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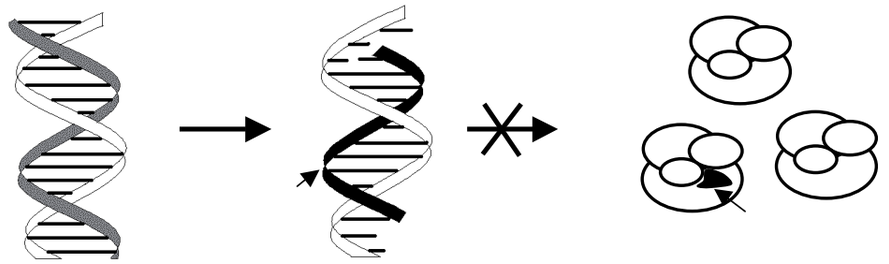

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TARGETING NUCLEIC ACIDS IN BACTERIA WITH SYNTHETIC LIGANDS

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In the Name of God, the Beneficent, the Merciful

I would like to dedicate this thesis to my dear late parents ***Fatemeh*** *and* ***Hassan***

Questions are the cure for human ignorance.
Let us ask what we do not know, and solve our
entire unknown (***Mohammad Taghi Jafari).***

ABSTRACT

There is a need for new antibacterial agents, and one attractive strategy is to develop nucleic acid ligands that inhibit pathogen genes selectively. Also, such ligands can be used as molecular biology probes to study gene function and nucleic acid structures. In this thesis, bacterial genes were selectively inhibited with antisense peptide nucleic acid (PNA) and the higher order structures formed by $(GAA)_n$ repeats were probed with the intercalator benzoquinoquinoxaline (BQQ).

A majority of bacterial genes belong to tight clusters and operons, and regulation within cotranscribed genes has been difficult to study. We examined the effects of antisense silencing of individual ORFs within a natural and synthetic operon in *Escherichia coli*. The results indicate that expression can be discoordinated within a synthetic operon but only partially discoordinated within a natural operon.

Bacteria use natural antisense mechanisms to regulate gene expression and an established model for sense/antisense RNA pairing is the *hok*/*sok* toxin–antitoxin (TA) locus. We aimed to test this model in cells and also the idea that sequestration of Sok-RNA could provide a novel antimicrobial strategy using antisense agents. The results support the *hok*/*sok* sense/antisense interaction model and the idea that PNA can out-compete this interaction and provide potent killing activity.

Certain antisense agents are effective in bacteria, yet it is unclear how these relatively large molecules overcome stringent bacterial barriers. Here we determined the transit kinetics of peptide-PNAs and observed an accumulation of cell-associated PNA in *E. coli* and slow efflux. Consistent with cell accumulation and retention, the post-antibiotic effect (PAE) of a PNA that targets the growth essential fatty acid biosynthesis gene *acpP* was greater than seven hours.

At the DNA level, polypyrimidine/polypurine rich sequences have the potential to form intramolecular triple helix structures (H-DNA). Triplet $(GAA)_n$ repeats are pathological in Friedreich's ataxia (FA) disease, and may form H-DNA. Here we probed for triplex structures in $(GAA)_n$ sequences using the triple-helix specific stabilizing compound BQQ. The results showed that *E. coli* plasmids carrying a $(GAA)_n$ repeat sequence form H-DNA, suggesting that these structures play a role in the pathology of FA.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Rikard Dryselius, **Abbas Nikravesh**, Agne Kulyte, Shan Goh and Liam Good
Variable coordination of cotranscribed genes in *Escherichia coli* following antisense repression
BMC Microbiology.2006;6(97)

II. Omid R. Faridani, **Abbas Nikravesh**, Deo Prakash Pandey, Kenn Gerdes, and Liam Good
Competitive inhibition of natural antisense Sok-RNA interactions activates Hok-mediated cell killing in *Escherichia coli*
Nucleic Acids Res. 2006;34(20):5915-22

III. **Abbas Nikravesh**, Rikard Dryselius, Omid R Faridani, Shan Goh, Majid Sadeghizadeh, Mehrdad Behmanesh, Anita Ganyu, Erik Jan Klok, Rula Zain and Liam Good
Antisense PNA Accumulates in *Escherichia coli* and Mediates a Long Post-antibiotic Effect
Mol Ther. 2007 Aug;15(8):1537-42

IV. **Abbas Nikravesh**, Helen Bergquist, Raquel Domingo Fernandez, Chi-Hung Nguyen, Liam Good & Rula Zain
Friedreich's ataxia $(GAA)_n$ triplex DNA structures are stabilized by benzoquinoquinoxaline derivatives
Submitted, 2007

CONTENTS

LIST OF ABBREVIATIONS

ACP	acyl carrier protein
AO	antisense oligonucleotide
CFU	colony forming unit
dsRNA	double-stranded RNA
EMSA	electrophoretic mobility shift assay
FA	Friedreich's ataxia
hok	host killing
LNA	locked nucleic acid
MIC	minimal inhibitory concentration
miRNA	microRNA
MP	methylphosphonate
mRNA	messenger RNA
MRSA	methicillin-resistant *Staphylococcus aureus*
ncRNA	non-coding RNA
ON	oligonucleotide
PBP	penicillin binding protein
PABA	para-aminobenzoic acid
PCR	polymerase chain reaction
PMO	phosphorodiamidate morpholino oligomers
PNA	peptide nucleic acid
PS	phosphorothioate
RISC	RNA induced silencing complex
RNAi	RNA interference
rRNA	ribosomal RNA
RT-PCR	real-time reverse transcriptase-PCR
SD	Shine-Dalgarno
snRNA	small nuclear RNA
sok	suppression of killing
TFO	triplex forming oligonucleotide
tRNA	transfer RNA
UTR	untranslated region

1 INTRODUCTION

1.1 BACTERIA AND HEALTH

Bacteria are involved in many aspects of ecology and health. It seems likely that all species both benefit and suffer from interactions with bacteria. For example, we use bacteria in making yogurt, cheese and other fermented foods. Also, large numbers of bacteria live on the skin and in the digestive tract. The human gut contains more than 1000 bacterial species, which are generally beneficial [1]. Gut bacteria synthesize vitamins such as folic acid, vitamin K and biotin, and they ferment complex indigestible carbohydrates. Other useful bacteria in gut flora include *Lactobacillius* species, which convert milk sugar to lactic acid. Also, bacteria play very important roles in medicine as vaccine components and in the production of drugs, hormones and antibodies. On the other hand, pathogenic bacteria cause enormous levels of spoilage, suffering and death through infection.

1.1.1 Bacterial Infection

The best known examples of harmful bacteria are those that cause infectious diseases. The most common bacterial disease is tuberculosis, caused by the bacterium *Mycobacterium tuberculosis*, a leading cause of death worldwide [2]. Each pathogenic species participates in a characteristic spectrum of interactions with its host. Some organisms such as *Staphylococcus* or *Streptococcus* species cause skin infection, and other forms of surface infection [3]. On the other hand, many of these organisms are also part of the normal flora and usually exist on the skin or in the nose without causing any disease at all. Obligate intracellular parasites, such as the *Rickettsia* and *Chlamydia*, are and able to grow and reproduce only within the host cells [4]. Finally, some species, such as *Pseudomonas aeruginosa*, are opportunistic pathogens and cause disease in individuals suffering from immune-suppression or cystic fibrosis [5, 6]. Suspected infections may be involved in a variety of other diseases including heart disease and multiple sclerosis [4].

