

Essential Gene(s) Targeted by Peptide Nucleic Acids Kills *Mycobacterium smegmatis* in Culture and in Infected Macrophages

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Abstract

Background: Antisense peptide nucleic acids (PNAs) exhibit growth inhibitory effects on bacteria by inhibiting the expression of essential genes and could be promising therapeutic agents for treating bacterial infections. A study was carried out to determine the efficacy of several antisense PNAs in inhibiting extracellular and intracellular growth of *Mycobacterium smegmatis*. **Methods:** Six PNAs obtained from a commercial supplier were tested to evaluate the inhibitory effect on bacterial growth by inhibiting the expression of the following essential genes: *inhA* (a fatty acid elongase), *rpsL* (ribosomal S12 protein), *gyrA* (DNA gyrase), *pncA* (pyrazinamidase), *polA* (DNA polymerase I) and *rpoC* (RNA polymerase β subunit) of *M. smegmatis*. Each PNA was tested at 20 μ M, 10 μ M, 5 μ M and 2.5 μ M concentrations to determine whether they caused a dose dependent killing of *M. smegmatis* cultured in Middlebrook 7H9 broth or in a J774A.1 murine macrophage cell line. **Results:** In Middlebrook broth, the strong growth inhibitory effect against *M. smegmatis* was observed by PNAs targeting the *inhA* and *rpsL* genes at all four concentrations. The PNAs targeting the *pncA*, *polA* and *rpoC* genes were found to exhibit strong growth inhibition against *M. smegmatis* but only at 20 μ M concentration. No growth inhibition of *M. smegmatis* was seen in pure culture when treated with PNAs targeting *gyrA* and a mismatch PNA targeting *dnaG* (DNA primase). All six PNAs showed killing of *M. smegmatis* in J774A.1 macrophage cell line that were statistically significant ($p < 0.05$). **Conclusion:** It may be concluded from this study that PNAs could be potential therapeutics for mycobacterial infections.

Keywords

Middlebrook 7H9 Broth Culture, J774A.1 Murine Macrophage Cell Line,

Antisense Therapy, Peptide Nucleic Acid, Cell Penetrating Peptide,
Mycobacterium

1. Introduction

Mycobacterium smegmatis is an avirulent bacterial genus present in the soil and smegma that shows many features similar to *Mycobacterium tuberculosis* (*Mtb*), responsible for causing tuberculosis in humans [1]. Tuberculosis is one of the major causes of human death in the world. Around one-third of the human population is estimated to be infected with *Mtb*. Antibiotics commonly used to treat the *Mtb* in humans require prolonged therapy and they have many toxic side effects. The antibiotics used against *Mtb* cannot readily penetrate through the lipid-rich mycobacterial cell wall that represents a hurdle in the treatment of clinical disease [2] [3]. Moreover, emergence of multidrug-resistant (MDR) strains of *Mtb* is a great challenge to treat tuberculosis with a combination of antibiotics. It is very essential to develop an effective treatment for MDR *Mtb* without toxicity and that enables highly targeted delivery of antimicrobials to their intracellular niche.

Antisense/antigene therapy using peptide nucleic acids (PNAs) has the potential to help control MDR bacterial infections. PNA is DNA mimic with a peptide backbone instead of sugar (deoxyribose) [4]. It binds to form double stranded like DNA and complexes with complementary DNA or RNA. Short antisense oligonucleotides (10 - 12 mers) PNAs can bind at start codon and upstream regions of essential genes. Addition of cell penetrating peptide (CPP) to the PNA transports it across cell membranes and bacterial cell envelopes, which is essential for its antimicrobial effects [5].

Recently PNA therapy has shown promising results in inhibiting the growth of *Salmonella typhimurium*, *Staphylococcus aureus* and *Escherichia coli*, by down regulating the functions of the essential genes of these bacteria [6] [7] [8]. A previous study showed antisense PNA targeting only *inhA* gene inhibited the growth of *M. smegmatis* in pure culture [9]. As *Mycobacterium* spp. are intracellular pathogens, the growth inhibitory effects of antisense PNA must be studied inside the macrophage. To the best of our knowledge, no study has been performed so far to evaluate the growth inhibitory functions of anti-sense PNAs against *Mycobacterium* targeting one of its essential genes inside the macrophage. In this study, we have designed 6 antisense PNAs complementary to the translation start codon region on the mRNA of six essential genes of *M. smegmatis*; these are *inhA*, *rpsL*, *gyrA*, *pncA*, *polA* and *rpoC*. PNAs were conjugated with cell penetrating peptide (CPP = KFFKFFKFFK) to aid cell membrane passage [10]. The objectives of this study were to evaluate the inhibitory function of six anti-sense PNAs against *M. smegmatis* in pure culture and inside the macrophages and to identify the most suitable gene targets of *Mycobacterium* for antisense therapy.

