Potentiating the Anti-Tuberculosis Efficacy of Peptide Nucleic Acids through Combinations with Permeabilizing Drugs

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ABSTRACT The emergence of antimicrobial resistance warrants for the development of improved treatment approaches. In this regard, peptide nucleic acids (PNAs) have shown great promise, exhibiting antibiotic properties through the targeting of cellular nucleic acids. We aimed to study the efficacy of PNA as an anti-tuberculosis agent. Since the efficacy of PNA is limited by its low penetration into the cell, we also investigated combinatorial treatments using permeabilizing drugs to improve PNA efficacy. Various concentrations of anti-inhA PNA, permeabilizing drugs, and their combinations were screened against extracellular and intracellular mycobacteria. 0.625 to 5 μM anti-inhA PNA was observed to merely inhibit the growth of extracellular M. smegmatis, while low intracellular bacterial load was reduced by 2 or 2.5 log-fold when treated with 2.5 or 5 μM PNA, respectively. Anti-inhA PNA against M. tuberculosis H37Ra exhibited bactericidal properties at 2.5 and 5 μM and enabled a slight reduction in intracellular M. tuberculosis at concentrations from 2.5 to 20 μM. Of the permeabilizing drugs tested, ethambutol showed the most permeabilizing potential and ultimately potentiated anti-inhA PNA to the greatest extent, reducing its efficacious concentration to 1.25 μM against both M. smegmatis and M. tuberculosis. Furthermore, an enhanced clearance of 1.3 log-fold was observed for ethambutol-anti-inhA PNA combinations against intracellular M. tuberculosis. Thus, permeabilizing drug-PNA combinations indeed exhibit improved efficacies. We therefore propose that anti-inhA PNA could improve therapy even when applied in minute doses as an addition to the current anti-tuberculosis drug regimen.

IMPORTANCE Peptide nucleic acids have great potential in therapeutics as anti-gene/anti-sense agents. However, their limited uptake in cells has curtailed their widespread application. Through this study, we explore a PNA-drug combinatorial strategy to improve the efficacy of PNAs and reduce their effective concentrations. This work also focuses on improving tuberculosis treatment, which is hindered by the emergence of antimicrobial-resistant strains of Mycobacterium tuberculosis. It is observed that the antibacterial efficacy of anti-inhA PNA is enhanced when it is combined with permeabilizing drugs, particularly ethambutol. This indicates that the addition of even small concentrations of anti-inhA PNA to the current TB regimen could potentiate their therapeutic efficiency. We hypothesize that this system would also overcome isoniazid resistance, since the resistance mutations lie outside the designed anti-inhA PNA target site.

KEYWORDS ethambutol, peptide nucleic acids, permeability, tuberculosis, intramacrophage

Through their anti-gene/anti-sense properties (1–4), peptide nucleic acids (PNAs) display antibacterial activity against many pathogenic bacteria, such as Escherichia coli (5–7), Klebsiella pneumonia (8), Pseudomonas aeruginosa (9), Salmonella enterica (10, 11), Staphylococcus aureus (12), and Brucella suis (13) when targeting essential genes like gyrA (8, 12), ftsZ (4, 9), acpP (9, 14), and rpoB (13). Their application in
therapeutics, however, is mainly limited by the low penetration capacity of PNA across cell membranes (3, 15), particularly bacterial membranes (16). PNA encapsulation in nanoparticles (17), linkage to antimicrobial peptides (18), and conjugation to cell penetrating peptides (CPPs) (5) have been explored to improve its uptake. PNA-CPP conjugates demonstrate 15- to 20-fold greater efficacy than PNA alone (14). The efficacious concentrations, however, remain high.

Treatment of in vitro and in vivo bacterial infections caused by permeable mutants of E. coli demonstrates a lower dosage requirement of PNA/PNA-CPP compared to treatment of infections with wild type (WT) strains (7, 14, 19). Combinations of PNA with a nonapeptide or conjugated to CPP prove to enhance efficacy only partially in WT strains, where permeable mutants continue to exhibit greater susceptibility (7, 19). Thus, a combinatorial therapy of CPP-PNA with a permeabilizing drug could enhance overall bactericidal efficacy.

Our study focuses on the evaluation of a PNA-CPP targeting an essential gene for treatment of tuberculosis (TB). Kulyté et al. (2005) demonstrated that anti-inhA PNA targeting the gene start site in Mycobacterium smegmatis could partially inhibit its growth at 5 μM over a 16-h period (20). We investigated the effect of permeabilizing drugs, colistin, ceftazidime pentahydrate, or ethambutol, on the efficacy of this anti-inhA PNA. Colistin, ceftazidime, and ethambutol target cations in the lipopolysaccharide layer (21), penicillin-binding proteins (22), and arabinogalactan/lipoarabinogalactan synthesis (23), respectively. Colistin and ethambutol have additionally been found to improve the efficacy of other anti-TB drugs (24, 25) while combinations of ceftazidime and avibactam are effective against the Mycobacterium avium complex (26). Thus, the permeabilizing activity of these drugs was hypothesized to improve the efficacy of anti-inhA PNA by enhancing PNA uptake.

We also test the efficacy of anti-inhA PNA designed to target the start site of the inhA in M. tuberculosis and examined its efficacy in combination with permeabilizing drugs against both extracellular and intramacrophage mycobacteria.