1.1.2 Treatment of bacterial infection

Antibacterial agents are used to treat bacterial infections and they have other important applications in hygiene and industry. Antibacterials include the well known antibiotics. Also, there are antibacterial essential oils, which are hydrophobic liquids containing volatile aroma compounds from plants. Other types of antibacterials include cationic

elements, such as ionic mercury, copper and silver, which are widely used in medicine and industry. Antibacterial agents can either be natural products or synthetic chemicals and they typically block a crucial molecular structure or function in bacteria.

The largest scale application of antibacterial agents outside of human medicine is in the animal industries. It has been estimated that millions of pounds of antibacterials are used each year in animal agriculture [7]. Through mechanisms that are not well understood, sub-therapeutic antibiotic doses promote the growth efficiency of animals and they have been used to improve production for about 50 years in many countries. The interactions between antibiotics and animal gut microbia are believed to be key in promoting growth rates [8]. Despite the benefits, there are obvious concerns about the use of antibacterial agents in animal fodder with respect to the spread of antibacterial resistance.

1.1.2.1 Antibacterial mechanism of action

A better understanding on the sequence of events leading to cell death from the wide range of antibacterials is needed for development of new antibacterial drugs. Issues surrounding drug-target interactions and whether the resultant molecular function is lethal to bacteria are the main criterion used to classify the antibacterial mechanism of action [9].

Antibacterial agents fall into two general categories: bactericidal drugs, which kill bacteria with an efficiency of > 99.9%, and bacteriostatic drugs, which merely inhibit growth [10]. One class of bactericidal antibiotics are the quinolones which target DNA gyrase complexed with DNA and blockDNA replication and repair, which drives double-strand DNA breaks and cell death. Cell-wall synthesis inhibitors interfere with normal cell-wall synthesis and induce lysis and cell death. For example, beta-lactams interact with penicillin-binding proteins and glycopeptides interact with peptidoglycan building blocks. Bacteriostatic drugs mainly inhibit ribosome function, targeting both the 30S and 50S ribosome subunits [11].

More recently it has been shown that the bactericidal antibiotics, having distinct drug-target interactions, stimulate the production of highly harmful hydroxyl radicals in Gram-negative and Gram-positive bacteria, which finally contribute to cell death. In contrast, the bacteriostatic drugs tested do not lead to the production of hydroxyl radicals [9]. Therefore, among antibacterials that have diverse primary targets there appears to be similarities in how they finally lead to cell death.

1.1.2.2 Antibacterial Resistance

Antibiotics and other antibacterial drugs have saved millions of lives and eased patients' suffering over many decades. However, over this period, bacteria have developed resistance to existing drugs, making infections difficult to treat. Resistance is the ability of a microorganism to bear the effects of an antibacterial drug. Resistance may develop via natural selection through random mutation, which occurs at a rate of about 10^{-8} per base pair per generation in bacteria [12]. Also, resistance can be quickly acquired as a fully functional gene from another organism in a horizontal fashion by genetic transfer.

Antibiotic action is an environmental pressure for bacteria, and those bacteria which have a resistance mutation or trait may survive and reproduce. Resistance traits are then passed to progeny, resulting in fully resistant generations. Patterns of antibiotic usage greatly affect the incidence of resistant organisms. For example, exposure to broad-spectrum antibiotics, such as second- and third-generation cephalosporins, speed the development of methicillin resistance. Other factors contributing towards resistance include incorrect diagnosis, unnecessary prescriptions, improper use of antibiotics by patients, and the use of antibiotics as food additives for growth promotion of domestic animals [13].

1.1.2.2.1 The history of antibiotic resistance

Staphylococcus aureus was the first bacterium in which penicillin resistance was discovered in 1947, just four years after the drug started being mass-produced [14]. Methicillin then became a widely used and methicillin-resistant *Staphylococcus aureus* (MRSA) strains were soon detected in Britain in 1961 and are now "quite common" in hospitals. MRSA was responsible for 37% of fatal cases of blood poisoning in the UK in 1999, up from 4% in 1991. Half of all *S. aureus* infections in the US are resistant to penicillin, methicillin, tetracycline and erythromycin. Vancomycin is often the only effective agent available at present. However, vancomycin-resistant *Staphylococcus aureus* (VRSA) was first identified in Japan in 1996. A new class of antibiotics, oxazolidinones represented by linezolid, became available in the 1990s. These new compounds are comparable to vancomycin in effectiveness against MRSA. However, linezolid-resistance in *Staphylococcus aureus* was reported soon after its introduction [15, 16]. These examples highlight the unfortunate fact that new antibacterial agents may quickly lose effectiveness.

1.1.2.2.2 Antibacterial resistance mechanisms

The four main mechanisms by which bacteria exhibit resistance to a drug are:

- Inactivation or cleavage of the drug: e.g. enzymatic deactivation of penicillin G in some penicillin-resistant bacteria through the production of ß-lactamases.
- Alteration of drug target site: e.g. alteration of the binding target site of penicillins (PBP), which underlies methicillin-resistant *Staphylococcus aureus* (MRSA) and other penicillin-resistant bacteria
- Alteration of metabolic pathways or drug target overexpression: e.g. some sulfonamide-resistant bacteria do not require para-aminobenzoic acid (PABA), an important precursor for the synthesis of folic acid and nucleic acids. Instead, like mammalian cells, they utilize preformed folic acid.
- Reduced drug accumulation: by decreasing drug uptake through cell wall modification and by increasing drug efflux [11].

These major forms of resistance mechanisms are key issues to be considered when aiming to improve existing drugs and in the development new antibacterials.

1.1.2.2.3 Possible solutions to the problem of resistance

There are several possible solutions to reduce the threat of antibacterial resistance. Several decades ago, research efforts provided new drugs and combination therapy in time to treat bacteria that became resistant to older antibiotics [14]. Due to resistance, infectious disease experts are now concerned that effective drugs may not be available to treat seriously ill patients. Unfortunately, the development of a new drug is very difficult and time consuming. Indeed, only two new antibiotics have been introduced over the past 30 years [17].

The resistance problem demands that renewed efforts be made to seek new antibacterial agents against pathogenic bacteria resistant to current antibiotics. One possible strategy towards this objective is to isolate additional metabolites from living organisms. In many cases, these substances serve as an organism's defense against micro-organisms [18]. This is not a new strategy, but the chemical diversity in nature has not been fully exploited. Other strategies include improved vaccines, bacteriophage therapy and attempts to synthesis new antibacterial agents that target additional cellular structures. Finally, avoiding the use of antibiotics in unnecessary situations can reduce the spread of antibiotics-resistant bacteria.

1.2 BACTERIA AS AN EXPERIMENTAL SYSTEM

Most of the work in this thesis involves experiments on the bacteria *Escherichia coli*, which is a pathogenic species and also a valuable model organism. A model organism is a species that is intensively studied to understand general aspects of biological phenomena, with the hope that discoveries made in the model organism holds true in other species. In particular, model organisms are widely used to explore potential causes and treatments for human disease when experimentation on humans would be unfeasible or unethical. This strategy is made possible by the common descent of all living organisms and the conservation of metabolic and developmental pathways.

