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14. ABSTRACT
As summarized in ARO Topic A09A-T004, resistance to antibiotics in current use continues to spread, while the pipeline of approved new antibiotics is shrinking. Antibiotic therapy today is a crude and increasingly ineffective weapon against infection. Current broad spectrum antibiotics notoriously stimulate the spread of resistance, and also open the way to secondary infections by MRSA and other pathogens. We offer here a solution that avoids stimulating resistance as well as collateral damage to the natural biome: a target selective antimicrobial therapy. As

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Report Title

Antimicrobial peptide-PNA conjugates selectively targeting bacterial genes

ABSTRACT

As summarized in ARO Topic A09A-T004, resistance to antibiotics in current use continues to spread, while the pipeline of approved new antibiotics is shrinking. Antibiotic therapy today is a crude and increasingly ineffective weapon against infection. Current broad spectrum antibiotics notoriously stimulate the spread of resistance, and also open the way to secondary infections by MRSA and other pathogens. We offer here a solution that avoids stimulating resistance as well as collateral damage to the natural biome: a target selective antimicrobial therapy. As a proof-of-concept project, this proposal seeks to explore the possibility that non-covalently linked combinations of peptide mimetics and peptide nucleic acids (PNA's) provide a basis for a strain-selective antibacterial therapy. Initial publications suggest that conjugates of cell penetrating peptides and PNA's can overcome the barrier in transporting the PNA's into cells. The strategy is to use (RW)4D and (RW)3, both designed in our lab, to guide a PNA sequence (conjugated or not) to the appropriate target RNA and thereby silence its expression. In initial testing, we used PNA sequences designed to inhibit growth of E. coli and MRSA cells, already reported in literature previously, but using our RW motif. Our preliminary results with this experiment are positive!

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NAME
Kallenbach, Neville R.
Total Number: 1

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Antimicrobial peptide-PNA conjugates selectively targeting bacterial genes

Abstract

As summarized in ARO Topic A09A-T004, resistance to antibiotics in current use continues to spread, while the pipeline of approved new antibiotics is shrinking. Multidrug resistance (MDR) among pathogens is a persistent problem, for civilians as well as military personnel wounded in the field, in hospitals where wounded personnel associate with other patients, and in community venues. Antibiotic therapy today is a crude and increasingly ineffective weapon against infection. Current broad spectrum antibiotics also open the way to secondary infections by MRSA and other pathogens that threaten patient life and health, including C. difficile. Thus, antibacterial strategies that provide timely and effective therapeutic countermeasures are urgently required for possible outbreaks of MRSA or other MDR infections.

As a proof-of-concept project, this proposal seeks to explore the possibility that non-covalently linked combinations of peptide mimetics and peptide nucleic acids (PNA's) provide a basis for a strain-selective antibacterial therapy. Initial publications suggest that conjugates of cell penetrating peptides and PNA's can overcome the barrier in transporting the PNA's into cells. The strategy is to use (RW)4D, a peptidodendrimer from our lab and (RW)3, a linear peptide, to guide a PNA sequence (conjugated or not) to the appropriate target RNA and thereby silence its expression. In initial testing, we used PNA sequences designed to inhibit growth of E. coli and MRSA cells, already reported by Nielsen's group and Luo's group(Bai, 2012), respectively, but using our RW motif. Our preliminary results with this experiment are positive!

We apologize for lateness of this report, but our laboratory has just been moved from 1166 Waverly to smaller space in 866 Waverly, with facilities up to now.

