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Inhibition of bacterial translation and growth by peptide nucleic acids targeted to domain II of 23S rRNA

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Abstract: The objective of this work was to study the inhibitory effects of antisense peptide nucleic acids (PNAs) targeted to domain II of 23S rRNA on bacterial translation and growth. In this paper, we report that PNA(G1138) or peptide-PNA(G1138) targeted to domain II of 23S rRNA can inhibit both translation *in vitro* (in a cell-free translation system) and bacterial growth *in vivo*. The inhibitory concentration (IC_{50}) and the minimum inhibiting concentration (MIC) are 0.15 and 10 μM, respectively. The inhibition effect of PNA(G1138) *in vitro* is somewhat lower than that of tetracycline ($IC_{50} = 0.12$ μM), but the MIC of peptide-PNA(G1138) against *Escherichia coli* is significantly higher than that of tetracycline (MIC = 4 μM). Further studies based on similar colony-forming unit (CFU) assays showed that peptide-PNA(G1138) at 10 μM is bactericidal, but the bactericidal effect is less effective than that of tetracycline. Nevertheless, the results demonstrated that the peptide-PNA(G1138) treatment is bactericidal in a dose- and sequence-dependent manner and that the G1138 site of 23S rRNA is a possible sequence target for designing novel PNA-based antibiotics. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide nucleic acid (PNA); peptide; antisense; 23S rRNA; cell-free translation

INTRODUCTION

Antibiotics are widely used in medicine, agriculture and industrial fermentations. However, bacterial drug resistance has become a serious problem, and research on new antibiotics is very slow. Many studies, including antisense inhibition, are going on to conquer microbes.

Antisense inhibition is a basic mode to regulate gene expression in microorganisms. Functional domains of mRNA and rRNA are the most common entry targets for antisense regulation. Translational inhibition by antisense oligonucleotide technology is very promising, but there are still many problems, e.g. sequence specificity, biological stability, solubility and cell uptake [1–4]. Peptide nucleic acid (PNA) is a DNA mimic that bears a pseudo-peptide backbone composed of achiral and uncharged *N*-(2-aminoethyl) glycine units (Figure 1(a)). PNA has been shown to hybridize in a sequence-specific manner and with high affinity to complementary sequences of single-stranded DNA or RNA molecules, while resistant to digestion and degradation by nucleases and proteases [5–8]. Homopyrimidine PNAs bind to complementary DNA(RNA) targets forming $(PNA)_2$/DNA(RNA) triplexes of very high thermal stability, two PNA chains of which combine with DNA(RNA) following the base pairing rules of Watson–Crick and Hoogsteen [9,10]. The potential of PNAs as specific inhibitors of translation has been studied, and it has been shown that PNAs targeted to domain V of 23S rRNA [11,12] and mRNA [12–19] can significantly inhibit protein synthesis in bacteria.

In 23S rRNA domain II, G1138, C1045 and A1067 sites are associated with GTPase and are close relatives to the rRNA peptidyl transferase center and the EF-G elongation factor [20,21]. Adjacent sequences of G1138, C1045 and A1067 sites are single-stranded (Figure 1(b)). Hanvey *et al.* [22] have demonstrated that in cells PNAs are difficult to bind with dsDNA because of high ionic concentration, but they can form highly stable hybrid compounds with single-stranded DNA.

PNA is inefficiently taken up by cultured cells and is slow to move across phospholipids vesicle (liposome) membranes [23]. Furthermore, the molecular weight of typical oligonucleotides exceeds the expected cut-off for efficient passive diffusion through the nonspecific porin channels that span the *E scherichia coli* cell wall. However, attachment of PNAs to the cell-permeabilizing peptide KFFKFFKFFK can dramatically improve antisense potency [19].

In this study, PNA(G1138), PNA(A1067) and PNA (C1045) were designed. Their effects on translation inhibition were studied in a cell-free translation system. PNAs that can effectively inhibit translation *in vitro* were then designed as peptide (KFFKFFKFFK)-PNAs to analyze their effects on suppressing *E. coli* growth *in vivo*.

