Tetrahedral DNA nanoparticle vector for intracellular delivery of targeted peptide nucleic acid antisense agents to restore antibiotic sensitivity in cefotaxime resistant *E. coli*.

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1. Abstract

The bacterial cell wall presents a barrier to the uptake of unmodified synthetic antisense oligonucleotides, such as peptide nucleic acids (PNAs), and so is one of the greatest obstacles to the development of their use as therapeutic anti-bacterial agents. Cell-penetrating peptides have been covalently attached to antisense agents, to facilitate penetration of the bacterial cell wall and deliver their cargo into the cytoplasm. Although they are an effective vector for antisense oligonucleotides, they are not specific for bacterial cells, and can exhibit growth inhibitory properties at higher doses. Using a bacterial cell growth assay in the presence of cefotaxime (CTX 16 mg/L), we have developed and evaluated a self-assembling non-toxic DNA tetrahedron nanoparticle vector incorporating a targeted anti-$bla_{\text{CTX-M-group 1}}$ antisense peptide nucleic acid (PNA4) in its structure for penetration of the bacterial cell wall. A dose-dependent CTX potentiating effect was observed when PNA4 (0 – 40 μM) was incorporated into the structure of a DNA tetrahedron vector. The minimum inhibitory concentration (to CTX) of an *E. coli* field isolate harbouring a plasmid carrying $bla_{\text{CTX-M-3}}$ was reduced from 35 mg/L to 16 mg/L in the presence of PNA4 carried by the DNA tetrahedron vector (40 μM), contrasting with no reduction in MIC in the presence of PNA4 alone. No growth inhibitory effects of the DNA tetrahedron vector alone were observed.

2. Introduction

Antisense oligonucleotides have been successfully utilised to inhibit the expression of a wide range of bacterial genes for research uses and as potential anti-microbial therapeutic strategies. They have been shown to be specific to their mRNA targets, and translational inhibition has been demonstrated [1]. The bacterial cell wall complex presents a major obstacle to delivery of antisense oligonucleotides into field and clinical isolates [2,3] which necessitates development of more efficient delivery strategies. Currently, a commonly used and effective delivery method is conjugation of a synthetic antisense molecule to a cell-penetrating peptide [4]. However, the use of a cell-penetrating peptide has certain significant problems such as inherent toxicity to both bacterial and mammalian cells. Additionally, the large-scale synthesis of the peptide conjugated antisense oligonucleotides is presently an expensive and time consuming procedure. Resistance to cell-penetrating peptides has also been observed, for example linked to mutations in a gene coding for the active transporter protein SbmA [5]. Most cell-penetrating peptides have also been shown to lack cellular tropism with no specificity to any cell type having been shown [6], further hindering their potential therapeutic application. Previous studies have found a synergistic growth inhibitory effect of certain antibiotics with cell-penetrating peptides [7] and an increase in bacterial cell wall permeability [4]. These studies may suggest cell wall disruption is a likely mechanism of cell penetration and would be a disadvantageous characteristic for the development of tissue/cell target-specific therapeutic agents.

The current study developed a potential alternative delivery vehicle for synthetic antisense oligonucleotides; a novel delivery mechanism was based on self-assembling 3D DNA tetrahedral structures. DNA is inherently non-toxic to cells [8], making it a highly suitable therapeutic delivery vector.

Setyawati *et al.* reported that a tetrahedral DNA structure was able to traverse the bacterial Gram-negative cell wall and enter the cytoplasm with no toxic effects of the structure alone [9]. Their DNA tetrahedron vector was designed to be self-assembling based on regions of complementarity between single-stranded DNA [10]. This DNA tetrahedron consisted of four 55-base ssDNA strands, each strand incorporating unique regions of complementarity with other strands resulting in, by Watson-Crick base pairing, the folding of the strands to form a stable 3-dimensional equilateral tetrahedron.

Adapting this approach to the design and construction of 3D structures based on ssDNA, the present study developed a DNA tetrahedron vector incorporating a peptide nucleic acid (PNA) into its structural design. This anti-*bla*$_{\text{CTX-M-group 1}}$ PNA (PNA4) has been previously shown to translationally inhibit the expression of β-lactamase CTX-M-group 1 in field and clinical *E. coli* isolates and partially restore cefotaxime sensitivity in strains with a reduced susceptibility phenotype [11].

3. Materials and Methods

3.1. Strains

E. coli strain LREC461 of human origin was obtained from the Public Health laboratory, UK and had reduced susceptibility to CTX which could be partially restored by PNA4 conjugated to a cell penetrating peptide [11].

3.2. PNA4 Antisense Oligomer

The 13-mer anti-$bla_{\text{CTX-M-15}}$ PNA4 was synthesized commercially by Cambridge Research Biochemicals (Cleveland, UK) with the base sequence of: ttccttattctgg (5' - 3').