1. Tasks

1.1. Synthesize antisense PNA sequences, RW peptides and conjugates

1.2. Microdilution assay of MRSA and E. coli cell growth in the presence of single PNAs, peptide, and PNA-peptide conjugates

1.3. Synergistic effect of PNA and RW-peptide mixture against bacterial growth

2. Results and Analysis

2.1. Synthesize antisense PNA and PNA-peptide conjugate

Antisense antibacterials are short (about 10 bases) synthetic DNA analogs that inhibit essential gene expression at the mRNA level in a sequence-specific manner (Rasmussen, 2007). The neutral and flexible PNA backbone offers important advantages in hybridization with a complementary nucleic acid: the Tm of complexes is higher than DNA-DNA for example, and less sensitive to ionic strength. Antisense inhibition by a PNA depends on the function of the targeted gene. Antisense inhibition of β-galactosidase shut down β-galactosidase synthesis with a well-calibrated colorimetric assay in E. coli based on the X-gal blue-white color change, (Good, 2000). In the case of MRSA, RNA polymerase σ□□ (encoded by gene rpoD) is a conserved prokaryotic factor essential for transcription initiation in exponentially growing MRSA, implying strong potential to achieve antisense inhibition (Paget, 2003).

Antisense oligomers, especially peptide nucleic acid (PNA) (Nielsen, 2010) possess favorable properties in light of antisense antibacterial application, including improved targeting specificity, binding affinity, biological stability and access to a variety of chemical modification. A major challenge for the application of PNA gene silencing technology to bacteria is the inefficient entry of PNAs into the targeted cell due to restrictions imposed by the bacterial membrane. Peptide (RXR)4XB and (KFF)3K, were previously reported as a potent permeabilizer against E. coli and MRSA cells (Mellbye, 2009). (RW)4D, a small dendrimeric AMP and (RW)3, a linear hexameric peptide, both designed in our lab, interact with wall polymers and cause penetration of the cell membrane at sub-lethal concentration (Liu, 2007).

Scheme 1. Synthesis of PNA-dendrimer conjugate. (a) (RW)4D-cysteine (b)Free PNA (C) PNA-(RW)4D conjugates.

MRSA RNA polymerase-rpoD-targeting antisense PNA (PNA 1) and E.coli β-galactosidase-lacZ-targeting antisense PNA (PNA 4) were synthesized by standard solid phase synthesis (SPPS) (Nielsen, 2010; Totsingan et al., 2010) (Table 1). Anti-rpoD PNA-(KFF)3K conjugates (PNA2), which showed bacteriocidal antisense effect against MRSA in previous study (Bai et al., 2012), was included as control. Anti-rpoD PNA-(RW)4D (PNA3) and Anti- lacZ PNA-(KFF)3K (PNA5) conjugates were

successfully synthesized by modifying the protocol reported in the literature (Good et al., 2000) (Nielsen, 2010) to accept self-penetrating peptides (Liu et al., 2007). All five PNAs were purified on RP-HPLC and confirmed with expected M.W. (Scheme 1, Figure 1, Table 1)

Fig. 1. ESI-MS of PNA1, PNA2 and PNA3. a) PNA1, H-TTTCTCGTCA-NH2, b) PNA2, Anti-rpoD PNA-(KFF)3K conjugates, c) PNA3, Anti-rpoD PNA-(RW)4D

Table 1: PNA sequences and the corresponding ESI-MS characterization

Designation
PNA sequence
#Bases
Strain ESI-MS data
(MW): Expected/found

PNA1
H-TTTCTCGTCA-NH2
10
MRSA (2668.6): 1335.3/1335.3 (MH22+); 890.5/890.7 (MH33+); 668.2/668.4 (MH44+);534.7/535.0 (MH55+)

PNA2
H-(KFF)3K-(β-ala)-TTTCTCGTCA-NH2
10
MRSA (4135.4): 1034.9/1034.9 (MH44+); 828.1/828.5 (MH55+); 690.2/690.5 (MH66+);591.8/592.2 (MH77+)

PNA3
(RW)4D-TTCTCGTCA-NH2
10
MRSA (4493.8): 1124.5/1124.8 (MH44+); 899.8/900.1 (MH55+); 750.0/750.3 (MH66+);643.0/643.3 (MH77+)

PNA4
H-TAGCTGTTTC-K-NH2
10
E coli K12 (2836.8): 946.6/946.3 (MH33+); 710.2/710.0 (MH44+); 568.4/568.2 (MH55+)