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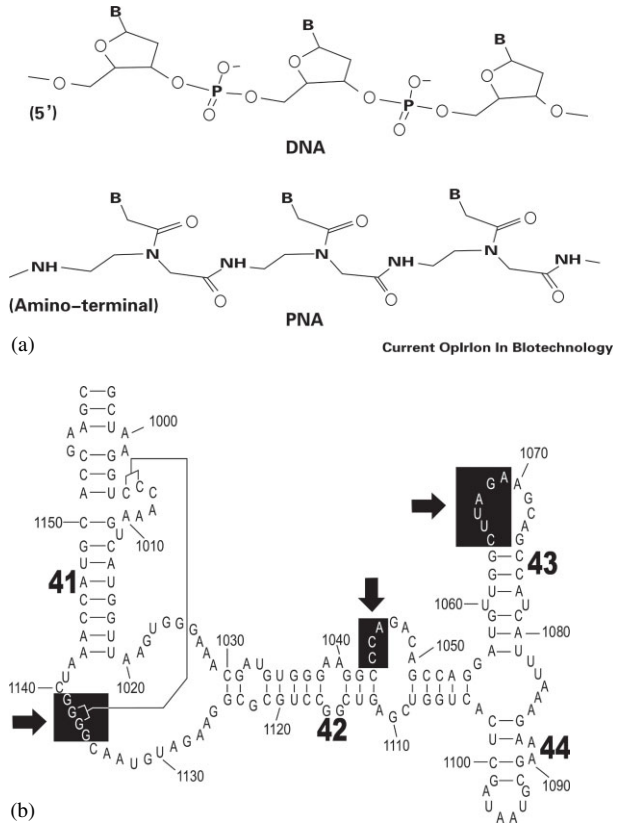

Figure 1 (a) Structural comparison between PNA and DNA; (b) Structure of 23S rRNA domain II and targeted sites of the PNAs. Arrows (from left to right) indicate the targeted sites of PNA(G1138), PNA(C1045) and PNA(A1067), respectively.

MATERIALS AND METHODS

Bacterial Strain, Plasmids and PNAs

E. coli DH5α and plasmid enhanced green fluorescent protein-N1 (pEGFP-N1) were from our department. *E. coli* BL21(DE3) and pET28a plasmid were kindly provided by the Jiangsu Institution of Parasitic Diseases. The PNAs (Table 1) were synthesized by Bio-Synthesis Inc.

Construction and Preparation of pET28a-EGFP Plasmid

Utilizing pEGFP-N1 plasmid as a template, full-length EGFP cDNA was amplified by polymerase chain reaction (PCR) with specific primers synthesized by Shanghai Biological Engineering Company (upstream primer: 5′-AAAGAATTC (*EcoR* I) TAA (termination codon) AAGG (RBS) AGATATATTATG (initiation codon) GTGAGCAAGGGCGAGGAG-3′; downstream primer: 5′-AAAAAGCTT (*Hind* III) ATGTGGTATGGCTGATTATGAT-3′). The EGFP cDNA digested with *EcoR* I, *Hind* III was ligated to pET28a vector digested with the same restriction enzymes, and then the resultant ligation product was transformed into *E. coli* DH5α. Positive clones were propagated and recombinant plasmids were extracted for further identification by sequencing. The sequence-confirmed recombinant plasmid was named pET28a-EGFP. Macropreparation of pET28a-EGFP plasmid was performed according to the handbook of QIAGEN-tip 2500 Kit. The concentration of the extracted pET28a-EGFP plasmid was 0.7mg/ml.

Cell-free Translation and Fluorescence Measurement

Cell-free translation system was carried out according to the instruction of *E. coli* T7 s30 Extract Kit (Promega, USA), except that each system was supplemented with different concentrations of PNAs (0, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 μM final concentration) and tetracycline (0, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 μM final concentration), respectively. Finally, 40 μl aliquots of the cell-free mixture were removed for fluorescence intensity measurements using a spectrofluorometer.