Model organisms are chosen on the basis that they are agreeable to experimental manipulation. This usually includes characteristics such as short life-cycle, small size, accessibility for genetic manipulation. One of the most popular model systems is the bacterium *E. coli*, a common component of the human digestive system. The bacterial system (especially *E. coli*) commonly provides material for recombinant DNA technology and is a suitable host for gene expression. The large amount of knowledge about *E. coli* makes it an excellent organism for testing new analytical technologies such as genome sequencing [19] and genome-wide microarrays [20].

1.3 NUCLEIC ACIDS AND GENE EXPRESSION REGULATION

Nucleic acids include DNA and RNA, and structural aspects of these molecules influence gene expression. Although a functional gene product may be RNA or a protein, the majority of known regulatory mechanisms alter the expression of protein coding genes. There are three general levels of gene regulation in cells; these are transcription, translation and post-translation control.

1.3.1 DNA structure and gene expression regulation

DNA is a long polymer composed of nucleotides. Two strands of DNA twist around one another to form a double helix with a minor groove and a major groove. In cells, DNA is mainly in the B-form, but this structure can change locally. Such alternative structures are discussed below and are the topic of paper IV. Also, within cells, DNA is bound by structural proteins such as histones to create highly organized structures that build-up the chromosomes.

Regulation of transcription controls the amount of RNA produced. The transcriptional control of a specific gene is determined by the ordered assembly of DNA-binding proteins on the regulatory or promoter regions, which are mainly located

upstream of the coding region [21]. Repressor and activator proteins bind to their DNA target sites using the binding domain and the regulatory domain inhibits or promotes interactions between RNA polymerases and promoters. The disruption or modulation of DNA-protein interactions is a critical aspect of transcriptional regulation [22].

1.3.1.1 Non-B-DNA structures

Several types of non-B-DNA conformations have been identified, and it is likely that other non-B conformations will be discovered. Sequences with the potential to adopt non-B-DNA conformations such as invert and direct repeats, repeating elements rich in guanosine residues and polypurine-polypyrimidine (pu-py) elements are very abundant in all species[23]. Repeat sequences appear to be major contributors to the formation of non-B-DNA structures, although unusual structures can form on a variety of other sequences. Sequences with the potential to form non-B-DNA are associated with nearly 30 hereditary disorders in humans [23]. These structures have been of great interest in biophysical studies; however, only in very few cases direct evidence is available for the involvement of non-B-DNA structures in biological processes or disease etiology.

1.3.1.1.1 Intramolecular triple helix DNA (H-DNA)

An intramolecular triple helix, or H-DNA, is a structure of DNA forming at polypurine-polypyrimidine regions containing mirror repeat symmetry. Within these sequences, a portion of the tract can dissociate and one of the single strands folds back and binds within the major groove of the neighboring duplex, forming a triple helix. In this three-strand nucleic acid structure, Hoogesteen or reverse Hoogsteen hydrogen bonds are formed by the polypurine (R) or polypyrimidine (Y) single strand and the polypurines (R) of Watson-Crick base pairs. Negative supercoiling, pH and divalent metal ions influence H-DNA formation [24, 25].

It has been difficult to demonstrate H-DNA in biological systems; however, there are hints that triplex structures are formed by pathological triplet expansions. For example, severe $(GAA)_n$ repeat expansions in the frataxin gene cause most forms of Friedriech's Ataxia (FA). There is evidence that H-DNA formed by expanded $(GAA)_n$ repeats stalls DNA polymerases during DNA synthesis *in vitro* [26]. Also, $(GAA)_n$ repeats interfere with *in vitro* transcription by both prokaryotic and eukaryotic enzymes [27]. H-DNA may inhibit the transcription of the Friedreich's ataxia gene and result in reduced frataxin protein [28].

1.3.2 RNA structure and gene expression regulation

Similar to DNA, RNA molecules consists of four nucleotides; however, in RNA the T nucleotides are replaced by uracils (U). An RNA strand is able to form a large variety of secondary and higher structures affecting its function. There are two main groups of cellular RNA called coding RNA (mRNA) and non-coding RNA (rRNA, tRNA, snRNA, snoRNA and natural antisense RNA). Many non-coding RNAs regulate gene expression [29, 30]. In the case of antisense regulation, the antisense RNA hybridises to a target RNA (a sense RNA). The effects of such complementary binding may be diverse; however, several antisense RNAs are known to inhibit translation.

Knowledge about the regulatory properties of RNA has led to the development of a number of nucleic acid analogues and mimics designed to regulate gene expression (Figure 1). Such analogues and mimics, also called antisense agents, usually possess higher stability and improved target binding properties relative to naturally occurring antisense RNA. In principle, antisense agents can be designed to target and regulate any gene, a property that makes them attractive both for studies of gene function and as drug to inhibit disease causing genes.

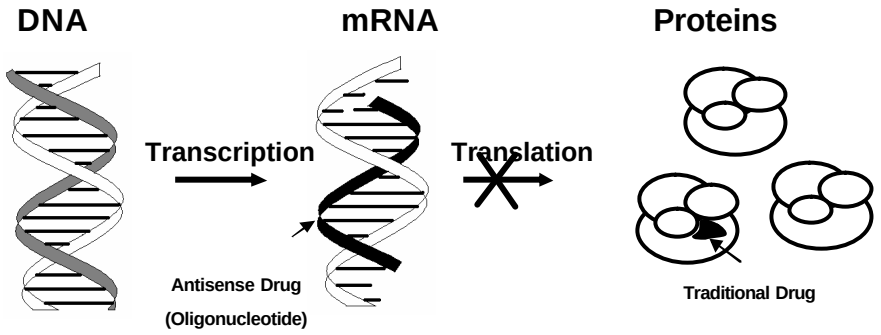

Figure 1. Models for antisense oligonucleotide and traditional drug mechanisms of action. The stored information in DNA is transcribed to mRNAs which are usually further translated into proteins. Antisense oligonucleotides bind to mRNA and interfere with translation, whereas traditional drugs typically bind to proteins and interfere with function.

1.3.2.1 RNA higher order structures

Single-stranded nucleic acids often fold back on themselves to form functional secondary and higher order structures [31]. The simplest and most common structural motif formed in RNA is a stem-loop, created when two complementary sequences within a single stranded RNA come together to form double-helical structures. In many cases, these double helices are made up entirely of Watson-Crick base pairs. In other cases, the structures include mismatched (bulged) bases [32]. Antisense agents can potentially prevent these interactions by disrupting higher order structures or their formation.

1.4 NUCLEIC ACID LIGANDS

Traditionally, it is mainly proteins that serve as molecular targets for therapeutic purposes. Although there are hundreds of essential genes in a bacterial cell, only a few have been successfully targeted using protein inhibitors. In theory, a more flexible strategy is to develop ligands to selectively target nucleic acids. There are several reasons to consider nucleic acids as a target. Relative to the large number of proteins in cells, there are fewer copies of mRNA and just one or two copies of DNA. Moreover, inhibition at the nucleic acids level would occur earlier in gene expression and could have longer lasting effects.

1.4.1 DNA ligands

DNA binding molecules include small molecule ligands, DNA-binding proteins and oligonucleotides (figure 2). Nature provides a variety of molecules that bind DNA. Therefore, DNA ligands have important biological and medical activities.

