Molecular Therapy
Nucleic Acids
Review

Therapeutic and diagnostic applications of antisense peptide nucleic acids

Victoria MacLelland,[1,2] Madeline Kravitz,[1,2] and Anisha Gupta[1]

[1]Department of Pharmaceutical Sciences, University of Saint Joseph, West Hartford, CT 06117, USA

Peptide nucleic acids (PNAs) are synthetic nucleic acid analogs with a neutral N-(2-aminoethyl) glycine backbone. PNAs possess unique physicochemical characteristics such as increased resistance to enzymatic degradation, ionic strength and stability over a wide range of temperatures and pH, and low intrinsic electrostatic repulsion against complementary target oligonucleotides. PNA has been widely used as an antisense oligonucleotide (ASO). Despite the favorable characteristics of PNA, in comparison with other ASO technologies, the use of antisense PNA for novel therapeutics has lagged. This review provides a brief overview of PNA, its antisense mechanisms of action, delivery strategies, and highlights successful applications of PNA, focusing on anti-pathogenic, anti-neurodegenerative disease, anti-cancer, and diagnostic agents. For each application, several studies are discussed focusing on the different target sites of the PNA, design of different PNAs and the therapeutic outcome in different cell lines and animal models. Thereafter, persisting limitations slowing the successful integration of antisense PNA therapeutics are discussed in order to highlight actionable next steps in the development and optimization of PNA as an ASO.

INTRODUCTION

The established central dogma of molecular biology states that DNA is transcribed into RNA, which is then translated into protein (Figure 1A).[1] The development of oligonucleotide-based nucleic acid analogs has become increasingly relevant in medicine and molecular biology research due to their ability to preferentially bind to and modulate specific regions of DNA or RNA, and consequently interfere with protein production.[1] Antisense oligonucleotides (ASO) interact with precursor messenger RNA (pre-mRNA) in the nucleus or cytoplasmic mRNA or noncoding RNA (ncRNA) via Watson-Crick hydrogen-bonding interactions, while antigene oligonucleotides interact with double-stranded DNA via Hoogsteen or reversed-Hoogsteen hydrogen-bonding interactions and the formation of triple-stranded complexes (Figures 1B and 1C).[1]

Antisense mechanisms targeting mRNA/ncRNA have unique benefits over antigene mechanisms that target chromosomal DNA. Although DNA appears to be a more stable target, RNA targets offer great structural variety, including mRNA, microRNA (miRNA), transfer RNA (tRNA), short interfering RNA (siRNA), ribosomal RNA (rRNA), and long noncoding RNA (lncRNA). Additionally, RNA serves as an attractive target when considering that over 80% of the genome is estimated to be transcribed.[2] Thus, through antisense mechanisms, ASO analogs hold great promise for the design of molecules that can be used to alter gene expression, selectively, at the RNA level. While unmodified ASOs have been used to target RNA with moderate success, newer generations of ASO (phosphorothioates [PS], phosphorodiamidate morpholino oligomers [PMO], locked nucleic acids [LNA], and peptide nucleic acids [PNA]) have been chemically modified to decrease hurdles associated with polarity and degradation, thereby increasing their efficacy.[3,4]

The unique physicochemical properties of PNA that confer its many advantageous biological characteristics over other modified ASOs can be attributed to the modifications made to its backbone.[2,3] Distinguishing itself from naturally occurring DNA and other DNA analogs, PNA has a neutral N-(2-aminoethyl)-glycine backbone in place of a phosphodiester backbone (Figure 1D).[2,3] Further comparing their structures, PNA and DNA are similar in their intramolecular spacing and geometry, yet different in their charge and chirality.[2–4] The neutrality of PNA exerts resistance to the enzymatic degradation that DNA is susceptible to in both serum and cell extracts, which prolongs its lifetime *in vitro* and *in vivo*, making it an ideal potential therapeutic agent.[4] The neutral charge of PNA is also responsible for its unique ionic strength and biological and chemical stability over a wide range of temperatures and pH.[4] More specifically, unlike DNA, PNA binds independently of medium salt concentration, remains stable at acidic pH, and exhibits a high thermal melting temperature.[4] Additionally, the low intrinsic electrostatic repulsion of PNA against its complementary oligonucleotides allow for it to hybridize with high affinity and sequence specificity.[4] The cost of synthesis of PNA is relatively less compared with the cost of synthesis of traditional ASOs. Traditional ASOs require phosphoramidite chemistry-based synthetic protocols, whereas PNAs can be synthesized by peptide chemistry-based methods. Further, cost analysis of PNA varies based on choice of monomers used for synthesis. Tertiary butoxy carbonyl (Boc)-protected monomers are costlier than fluorenylmethoxycarbonyl (Fmoc)-protected PNA monomers. The cost of particular PNAs and ASOs is dependent on the length of oligomers

https://doi.org/10.1016/j.omtn.2023.102086.