Autoradiography and TCA Protein Precipitation Assay

Cell-free translation system was performed as described above, with the exception that each system was supplemented with 1 μl [^{35}S]-methionine (1200 Ci/mmol). Autoradiography was performed according to 'Molecular Cloning: A Laboratory Manual' protocol. Gel Drying Kit (Promega) and BioMax MR Film (Kodak) were used. TCA protein precipitation assay was performed according to protocol of *E. coli* T7S30 Extract Kit using a Beckman LS-600 liquid scintillation counter.

E. coli Growth and Inhibition

E. coli DH5α were pregrown overnight in 1ml LB medium at 37°C with constant shaking at 200 rpm. Using the LB liquid medium at 10% of normal strength as dilution, the culture was diluted to 10^6 bacteria per milliliter. Ninety microliters of LB media at 10% strength containing different concentrations of PNAs (0, 2, 5, 10, 20, 50 μM final concentration) or tetracycline (0, 1, 2, 3,4, 5 μM final concentration) was added to 96-well plates with lid, and then 10 μl diluted cultures corresponding to 10^4 cells were withdrawn into the wells in a 100 μl total culture volume. The 96-well plates were incubated for 24h at 37°C with constant shaking at 200 rpm. The turbidity was recorded at 550 nm at 3-h intervals (0, 3, 6, 9, 12, 15, 18 h). LB agar plates were inoculated with 100 μl aliquots exposed to 10 μM peptide-PNA(G1138) or 4 μM tetracycline at different times (i.e. 0, 15, 30, 45, 60, 120, 180, 240 min). The agar plates were cultured overnight at 37°C for colony-forming units (CFUs) assay.

RESULTS

Variation of Fluorescence Intensity

To establish a reporter system for our studies, a pET28a-EGFP plasmid was constructed that expresses EGFP under the control of a T7 promoter. EGFP was used as the reporter molecule, as it facilitates convenient measurement for fluorescence intensity variation in a cell-free translation system. The cell-free translation system containing pET28a-EGFP plasmid and different concentrations of PNA(G1138) were incubated together at 37°C for 2 h. Fluorescence intensity was

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Table 1 PNAs and inhibitory concentrations

PNAs	Target region	Inhibitory concentrations (μM)	
		Cell-free translation (IC_{50})	Cell growth (MIC)
Duplex-forming			
PNA2141 H-ATATGAATC-NH2	Control	>1	
PNA(C1045) H-CTGTCTGGGCC-NH_2	23S rRNA domain II	>1	
Triplex-forming			
PNA(G1138) H-JJJJ-$(egl)_3$-CCCCGTT-NH_2	23S rRNA domain II	0.15	
PNA(A1067) H-TJTTJ-(egl)3-CTTCTAA-NH2	23S rRNA domain II	>1	
Peptide-PNA			
Peptide-PNA(G1138) H $(KFF)_3$K-L-JJJJ-$(egl)_3$-CCCCGTT-NH_2	23S rRNA domain II		10
Peptide-PNA(mismatch G1138) H $(KFF)_3$K-L-JJJJ-$(egl)_3$-CACAGTT-NH_2	Control		>50
Peptide-PNA(Unrelated G1138) H$(KFF)_3$K-L-JJJJ-$(egl)_3$-ACGGCTTGA-NH_2	Control		>50
Tetracycline (known translation inhibitor)		0.12	4

The PNAs are written from their amino to carboxyl termini, with the carboxy termini containing an amide. Egl stands for ethylene glycol linker; J is pseudo-isocytosine.