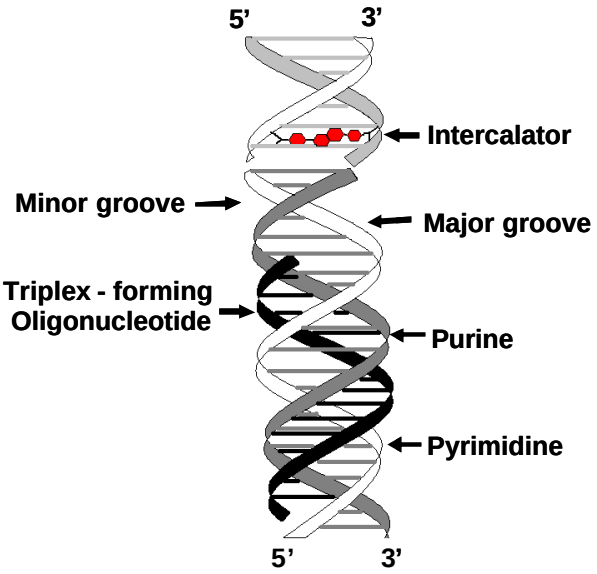

Figure 2. Ligand binding sites on DNA. The grey lines indicate Watson-Crick hydrogen bonds between two strands of DNA. A triplex forming oligonucleotide (TFO) establishes Hoogsteen or reverse Hoogsteen hydrogen bonds with the purine strand in the major groove, indicated with the black line. Intercalators such as anthracycline fit between base pairs of DNA, whereas other ligands, such as berenil and distamycin A bind within the minor groove.

1.4.1.1 Small molecule ligands

A variety of compounds have been discovered that bind double-stranded DNA. The mode of binding can be classified as electrostatic interactions, intercalation, and minor and major groove binding. However, this classification is not strict as many compounds operate through more than one mode of binding. Also, DNA ligands can be classified from a functional perspective: alkylating agents, inducers of single or double-strand DNA breaks, intercalators and minor groove binders [33, 34].

DNA binding molecules were mainly discovered during screening for antibacterial or antiproliferative activity of compounds or extracts isolated from natural sources. The oldest class of compounds with a therapeutic use and known to interact with DNA are alkylating agents, such as cyclophosphamide, cisplatin, busulfan, or carmustine. Sulfur mustard was first synthesized in 1854 and the antimicrobials properties were discovered more than 30 years later [32, 35]. Intercalators such as acridines, actinomycin D and doxorubicin are another class of DNA binding molecules. In the

1940s, simple acridine-based compounds such as 9-aminoacridine and proflavine were used as antibacterial agents. The antibacterial activity of acridine is directly related to its interaction with DNA [36].

1.4.1.1.1 Small molecule ligands as anticancer agents

Some anticancer agents are actually antibiotics, including members of the anthracycline group and actinomycin D. Anthracycline intercalates between the base pairs of DNA, thus preventing the replication of rapidly growing cancer cells. Actinomycin D is primarily used as an investigative tool in cell biology to inhibit transcription. It does this by binding to DNA within the transcription initiation complex, which blocks elongation of RNA polymerase [37].

Alkylating agents that covalently bind an alkyl group are well known anticancer drugs. These include nitrogen and sulfur mustards, nitrosoureas, trizenes, and ethyleneimines [38]. The main site of alkylation on DNA has been identified as N (7) on guanine for nitrogen mustards and N (3) on cytidine for nitrosoureas. Following the formation of a covalent bond with a nucleotide, the secondary interactions with DNA may lead to the formation of inter- or intra-strand cross-links. It has been suggested that this type of damage may be harmful for dividing cells [39, 40].

1.4.1.1.2 Small molecule ligands as antimicrobials

There has been an alarming increase in resistance of bacterial strains to different classes of antibiotics. Thus, there is an increasing need for novel classes of antimicrobials. In particular, there is a need for compounds with a novel mechanism of action and less susceptibility to resistance mechanisms [41]. Bacterial DNA could potentially serve as an attractive and novel target for the development of new drugs. For antimicrobial applications in the clinic, it is important to preferentially inhibit bacterial DNA over human DNA to minimize toxicity. Chemical agents able to interfere with microbial DNA are widespread in nature and show outstanding therapeutic efficacy in infectious diseases [42]. The minor groove binders are one of the most widely studied classes of nucleic acid ligands and they possess several biological activities, such as antiprotozoal, antiviral and antibacterial effects.

There are several examples of antimicrobial DNA ligands that are clinically useful. Minor groove binding DAPI and berenil proved to be therapeutically useful agents against several protozoic diseases [43]. DAPI is a well known fluorescent counter stain used in cell biology and is also a useful agent against *Trypanosome congolese* [44].

Berenil is trypanocidal and is used in veterinary medicine for treatment of trypanosomiasis [43, 45]. These compounds are characterized by a high affinity for AT-rich sequences [46]. Also distamycin A analogous have been used to treat severe infections caused by drug-resistant Gram-positive bacteria. It has been shown that distamycin A analogs bind to AT rich target sequence that are commonly found in bacterial promoters and replication origin sequences [47]. Unfortunately, due to poor sequence selectivity or species selective effects, there are often toxic effects associated with current nucleic acid ligand drugs.

1.4.1.1.3 Natural and synthetic source of small molecule ligands

The first DNA-binding molecules to be studied were mainly natural products, which were discovered as biological metabolites. Their development as drugs has been restricted because of lack of selectivity and toxicity. The emergence of experimental techniques suitable for the determination of their DNA-binding selectivity, such as footprinting experiments, X-ray crystallography, NMR and molecular modeling, has advanced understanding of drug-DNA interactions. Also, this knowledge permitted the synthesis of selective target inhibitors that have improved *in vitro* and *in vivo* properties [48, 49]. For example, substitutions on anthramycin have provided derivatives with increased cytotoxicity on tumour cells and less cardiotoxic side effects [49]. In recent years, many efforts have been made to combine DNA ligands with other anti-tumor compounds to improve cancer therapy [50].

1.4.1.2 DNA binding proteins

DNA-protein interactions can be non-specific, or specific. Chromosome structural proteins such as histones are examples of non-specific binding proteins, whereas transcription factors that control gene expression are examples of specific DNA binding proteins. Gene expression is regulated by the interaction of various protein factors with DNA and RNA. The major groove of the DNA double helix is the main site for protein sequence specific interactions. Zinc finger proteins are an interesting group among proteins that bind DNA. These structurally defined fingers can be manipulated to create proteins with similar structures but altered binding preferences. The use of combinatorial technologies and/or structure-based rational design [51-53] has been used to create new zinc finger proteins that recognize target sites of up to 18 base pairs with high affinity and sequence selectivity.

1.4.1.3 Oligonucleotides

Oligonucleotides can recognize DNA at specific sequences by forming triple-helix or strand invasion complexes. Targeting oligonucleotides to the gene itself (antigene) presents several advantages as compared to messenger RNA (antisense). Blocking mRNA translation using antisense agents does not prevent the corresponding gene from being transcribed. Preventing gene transcription using an antigene approach is expected to prevent the production of mRNA [54]. In practice, however, antigene strategies have proven relatively difficult.