PNA5 H-(KFF)3K-(□-Ahx)- TAGCTGTTTC-K-NH2
10
E coli K12 (4345.7): 870.1/870.2 (MH55+); 725.3/725.4 (MH66+); 621.8/621.9 (MH77+); 544.2/544.4 (MH88+)

2.2. Growth assays and susceptibility testing in the presence of constructs

The antibacterial activity of free PNAs, PNA-peptide conjugates and PNA/peptide mixtures were tested by following standard broth microdilution protocols recommended by the National Committee for Clinical Laboratory Standard (NCCLS). The growth inhibitory effect of PNA-Peptide conjugates were evaluated against MRSA and E coli K12 (Table 2). As expected, poor cellular uptake was confirmed with free PNAs (PNA1 and PNA4), which showed no growth inhibition effect at the highest concentrations tested (40 μM). Anti-rpoD PNA-(RW)4D (PNA3) and anti-rpoD PNA-(KFF)3K (PNA2) are potent and exhibit the same MIC values (15 μM) against MRSA. In the case of E.coli K12 strain. Despite the fact that the chosen sequence occurs in several copies in the chromosome and is not unique to the lac promoter, the PNA alone has no detectable activity below 40 μM. The sequence conjugated to (KFF)3K does, despite its selection to hybridize with lac mRNA.

Table 2: Summary of PNAs activity against MRSA and E coli K12.

Drug	Strains	MIC, μM
(RW)4D	MRSA	22±3.5
(KFF)3K	MRSA	>40
PNA1	MRSA	>40
PNA2	MRSA	15±2.3
PNA3	MRSA	15±1.9
PNA4	E coli K12	>40
PNA5	E coli K12	10 ±1.6

2.3. Synergistic effect of PNA and peptide mixture against bacterial growth
Relative to antisense PNA alone, mixture of various PNAs with RW-peptide showed 3-4 time increased growth inhibitory effects against MRSA and E. coli. and are even more potent than the corresponding RW-peptide (Figure 2 and Figure 3). The results demonstrate that RW-peptide and PNAs may act in a synergistic way.

Fig. 2. Antimicrobial activity against MRSA of Anti-rpoD PNA (PNA1), Anti-rpoD PNA-(RW)4D (PNA3) and PNA1/(RW)4D mixtures. Preliminary results showing active synergy between membrane penetrating (RW)4D peptide and decameric anti-rpoD PNA sequences in MRSA.

Fig. 3. Antimicrobial activity against E coli K12 of Anti-lacZ PNA (PNA4) and PNA4/(RW)3 mixtures. Preliminary results showing active synergy between membrane penetrating RW peptide and a decameric PNA sequence present in multiple copies in E. coli K12.

The linear antimicrobial peptide (RW)3 is active, albeit only moderately so. An equimolar mix of (RW)3 and the PNA shows about two fold increase in activity. This implies that covalent conjugation may not ensure the best balance between the components. Based on the results, we believe we need to further optimize the ratios of the peptide and PNA to compare these to the conjugated chains. It is not clear that 1:1 ratios are close to the optimum.

3. Conclusions
The results so far confirm our hypothesis that conjugation of a cationic antimicrobial agent to a PNA might not offer the best approach for designing a target selective anti-infective. Instead we will monitor various ratios of the PNA vs AMP in order to find the optimum penetration/inactivation ratio.

3.1. (RW)4D and (RW)3 peptides conjugated to 10-mer PNAs (Anti-rpoD PNA and anti-LacZ PNA) have been successfully synthesized with expected M.W.
3.2. PNA conjugates show micromolar-range growth inhibitory effects against MRSA and E. coli in a concentration-dependent manner. PNA/peptide mixtures show increased growth inhibitory effects against MRSA and E. coli.
3.3. Combination of two agents, one that breaches the wall and membrane, the other that targets gene expression in a pathogen, offers an approach to a narrow spectrum anti-infective therapy that merits detailed investigation.

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Technology Transfer