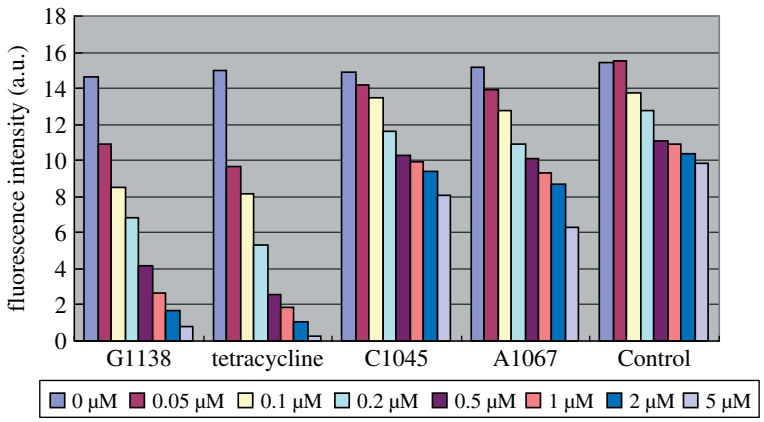

Figure 2 Fluorescence intensity measurement by a spectrofluorometer in the presence or absence of PNAs and tetracycline. The values represent the means from two independent experiments.

measured by a spectrofluorometer. The data indicated that the fluorescence intensities decreased as the PNA(G1138) concentration increased (Figure 2), and the inhibitory effect was similar to that of tetracycline. Other PNAs had no evident inhibitory effects.

Expression and Inhibition of EGFP

Because the EGFP present in the cell-free translation systems is not 100% active [24], to demonstrate that the decline of fluorescence intensity is due to reduction of EGFP expression, we employed autoradiography to analyze the inhibitory effects of PNAs and tetracycline. PNAs or tetracycline was added to the cell-free translation systems containing [^{35}S]-methionine and pET28a-EGFP. After 2 h of incubation at 37 °C, the reduction of EGFP was confirmed by autoradiography. Results demonstrated that PNA(G1138) can effectively inhibit EGFP synthesis, as with tetracycline (Figure 3(c)). The inhibitory effect appeared in a dose-dependent manner (Figure 3(b)). On the contrary, the other PNAs did not show any inhibitory effect on the EGFP expression (Figure 3(a)).

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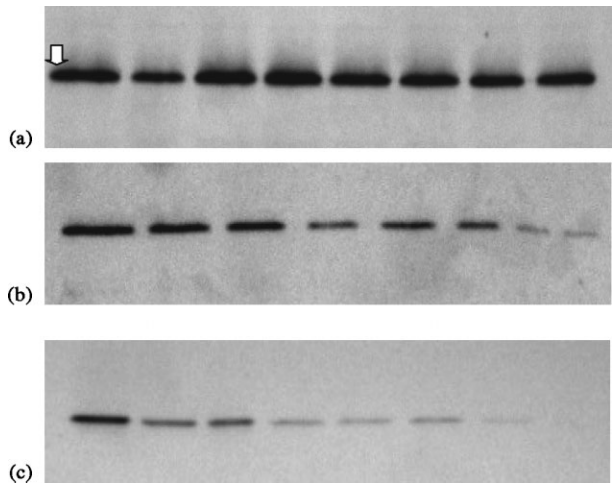

Figure 3 Autoradiography demonstrating the reduction of EGFP expression in a cell-free translation system (a) the inhibition result of ineffective PNAs; (b) the inhibition result of PNA(G1138); (c) the inhibition result of tetracycline. The arrow indicates the EGFP strap. The inhibitory concentrations of PNAs and tetracycline are 0, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 μM from left to right.

Relative Incorporation of (^{35}S) methionine

To determine quantitatively the inhibitory concentrations of antisense PNAs, TCA protein precipitation assay was used to measure the relative incorporation of [^{35}S]-methionine. PNAs and tetracycline respectively were added to the cell-free translation systems containing [^{35}S]-methionine at different concentrations. After 2 h of incubation at 37°C, relative [^{35}S]-methionine incorporations were measured by a liquid scintillation counter. As shown in Figure 4, when the concentrations of PNA(G1138) and tetracycline were 0.2 and 0.12 μM respectively, the relative incorporation of [^{35}S]methionine decreased by 50%. The inhibition effects of PNAs are shown in Table 1.