[2]These authors contributed equally

Corresponding author:
E-mail: agupta@usj.edu

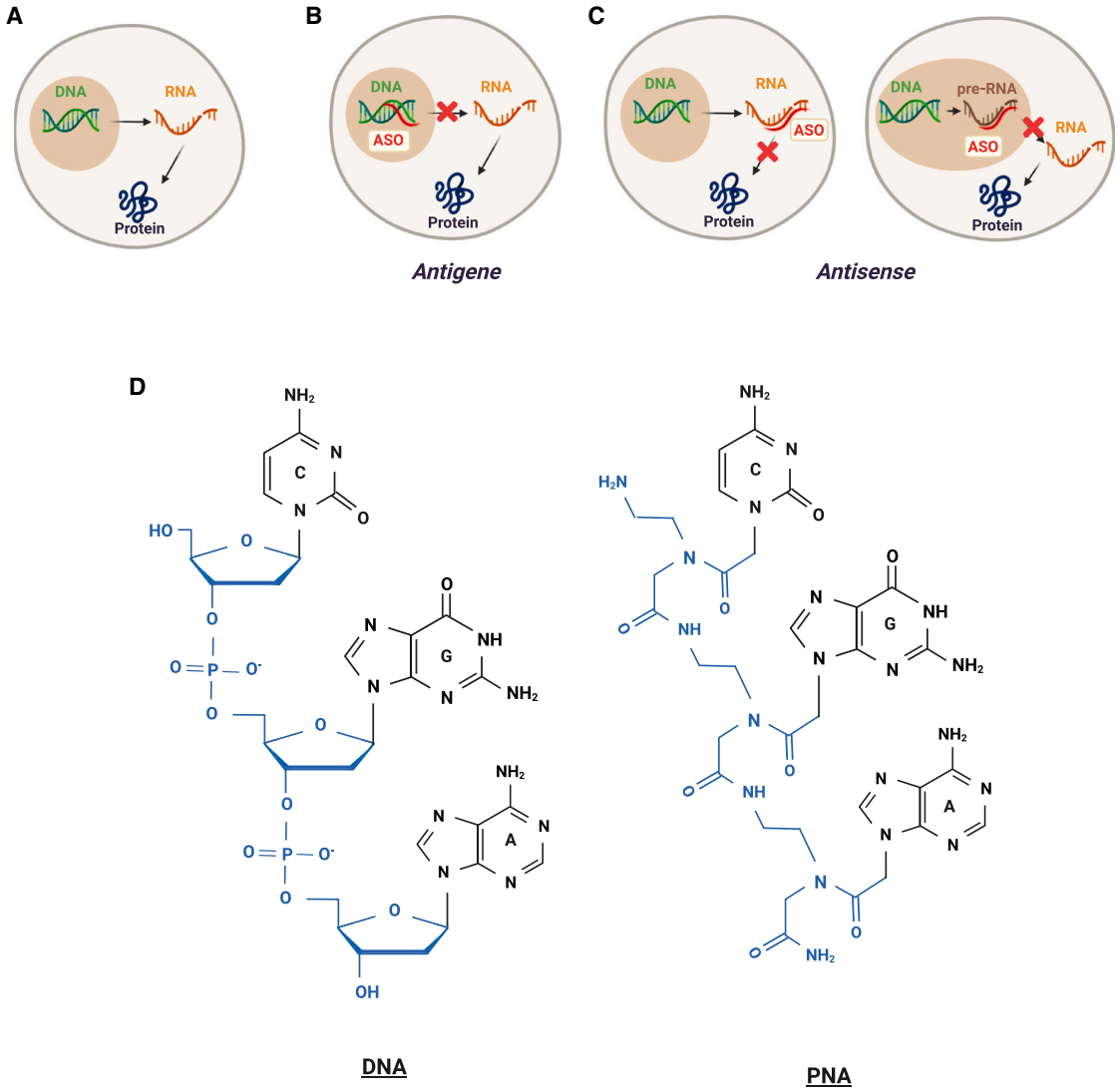

Figure 1. Antigene vs antisense and structures of DNA and PNA

(A–C) Antisense versus antigene mechanisms of PNA. (D) Structural comparison of DNA and PNA, with the backbones depicted in blue showing DNA's negatively charged phosphate backbone versus PNA's neutral N-(2-aminoethyl) glycine backbone.

and whether the PNAs or ASOs are required with covalently conjugated fluorophores for biodistribution and cellular uptake studies.