## RESULTS

Through the targeting of an essential gene, it has been proven that peptide nucleic acids (PNAs) can have antibacterial effects (27–30). InhA, encoding an NADPH-dependent enoyl-acyl carrier protein reductase, is an essential gene for the synthesis of the fatty acids that constitute the mycolic acid in the mycobacterial cell wall (31). InhA inhibition or downregulation is known to effectively inhibit survival of Mycobacterium (32, 33). Thus, inhA was selected for our study. Anti-inhA PNA designed against M. smegmatis has a growth-inhibitory effect against this bacterium. However, efficacy was observed to be achieved at concentrations of no less than 5 μM (20). We selected this predesigned anti-inhA PNA for our study and also designed an anti-inhA PNA against M. tuberculosis (Table 1).

### Anti-mycobacterial effect of anti-inhA PNA and permeabilizing agents against M. smegmatis

The treatment of 10^6 CFU/mL of M. smegmatis with 0.625 to 5 μM of anti-inhA PNA over 48 h was observed to have growth-inhibitory effects, maintaining a constant cell density throughout the treatment time (Fig. 1a). This was consistent with the reports by Kulyté et al. (2005) which demonstrated that 2 and 5 μM anti-inhA PNA displayed similar growth inhibitory profiles at 16 h (20).

To facilitate the entry, and therefore the efficacy, of the anti-inhA PNA, we propose a combinatorial treatment with a permeabilizing drug. The MICs of colistin, ceftazidime pentahydrate, and ethambutol were therefore determined. Colistin was found to have

| CPP-PNA sequence | Bacterial target | Size (bp) | Reference |
|------------------|-----------------|-----------|-----------|
| (KFF)3K-GTCATTGGT-NH₂ | inhA M. smegmatis | 10 | (20) |
| (KFF)3K-CTGTCATGTGCG-NH₂ | inhA M. tuberculosis | 12 | This study |
| (KFF)3K-GTGTCATGTGCG-NH₂ | Mismatch M. tuberculosis | 12 | This study |

### TABLE 1 Anti-inhA PNA sequences used in this study to target either M. smegmatis or M. tuberculosis
an MIC of 16 mg/L, while ceftazidime pentahydrate and ethambutol exhibited MICs of 256 mg/L and 1.6 mg/L, respectively (Fig. 1b). It is expected that the bacteriostatic concentrations of these drugs would be optimal for altering cell membrane permeability without causing cell death.

**Effect of permeabilizing drugs on membrane permeability of *M. smegmatis***. To confirm the permeabilization of *M. smegmatis* cells treated with inhibitory concentrations of colistin, ceftazidime pentahydrate, and ethambutol, an NPN assay was performed. The hydrophobic dye NPN (1-N-phenylnapthylamine) exhibits low fluorescence in an aqueous environment. Entry into lipid-rich cell membrane causes an increase in fluorescence intensity. Any alteration of cell membrane integrity increases entry of the dye via the cell membrane, resulting in increased fluorescence (34). Confocal imaging of NPN-stained cells after 4 h of permeabilizing drug treatment showed that these cells had a higher fluorescence compared to the untreated control (Fig. 2), confirming that the treatment enhanced the permeability of *M. smegmatis* cells. Note that cell density prior to NPN staining was maintained constantly across all samples.

**Combinatorial effect of permeabilizing drugs and various anti-inhA PNA concentrations.** MICs of each drug were combined with incrementally increasing concentrations of anti-inhA PNA. The combined efficacy was determined after 48 h of treatment. Treatment with 16 mg/L of colistin improved the efficacy of 5 µM anti-inhA PNA by 2.5 log-fold. At this concentration, however, colistin had no effect on the efficacy of 0.625 to 2.5 µM anti-inhA PNA (Fig. 3a). Treatment with 256 mg/L of ceftazidime pentahydrate in combination with 1.25, 2.5, and 5 µM anti-inhA PNA resulted in 2.6, 2.6, and 3.6 log-fold reductions in bacterial load, respectively (Fig. 3b). Ethambutol, which demonstrated the highest effect on membrane permeability in the NPN assay, enhanced the efficacy of 1.25 to 5 µM anti-inhA PNA, resulting in a 3 to 4 log-fold greater reduction in bacterial load (Fig. 3c). Thus, permeabilizing drugs do improve the efficacy of anti-inhA PNA, and the extent of this increase could depend on the drug’s mode of action and/or its efficiency in disrupting the bacterial membrane.

Since the anti-inhA PNA targets the same cellular process as the anti-TB drug isoniazid (INH), we also investigated the combinatorial efficacy of permeabilizing drugs with INH through a disk diffusion assay. We used a disk diffusion assay here since INH in liquid culture (Middlebrook 7H9 broth) did not exhibit an MIC against *M. smegmatis* up to concentrations as high as 1024 mg/L. In this study, colistin, ceftazidime pentahydrate, and ethambutol were loaded, individually or in combination with INH, on sterile discs placed on LB agar spread-plated with *M. smegmatis* culture. The clearance zone indicated the efficacy of the loaded antibiotic(s). We observed increased efficacy only for combinatorial treatments of INH (32 µg) with ceftazidime pentahydrate (512 and 1024 µg), where inhibition zone diameters increased from ≤10 mm to 13.5 to 14.5 mm.
Toxicity of permeabilizing drugs to macrophage cells. Translation of this combinatorial therapy for treatment of intracellular pathogens requires that the compounds exert minimal toxicity toward the host. The toxicity of the permeabilizing drugs toward the macrophage cells was therefore monitored over 48 h. At 48 h, colistin exhibited no toxicity at concentrations up to 32 mg/L. Macrophage viability was reduced to 22% on treatment with 128 mg/L of colistin (data not shown). Ceftazidime pentahydrate exhibits a slight toxicity at 1024 mg/L over 48 h, where the relative viability dropped to 65% (data not shown). Treatment of 4 to 16 mg/L of ethambutol was found to be nontoxic over 48 h (data not shown). Thus, colistin is considered to be nontoxic at concentrations of ≤64 mg/L, ceftazidime pentahydrate exerts a toxicity at concentrations of ≥1024 mg/L, and ethambutol is nontoxic at concentrations of ≤16 mg/L.