Effects of Growth and Inhibition of *E. coli* DH5α

We designed the peptide (KFFKFFKFFK)-PNA(G1138) to study the bacteriostatic effect compared to that of PNA(G1138) and tetracycline. PNA(G1138), peptide-PNAs and tetracycline were added to *E. coli* DH5α cultures at different concentrations. Cell growth was indicated by increased turbidity using OD_{550} measurements at different times. The results showed that the inhibition effect of PNA(G1138) was unexpected, and the bacteriostatic effect of peptide-PNA(G1138) became more obvious with the increase in concentration and that of tetracycline was found to be more apparent. Their minimum inhibiting concentrations (MICs) are approximately >50 μM (Figure 5(e)), 10 μM (Figure 5(a)) and 4 μM (Figure 5(d)), respectively. The results also demonstrated that the mismatch and unrelated peptide-PNA(G1138) at 50 μM did not inhibit the growth of *E. coli* DH5α (Figure 5(b) and (c)).

Colony-forming Units Assay

The bactericidal effect of antibacterial peptide-PNA (G1138) conjugates on *E. coli* DH5α and tetracycline were further examined by a cell viability study. With exposure to 10 μM peptide-PNA(G1138) or 4 μM tetracycline, the number of CFUs were determined by plating at different time points. For peptide-PNA(G1138), the number of CFUs decreased appreciably from 10^5/ml, and no viable cells were detected after 4 h, but for tetracycline, the period was only 1 h (Figure 6).

DISCUSSION

Studies of bacterial growth and inhibition have shown that the use of sequence-specific peptide-PNAs targeted to domain V of 23S rRNA [11,12] and mRNA [12–19] is a possible and effective approach to suppress bacterial growth. However, less emphasis has been placed on

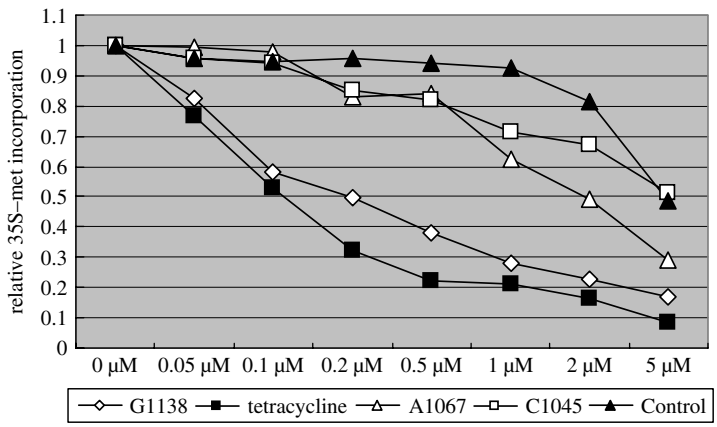

Figure 4 Translation in cell-free system assayed by measuring the relative [35S] methionine incorporation in the presence or absence of PNAs and tetracycline. The values represent the means of two independent experiments.