Reviews to date focus primarily on the applications of modified ASO, overlooking the potential of PNA exclusively.[5] Moreover, many existing reviews on PNA tend to focus on their antigene applications, and very few emphasize antisense applications.[3,4,6,7] Despite the potential of antisense PNAs to be applied across numerous clinical therapeutics and diagnostics, they have not been successfully approved or brought to market. The largest barrier to the development of antisense PNA is increasing cell permeability to maximize delivery of the PNA specifically into target cells at physiological levels while decreasing off-target toxicity. This review discusses the potential applications of antisense PNA as anti-pathogenic, anti-neurodegenerative, anti-cancer, and diagnostic agents, while distinguishing between areas that pose significant clinical benefits and areas that warrant continued research.

MECHANISMS OF ACTION OF PNA

Antisense PNA exerts its actions via splicing modulation or interruption of translation. Based on the target site, PNA exerts its actions by targeting pre-mRNA in the nucleus (splicing modulation), coding RNA (mRNA) and noncoding RNA (ncRNA) in the cytoplasm (translational arrest or translational block) (Figure 2).[4,5]

Splicing modulation

Once DNA is transcribed into pre-mRNA, it must be spliced to generate the correct, mature mRNA.[4,5] Normal RNA splicing consists of the exclusion of nonfunctional introns and joining of exons, which

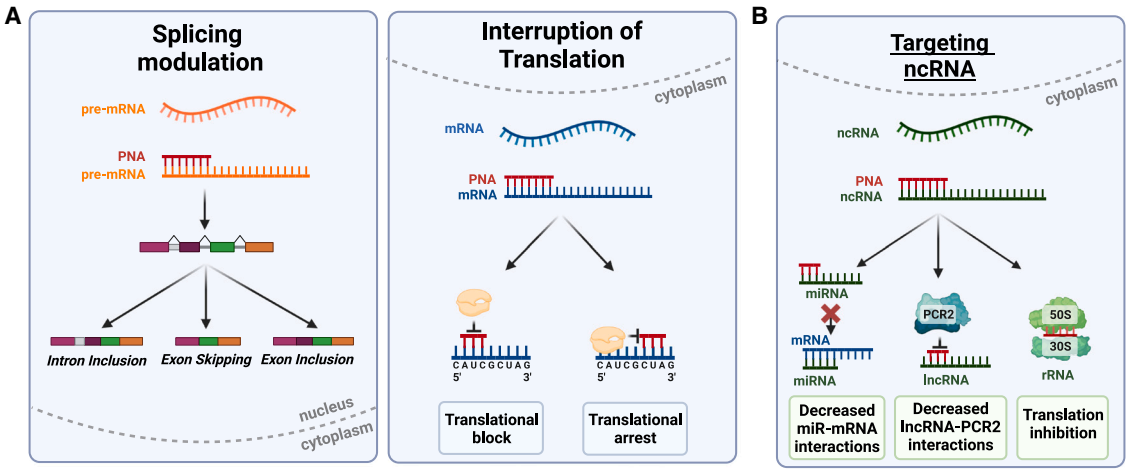

Figure 2. Schematic showing mechanisms of action of PNA

(A) PNA targeting pre-mRNA through splicing modulations and targeting mRNA through translational block and translational arrest. (B) PNA targeting various ncRNAs. PNA binds to ncRNA and prevents its interaction with rRNA and mRNA binding sites.