Effect of anti-\(\text{inhA}\) PNA and cell wall-targeting drug-PNA combinations on the survival of intracellular \(M.\ smegmatis\). To evaluate the efficacy of anti-\(\text{inhA}\) against intramacrophage \(M.\ smegmatis\), we infected differentiated THP-1 cells with \(M.\ smegmatis\). The intracellular bacterial load achieved was \(10^5\) CFU/mL. Treatment of intracellular \(M.\ smegmatis\) cells with anti-\(\text{inhA}\) PNA resulted in a time- and concentration-dependent reduction in intracellular bacterial load. The highest concentration tested, 5 \(\mu\)M anti-\(\text{inhA}\) PNA, resulted in 1.5 and 2.5 log-fold reductions in intramacrophage \(M.\ smegmatis\) loads over 12 and 24 h, respectively (Fig. 4a). \(M.\ smegmatis\)-infected macrophage cells were treated with colistin, ceftazidime pentahydrate, ethambutol, or a combination of each permeabilizing drug and anti-\(\text{inhA}\) PNA for 24 h. The concentration for each permeabilizing drug was selected such that it was nontoxic to macrophage cells and only minimally affected intracellular bacterial survival, i.e., intracellular bacterial loads were not affected by more than 1 log-fold. Four mg/L of colistin in

![Image of confocal imaging](https://www.microbiolibrary.com/fig2.png)

**FIG 2** Analysis of the effects of permeabilizing drugs at their MICs on the membrane permeability of \(M.\ smegmatis\) at 4 h. Confocal imaging of (a) nontreated control, (b) SDS-treated control, and (c) colistin-, (d) ceftazidime pentahydrate-, and (e) ethambutol-treated \(M.\ smegmatis\) stained with NPN dye. FU (fluorescence units) indicates corrected total cell fluorescence.
combination with 2.5 or 5 μM anti-inhA PNA enabled identical intracellular bacterial clearance to that of the corresponding free anti-inhA PNA treatment. This indicates that colistin does not have any effect, either beneficial or adverse, on the intracellular efficacy of PNA (Fig. 4b). Ceftazidime pentahydrate employed at a concentration of 128 mg/L improved the efficacy of 5 μM anti-inhA PNA, i.e., a 0.91 log-fold enhanced reduction in bacterial load compared to treatment with PNA alone (Fig. 4c). Additionally, 0.4 mg/L of ethambutol exhibited similar effects, where a combination of 0.4 mg/L ethambutol and 5 μM anti-inhA PNA achieved higher intracellular bacterial clearance (0.8 log-fold enhancement) than that achieved with PNA alone (Fig. 4d). Statistical significance determined through the Tukey-Kramer analysis of variance (ANOVA) post hoc test, however, indicated that even the combinations of ceftazidime pentahydrate or ethambutol with 5 μM anti-inhA PNA did not exhibit significance with 95% confidence. This is possibly due to the reduced accuracy at the detection limit of the drop-plating assay (35).

To investigate the efficacy of anti-inhA PNA and permeabilizing drug combinatorial treatments in tuberculosis therapy, similar experiments were carried out against M. tuberculosis H37Ra. For this purpose, we designed a PNA sequence complementary to the start site of the inhA gene in M. tuberculosis. The PNA was purchased as a PNA-CPP conjugate, where the CPP used was (KFF)3K.

Efficacy of anti-inhA PNA, permeabilizing drugs, and their combinations against M. tuberculosis H37Ra. The antibacterial efficacy of anti-inhA PNA was screened against M. tuberculosis H37Ra over a range of concentrations. Low concentrations of 0.625 and 1.25 μM were found to be ineffective, while 2.5 and 5 μM anti-inhA PNA resulted in 1 and 2.4 log-fold reductions in bacterial load, respectively (Fig. 5a). A mismatch PNA sequence, having no binding site in the M. tuberculosis genome, was also tested as a control, and was found to have no effect on bacterial growth at a concentration of 5 μM (Fig. 5a). Prior to testing the combinatorial effects of permeabilizing drugs and PNA against M. tuberculosis, the MIC of each permeabilizing drug was determined after
a 7-day treatment time. *M. tuberculosis* H37Ra growth inhibition was observed at 64 mg/L of colistin, 32 to 64 mg/L of ceftazidime pentahydrate, and 1.6 mg/L of ethambutol. These results were in correlation with previous reports showing the MICs of colistin, ceftazidime, and ethambutol to be 64 mg/L (25), 32 mg/L (26), and 2.5 to 5 mg/L (36), respectively, against *M. tuberculosis*. A combinatorial treatment of colistin or ceftazidime pentahydrate and anti-inhA PNA showed no overall benefit compared to the individual treatment with drug or PNA alone (Fig. 5c and d). This is likely due to a reduced ability of colistin and ceftazidime pentahydrate to permeabilize the cell membrane of *M. tuberculosis* H37Ra. Ethambutol in combination with 1.25 or 2.5 μM anti-inhA PNA, however, does improve the overall efficacy, causing a 2.8 or 1.3 log-fold enhanced reduction in bacterial load, respectively (Fig. 5e). Being an anti-TB agent itself, ethambutol effectively targets and permeates the cell wall of *M. tuberculosis*, enabling an enhanced efficacy in combinatorial treatment with anti-inhA PNA. Thus, similar to our observations for *M. smegmatis*, permeabilizing drugs can improve the efficacy of anti-inhA PNA against *M. tuberculosis* H37Ra. The efficacy of such a combinatorial therapy could be dependent on the permeabilizing potential of the drug.