In triple-helix formation, oligonucleotides recognize duplex DNA by binding within the major groove through Hoogsteen or reverse Hoogsteen base pairing. An optimal target sequence contains consecutive purines on the same strand since only purines bases are able to establish two Hoogsteen or reverse Hoogsteen type hydrogen bonds [21, 55]. Triplex forming oligonucleotides are still largely restricted to polypurine-polypyrimidine target duplexes [56, 57].

Natural DNA oligonucleotides appear to have limited potential for both triple helix formation and strand invasion inside cells. In addition to the issues described above, practical limitations include poor delivery and compartmentalization in cells, low binding affinity under physiological conditions, lack of oligonucleotide stability against nuclease degradation and target accessibility in the chromatin context. These limitations have reduced the initial excitement of oligonucleotides as tools to control gene expression under physiological conditions. Selected chemical modifications are being pursued by chemists to improve some of these properties [58, 59].

In the next section, the focus is on the antigene application of triplex forming oligonucleotide (TFO). The properties and application of peptide nucleic acid (PNA) will be discussed in details in later sections.

1.4.1.3.1 Antigene applications of triplex forming oligonucleotide

The *c-myc* proto-oncogene is the first gene for which expression was modulated *in vitro* [60] and in cell culture [61] using the triplex strategy. TFOs have been shown to form triplexes in various gene promoters and prevent regulatory protein and transcription factor binding [62]. Also, there is evidence that triplexes can block polymerases and inhibit replication or transcription elongation [63]. A number of experiments have demonstrated the ability of TFOs to inhibit gene expression on plasmid targets *in vitro* and *in vivo* [64, 65]. It has been shown that a TFO targeted to the promoter of interleukin-2 receptors alpha (IL-2Ra) gene could inhibit transcription of the

endogenous gene in lymphocytes [66], and transcriptional inhibition by TFOs has been observed at other loci [61, 67]. An alternative antigene approach is to use TFOs to induce site-specific mutation [54]. TFOs can stimulate recombination in mammalian cells [68, 69] and this can be used for gene correction at a low frequency. TFOs can also be used to direct the delivery of DNA reactive molecules to a particular sequence. Daunomycin-TFO conjugates have been used to induce site-specific DNA damage in *c-myc* promoters and inhibit growth in prostate cancer cells [70].

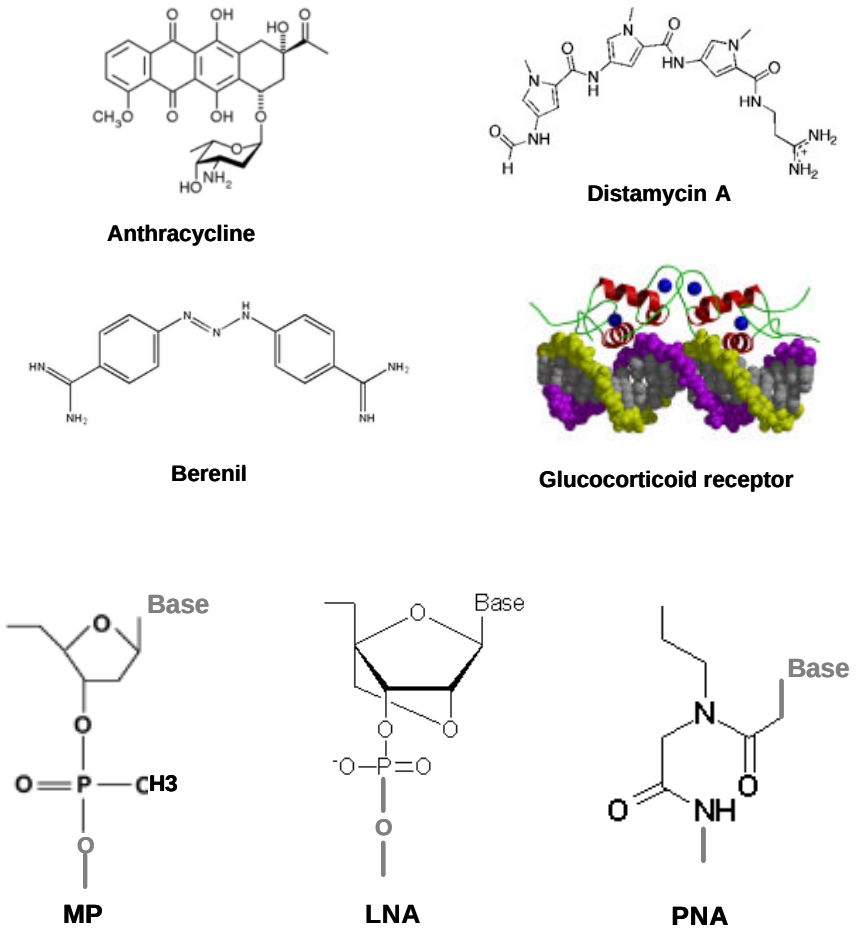

Figure 3. DNA Ligands structures. Some representative examples of DNA binding ligands. Small molecule ligands bind through the minor groove (Berenil, Distamycin A) or intercalate into DNA (Anthracycline) (see Figure 2). DNA-binding proteins bind via the major groove (Glucocorticoid receptor, taken from

www.biochem.arizona.edu/dept/GluRecep-miesfel...). Also oligonucleotides bind to DNA either by triplex formation or strand invasion (methylphosphonate=MP, locked nucleic acid= LNA, peptide nucleic acid=PNA).

1.5 ANTISENSE OLIGONUCLEOTIDES

Antisense oligonucleotides are natural or synthetic polymers that are complementary to target sense sequences, with mRNA being the usual target. Antisense was first envisioned in 1967 [71] and demonstrated *in vitro* in 1977 [72]. The definition of antisense logically follows the definition of the sense strand as the coding DNA or RNA sequence. One strand from double strand DNA serves as a template for synthesis of a complementary RNA strand. This template DNA strand is called the transcribed strand and the mRNA transcript is said to be the sense sequence. The usual target in antisense strategies is mRNA, and most cases of natural antisense also involve mRNA as the target (figure 4).

However, many functional RNAs targeted by antisense sequences are non-coding and, therefore, are not strictly sense RNA. At the mechanistic level, antisense sequences can act through steric hindrance or degradation of target sequences. The antisense sequence can be DNA or RNA and their chemical derivatives. Nucleic acid mimics with synthetic backbone structures are considered antisense sequences although they are not formally nucleic acids. Therefore, within the definition of antisense there can be various target sequences, mechanisms of inhibition and antisense polymers. This variety underlies a wide range of antisense mechanisms and effects.

Figure 4. MessengerRNA (mRNA) regions typically susceptible to translation repression by steric hindrance through antisense. The translation start codon as well as 5' and 3' untranslated regions (UTRs) are sites on mRNA where antisense oligonuleotides (AO) sequences bind and repress translation. Antisense agents that trigger decay mechanisms can be effective when targeting other accessible regions.

1.5.1 Natural Antisense RNA

The first naturally occurring antisense RNA was discovered in 1981 through the study of plasmid replication in *E .coli* [73]. It has since become clear that antisense RNAs are

involved in the regulation of diverse and complex phenomena. In prokaryotes, antisense RNAs regulate plasmid replication and incompatibility, transposon transposition, lambda phage development and many other processes. In eukaryotes, antisense RNAs are involved in RNA splicing, RNA editing, development and several other processes.