2. Materials and Methods

2.1. Bacterial Strain

M. smegmatis was obtained from the laboratory repository of the Center for Molecular Medicine and Infectious Disease (CMMID) now called Center for One Health Research (COHR), Virginia-Maryland College of Veterinary Medicine at Virginia Tech.

2.2. Bacterial Growth in Pure Culture

A frozen stock of *M. smegmatis* was diluted in Middlebrook 7H9 broth (Sigma) enriched with 10% ADC incubated at 37°C aerobically for 48 h on a shaker incubator until the OD₆₀₀ 0.5 was reached.

2.3. Peptide Nucleic Acids

The list of PNAs shown in **Table 1** was synthesized by Panagene Inc. (Daejeon, Republic of Korea). PNAs conjugated with cell penetrating peptide (KFF)₃K, where K is lysine and F is phenylalanine, to facilitate PNAs uptake through bacterial cell envelope.

2.4. Growth of *M. smegmatis* in Pure Culture

An overnight culture of *M. smegmatis* was diluted in 7H9 broth to obtain 6 × 10⁴ colony forming units (CFUs)/ml and incubated with either 20 μM, 10 μM, 5 μM or 2.5 μM concentration PNAs (volume = 100 μl) in triplicate using a 96 well, low adhesion microtiter plate (Corning Inc. Cat. # 3474). The plate was sealed with an adhesive lid (Microseal B, Bio-Rad) and incubated at 37°C for 48 h on a SPECTRA max 340PC with shaker incubator; growth was monitored by measuring optical density of the culture spectrophotometrically (600 nm). All PNA

Table 1. *M. smegmatis* targeted genes and PNA-specific sequence.

Gene target	Gene accession no.	CPP-PNA sequence sequence ^a	Proposed functions/Drug
<i>inhA</i>	MSMEG_3151	H-KFFKFFKFFK-O-gt cat ttggt-NH ₂	Isoniazid Enoyl-ACP reductase (acyl carrier protein) of fatty acid synthase II needed to elongate long chain fatty acids for the synthesis of mycolic acid/Isoniazid (INH)
<i>rpsL</i>	MSMEG_1398	H-KFFKFFKFFK-O-ttgg cat gta-NH ₂	Ribosomal protein S12/Streptomycin
<i>gyrA</i>	MSMEG_0456	H-KFFKFFKFFK-O- cat atcgtcgga-NH ₂	DNA gyrase subunit A/Fluoroquinolones
<i>pncA</i>	MSMEG_6506	H-KFFKFFKFFK-O- cat gcctcga-NH ₂	PZA inhibits the activity of FAS I (palmitic acid) in PZA-susceptible <i>M. smegmatis</i> strains/Pyrazinamide (PZA)
<i>polA</i>	MSMEG_3839	H-KFFKFFKFFK-O-tt cat gcctgt-NH ₂	DNA polymerase I
<i>rpoC</i>	MSMEG_1368	H-KFFKFFKFFK-O- cat cgtaactc-NH ₂	RNA polymerase subunit β
<i>dnaG</i> (negative control PNA)	BR_1480	H-KFFKFFKFFK-O- cat tacagatt-NH ₂	Mismatch PNA for DNA primase

^aThe PNAs and CPP were synthesized (Panagene Inc., Daejeon, South Korea) and joined by an ethylene glycol linker designated as “O”; a glycol linker of nine atoms used to distance the hybridization portion of the molecule from the CPP. The bolded “cat” indicates the PNA sequence complementary to ATG start codon contained in the specified gene.

treated cultures of *M. smegmatis* were removed after 48 h and were serially diluted and plated on 7H10 Middlebrook agar. The plates were incubated at 37°C for 2 to 4 days to determine the number of viable cells (CFU/ml).