**Effect of anti-inhA PNA and its combinations on the survival of intramacrophage *M. tuberculosis* H37Ra.** The efficacies of anti-inhA PNA and its combinations with permeabilizing drugs against intracellular *M. tuberculosis* were tested. It was observed that 2.5 μM anti-inhA PNA achieves effects similar to those observed for the untreated control, with higher PNA concentrations of 5, 10, and 20 μM enabling log reductions of 0.51, 0.55, and 0.75, respectively (Fig. 6a). Treatment with 16 mg/L colistin in combination with 2.5 or 5 μM anti-inhA has no effect on the efficacy of PNA (Fig. 6b). Treatment with 8 mg/L of ceftazidime pentahydrate improves the efficacy of 5 μM anti-inhA PNA, reducing the intracellular bacterial load by 1.2 log-fold (Fig. 6c). Treatment with 0.8 mg/L of ethambutol combined with 2.5 or 5 μM anti-inhA PNA showed 1.3 and 1.1

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**FIG 4** Efficacy of anti-inhA PNA alone and in combination with permeabilizing drugs against intramacrophage *M. smegmatis*. Intracellular bacterial load post treatment with (a) anti-inhA PNA alone over 12 and 24 h and (b) colistin, (c) ceftazidime pentahydrate, and (d) ethambutol combined with 0 to 5 μM anti-inhA PNA over 24 h. Data were obtained from 3 biological replicates and error bars indicate the standard deviation of each sample. Significance is indicated by * for P-value ≤ 0.05, ** for P-value ≤ 0.01, and *** for P-value ≤ 0.001.
log-fold reductions in intracellular bacterial load, respectively (Fig. 6d). Thus, the combinatorial treatment achieves 7- to 20-times greater bacterial clearance than treatment with 2.5 to 5 μM anti-
inhA PNA alone, respectively.

**DISCUSSION**

Tuberculosis is the leading cause of deaths resulting from a single infectious agent after COVID-19, with the mortality rate having increased between 2019 and 2020 (37). The emergence of antimicrobial-resistant strains of *M. tuberculosis* further hinders treatment efforts and necessitates the development of improved therapies (37, 38). PNA, by targeting *inhA* in *M. smegmatis*, has anti-mycobacterial properties (20). However, its efficacy against intracellular bacteria and *M. tuberculosis* (the causative agent of tuberculosis) remained unknown. Furthermore, its antimicrobial efficacy was observed at concentrations of 5 μM and greater. Our study therefore aimed to investigate the therapeutic potential of anti-
inhA PNA against *M. smegmatis* and *M. tuberculosis*, both in culture and intramacrophage. We also investigated a combinatorial treatment approach to potentiate PNA activity and reduce efficacious PNA concentrations.

This study confirmed that anti-
inhA PNA of 10 to 12 bp which target the start site of *inhA* in *M. smegmatis* or *M. tuberculosis* exhibit anti-mycobacterial properties. Anti-
inhA PNA inhibited *M. smegmatis* growth at concentrations of 0.625 to 5 μM, while concentrations of 2.5 μM and greater exhibited bactericidal effects against *M. tuberculosis*. This antimycobacterial ability was attributed to its optimal length (6, 39, 40), CPP conjugation that promoted its uptake into the cell (5, 14, 41), and target start site-binding which enabled an efficient anti-sense effect (40). Low intracellular bacterial loads of *M. smegmatis* were partially cleared upon treatment with anti-
inhA PNA, in a concentration- and time-dependent manner.
manner. However, concentrations as high as 20 μM only minutely affected intracellular M. tuberculosis survival. This reduced efficacy against intracellular bacteria could be attributed to compartmentalization of the internalized PNA making it less accessible to the intracellular bacteria (42). The overall high PNA concentration requirements for treatment could be due to the poor penetration capacity of PNA, since permeable mutants are more susceptible than wild-type (WT) strains to anti-sense PNA (7, 19). Hence, we hypothesized that combinatorial treatments with a permeabilizing drug and PNA could enable reductions in efficacious PNA concentrations.

Combining anti-inhA PNA with permeabilizing drugs enhances its antibacterial efficacy, with combinations with ethambutol showing the most promise. This corresponded with the highest increase in permeability observed when M. smegmatis cells were treated with ethambutol. Further, our study also confirmed an improved anti-inhA PNA efficacy against intracellular M. smegmatis/M. tuberculosis when it was combined with ethambutol. Studies by Dryselius et al. (2005) and Pantenge et al. (2013) reported that combinations of PNA and antibiotics inhibiting the same target through mRNA and protein inhibition result in synergistic responses in E. coli, S. aureus, and Streptococcus pyogenes (43, 44). Thus, the addition of an anti-inhA PNA to the current anti-TB regimen, where first-line drugs include rifampicin, isoniazid, pyrazinamide, and ethambutol (45), could possibly improve therapeutic efficacies. The presence of ethambutol in the existing treatment would also enable a low-concentration requirement of PNA for achieving desirable efficacy.