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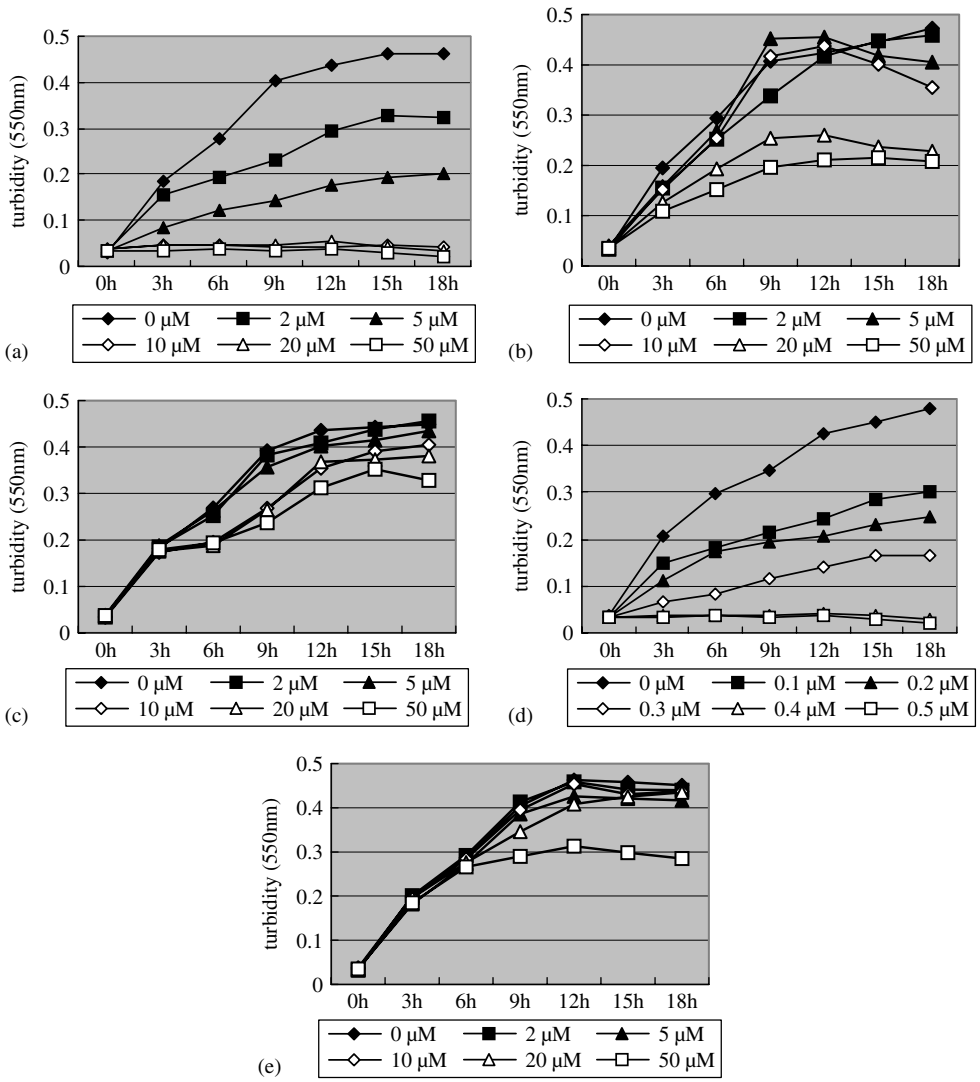

Figure 5 *E. coli* DH5α growth and inhibition using PNAs or tetracycline of different concentrations. PNAs were added to *E. coli* DH5α cultures at 2, 5, 10, 20 and 50 μM and compared to cultures lacking peptide-PNAs. Tetracycline was added to *E. coli* DH5α cultures at 0, 1, 2, 3, 4 and 5 μM. (a) Dose-dependent growth inhibition of *E. coli* DH5α by peptide-PNA(G1138). (b) Dose-dependent growth inhibition of *E. coli* DH5α by peptide-PNA(mismatch G1138). (c) Dose-dependent growth inhibition of *E. coli* DH5α by peptide-PNA(unrelated G1138). (d) Dose-dependent growth inhibition of *E. coli* DH5α by tetracycline. (e) Dose-dependent growth inhibition of *E. coli* DH5α by PNA(G1138).

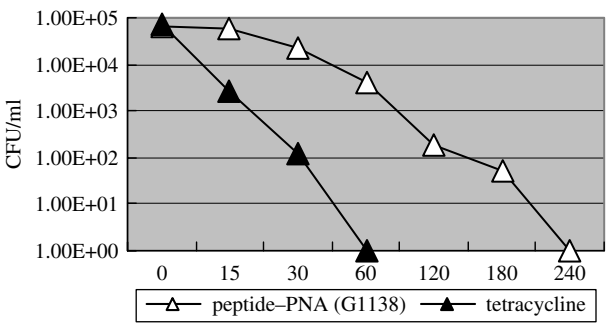

Figure 6 Bactericidal antisense effects of antibacterial peptide-PNA(G1138) and tetracycline against *E. coli* DH5α. Cultures of *E. coli* DH5α were established in a 10% LB medium at 106 CFU/ml and treated with peptide-PNA(G1138) at 10 μM or tetracycline at 4 μM.