is modulated by proteins and small nuclear RNA in the spliceosome, conserved sequences in the splice junctions, and enhancer and silencer sequences in both the introns and exons. PNA can silence gene expression by inhibiting or altering RNA splicing to result in exon inclusion, exon exclusion (skipping), or intron inclusion. To highlight the therapeutic purposes of splicing modulation, PNA can block splicing machinery from inappropriately deleting certain sequences in pre-mRNA, resulting in the inclusion of the right exons in the mRNA sequence, production of the correct protein, and potential amelioration of some genetic diseases. While exon inclusion prevents the spliceosome and splicing factors from accessing the transcript sites on the pre-mRNA, exon skipping corrects disrupted reading frames and produces short, yet functional, alternative proteins. Additionally, PNA can also be used to correct aberrant splicing in certain disorders via intron inclusion to produce nonfunctional mRNAs that are susceptible to destabilization. Exon inclusion, exon skipping, and intron inclusion result from the steric hindrance that is achieved when PNA binds to the pre-mRNA.[4,5]

Translational block and translational arrest

The steric hindrance caused by PNA is directly related to the binding affinity it has for mRNA targeted sequences, with an increase in binding affinity resulting in superior hybridization and consequent translational block or arrest.[4,5] PNA can block or arrest translation in one of three ways: by inhibiting the transport of RNA to the cytoplasm; by inhibiting ribosome assembly; or by inhibiting the elongation process. PNA can form duplexes or triplexes with mRNA. Duplex-forming PNA causes translational block by inhibiting ribosome assembly via targeting of the AUG start codon region or 5′ untranslated region (5′UTR) of mRNA. Duplex-forming or triplex-forming PNA causes translational arrest by inhibiting the elongation process via targeting of the coding region of mRNA.[4,5] Given the accessibility of PNA to cognate regions of mRNA, utilizing steric hindrance to block or arrest translational machinery serves as an appealing mechanism. Additional examples of PNA leading to translational block and translational arrest are provided in the sections entitled "antisense applications as anti-pathogenic agents" and "antisense applications in neurodegenerative diseases."

INHIBITION OF NONCODING RNA FUNCTION

Human genome sequencing results reveal that only 2% of the human genome is made up of coding and 98% is noncoding RNA (ncRNA). Hence, targeting ncRNA can open new avenues for the PNA-based antisense applications. NcRNAs are a diverse group of RNA species generated from the larger part of the genome that produces noncoding transcriptions responsible for the regulation of gene expression and protein function.[8] NcRNA are thought to be important contributors to human disease, as they have been linked to many complex biological processes, such as immune cell development and function, neural development, immune disorders, neurological diseases, and cancer.[8] With this, a promising approach for the treatment of various diseases is the utilization of PNA to target naturally occurring ncRNA such as miRNA, lncRNA, and rRNA. Below are the mechanisms by which PNAs target different ncRNAs.

Decreased miRNA-mRNA interactions

MiRNA has gained much interest, given its physiological role in various biological processes. MiRNAs are small (∼22–24 nucleotide) ncRNAs that bind with mRNA transcripts with partial complementarity and recruit the RNA-induced silencing complex (RISC) complex to the target site resulting in mRNA degradation and translational repression. Hence, miRNAs are responsible for the regulation of gene expression at the post-transcriptional level via sequence-selective targeting of mRNA.[9,10] Given that a single miRNA can target several mRNAs, it is estimated that up to 40% of human mRNAs contain miRNA binding sites. Depending on the complementarity between miRNA and mRNA target sequences, targeting miRNA can result in translational repression. miRNAs typically interact

with the 3′ untranslated region (3′UTR) of mRNA. Some studies also reported binding to coding sequences and 5′UTR. One example of mRNA target recognition involves the incorporation of miRNA into the multimeric protein-RNA complex, RISC. Anti-miRNA PNA can bind miRNA and consequently prevent its interaction with mRNA sites. Upregulation of miRNA expression is seen in several diseases and cellular processes, such as carcinogenesis and immune development.[9,10] miR-155 is upregulated in various cancers like lymphoma and leukemia.[11] mi-21 is upregulated in glioblastoma.[12] Hence, modulating the expression of these miRNAs using anti-miR-based synthetic nucleic acid analogs can be a potential therapy for various diseases, including cancer. PNAs and chemically modified PNAs have been extensively used to target miRNAs like miR-155, and miR-21. Anti-miRNA PNA binds to miRNA and acts as a "sponge" to block the ability of the miRNA to interact with its mRNA targets.

Thus, the noted ability of PNA to alter biological functions of miRNA, both *in vitro* and *in vivo*, offers a novel mechanistic approach for the development of several disease therapeutics. Additional examples of PNA leading to decreased miRNA-mRNA interactions are provided in the section discussing antisense applications as anti-cancer agents.