FIG 6 The survival of intramacrophage M. tuberculosis H37Ra upon treatment with anti-inhA PNA, permeabilizing drugs, and drug-PNA combinations. (a) Intracellular bacterial load at 0 h and 5 day after treatment with anti-inhA PNA at 37°C, 5% CO2. Intracellular bacterial load at 5 day after treatment with (b) colistin, (c) ceftazidime pentahydrate, or (d) ethambutol and their combination with anti-inhA PNA at 37°C, 5% CO2. The data were plotted from a sample size of n = 3, where error bars indicate the standard deviation of each sample. Significance of the combinatorial treatments with respect to individual drug and PNA treatments is denoted by *, P ≤ 0.05.
When testing permeabilizing drugs in combination with INH, an anti-TB drug targeting the same mycolic acid synthesis step, INH exhibited enhanced efficacy only in combination with ceftazidime pentahydrate against \( M. \) \textit{smegmatis}. However, colistin and ethambutol were shown to have synergistic responses with INH against \( M. \) \textit{tuberculosis} (25, 46). This difference in combinatorial efficacy could be attributed to differences between the \( M. \) \textit{smegmatis} and \( M. \) \textit{tuberculosis} strains in the cell wall (47) and the presence of regulatory protein (believed to be involved in synergy between ethambutol and INH in \( M. \) \textit{tuberculosis}) (46). These results also deviated from those of our permeabilizing drug-PNA combinatorial studies, which may be due to the inability of colistin and ethambutol to sufficiently increase intracellular INH concentrations to the levels required to achieve a visible change in INH efficacy. In contrast, PNAs are known to be resistant to efflux mechanisms (7, 48) and hence their accumulation in the cell is only affected by factors which affect its influx. We showed that these permeabilizing drugs, at their inhibitory concentrations, facilitate the entry of substances into the cell. Thus, we believe that colistin, ceftazidime pentahydrate, and ethambutol potentiated the efficacy of anti-\textit{inhA} PNA by promoting its entry into the mycobacterial cell.

The anti-\textit{inhA} PNA designed against \( M. \) \textit{tuberculosis} is also hypothesized to overcome isoniazid resistance, since isoniazid resistance has been demonstrated to be primarily through mutations in the katG gene (33). Furthermore, the anti-\textit{inhA} PNA is designed to bind to the −5 bp to +7 bp region in \textit{inhA}. This region of the \textit{inhA} promoter in \( M. \) \textit{tuberculosis} reports no mutation frequency among resistant species (49). This anti-\textit{inhA} PNA could, therefore, also have the potential to overcome drug resistance.

In conclusion, combinatorial therapies involving the use of essential gene-targeting PNAs and permeabilizing drugs like ethambutol present a new avenue of therapy for diseases such as tuberculosis which urgently need more effective antibacterial treatment approaches. The permeabilizing drugs increase cell wall permeability and, in turn, possibly enable the uptake of the anti-\textit{inhA} PNA. This allows enhanced efficacy of the anti-\textit{inhA} PNA such that its effective concentrations can also be reduced. Furthermore, such a system is effective against both extracellular and intramacrophage \( M. \) \textit{smegmatis} and \( M. \) \textit{tuberculosis} H37Ra. It is also our belief that the anti-\textit{inhA} PNA designed against \( M. \) \textit{tuberculosis} will continue to show promise against drug-resistant strains of \( M. \) \textit{tuberculosis}.

**MATERIALS AND METHODS**

**Bacterial strains and culturing.** The bacterial strains used in this study, \textit{Mycobacterium smegmatis} mc\(^{2}\) 155 and \textit{Mycobacterium tuberculosis} H37Ra, were obtained from Jaya Tyagi at the All India Institute of Medical Sciences, Delhi, and from Vinay Nandicoori at National Institute of Immunology, Delhi, respectively. \textit{M. smegmatis} was cultured to an OD\(_{600}\) (optical density at 600 nm) of 0.5 in Middlebrook 7H (HiMedia) broth, supplemented with 0.44% glycerol (Rankem) and 0.15% Tween 80 (HiMedia), at 37°C and 200 rpm. \textit{M. tuberculosis} H37Ra, cultured in a biosafety level 2 + facility, was grown in Middlebrook 7H9 medium, supplemented with 0.44% glycerol, 0.15% Tween 80, and 10% albumin-dextrose-NaCl solution (ADN), at 37°C and 180 rpm. The 0.5-OD\(_{600}\) cultures were used for testing the efficacy of compounds, membrane permeability assays, and infection.

**Human cell line and culturing.** THP-1 cells were received from Vinay Nandicoori at the National Institute of Immunology (Delhi). THP-1 cells were passaged in complete RPMI medium (RPMI medium supplemented with 10% fetal bovine serum (FBS), HiMedia), penicillin (100 IU, Sigma-Aldrich), and streptomycin (100 mg/L, Sigma-Aldrich) and cultured at 37°C and 5% CO\(_{2}\). The THP-1 monocytes were differentiated to macrophages by treatment with 0.025 mg/L phorbol 12-myristate acetate (PMA, Sigma-Aldrich) for 24 h at 37°C and 5% CO\(_{2}\). Differentiated THP-1 cells were then cultured in fresh complete RPMI medium for 24 h prior to further treatment or infection.