The first chromosomally expressed antisense RNA to be identified was the micF RNA in *E. coli* [29]. It was found that micF RNA inhibits *ompF* expression by hybridization to the *ompF* mRNA. Following the identification of micF as an antisense RNA, many other chromosomally encoded RNAs have been detected in prokaryotes.

Naturally occurring antisense RNAs in eukaryotes and their potential roles in posttranscriptional regulation of gene expression have been reviewed [74-76]. In *C. elegans*, the 22 nt-long lin-4 antisense RNA regulates developmental timing of larval molts by binding to the 3'- UTRs of target mRNAs encoded by the *lin-14* and *lin-28* genes [77]. There are further examples, such as let7, which encodes a 21-nt antisense RNA that is present in *C. elegans*, *Drosophila* and vertebrates [78]. These antisense RNAs are now known as micro-RNAs.

MicroRNA precursors (pre-microRNAs) are cleaved into short fragments of 21-23 nucleotides called mature miRNAs by an enzyme named Dicer. One of the two strands of each fragment is then incorporated into the RNA Induced Silencing Complex (RISC) and base-pairs with complementary target RNA, leading to the cleavage or translational repression of mRNA [79]. It is know known that microRNAs are part of RNA interferences mechanisms. Fire and Mello first described RNAi in the worm *C. elegans* in 1998 [80].

1.5.2 Synthetic Antisense agents

Some decades ago it was shown that translation can be inhibited when a poly (A) RNA hybridises to a poly (U) template RNA [81]. This finding was expanded to use DNA fragments for hybridization with target mRNAs in order to identify genes; the method was called hybrid-arrested gene identification [72]. Although, the attempts to synthesize nucleoside and dinucleoside phosphate derivatives, which base pair to complementary nucleotide sequences, was made in 1960s [71], the first finding that small oligonucleotides (ONs) could be used to specifically inhibit gene expression was shown in late 1970s [82, 83]. This conclusion was made from the observation that a specific 13-mer oligonucleotide can act as a hybridization competitor to 35S RNA and inhibit Rouse sarcoma viral RNA replication and protein translation in cell culture [82,

83]. This gave hope for antisense applications in gene functional studies and in the development of therapeutics that specifically silence expression of disease causing genes.

It soon became apparent that unmodified DNA/RNA oligonucleotides are susceptible to cellular nucleases. This restricts practical applications, particularly *in vivo,* so the production of chemically modified synthetic antisense increased sharply since the mid-1980s. A large number of chemically modified forms of nucleic acid analogues have been developed to help increase both nuclease stability and their affinity for target RNA.

Cell uptake of synthetic antisense agents is another critical issue. To improve uptake, carrier molecules or transfection aids have been used extensively. The most popular transfection aids include Ca^{2+}, cationic lipids and liposome complexes. Also, microinjection and electroporation are being used to increase cellular delivery [84].

Inactivation of the target RNA can occur through RNase H mediated cleavage or steric hindrance. RNase H is a ubiquitous enzyme that hydrolyses the RNA strand in the DNA/RNA duplex. Many synthetic antisense agents especially those with a structure that is similar to native DNA are able to induce RNase H cleavage of target RNA. In contrast, other synthetic antisense ONs that form RNA/ RNA like hybrids rather than RNA/DNA hybrids do not activate RNase H. It is thought that these ONs sterically hinder mRNA translation. For example, hindering antisense ONs can either arrest a ribosome elongation or more commonly block initiation of translation. It is not known whether sterically hindering antisense ONs can act catalytically, although this is possible if they are able to trigger decay mechanisms in cells [85]. Interestingly, the results from Paper I suggest that hindrance can trigger intrinsic decay mechanisms.

1.5.2.1 Nucleic acid analogues

In recent decades many nucleic acid analogues have been introduced. Some of the most popular examples will be described briefly here. Phosphorothioate (PS) oligonucleotides are a class in which one of the oxygen atoms in the phosphate group is replaced with a sulfur atom. It has been shown that PSs have a broad range of activity [86]. The methylphosphonate (MP) is highly nuclease resistant molecule, where one of the non-bridging oxygens is replaced by a methyl group at the phosphodiester backbone [87]. Locked nucleic acid (LNA) is a ribonucleotide that contains a methylene bridge between the 2'-oxygen and the 4'-carbon on the ribose and was first described in 1998 (figure 3) [88].

1.5.2.2 Nucleic acid mimics

There are two popular types of DNA mimics, Phosphorodiamidate morpholino oligomers (PMOs) and peptide nucleic acid (PNA) (figure 3). PMO were developed as antisense agents that should act by blocking translation through steric hindrance [89]. The sugar ring is replaced by a morpholino moiety and a phosphorodiamidate linkage is applied instead of phosphodiesters. This results in an uncharged molecule that is unable to induce RNase H [90, 91].

Peptide Nucleic acid (PNA) is nucleic acid mimic with natural nucleobases attached to a backbone composed of N-(2- aminoethyle) glycine [92]. The PNA backbone displays favourable hybridization properties, following Watson/Crick base pairing rules. PNA has been used in a number of diagnostic applications [93]. For example, rapid detection of the chromosomal localization of specific genes by fluorescence in situ hybridization (FISH) using fluorophore labeled PNAs are now possible [94].

1.5.2.2.1 Chemical properties of PNA

The distances between the nucleobases in the pseudopeptide backbone of PNA are similar to those found in natural DNA and RNA. This permits efficient pairing with complementary oligonucleotides. PNA has a charge neutral backbone and this provides high binding affinity to complementary nucleic acids. The thermal stabilities increase in following order, DNA/PNA < RNA/PNA < PNA/PNA [95, 96]. In addition, an important property for target specificity is that the sequence discrimination is more efficient for PNA recognition of DNA than DNA recognition of DNA [97, 98]. Taking advantage of these properties, PNA can be used to block PCR amplification for direct analysis of single base mutations [97].

1.5.2.2.2 Activity of PNA in Biological systems

Unmodified PNA typically shows very poor cellular uptake properties in cell culture *in vitro*. Chemical modifications on PNA itself or conjugation with positively charged peptides like $(KFF)_3K$ increase cellular uptake [99, 100]. Such modifications have enhanced many biological applications of PNA. Despite the uptake problem, carefully designed PNAs can be active in cell culture systems [101] and in animal models [102, 103].

When targeting RNA, a PNA/RNA duplex does not induce cellular nucleases such as RNase H [104] or RISC complex components [93], due to the unnatural backbone of

PNA. Rather, PNA inhibits mRNA translation selectively through steric hindrance by binding to the translation initiation region [105]. The ability of PNA to reach the nucleolus makes it a good candidate to alter mRNA splicing processes [106]. The potential of PNA to alter mRNA splicing patterns has been reported *in vitro* [107] and *in vivo* [108]. Also, viral genomic RNA presents obvious targets for PNA. Several laboratories have shown that PNA inhibits elongation of viral reverse transcription on RNA templates and replication as well as in infected cells [109, 110].