Decreased lncRNA-PRC2 interactions

lncRNAs are large transcripts (>200 nt in length) that contain two types of functional elements: interactor elements, which interact with other nucleic acids, proteins, or lipids, and structural elements, which form secondary and/or tertiary 3D RNA structures for functional interactions.[13,14] Allowing lncRNA to function in more variable ways than miRNA is their ability to interact with DNA, RNA, and proteins via base pairing in their linear form or chemical interactions in their secondary structure. lncRNAs regulate many key biological processes such as nuclear transport, miRNA activity, and epigenetic regulation. One of the most significant roles of lncRNA is the regulation of gene expression via a mechanism involving interaction with the epigenetic silencing complex, polycomb repressive complex 2 (PRC2). The PRC2 complex is unable to target and silence genomic regions by itself and relies on lncRNA to guide silencing at specific regions of the genome where they associate. Given the ability of lncRNA to direct epigenetic silencing through PRC2 association, their overexpression has been linked to the silencing of tumor suppressor genes and production of malignant cancerous phenotypes. The PRC2 complex contains three core protein subunits (EZH2, EED, and SUZ12) that aid in methyltransferase activity associated with transcriptional repression.[13,14] Thus, PNA can be used to inhibit the binding interactions between the subunits of the PRC2 core catalytic heterotrimer (EZH2-EED-SUZ12) and PRC2-interacting lncRNA, which serves as a promising approach for the development of small molecule interventions for cancer therapy. Additional examples of PNA leading to decreased lncRNA-PRC interactions are provided in the section discussing antisense applications as anti-cancer agents.

Translational inhibition due to PNA-rRNA interaction

Natural antibiotics exert their anti-microbial effects by binding to ribosomal RNA (rRNA) and disrupting protein synthesis.[15,16] To date, several studies have demonstrated bacterial cell growth inhibition via the binding of PNA oligomers to various functional rRNA domains. Three-dimensional structures of bacterial ribosomes, determined by X-ray crystallography, have aided in the identification of potential PNA target regions within rRNA domains.[15] The ribosomal subunits, 30S and 50S in bacteria, interact non-covalently via several intersubunit connections.[16] These connections are primarily based on RNA-RNA interactions and facilitate the association and signal transmission between both subunits. With respect to intersubunit interference, PNA can strand-invade and disrupt the peptidyl transferase center (PTC) and α-sarcin loop of 23S rRNA in the 50S subunit, resulting in an alteration of enzymatic function and inhibition of translation. PNA can also target the Helix 69 (H69) fragment of 23S rRNA, which plays an essential role in ribosome functioning and cell survival. Translational inhibition has also been observed by PNA targeted against the mRNA binding site of 16S rRNA in the 30S subunit, and many other 16S rRNA regions that interact with translation factors. Many regions in both subunits of rRNA serve as potential targets for sequence-specific binding and inhibition of ribosome function by PNA. PNA possesses the ability to block DNA and RNA polymerase, ribosome progression, and telomerase activity, making them ideal therapeutics as anti-infective agents.[15–17] Additional examples of PNA leading to translational inhibition are provided in the section discussing antisense applications as anti-pathogenic agents.

CELLULAR DELIVERY AND UPTAKE OF PNA

Oligonucleotides are large and hydrophilic, meaning they do not readily pass through plasma membranes, which is essential to their function intracellularly.[18] A large barrier to effective delivery of PNA to target sites has been penetrating the cellular membranes of target cells. There are various methods being explored to increase cellular uptake of PNA in order to increase their clinical applications (Figure 3).

Cell-penetrating peptides

When conjugated to PNA, cell-penetrating peptides (CPPs) represent a reliable mechanism of transporting PNA across cellular membranes and were the first method used successfully for PNA delivery.[7] There are both naturally and synthetically derived CPPs. CPPs aid in the cellular uptake of PNA via numerous mechanisms, which are dependent on the specific organism that they are entering. There is evidence for endocytosis and energy-independent processes for CPP transport; it is currently believed that endocytosis is the most common mechanism at low concentrations.[19]

Natural CPP

Natural peptide-derived CPPs include penetratin from the *Antennaphedia homeodomain,* Tat from the human immunodeficiency virus (HIV)-1 TAT protein, transportan from *galanin* and *mastroparan,* and nuclear localization signal (NLS) peptide.[7] Different peptides preferentially localize in different regions within target cells. For example, transportan localizes in membranous structures of cells, whereas penetratin and NLS localize within the nucleus. Additionally, pH-low insertion peptides (pHLIP) translocate PNA across membranes of cells expressing a low pH of 6.2, and have been used in targeting tumors