**Peptide nucleic acid design.** The 10-bp anti-\textit{inhA} PNA sequence was selected from the study by Kulyte et al. (20) and its target site was confirmed bioinformatically using the blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and SnapGene (v5.2, http://www.snapgene.com) tools. An anti-\textit{inhA} PNA sequence was also designed to target the gene start site of \textit{M. tuberculosis} H37Ra, \textit{M. tuberculosis} H37Rv, and \textit{M. bovis}. For experimentation, the PNAs were obtained from PANAGENE (Daejeon, Republic of Korea) as PNA-CPP (KFFKFPKFPPK) conjugates.

**Efficacy testing using a broth microdilution assay.** Here, 100-\(\mu\)L aliquots of a 10-fold diluted, 0.5-OD\(_{600}\) \textit{M. smegmatis} culture were treated with 0.625 to 5 \(\mu\)M anti-\textit{inhA} PNA or permeabilizing drugs (4 to 16 mg/L colistin, 128 to 512 mg/L ceftazidime pentahydrate, or 0.8 to 3.2 mg/L ethambutol) for 48 h at 37°C. All three permeabilizing drugs were purchased from HiMedia. Post-treatment, the cultures were drop-plated to determine the drop count in CFU/mL (equation below). Briefly, 10-\(\mu\)L drops of serially
diluted samples were distinctly loaded on a Luria Bertani (LB) agar (HiMedia) plate and incubated for 48 h at 37°C. The colony count and corresponding dilution factor were noted. The lowest concentration of antibacterial agent that resulted in no change in bacterial count compared to the initial cell count was determined as the MIC.

\[
\text{Drop count (CFU/mL)} = \frac{\text{Colonies counted} \times \text{dilution factor}}{0.01}
\]

The efficacies of anti-inhA PNA (0.625 to 5 μM) and the permeabilizing drugs [colistin (64 to 256 mg/L), ceftazidime pentahydrate (32 to 128 mg/L), and ethambutol (1.6 to 6.4 mg/L)] were tested against M. tuberculosis H37Ra over 5 days at 37°C and 5% CO₂. Viable cell densities after treatment were determined through a drop plating assay carried out on Middlebrook 7H11 agar and incubated for 30 days. Permeabilizing drug-PNA combination studies were carried out in an identical manner, combining the MIC of the permeabilizing drug with varied concentrations of PNA.

**Efficacy testing using the disc diffusion assay.** Briefly, 10⁶ cells of a 0.5 OD₆₀₀ M. smegmatis culture was spread plated on to an LB agar plate. Sterile cellulose discs were distinctly placed on the agar plate and colistin, ceftazidime pentahydrate, ethambutol, or isoniazid (INH) were loaded on the discs as desired. The agar plates were incubated at 37°C for 48 h and the diameters of the clearance zones, observed after incubation, were measured.

**Membrane permeability.** The membrane permeability was monitored through an NPN (1-N-phenyl-naphthylamine, HiMedia) assay (50). Briefly, M. smegmatis cells were treated with 16 mg/L of colistin, 256 mg/L of ceftazidime pentahydrate, and 1.6 mg/L of ethambutol for 4 h at 37°C and 200 rpm. The 0.5-OD₆₀₀ treated cells were pelleted out from each sample and resuspended in 1 mL sterile ice-cold 5 mM HEPES (HiMedia) buffer. A 100-μL volume of these cells was aliquoted in a sterile microcentrifuge tube and mixed with 100 μL of ice-cold 50 μM NPN prepared in HEPES buffer. These NPN-stained cells were immediately imaged in a laser scanning confocal microscope (LSM 780, Carl Zeiss) using a 63× apochromatic oil objective. The excitation wavelength used was 405 nm and the emission was detected using a photon multiplier tube detector in a range of 410 to 500 nm. Approximately 70 single cell images were further analyzed using ZenBlue and ImageJ softwares to determine the corrected total cell fluorescence.

**Toxicity.** Differentiated THP-1 cells were washed and acclimatized in fresh complete RPMI medium for 24 h. These cells were then treated with 32 to 128 mg/L of colistin, 128 to 1024 mg/L of ceftazidime pentahydrate, and 4 to 16 mg/L of ethambutol for 24 and 48 h. Post-treatment, the cells were trypsinized and cell counts were quantified using a Trypan Blue (0.4%) solution for manual cell counting in a hemocytometer.

**Macrophage infection.** Differentiated THP-1 cells were infected with M. smegmatis/M. tuberculosis H37Ra at an MOI of 10:1, where the bacterial culture was prepared to 0.5 OD₆₀₀ in complete RPMI medium after syringe treatment to break up bacterial clumps. The macrophages and mycobacteria were cocultured for 3 h at 37°C and 5% CO₂, after which the extracellular bacteria were removed and washed off thrice with phosphate-buffered saline supplemented with Ca-Mg. Complete RPMI medium containing 200 μg/mL amikacin was added to each well after the final wash, and the cells were incubated for 4 h at 37°C and 5% CO₂. This ensured the complete elimination of extracellular bacteria. The amikacin was then washed off. This is considered the 0-h time point of infection. Fresh complete RPMI medium was added to the treatment wells, while the macrophages in the representative wells were lysed using 0.05% SDS to determine the 0-h bacterial load. The infected cells were then treated with PNA (0.625 to 5 μM), colistin, ceftazidime pentahydrate, ethambutol, or selected combinations of a permeabilizing drug and PNA for 12 or 24 h (M. smegmatis infection) or for 5 days (M. tuberculosis H37Ra infection).