PNAs targeted to domain II of 23S rRNA. Here, we utilized a cell-free translation system to illustrate that PNAs targeted to domain II of 23S rRNA can effectively inhibit protein synthesis *in vitro*, and further studied the growth inhibition of peptide-PNAs targeted to domain II of 23S rRNA on *E. coli* DH5α.

The data demonstrated that PNA(G1138) can efficiently suppress EGFP expression in a cell-free translation system. The inhibition effect of PNA(G1138) is somewhat lower than that of the tetracycline, and PNA(C1045) and PNA(A1067) have no conspicuous inhibition effects. Why can PNA(G1138) inhibit bacterial translation *in vitro* but other PNAs cannot? It is possible that nucleotide G1138 is a component of the pseudoknot involving ntG1137–G1138 and C1005–C1006,

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and disruption of this pseudo-knot has been shown to inactivate the 50s subunit by eliminating its ability to associate with the 30s subunit [21]. Triplex-forming of PNA(G1138) may effectively inhibit pseudo-knot formation, thereby inhibiting protein synthesis. Alternatively, as shown in Figure 1(b), it is possible that it is easier for the PNA to access the G1138 site because it is more accessible in terms of space, while in A1067 and C1045 sites there is no such feature.

Studies have shown that the PNAs bind preferentially to their complementary targets, and the binding is weaker if a target contains one mismatch, whereas no binding takes place to targets containing two mismatches [25,26]. Results presented here have also implied that peptide-PNA(G1138) treatment can inhibit *E. coli* growth in a dose- and sequence-dependent manner.

Although the peptide-PNA(G1138) is bactericidal, its effective concentration is much higher than that of tetracycline. Moreover, peptide-PNA(G1138) tends to act more slowly than tetracycline. Why is the inhibitory effect of PNA(G1138) only slightly lower than that of tetracycline in a cell-free translation system, while the peptide-PNA(G1138) is less effective and slower than tetracycline in inhibiting *E. coli* growth? There are several possibilities: First, in a cell-free translation system, there is no barrier to prevent the PNAs from entering the cells, so the problem of cellular uptake does not exist. Second, although PNA(1138) delivery into *E. coli* can be enhanced using the attached cationic carrier peptide KFFKFFKFFK, the effect of peptide-PNA(G1138) uptake into the cell may be far from ideal. Third, since the action of peptide-PNA(G1138) is apparently slower than that of antibiotic tetracycline, it may imply a transportation problem. Altogether, these results demonstrate that the biggest obstacle for peptide-PNA(G1138) becoming an effective antisense antibacterial drug may be attributed to its ineffectiveness in cellular entry.

On the basis of comparison of the bactericidal concentration of peptide-PNA(G1138) and tetracycline, a comparison was made of the MICs between the peptide-PNA(G1138) and the peptide-PNAs targeted to mRNA and α-sarcin of rRNA in *E. coli* K12 [12], and it was found that the MIC of peptide-PNA(G1138) is also very high (about fivefold higher). Apart from a different target, are there any other factors that may be responsible for the weaker bactericidal effect of peptide-PNA(G1138)? One possible explanation is that the *E. coli* strain used in our study is different from that in Ref. 12.

To conclude, our results have clearly demonstrated that the G1138 site of 23S rRNA is a good target site upon which antisense agents may act. Confirmation that the peptide-PNA(G1138) treatment is bactericidal in *E. coli* DH5α and that the effect was dose- and sequence-dependent were established. When compared to tetracycline and with other studies, the relatively weak inhibitory effect of peptide-PNA(G1138) however requires further research.

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