2.5. Growth of *M. smegmatis* inside J774A.1 Murine Macrophage

Intracellular activity of PNAs was evaluated in J774A.1 murine macrophage cell line (ATCC, Manassas, VA). J774A.1 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) supplemented with heat inactivated 10% fetal bovine serum. The cells were grown at 37°C in an atmosphere containing 5% CO₂. J774A.1 cells were plated in 200 µl of DMEM at a concentration of 6.5×10^4 cells per well in 96-well tissue culture white plates with clear bottoms (Corning Inc. Cat. #3596) and allowed to adhere overnight. For the infection, mid-log phase *M. smegmatis* (OD₆₀₀ = 0.5) were washed twice with PBS, centrifuged at $2800 \times G$ for 15 min and then resuspended in DMEM and sonicated for 2×30 seconds in a bath sonicator to disperse clumps. The bacteria in the supernatant were then diluted in DMEM to a concentration of 1.6×10^6 CFUs; 100 µl of bacterial suspension was added to each well of the J774A.1 cells. After 1h of infection at 37°C in 5% CO₂, J774A.1 cells were washed twice with gentamicin (10 µg/ml) to eliminate any extracellular bacteria. Cells were treated with PNAs designed to target *inhA*, *rpsL*, *pncA*, *rpoC*, *polA*, *gyrA* and *dnaG*. The concentrations of each PNA were 0.2 µM, 2 µM and 20 µM; rifampin concentration was 16 µg/ml. 100 µl of each PNA concentration or rifampin was added to infected J774A.1 cells in triplicate and incubated at 37°C under 5% CO₂ for 24 h. The infected macrophages were lysed with 100 µl of 0.1% TritonX-100 (Sigma) and serially diluted in PBS-Tween and 25 µl lysed cells were inoculated onto Middlebrook 7H10 agar (Difco) plates for viable count determination. The CFU was determined just after the infection (day 0, *i.e.*, post gentamicin treatment) and at the end of the experiment.

2.6. Macrophage Viability and Toxicity Assay

Cell viability assay using tetrazolium compound [CellTiter 96[®] Aqueous Non-Radioactive Cell Proliferation Assay (MTS); Promega, Fitchburg, WI] was conducted to measure the growth inhibitory effects of PNAs linked with CPP on J774A.1 cells. The macrophages were treated with 20 µM PNA linked with CPP and incubated at 37°C under 5% CO₂ for 24 h. The viable cell numbers were determined at 490 nm absorbance due to the formazone product. The trypan blue exclusion assay was used to measure cell viability after 24 h of PNA treatment. The number of viable macrophage cells was assessed using a hemocytometer. The percentage of living cells was determined by counting at least 100 cells.

2.7. Statistical Analysis

CFU data of PNAs treated and untreated infected cells were analyzed for statistical significance using student's *t* test (Microsoft Excel version 2010, USA). A P

value of ≤ 0.05 was considered to be statistically significant.

3. Results

3.1. Inhibition of Growth in Pure Culture

The results of growth inhibitory assays of six PNA showed that only two PNAs (*inhA* and *rpsL*) out of six essential genes were found to exhibit strong growth inhibition at all concentrations. Three PNAs (*pncA*, *polA* and *rpoC*) showed growth inhibitory effect only at the highest concentration used *i.e.*, 20 μM concentration. One PNA (*gyrA*) and a mismatch PNA did not show any growth inhibitory effect. The growth inhibition effects of PNAs are summarized in **Table 2**.

3.2. Inhibition of Growth inside Murine Macrophage

Growth inhibitory effects of PNAs were measured at 24 h post-treatment. The results show that all six PNAs significantly decreased the intracellular growth of *M. smegmatis* ($p > 0.05$). PNAs targeting *inhA* and *rpsL* showed concentration dependent growth inhibition as compared to remaining four PNAs. Complete growth inhibition of intracellular growth was observed for *rpsL* PNA at 20 μM concentration. The ability of PNAs to inhibit growth of *M. smegmatis* inside murine macrophages is shown in **Figure 1**.