**Statistical Analysis.** Individual treatments with anti-inhA PNA or permeabilizing drugs were analyzed using the Student’s t test. Statistical significance for combinatorial treatment studies was determined through ANOVA followed by the Tukey-Kramer post hoc test.

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The authors have no conflicts of interest to declare.

**REFERENCES**

1. Lee HT, Kim SK, Yoon JW. 2019. Antisense peptide nucleic acids as a potential anti-infective agent. J Microbiol 57:423–430. https://doi.org/10.1007/s12275-019-8635-4.

2. Nielsen PE. 2001. Peptide nucleic acids as antibacterial agents via the antisense principle. Expert Opin Invest Drugs 10:331–341. https://doi.org/10.1517/13543784.10.2.331.

3. Quijano E, Bahal R, Ricciardi A, Saltzman WM, Glazer PM. 2017. Therapeutic peptide nucleic acids: principles, limitations, and opportunities. Yale J Biol Med 90:583–598.

4. Narenji H, Teymournejad O, Rezaee MA, Taghzadeh S, Mehramuz B, Aghazadeh M, Asgharzadeh M, Madhi M, Gholizadeh P, Ganbarov K, Yousefi M, Pakravan A, Dal T, Ahmadi R, Samadi Kafli H. 2020. Antisense
peptide nucleic acids against fhNZ and eflA4 genes inhibit growth and biofilm formation of Enterococcus faecalis. Microbiot Pathog 139:e103907. https://doi.org/10.1016/j.micpath.2019.103907.

5. Eriksson M, Nielsen PE, Good L. 2002. Cell permeabilization and uptake of antisense peptide nucleic acid (PNA) into Escherichia coli. J Biol Chem 277:7144–7147. https://doi.org/10.1074/jbc.M106624200.

6. Goltermann L, Yavari N, Zhang M, Ghosal A, Nielsen PE. 2019. PNA length restriction of antibacterial activity of peptide-PNA conjugates in Escherichia coli through effects of the inner membrane. Front Microbiol 10: 1032. https://doi.org/10.3389/fmicb.2019.01032.

7. Good L, Sandberg R, Larsson O, Nielsen PE, Wahlestedt C. 2000. Antisense PNA effects in Escherichia coli are limited by the outer membrane LPS layer. Microbiology (Reading) 146:2665–2670. https://doi.org/10.1099/00221287-146-10.2665.

8. Kurupati P, Tan KS, Kumarasinghe G, Poh CL. 2007. Inhibition of gene expression and growth by antisense peptide nucleic acids in a multiresistant beta-lactamase-producing Klebsiella pneumoniae strain. Antimicrob Agents Chemother 51:805–811. https://doi.org/10.1128/AAC.00709-06.

9. Maekawa K, Azuma M, Okuno Y, Tsukamoto T, Nishiguchi K, Setsukinai K, Maki H, Numata Y, Takemoto H, Rokusmusama K. 2015. Antisense peptide nucleic acid-peptide conjugates for functional analyses of genes in Pseudomonas aeruginosa. Bioorg Med Chem 23:7234–7239. https://doi.org/10.1016/j.bmc.2015.08.020.

10. Goh S, Boberek JM, Nakashima N, Stach J, Good L. 2009. Concurrent growth rate and transcript analyses reveal essential gene stringency in Escherichia coli. PLoS One 4:e6061. https://doi.org/10.1371/journal.pone.0006061.

11. Mondhe M, Chessher A, Goh S, Good L, Stach JE. 2014. Species-selective killing of bacteria by antimicrobial peptide-PNAs. PLoS One 9:e89092. https://doi.org/10.1371/journal.pone.0089092.

12. Nekhotaeva N, Svensson K, Nielsen PE, Good L. 2004. Inhibition of Staphylococcus aureus gene expression and growth using antisense peptide nucleic acids. Mol Ther 10:652–659. https://doi.org/10.1038/sj.1002476.

13. Rajasekaran P, Alexander JC, Seleem MN, Jain N, Srinanganathan N, Wattam AR, Setubal JC, Boyle SM. 2013. Peptide nucleic acids inhibit growth of Brucella suis in pure culture and in infected murine macrophages. Int J Antimicrob Agents 41:358. https://doi.org/10.1016/j.ijantimicag.2012.11.017.

14. Good L, Awasthi SK, Drysellus R, Larsson O, Nielsen PE. 2001. Bacterial essential antisense effects of peptide-PNA conjugates. Nat Biotechnol 19:360–364. https://doi.org/10.1038/68753.

15. Montazereshaeib H, Hejazi MS, Nozad Charoudeh H. 2018. Potential of pepRNA and pepPNA compounds. Polish J Microbiology 54:5–11.

16. Kulyte A, Nekhotiaeva N, Awasthi SK, Good L. 2005. Inhibition of gene expression and growth by antisense peptide nucleic acids in a multiresistant beta-lactamase-producing Klebsiella pneumoniae strain. Antimicrob Agents Chemother 51:805–811. https://doi.org/10.1128/AAC.00709-06.

17. Montazereshaeib H, Hejazi MS, Nozad Charoudeh H. 2018. Potential of pepRNA and pepPNA compounds. Polish J Microbiology 54:5–11.