When targeting DNA, PNA may act either as a transcription activator or inhibitor (antigene) [111]. PNA can bind dsDNA to form a triplex structure and the complex may block gene expression [112] *in vitro*, in cell culture and in animal studies. In eukaryotic systems, specific down regulation of *c-myc* transcription using a PNA complementary to noncoding region upstream of an oncogene has been reported [113]. Also, PNA binds certain dsDNA sequences via strand displacement, forming a single-stranded DNA loop (D-loop). These structures can facilitate transcription of downstream genes both *in vitro* and *in vivo* [114-116]. For example, *E. coli* and T7 RNA polymerases possess a high affinity for D-loops generated by 10-12 bases long PNAs and initiate transcription *in vitro* [117]. Also, transcription activation by a PNA-peptide chimera in a mammalian nuclear extract has also been reported [118].

2 AIMS

Aims addressed in this thesis include:

Determine how antisense inhibition affects the expression of genes that are cotranscribed in *E. coli* operons.

Study the ability of PNAs to competitively inhibit a small non- coding regulatory RNA in *E. coli*.

Determine the uptake and efflux kinetics of peptide-PNA and its post-antibiotic effect (PAE) in *E. coli.*

Probe DNA higher order structures in $(GAA)_n$ repeats using ligands that recognize and stabilize triple-helix DNA structures.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Variable coordination of cotranscribed genes in *Escherichia coli* following antisense repression.

A majority of bacterial genes are within tight clusters and operons. For example, over half of the genes in *E. coli* are expressed from multigene operons. This complicates gene functional studies. To understand how antisense inhibition affects expression of genes that are cotranscribed, we constructed an artificial operon by inserting a stop codon between *gfp* and *DsRed* coding regions in a double reporter plasmid pGRFP [119]. *E. coli* AS19 containing this artificial operon was treated with peptide-PNA that targets GFP and RFP. We found near complete discoordination of expression between *gfp* and *DsRed* genes.

We extended this study to the endogenous *lac*-operon. This operon includes three structural genes *lacZ, lacY* and *lacA*. Protein expression data showed variable coordination of *lac*-operon ORFs following antisense repression. Antisense repression of upstream gene (*lacZ*) resulted in partial silencing of downstream genes (*lacY* and *lacA*). In contrast, peptide-PNA targeting upstream gene (*lacY*) resulted in complete silencing of downstream gene (*lacA*) and little silencing of the upstream gene (*lacZ*). For the naturally occurring *lac*-transcript, and also for many other endogenously expressed polycistronic transcripts, a variety of regulatory mechanisms may have evolved to balance gene expression according to the cell needs. The *gfp-DsRed* transcript, however, is not a natural component for bacteria and is not likely to be affected by such specific regulatory mechanisms.

To better understand the protein level results, we studied the impact of antisense PNA inhibition at the mRNA level using RT-PCR. We found that mRNA reduction is dose dependent and there is a coupled reduction between protein and mRNA. It has been reported that ribosomal binding influences mRNA stability [120-122]. Therefore, we suggested that antisense PNA inhibition of translation leads to mRNA destabilization through intrinsic decay mechanisms that operate on un-used transcripts.

3.2 PAPER II

Competitive inhibition of natural antisense Sok-RNA interactions activates Hok-mediated cell killing in *Escherichia coli*

Bacteria use natural antisense mechanism widely, yet there is little evidence for direct sense/antisense interaction in cells. Also, there have been few attempts to directly probe these structures [123]. An established model of sense/antisense interactions is the *hok/sok* toxin-antitoxin (TA) locus that stabilizes R1 plasmids in *E. coli* [124]. To test this model and also to test whether an antisense agent that sequesters Sok -RNA could induce Hok mediated killing, we treated *E. coli* carrying the *hok/sok* locus with anti-Sok PNAs. The aim was to out-compete *hok* mRNA::Sok-RNA interaction and stimulate Hok toxin synthesis in bacteria. Monitoring treated bacteria cell viability and morphology showed that anti-Sok PNAs sequester Sok-RNA in a sequence specific manner and activate Hok toxin synthesis. Interestingly, anti-Sok PNA is more potent than the antibiotic rifampicin. This result supports the *hok/sok* model. Also, according to this model, blockage of Sok-RNA activity should lead to accumulation of the mature 3' end truncated *hok* mRNA. Northern blot experiments detected the truncated, mature isoform of *hok* mRNA in PNA-treated cells.

In the next step, we determined the binding-rate of *hok* mRNA::Sok-RNA and PNA::Sok-RNA complexes. The comparison of the second-order binding-rate constant (k_2) of interactions showed that PNA::Sok-RNA interactions occur less quickly than *hok* mRNA::Sok-RNA interactions *in vitro*. These *in vitro* results suggest that efficient competition for Sok-RNA in cells requires an excess of PNA relative to *hok* mRNA. In this respect, it is interesting to consider that accumulation of PNA (paper III) may provide the excess levels needed. Finally, we found it is interesting to assess the phylogenetic distribution of antisense RNA-regulated toxin-encoding genes. BLAST analysis of almost 200 bacterial genomes revealed that many enteric bacteria have multiple *hok*/*sok* homologous and analogous RNA-regulated toxin-antitoxin loci.

3.3 PAPER III

Antisense PNA accumulates in *Escherichia coli* and mediates a long Post-antibiotic Effect

PNAs are surprisingly effective in various cell killing assays both *in vitro* and *in vivo* relative to conventional antibacterials [125]. What is the explanation for this potent killing activity? To study the mechanism behind this bactericidal efficacy, we investigated the cellular effects and transit kinetics of PNAs. First, we determined the minimal inhibitory concentrations (MICs) and the rate of cell killing by equimolar doses (2µm) for the anti-*acpP* peptide-PNA, a control sequence scrambled version of the anti-*acpP* peptide-PNA and four conventional antibiotics in parallel against *E. coli*.

To ensure sequence and gene selectivity of the anti-*acpP* peptide-PNA, we determined the effect of the targeted and control peptide-PNAs on *acpP* mRNA abundance in growing cells treated with sub-lethal concentrations using real-time reverse transcriptase-polymerase chain reaction (RT-PCR). The results are consistent with mRNA reduction in the presence of antisense PNAs (in paper I) and support a target selective antisense mechanism. Therefore, we conclude that an anti-*acpP* peptide-PNA is more potent antibacterial agent on molar basis than ampicillin, chloramphenicol, trimethoprim but not streptomycin under the test conditions.

Cell morphology and viability tests indicated that the peptide-PNA-treated cells remain intact, non-viable and elongated. Given that the anti-*acpP* peptide-PNA is bactericidal but does not lyse cells, we considered the possibility that PNAs may accumulate in bacteria or show a long retention period following exposure. The anti-*acpP* peptide-PNA targets an essential fatty acid biosynthesis gene, and cells that retain PNA may be unable to divide and may die from a lack of fatty acid biosynthesis even after the growth environment has been depleted of PNA. To address this question, we used two experimental techniques. First, *E. coli* was treated with a non-toxic Cy5 labeled anti-*lacZ* peptide-PNA. This experiment showed that a proportion of the PNA localizes in the bacterial cytoplasm. Second, to assess peptide-PNA uptake and efflux rates using a quantitative method, we established a gel shift assay for cell-associated peptide-PNA. A standard curve for PNA in cell lysates was generated and applied to quantify cell-associated PNA in the uptake and efflux kinetics analysis. The kinetic analysis showed that cell associated PNA exceeded the initial medium concentration within 30 min and increased further up to 5 hours. Also, PNA accumulates to 7.3 µM after 20 hours. PNA accumulation could involve target affinity, carrier peptide decay, or an inability to exit across asymmetric cell membrane structures and charge gradients.