3.3. Macrophage Viability and Toxicity Assay

The viability and reproductive capacity of J774A.1 macrophage cell line was tested by treating the cell line with PNAs linked or unlinked to CPP or by CPP only at 24 h post treatment. No visible toxic effects were seen both in trypan blue exclusion assay and MTT assay (data not shown). None of the tested concentrations of PNAs affected either morphology or doubling time of J774.A1 cells (data not shown).

Table 2. The ability of PNAs to inhibit *M. smegmatis* in pure culture.

Gene target	PNA concentration			
	20 μM	10 μM	5 μM	2.5 μM
<i>dnaG</i> *	-	-	-	-
<i>inhA</i>	++++	++++	++++	++++
<i>rpsL</i>	++++	++++	++++	++++
<i>gyrA</i>	-	-	-	-
<i>pncA</i>	++++	-	-	-
<i>polA</i>	++++	-	-	-
<i>rpoC</i>	++++	-	-	-

Abbreviations: +++++, Strong inhibition of growth; -, No growth inhibition, *, mismatch control PNA.

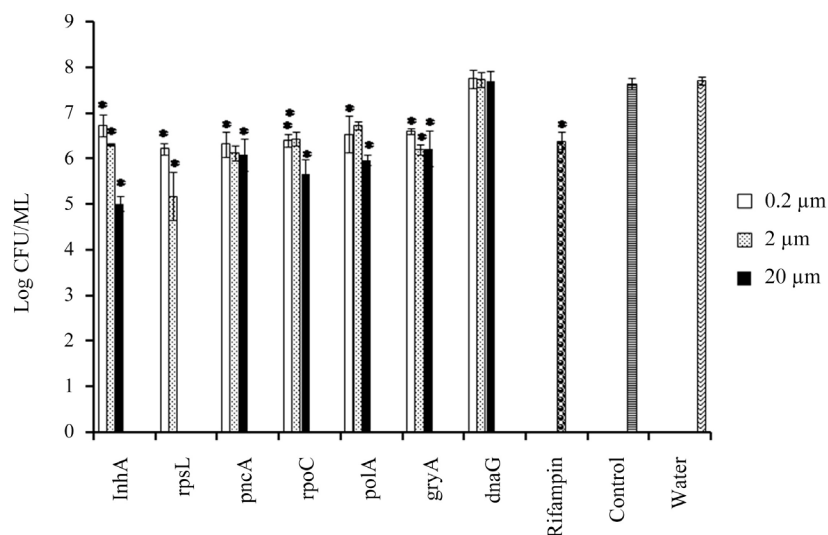


Figure 1. Bacteriocidal activity of PNA targeting *inhA*, *rpsL*, *pncA*, *rpoC*, *polA* and *gyrA* against *M. smegmatis* inside J774A.1 murine macrophage cells. Macrophages in 100 μ l of DMEM were infected with *M. smegmatis* at a MOI of 100 (seeding density and infection dose per well were 7.0×10^4 and 7.0×10^6 respectively). After 1 h post infection, extracellular bacteria were washed twice with 100 μ l of PBS + gentamicin (10 μ g/ml). Infected cells were resuspended in 100 μ l of DMEM treated with *inhA*, *rpsL*, *pncA*, *rpoC*, *polA* and *gyrA* (at 0.2, 2 and 20 μ M). Three wells were untreated controls and three wells were treated with water and three wells with rifampin (16 μ g/ml). Cells were lysed at 24 hr post infection, serially diluted and plated onto Middlebrook 7H9 agar and incubated at 37°C for 48 hr for measuring CFU. Results were expressed as mean Log CFU of three wells \pm SD. Complete killing of bacteria was observed in wells treated with *rpsL* at 20 μ M concentration. CFU data of treated and untreated cells were analyzed for statistical significance by student's *t* test. Statistically significant difference between treated and untreated cells were indicated by asterisk (**p* < 0.05).

