_3K were found to be efficient mediators of cell entry as well (16). The use of non-cationic carrier peptides could also alleviate the problem of aggregation and precipitation of conjugates consisting of anionic oligonucleotides and cationic peptides (34). Another perspective is the synthesis of disulfide-linked oligomer-peptide conjugates that are cleaved in the more reductive milieu of the bacterial cell to release the antisense oligomer. In summary, the development of carrier peptides with optimal performance in terms of bacterial uptake efficiency, delivery to the target, preference for bacterial membranes, and low toxicity in the host will be in the focus of future studies.

In a related study, a PNA-peptide conjugate directed against the essential \textit{fmnhB} gene suppressed growth of \textit{S. aureus} at 10 \( \mu \text{M} \) (16), similar to what we have observed. This inhibitor concentration was about 13 times higher than the I_{50} values for PNA-peptide-based inhibition of the non-essential \textit{gfp} gene (16). A major reason for such discrepancies could be that I_{50} values for the inhibition of a non-essential enzyme are different from concentrations required to fully suppress bacterial growth. Complete suppression of bacterial growth is expected to only be reached when the intracellular activity level of an essential gene product is depleted far below 50% of its normal level.

PNA-based inhibition of stable RNAs has been explored to some extent for ribosomal RNA as the target (35). Two triplex-forming 7- or 12-mer PNA strands connected by an AEEA linker were tested, one directed against a single-stranded heptanucleotide sequence in the \( \alpha \)-sarcin loop and the second against a dodecameric sequence in the peptidyl transferase center of \textit{E. coli} 23 S RNA. In the latter case and similar to the RNase P situation reported here, triplex formation required some disruption of local 23 S rRNA structure; the I_{50} value for inhibition of AS19 cells in 0.1 \% LB broth was reported as 5 \( \mu \text{M} \) (35). With respect to the triplex-forming PNA conjugate directed against the \( \alpha \)-sarcin loop, attachment of the (KFF)_3K carrier peptide improved the inhibitory effect on the growth of \textit{E. coli} K12 cells by a factor of seven (7). These findings, combined with those presented here, are encouraging in the further progression toward the development of stable RNA-specific drugs that exploit the hydrogen bonding capacities of nucleobases.

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REFERENCES

1. Frank, D. N., and Pace, N. R. (1998) \textit{Annu. Rev. Biochem.} \textbf{67}, 153–180

2. Dong, H., Kirsebom, L. A., and Nilsson, L. (1996) \textit{J. Mol. Biol.} \textbf{261}, 303–308

3. Kirsebom, L. A., and Svärd, S. G. (1994) \textit{EMBO J.} \textbf{13}, 4870–4876

4. Willkomm, D. K., Grueßgesiepe, H., Goudinakis, O., Kreitkner-Kazemi Far, R., Bald, R., Erdmann, V. A., and Hartmann, R. K. (2003) \textit{ChemBiochem} \textbf{4}, 1041–1048

5. Grueßgesiepe, H., Willkomm, D. K., Goudinakis, O., and Hartmann, R. K. (2003) \textit{ChemBioChem} \textbf{4}, 1049–1056

6. Childs, J. L., Poole, A. W., and Turner, D. H. (2003) \textit{RNA} \textbf{9}, 1437–1445

7. Good, L., Awasti, S. K., Dryselius, R., Larsson, O., and Nielsen, P. E. (2001) \textit{Nat. Biotechnol.} \textbf{19}, 360–364

8. Kurreck, I. (2003) \textit{Eur. J. Biochem.} \textbf{270}, 1628–1644

9. Wahlestedt, C., Salmi, P., Good, L., Kela, J., Johnsson, T., Hökfelt, T., Broberger, C., Porreca, F., Lai, J., Ren, K., Ossipov, M., Koshkin, A., Jakobsen, N., Skouv, J., Oerum, H., Jacobsen, M. H., and Wengel, J. (2000) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{97}, 5633–5638

10. Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Freier, S. M., Driver, D. A., Berg, R. H., Kim, S. K., Norden, B., and Nielsen, P. E. (1993) \textit{Nature} \textbf{365}, 566–568

11. Tomac, S., Sarkar, M., Ratilainen, R., Wittung, P., Nielsen, P. E., Nordén, B., and Gräsland, A. (1996) \textit{J. Am. Chem. Soc.} \textbf{118}, 5544–5552

12. Singh, S. K., and Wengel, J. (1998) \textit{Chem. Commun.} \textbf{12}, 1247–1248
Antisense Inhibition of Bacterial RNase P

13. Childs, J. L., Disney, M. D., and Turner, D. H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 11091–11096
14. Nielsen, K. E., Rasmussen, J., Kumar, R., Wesper, J., Jacobsen, J. P., and Petersen, M. (2004) Bioconjugate Chem. 15, 449–457
15. Elayadi, A. N., Braasch, D. A., and Corey, D. R. (2002) Biochemistry 41, 9973–9981
16. Nekhotiaeva, N., Awasthi, S. K., Nielsen, P. E., and Good, L. (2004) Mol. Ther. 10, 652–659
17. Schmidt, K. S., Borkowski, S., Kurreck, J., Stephens, A. W., Bald, R., Hecht, M., Friebe, M., Dinkelborg, L., and Erdmann, V. A. (2004) Nucleic Acids Res. 31, e119
18. Smith, D., Burgin, A. B., Haas, E. S., and Pace, N. R. (1992) J. Biol. Chem. 267, 2429–2436
19. Sekiguchi, M., and lida, S. (1967) Proc. Natl. Acad. Sci. U. S. A. 58, 2316–2320
20. Hardt, W. D., Schlegl, J., Erdmann, V. A., and Hartmann, R. K. (1993) Nucleic Acids Res. 21, 3521–3527
21. Dias, N., Sénamaud-Beaufort, C., le Forestier, E., Auvèn, C., Hélène, C., and Saison-Behmoaras, T. E. (2002) J. Mol. Biol. 320, 489–501
22. Geller, B. L., Deere, J. D., Stein, D. A., Kroecker, A. D., Moulton, H. M., and Iversen, P. L. (2003) Antimicrob. Agents Chemother. 47, 3233–3239
23. Rebuffat, A. G., Nawrocki, A. R., Nielsen, P. E., Bernasconi, A. G., Bernal-Mendez, E., Frey, B. M., and Frey, F. J. (2002) FASEB J. 16, 1426–1428
24. Vaara, M., and Porro, M. (1996) Antimicrob. Agents Chemother. 40, 1801–1805; Correction (1997) Antimicrob. Agents Chemother. 41, 496
25. Waugh, D. S., and Pace, N. R. (1990) J. Bacteriol. 172, 6316–6321
26. Wegscheid, B., Condon, C., and Hartmann, R. K. (2006) EMBO Rep. 7, 411–417
27. Brännvall, M., Pettersson, B. M. F., and Kiresebom, L. A. (2003) J. Mol. Biol. 325, 697–709
28. Tackett, A. J., Corey, D. R., and Raney, K. D. (2002) Nucleic Acids Res. 4, 950–957
29. Eckardt, S., Romby, P., and Sczakiel, G. (1997) Biochemistry 36, 12711–12721
30. Kolb, F. A., Engdahl, H. M., Slagter-Jager, J. G., Ehresmann, B., Ehresmann, C., Westhof, E., Wagner, E. G., and Romby, P. (2000) EMBO J. 19, 5905–5915
31. Nissen, P., Hansen, J., Ban, N., Moore, P. B., and Steitz, T. A. (2000) Science 289, 920–930
32. Zasloff, M. (2002) Nature 415, 389–395
33. Turner, J. J., Arzumanov, A. A., and Gait, M. J. (2005) Nucleic Acids Res. 1, 27–42
34. Good, L., and Nielsen, P. E. (1998) Proc. Natl. Acad. Sci. U. S. A. 5, 2073–2076