18. Eriksson M, Nielsen PE, Good L. 2002. Cell permeabilization and uptake of antisense peptide nucleic acid (PNA) into Escherichia coli. J Biol Chem 277:7144–7147. https://doi.org/10.1074/jbc.M106624200.

19. Goltermann L, Yavari N, Zhang M, Ghosal A, Nielsen PE. 2019. PNA length restriction of antibacterial activity of peptide-PNA conjugates in Escherichia coli through effects of the inner membrane. Front Microbiol 10: 1032. https://doi.org/10.3389/fmicb.2019.01032.

20. Good L, Sandberg R, Larsson O, Nielsen PE, Wahlestedt C. 2000. Antisense PNA effects in Escherichia coli are limited by the outer membrane LPS layer. Microbiology (Reading) 146:2665–2670. https://doi.org/10.1099/00221287-146-10.2665.

21. Kurupati P, Tan KS, Kumarasinghe G, Poh CL. 2007. Inhibition of gene expression and growth by antisense peptide nucleic acids in a multiresistant beta-lactamase-producing Klebsiella pneumoniae strain. Antimicrob Agents Chemother 51:805–811. https://doi.org/10.1128/AAC.00709-06.

22. Rajasekaran P, Alexander JC, Seleem MN, Jain N, Srinanganathan N, Wattam AR, Setubal JC, Boyle SM. 2013. Peptide nucleic acids inhibit growth of Brucella suis in pure culture and in infected murine macrophages. Int J Antimicrob Agents 41:358–362. https://doi.org/10.1016/j.ijantimicag.2012.11.017.

23. Good L, Awasthi SK, Drysellus R, Larsson O, Nielsen PE. 2001. Bacterial essential antisense effects of peptide-PNA conjugates. Nat Biotechnol 19:360–364. https://doi.org/10.1038/68753.

24. Montazereshaeib H, Hejazi MS, Nozad Charoudeh H. 2018. Potential of pepRNA and pepPNA compounds. Polish J Microbiology 54:5–11.

25. Kulyte A, Nekhotiaeva N, Awasthi SK, Good L. 2005. Inhibition of gene expression and growth by antisense peptide nucleic acids in a multiresistant beta-lactamase-producing Klebsiella pneumoniae strain. Antimicrob Agents Chemother 51:805–811. https://doi.org/10.1128/AAC.00709-06.

26. Eriksson M, Nielsen PE, Good L. 2002. Cell permeabilization and uptake of antisense peptide nucleic acid (PNA) into Escherichia coli. J Biol Chem 277:7144–7147. https://doi.org/10.1074/jbc.M106624200.

27. Goltermann L, Yavari N, Zhang M, Ghosal A, Nielsen PE. 2019. PNA length restriction of antibacterial activity of peptide-PNA conjugates in Escherichia coli through effects of the inner membrane. Front Microbiol 10: 1032. https://doi.org/10.3389/fmicb.2019.01032.

28. Good L, Sandberg R, Larsson O, Nielsen PE, Wahlestedt C. 2000. Antisense PNA effects in Escherichia coli are limited by the outer membrane LPS layer. Microbiology (Reading) 146:2665–2670. https://doi.org/10.1099/00221287-146-10.2665.
44. Patenge N, Pappesch R, Krawack F, Walda C, Mraheil MA, Jacob A, Hain T, Kreikemeyer B. 2013. Inhibition of Growth and Gene Expression by PNA-peptide Conjugates in Streptococcus pyogenes. Mol Ther Nucleic Acids 2:e132. https://doi.org/10.1038/mtna.2013.62.
45. World Health Organization (WHO). 2010. Standard treatment regimens. In Treatment of tuberculosis: guidelines, 4th ed. World Health Organization, Geneva, Switzerland.
46. Zhu C, Liu Y, Hu L, Yang M, He ZG. 2018. Molecular mechanism of the synergistic activity of ethambutol and isoniazid against Mycobacterium tuberculosis. J Biol Chem 293:16741–16750. https://doi.org/10.1074/jbc.RA118.002693.
47. Yamada H, Yamaguchi M, Igarashi Y, Chikamatsu K, Aono A, Murase Y, Morishige Y, Takaki A, Chibana H, Mitarai S. 2018. Mycolicibacterium smegmatis, basonym Mycobacterium smegmatis, expresses morphological phenotypes much more similar to Escherichia coli than Mycobacterium tuberculosis in quantitative structome analysis and CryoTEM examination. Front Microbiol 9:1992. https://doi.org/10.3389/fmicb.2018.01992.
48. Nikravesh A, Dryselius R, Faridani OR, Goh S, Sadeghizadeh M, Behmanesh M, Ganyu A, Klok EJ, Zain R, Good L. 2007. Antisense PNA accumulates in Escherichia coli and mediates a long post-antibiotic effect. Mol Ther 15:1537–1542. https://doi.org/10.1038/sj.mt.6300209.
49. Seifert M, Catanzaro D, Catanzaro A, Rodwell TC. 2015. Genetic mutations associated with isoniazid resistance in Mycobacterium tuberculosis: a systematic review. PLoS One 10:e0119628. https://doi.org/10.1371/journal.pone.0119628.
50. Padwal P, Bandyopadhyaya R, Mehra S. 2015. Biocompatible citric acid-coated iron oxide nanoparticles to enhance the activity of first-line anti-TB drugs in Mycobacterium smegmatis. J Chem Technol Biotechnol 90:1773–1781. https://doi.org/10.1002/jctb.4766.