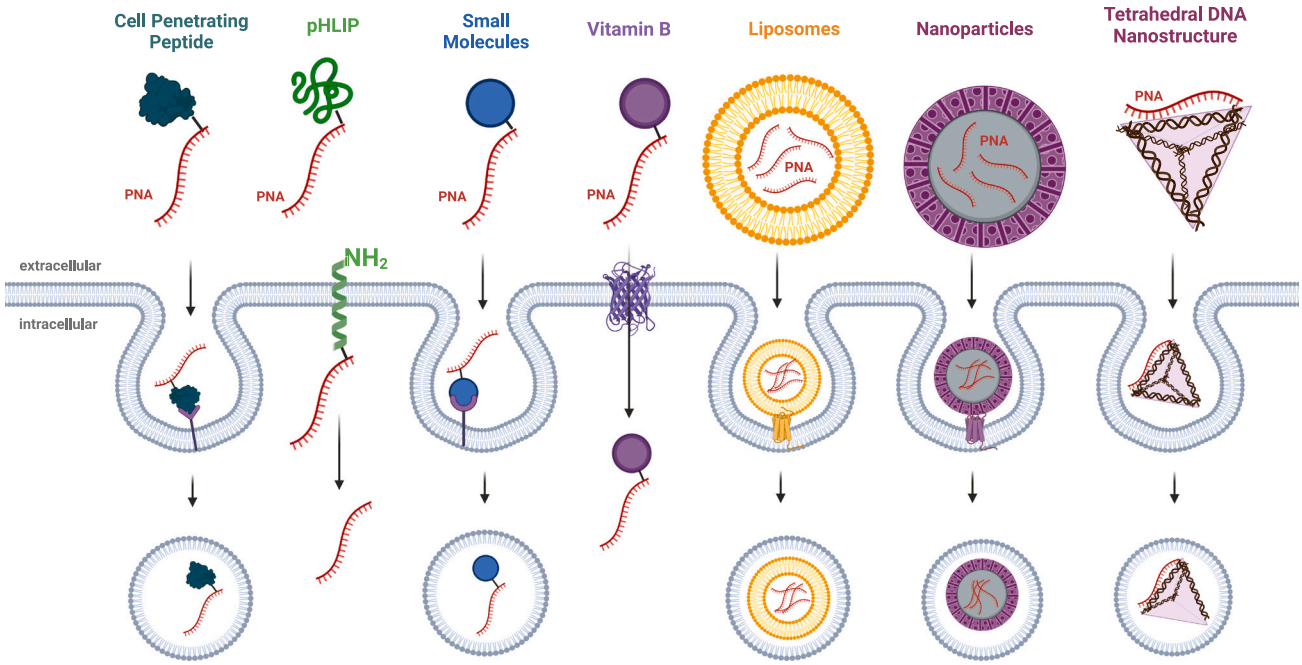

Figure 3. Methods of assisted PNA entry across cellular membranes

Methods of assisted PNA entry across cellular membranes include conjugation to cell-penetrating peptides (CPPs) (both natural and synthetically derived), conjugation to small molecules and vitamin B, liposomal encapsulation, nanoparticle encapsulation, and association with tetrahedral DNA nanostructures (TDN).

in vivo, which inherently express an acidic microenvironment.[7,20,21] PHLIP undergoes cellular translocation via the formation of alpha helical conformation in the acidic tumor surface. Traditionally, drug-like molecules are restricted to sizes of ~500 Da due to limitations crossing the membrane; however, with the addition of facilitating pHLIP conjugations, it is possible to deliver PNA up to ~7 kDa, in a pH-dependent manner, while retaining PNA's tumor-targeting capabilities.[21] Initially, Cheng et al. showed that when pHLIP was conjugated to antimiR-155 PNA, it resulted in effective inhibition of miR-155 oncomiRNA yielding improved phenotypic effects in a mouse lymphoma model.[22] Kaplan et al. established the efficacy of pHLIP-PNA in targeting non-homologous end-joining factor Ku80, which is involved in repairing double-strand breaks within DNA.[20] PHLIP-PNA, when conjugated with a partial mini-PEG sidechain substitution at the gamma position (γPNA) was able to deliver specifically to acidic cancer cells and tumor growth was substantially reduced, with no evidence of acute toxicities observed.[20]