4. Discussion

The identification of essential genes of microbes through microbial genomics helps selecting potential targets for antisense inhibition. Antisense therapy using PNAs has become an attractive tool for controlling bacterial growth. PNAs can produce strong complexes with complementary strands of DNA or RNA and inhibit expression of microbial genes required for their growth in dose dependent manner at low micromolar concentration [11] [12]. Bacteria living within eukaryotic host tissue constitute a significant challenge for antisense treatment [13]. The PNAs linked with cell penetrating peptides are found effective in inhibiting growth of microbes both *in vitro* and *in vivo* [8]. The high prevalence of extreme drug resistant *Mtb* leaves no treatment option for patients that leads to increased morbidity and mortality rate worldwide. In this situation there is an urgent need to develop new drugs against *Mtb* that could be alternatives to traditional antibiotics. The present study evaluated six PNAs against *M. smegmatis* both *in vitro* and *in vivo*. In this study six PNAs were selected using the bioinformatics resources and tools. The PNAs were linked with cell penetrating peptide (CPP): CPP-O-PNA. These were significantly more effective in inhibiting expression of

their targets as compared to non-CPP linked PNAs [8]. Cationic CPPs are known to bind with proteoglycans of eukaryotic cells and then enter into the cells by endocytosis [14] [15]. To develop a new antimicrobial, selection of essential genes as targets in bacteria for antisense PNAs is important [16]. The present study selected six essential genes (*inhA*, *rpsL*, *gyrA*, *pncA*, *polA* and *rpoC*) of *M. smegmatis* for development of effective antisense treatment targets. PNAs in this study were found to be effective in inhibiting the growth of *M. smegmatis* both in pure culture and murine macrophages. The antibacterial effect of PNA depends on bioavailability of PNA inside the bacterial cell while the lipid enriched cell wall is the major barrier of entrance to PNA inside *Mycobacterium* [2] [6]. Although *gyrA* PNA did not show growth inhibition of *M. smegmatis* in pure culture, it was found to have growth inhibitory effect against *M. smegmatis* inside murine macrophage. Growth inhibitory effects of *gyrA* PNA against *Klebsiella pneumonia* were observed both in pure culture and inside human epithelial cells at 20 μ M concentration [17]. PNAs targeting *rpsL* and *inhA* were found to be most effective in killing *M. smegmatis* as compared to other essential gene targets. A dose dependent growth inhibition of *M. smegmatis* was observed for both *rpsL* and *inhA* PNAs. The susceptibility of *M. smegmatis* by *inhA* PNA to growth inhibition was originally shown by Kulyte *et al.*, [9] and occurred at concentrations between 2 and 15 μ M.

There was no detectable toxic effect on macrophages noticed either in a trypan blue exclusion assay or in the cell viability MTS assay even at the highest concentration (20 μ M) of PNA used suggesting that the PNA is specific for *M. smegmatis*.

Antisense oligonucleotides are designed to bind the target mRNA to prevent translation or bind DNA to prevent gene transcription respectively. They can bind to the specific sequence of RNA and DNA by Watson-Crick base pairing which leads to a variety of post-binding events [17] [18]. Data from this study indirectly indicates that PNAs exerted antimicrobial activity by inhibiting the expression of essential gene(s) involved in different types of macromolecular synthesis.

Sequence homology is necessary for PNA binding in order to selectively hybridize to complementary nucleic acids of either chromosomal or messenger RNA of *M. smegmatis*.⁶ This study demonstrated sequence specific binding of PNAs with the gene targets since mismatch a PNA lacking sequence homology did not show growth inhibition against *M. smegmatis* both in pure culture and in *M. smegmatis* infected J774A.1 cells. The antimicrobial effect observed against *M. smegmatis* by antisense peptide PNAs in murine macrophage cell line shows the potential for the development of PNA based antibiotic against mycobacterial infection in humans.

5. Conclusion

M. smegmatis is susceptible in pure culture to PNA mediated growth inhibition by targeting genes involved in cell envelope synthesis, DNA and mRNA synthe-

sis, and protein synthesis. *M. smegmatis* growing in a macrophage cell line is also susceptible to PNA targeting genes involved in cell envelope and nucleic acid synthesis of the bacterium. Data from this study suggest that PNAs targeting *inhA* and *rpsL* could be useful as antisense therapy for *Mycobacterium* infections. Further studies to examine the effect of PNAs administered as nanoparticles targeted to macrophages in mice infected with *M. smegmatis* will need to be carried out.

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Ethical Approval

Not required.

Disclosure

A poster with interim findings was presented at the Euro-Scicon conference 2013 held on 21 March 2013 at the Royal College of Pathologist, London, UK. The poster's abstract was published in <https://www.euroscicon.com/documents/21March2013TB.pdf>.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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