Efflux pumps remove many antimicrobials from cells and provide multidrug resistance. To test whether PNAs are substrates for efflux pumps, we treated *E. coli* with a general efflux pump inhibitor, Phe-Arg-ß-naphthylamid (PAßN), in the presence anti-*acpP* peptide-PNA or rifampicin. Rifampicin potency was increased dramatically by PAßN, but PNA potency was altered very little or not at all. We conclude that PNAs are likely to be poor substrates for efflux pumps that are inhibited by PAßN. A long period of cellular retention suggested that peptide-PNAs would display a long post-antibiotic effect (PAE). A standard PAE assay showed that *E. coli* growth was severely delayed when exposed to anti-*acpP* peptide-PNA and streptomycin. Indeed, we observed a PAE of >11 hours, which to our knowledge is longer than other examples

reported in the literature. The delivery of antisense agents into bacteria remains a significant challenge; once delivered, however, the molecule is well retained, and this may contribute to potent antimicrobial activity and a long PAE. These results may explain the cell killing efficiency reported in paper II.

3.4 PAPER IV

Friedreich's ataxia $(GAA)_n$ triplex DNA structures are stabilized by Benzoquinoquinoxaline derivatives

We aimed to test whether intramolecular triple-helix DNA (H-DNA) structures exist in the $(GAA)_n$ expansion within pathological human frataxin intron one sequences. Triple helix DNA is proposed to form H-DNA structures within $(GAA)_n$ expanded repeats in the frataxin gene. It has been shown that BQQ derivatives stabilize H-DNA formed in plasmid DNA in growing *E. coli* and *in vitro* [126]. To probe for the presence of triplex DNA in $(GAA)_n$ repeat sequences we used triplex selective probe BQQ.

First, we tested the ability of synthetic $(GAA)_n$ duplex DNA and triplex forming oligonucleotides (TFO) to form purine intermolecular triplex structure (Y·R·R) using EMSA. Despite conducting this experiment under many conditions, including variations in the length and amount of TFOs and presence of mono and divalent cations in a variety of buffers, we could not observe Y·R·R structures. In contrast, we readily observed a $(GAA)_n$ pyrimidine triple helix (Y·R·Y) using the same DNA target as above. BQQ stabilizes pyrimidine triplex structure at pH 7.1, and more efficiently at pH 6.5. The apparent dissociation constant (K_D) measurement confirmed that BQQ stabilized this structure. We suggest that pyrimidine triplex formation occurs within FA $(GAA)_n$ repeats, although these results do not directly exclude Y.R.R motif H-DNA formation in FA plasmids.

In the next step, we tested the ability of natural $(GAA)_n$ repeats to form an intramolecular triplex structure (H-DNA) in supercoiled plasmid using the BQQ derivative (BQQ-OP). This conjugate binds to H-DNA structures and causes double strand DNA cleavage at the site of triplex formation in the presence of Cu^{2+} and a reducing agent. Control plasmid and $(GAA)_{115}$ containing plasmid including the flanking sequences of intron one of the human frataxin gene, were subjected to BQQ-OP cleavage. The $(GAA)_{115}$ containing plasmid was cleaved while the control plasmid was not cleaved. Therefore, $(GAA)_n$ repeats form H-DNA within supercoiled plasmids.

To test weather BQQ stabilizes H-DNA in cells; we attempted to probe triplex structures in *E. coli* using a fluorescently labeled BQQ conjugate. Despite many

attempts using a variety of conditions, we failed to observe selective staining of cells that contained the $(GAA)_n$ repeat plasmids over control cells. Also, we could not observe a selective phenotypic effect on cell growth or plasmid abundance when cells were grown in the presence of BQQ. Finally, we tested weather BQQ would promote the formation of "sticky DNA" in preparations of $(GAA)_n$ repeat containing plasmids. Sticky DNA is a higher order structure formed by the interaction of two $(GAA)_n$ repeat regions, and this interaction may involve triplex DNA structures. We added BQQ to solutions of plasmid containing 115 and 90 repeats, and control plasmids that lack the repeat or contain 9 repeats. Nine repeats are in the range of repeats found in healthy individuals. The stabilization of sticky DNA by BQQ was seen as an increase in the intensity of retarded bands. Our finding shows that the higher order structure "sticky DNA" includes intramolecular triple helix. The probed structure may play a role in the pathology of FA.

4 CONCLUSIONS

The conclusions from paper I are that antisense mediated repression of genes within operons affect cotranscribed genes to a variable degree. Target transcript stability appears to be closely related to inhibition of translation and presumably depends on translating ribosomes protecting the mRNA from intrinsic decay mechanisms. Therefore, for genes within operons and clusters it is likely that the nature of the target transcript will determine the inhibitory effects on cotranscribed genes. Consequently, no simple and specific methods for expression control of a single gene within polycistronic operons are available, and a thorough understanding of mRNA regulation and stability is required to understand the results from both knock-down and knock-out methods used in bacteria.

The implications of Paper II are that antitoxin antisense depletion can provide a novel antimicrobial strategy by activating suicide in bacteria. Anti Sok peptide-PNAs were bactericidal to *E. coli* containing a *hok/sok* plasmid and induced morphology properties consistent with cell killing by Hok protein. Therefore, antisense agents can be used as competitive inhibitors of RNA::RNA interaction in bacteria. We extended the previous knowledge of RNA toxin-antitoxin (TA) systems distribution in Enterobacteria.

From paper III, we conclude that cell uptake of PNA is relatively rapid and efflux is relatively slow in *E. coli*. PNA accumulation helps to maintain excess levels. Also, PNAs display a very long PAE, which likely reflects their cell accumulation and retention properties. Finally, we can conclude that PNAs accumulate in *E. coli* and this appears to explain the bactericidal effects observed *in vitro* and *in vivo*.

The implications of paper IV are that the pathological $(GAA)_n$ repeats associated with FA disease form both intermolecular and intramolecular triplex DNA structures. We obtained evidence for only the pyrimidine motif triplex and not for the purine motif triplex. Furthermore, stabilization of triple helix DNA led to an increase in the amount of "sticky DNA" structures within DNA plasmids containing expanded $(GAA)_n$ repeats.

5 FUTURE PERSPECTIVES

In the future, antisense strategies may be used as tools to control the expression of individual genes within operon and to study operon regulation. In a similar manner, competitive inhibition could be used to study the function of a wide range of small antisense regulatory RNAs. Possible practical applications of antisense agents are as molecules that provide new types of antimicrobial agents, particularly in indications where a long PAE would provide advantages. In particular, it may be worth considering indications that involve surface or local infections.

The experiments on (GAA)n repeat sequences described in this thesis were restricted to *in vitro* studies and probing of *E. coli* plasmid DNA. Nevertheless, the evidence that we provide for the existence of triplex structures within FA (GAA)n repeats suggests that destabilization of these structures could provide a possible therapeutic strategy for patients. Whereas BQQ stabilizes triplex structures, other DNA ligands may destabilize triplex structures. Such strategies may offer hope for developing small molecule DNA ligands to treat FA or other conditions that involve triplex DNA structures that arise from tri nucleotide repeat expansions.

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