3.3. Design of a 108 bp tetrahedron nanoparticle to carry a single 13-mer PNA (PNA4)

A DNA/PNA tetrahedron, designated PT01 (PNA4-carrying tetrahedron 01), was designed to self-assemble from three 54 nt single-stranded DNA (ssDNA) oligonucleotides (PT01-S1 - 3), one 41 nt ssDNA (PT01-S4) oligonucleotide and one 13-mer PNA (Figure 1a). Sequences of structural PT01 component ssDNA strands are as follows (5' – 3'):

PT01-S1: cgcgacttaggtccataatcaaggggccggtgagatgggagtgaacgggtctgg;

PT01-S2: tagcgttaggacaacggaatctcaccggccccttgatacgtgcgggtctgataa;

PT01-S3: ttatggacctaagtcgcgagtccagaataaggaactttatcagacccgcacgta;

PT01-S4: actccagacccgttcactccctccgttgtcctaacgctaag.

Tetrahedron component ssDNA strands were checked for unwanted hetero and homo dimerisation with Multiple Primer Analyzer [12]. Figure 1b is an illustrative representation of a DNA tetrahedron carrying a PNA after self-assembly.

3.3.1. Assembly of DNA tetrahedral PNA4 delivery vehicle

Using an adapted method described by Pei *et al*.[10], equimolar concentrations of ssDNA structural oligonucleotides and PNA4 were mixed in an annealing buffer (20 mM Tris, 50 mM $MgCl_2$, pH 8.0). The mixture was heated to 95° C for 4 minutes and cooled rapidly on ice for 30 minutes. Assembly of the tetrahedron was verified by agarose gel electrophoresis.

3.3.2. Verification of assembly of DNA tetrahedral PT01 delivery vehicle by agarose gel electrophoresis

Assembly of the tetrahedron was verified on a 2% w/v TBE agarose gel, and 4-5 V/ cm applied until sufficient separation was observed. Individual DNA strands, and combinations of strands e.g. PT01-S1 + PT01-S2, PT01-S1 + PT01-S2 + PT01-S3, and PT01-S1 + PT01-S2 + PT01-S3 + PT01-S4 + PNA4, were visualised by agarose gel electrophoresis (Figure 2), and single discreet bands of increasing size were regarded as evidence of successful annealing and complete tetrahedron formation. A 100 bp DNA ladder (Promega) with markers at 100 bp intervals between 100 and 1000 bp was used to estimate sizes of combinations of tetrahedron component strands. A fully assembled tetrahedron was expected to yield a discreet band of around 108 bp.

3.4. Cell growth assay for assessing the activity of anti-*bla*$_{\text{CTX-M-15}}$ antisense PNA oligonucleotides

A previously described *E. coli* cell growth assay in the presence of CTX was used for the evaluation of anti-*bla*$_{\text{CTX-M-15}}$ activity of the PNA delivery DNA tetrahedron vector [11]. Briefly, bacterial cultures were typically grown from glycerol stocks at 37^{o} C in a shaking incubator in MHB media containing CTX (2 mg/L) until early log phase (0.1 – 0.2 O.D. $_{600\ nm}$) was achieved. The culture was diluted to achieve a final cell density of approximately 100,000 CFU/ml, previously established empirically to be optimal for assessing the effect of antisense oligonucleotides, and for consistency across experiments. Diluted cell suspension (50 µl) was incubated with MHB (50 µl) growth medium supplemented with antibiotics and inhibitory agents where required, and transferred to a 96 well microtiter plate (Falcon). Bacterial cell growth was measured in a BMG Labtech FluoStar automated spectrophotometer (BMG LABTECH GmbH, Ortenberg, Germany) and optical density readings (600 nm) taken over 18-24 hours. The O.D. $_{600\ nm}$ readings were taken over 250

cycles, with 15 flashes per well per cycle approximately every 5 minutes. All replicates were independent cultures. Growth curves and statistical analyses were produced using Prism® 6 software (GraphPad).

3.5. Establishment of minimum inhibitory concentration (MIC) values for *E. coli* strains

E. coli strains were cultured in 96-well microtiter plates and incubated at 37°C. Growth was monitored at O.D. $_{600\ nm}$ for 18 – 24 hours and MIC values were reported as the minimum concentration of an agent, or combination of agents, required to inhibit growth (O.D. $_{600\ nm}$ < 0.1) in a minimum of 50% of replicates after 18 hours incubation at 37° C.

3.6. Experimental controls used for the evaluation of inhibition of β-lactamase activity by unmodified and peptide-conjugated PNA4

Specificity of unmodified and peptide-conjugated PNA4 was demonstrated in previous studies by Readman *et al*. [11]: briefly, anti-$bla_{\text{CTX-M-group 1}}$ specific activity was initially demonstrated in a cell-free translation/transcription coupled system followed by cell growth studies with a CTX resistant strain with a compromised cell wall. In field isolates, peptide-conjugated PNA4 control studies were undertaken with a cell-growth assay with an *E. coli* strain harbouring $bla_{\text{CTX-M-14}}$, with which PNA4 shared 15% (2 out of 13 bases) sequence complementarity. In the current study, control experiments were undertaken to demonstrate the lack of effect on growth of LREC461 to PT01 in the absence of CTX. Any effects of the tetrahedral structure itself were determined by cell-growth assays in the presence of a DNA control tetrahedron assembled from PT01 component ssDNA strands and a 13nt ssDNA oligonucleotide of the same sequence as PNA4.

4. Results

4.1. Design and assembly of a PNA-carrying tetrahedral DNA nanoparticle

The sequence of ssDNA forming the structural frame of the 3D tetrahedral vector consisted of terminal areas on each strand which were complementary with regions on the other structural strands. These regions of complementarity facilitated the formation of the 3D double-stranded DNA tetrahedron structure. After self-assembly, this was designed to form a tetrahedron with a total size of 108 bp, each edge being 18 bp in length, and PNA4 carried by a region of complementarity with PT01-S3 (**Error! Reference source not found.**1a). Assembly of the DNA tetrahedron PT01 was evaluated by visualisation of migration and gel-shift patterns of the component oligonucleotides, and intermediate and final products of the tetrahedron annealing process. As shown in Figure 2 the strands annealed to form progressively larger DNA complexes with slower electrophoretic migration patterns and; the final annealed product comprising of all components necessary for tetrahedron formation yielded a single discreet band, in a position on the gel consistent with a larger DNA molecule than the individual component parts.

4.2. Anti-*bla*$_{\text{CTX-M-3}}$ activity of PT01

E. coli field isolate LREC461 was incubated in the presence of a combination of CTX (16 mg/L - previously shown to be a suitable concentration for observation of CTX potentiation by PNA4) and PT01 (0 – 30 μM), and a significant synergistic (fractional inhibitory concentration index < 0.5) dose dependant effect was observed (Figure 3A), evidenced by the increasing time taken for the culture to achieve logarithmic growth. The MIC (to CTX) was reduced in the presence of PT01 (40 μM) from 35 mg/L to 16 mg/L (see Supplementary Fig. 1). Possible inherent toxicity of PT01 was evaluated by culturing LREC461 in the absence of CTX and in the presence of PT01 (10 – 30 μM), and no significant effects were observed (Figure 3B). A control DNA tetrahedron was assembled

substituting a 13 nt ssDNA oligonucleotide sharing 100% sequence identity with PNA4 for PNA4, and assembled using the components PT01-S1-4. No significant effects on growth were observed when field isolate LREC461 was cultured in the presence of the DNA control tetrahedron alone (10 – 30 μM; Figure 3C). Small but significant ($P < 0.05$) negative effects on growth were observed when field isolate LREC461 was cultured in the presence of the DNA control tetrahedron (10 μM) and CTX (16 mg/L). No significant effect was observed with PNA4 without the DNA tetrahedron (Figure 3D).

5. Discussion

This study demonstrated the application of a DNA tetrahedron vector to deliver a 13-mer PNA antisense oligonucleotide within a bacterial cell using a self-assembling complementary base pairing assembly approach. The delivery of the active synthetic antisense oligomer component of the tetrahedron relied upon post-cell penetration dissociation of the PNA from the DNA structure, or the DNA components of the structure being degraded either wholly or partially by endonucleases upon entering the bacterial cytoplasm, releasing the synthetic antisense agent from the DNA tetrahedron [9]. Setyawati *et al*. found that DNase I was sufficient to degrade a similar dsDNA structure in *Staphylococcus aureus* [9], whilst Li *et al*. found that such a structure was relatively stable in bovine serum albumin where DNase I concentrations were typically lower than intracellular concentrations [8]. The specific activity of PNA4 against $bla_{\text{CTX-M-15}}$ had been previously demonstrated by Readman *et al.* [11], this included the quantification of β-lactamase activity by an HPLC assay measuring the degradation of CTX in the presence and absence of PNA4 in a cell-free translation/transcription coupled system. Also the cell-wall compromised mutant strain AS19 was transformed with a $bla_{\text{CTX-M-15}}$ producing plasmid and cultured with CTX and the presence and absence of PNA4 and growth measured by spectrophotometry over 18 – 24 hours. As $bla_{\text{CTX-M-15}}$ is a non-essential gene, the use of PNA4 in the absence of CTX provided a suitable control for the isolation of the effects of PNA4 on growth. Finally a field isolate harbouring $bla_{\text{CTX-M-14}}$ to which PNA4 was not complementary was cultured with CTX in the presence and absence of PNA4 conjugated to a cell-penetrating peptide. This was the equivalent of a scrambled PNA control [11].

Assembly of the tetrahedral structures were verified by native agarose gel electrophoresis with a series of stepwise incremental DNA structural components. The number of nucleotides will not be related to the calibration standards because of the three dimensional nature of the

partially and fully assembled tetrahedron, however, progressively slower migrating discreet bands represent evidence of successful assembly.

A control DNA tetrahedron was constructed, substituting ssDNA, of the same sequence, for the antisense oligonucleotide. There were no observable effects in field isolates incubated in the presence of a DNA control tetrahedron in the absence of CTX. In the presence of CTX and DNA control tetrahedron combination, a small increase in CTX sensitivity was observed, potentially attributable to opportunistic co-translocation of CTX with the control DNA tetrahedron. Control data (figure 3C) for the tetrahedron demonstrated no inherent antimicrobial properties of the structure. In contrast, CPPs do have well documented growth inhibitory and toxic effects in bacteria and mammalian cell cultures. CPPs may enable uptake by membrane perturbation and thereby have toxic effects through a loss of structural integrity and opportunistic co-translocation of extra-cellular material; CPPs have been shown to have a synergistic effect with certain antibiotics [4,7]. The observed lack of inherent toxicity or a CTX potentiating effect of the control tetrahedral structure may indicate that membrane perturbation was not the primary mechanism of cell penetration and may thereby implicate an alternative uptake pathway. Bacterial natural competence is well known to exist across a range of species, although not well characterised in *E. coli*. Bacteria commonly express DNA receptor sites at the cell surface, and Gram-negative bacteria such as *Haemophilus parainfluenzae* and *Neisseria gonorrhoeae* have been shown to recognise sequence specific DNA fragments [13-15]. Mechanisms of DNA uptake in *E. coli* require further study, and it may be plausible to suggest that the shape and size of three dimensional DNA structures may enable cell penetration via an alternate hitherto unidentified pathway.

The observed re-sensitisation of field isolates to previously sub-lethal concentrations of CTX suggest that a DNA tetrahedron is able to penetrate a bacterial cell wall and deliver its cargo, which is able to specifically bind to its target and inhibit CTX-M protein expression. No

significant inhibitory effects were observed when field isolate LREC461 was treated with PNA-carrying tetrahedron PT01 in the absence of CTX. Further studies are required to optimise tetrahedron size and isolate the mechanism of penetration.

The CTX-potentiating effects of PT01 were smaller than the previously reported equivalent peptide-conjugated antisense oligonucleotides [11] which yielded a reduction in MIC (to CTX) in strain LREC461 from 35 mg/L to 8 mg/L in the presence of PNA4 conjugated to a cell-penetrating peptide with an amino acid sequence of $(KFF)_3K$ (3.2 µM), potentially suggesting a comparatively lower efficient penetration efficiency of the tetrahedron. However, the cell-penetrating peptide portion of the CPP-antisense oligonucleotide conjugate ($(KFF)_3K$) has been previously shown to have synergy with certain antibiotics [4,7], as well as some potential inherent toxicity, contributing to the overall growth inhibitory effect. Further studies are required to determine the range of bacterial strains susceptible to antisense oligonucleotide mediated protein expression inhibition carried by DNA tetrahedrons. Studies to investigate the discriminatory potential of tetrahedral delivery vehicles would also be potentially advantageous – cell-penetrating peptides commonly lack specificity for any cell types and their inherent toxicity to bacterial and mammalian cell cultures is well documented. A similar tetrahedral delivery vehicle was constructed and found to have no inhibitory effects against a mammalian cell culture, however, Walsh *et al.* reported substantial uptake of a DNA tetrahedral structure in mammalian cells [16]. Further investigations of the mechanism of uptake, and relative uptake efficiency would be required to elucidate this important factor. It is likely that shape, size and nucleotide sequence of the structure will impact uptake efficacy in different cell types; a delivery vector that was specific to bacterial cells and did not have inherent negative effects on cell growth would be desirable characteristics for a targeted vector carrying antimicrobial agents.

With a demonstrated ability to both penetrate a bacterial cell wall and deliver an active targeted synthetic antisense oligonucleotide, this approach has a large range of optimisation options, and a variety of potential applications. The low cost, simplicity and speed of assembly would appear to make this a potentially viable alternative antisense oligonucleotide delivery vehicle.

6. Author Disclosure Statement

Research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest; therefore no competing financial interests exist.

7. References

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