Synthetic CPP

It is also possible to derive CPP from synthetic sources. Linking cationic lysines to the C terminus of PNA molecules increases cellular uptake of PNA.[7] Further, increasing the number of lysines increases activity of PNA *in vitro*. In studies investigating the importance of the number of lysines added to the C terminus, Wright et al. found that reducing the lysine tail to four repeats reduces antisense activity of PNA by 2-fold, and further decreasing to two lysine repeats is correlated with a complete elimination of antisense activity.[7,23] In the same study, coadministration of lysine-conjugated PNA with protective antigen of anthrax (PA protein) was associated with further increased antisense PNA activity.[23] When conjugated to anti-bacterial PNA, the synthetic anti-bacterial peptide $(KFF)_3K$, which contains three repeating units of lysine and phenylalanine followed by another lysine amino acid has proven to promote cellular entry and antisense PNA activity.[24] In HeLa cells infected with *Escherichia coli*, PNA targeting the mRNA and rRNA of acyl carrier protein (acpP) and conjugated with $(KFF)_3K$ prevents cellular growth, without any apparent toxicity to the HeLa cells.

Small molecules

Similar to CPPs, the covalent conjugation of PNA to small molecules has proven to be a successful method for promoting cellular delivery. N-acetyl galactosamine (GalNAc) is a molecule that targets the asialoglycoprotein or galactose receptors, which are abundantly present on hepatocytes within the liver. Upon binding to the receptor, GalNAc undergoes clathrin-mediated endocytosis to enter the cell. GalNAc conjugates have successfully been facilitated within antisense and RNAi-based oligonucleotide therapies including gicosiran, lumasira, vitrisiran, and inclisiran.[25] Recently, Kumar et al. described *in vitro* and *in vivo* studies of di lactobionic acid (diLBA) and tris N-acetyl galactosamine (tGalNAc) conjugated to PNA targeting miR-122, which is overexpressed in hepatic tissue.[26] DiLBA and tGalNAc conjugates showed significant retention in hepatocytes following systemic, subcutaneous delivery. The full-length PNA supported the downregulation of miR-122 levels and subsequent de-repression of downstream targets, with no toxicities observed.[26] A successful method of delivery across a cellular membrane is to link the PNA

to a substance that is essential for the survival of the target cells. This has been validated via covalently conjugating vitamin B_{12} and PNA through a cleavable disulfide link.[7] Bacterial cells are not capable of creating their own vitamin B_{12} and rely on environmental sources. This intrinsic need for environmental vitamin B_{12} uptake can be used as a method for transporting PNA into cells. Within *E. coli*, vitamin B_{12} transport occurs via a TonB-dependent transport system and is recognized by the Vitamin B_{12}-specific receptor, BtuB.[27] By covalently linking vitamin B_{12} to a PNA targeting the mRNA of a reporter gene, Rownicki et al. demonstrated that vitamin B_{12} transported PNA into *E. coli* cells via a rapid energy-independent phase in which the molecule associates with a receptor on the outer membrane. The initial energy-independent phase is followed by a slower, energy-dependent phase, facilitating additional membrane proteins. This transport method was noted to occur more efficiently than the widely accepted synthetic CPP, $(KFF)_3K$.[28] It was noted that the attachment of vitamin B_{12} could negatively impact PNA hybridization with a targeted mRNA transcript.[28] However, the antisense activity of PNA was still visible and effects on hybridization were similar to what is observed for the $(KFF)_3K$ carrier.

Liposomes

PNA can be encapsulated in liposomal formulations, which can transport large oligomers across cellular membranes via endocytosis, either through caveolae or clathrin receptors present on the cell surface. PNA oligomers encapsulated in a liposomal formulation composed of egg phosphatidyl choline (PC)/cholesterol/DSPE-poly ethylene glycol 2000 (PEG) showed promising results in human erythroleukemic K562 cells.[29] In these liposomes, the egg PC represents a type of phospholipid forming the membrane, cholesterol serves to stabilize the membrane and add fluidization effects, and the PEG chains add flexibility to the liposomal surface while also preventing liposomal interaction with macrophages, providing stealth characteristics and increased circulation times.[29] Liposomes loaded with PNA or fluorescent PNA oligomers resulted in efficient and quick uptake by the K562 cells and downregulated target miR-210.[29]

Nanoparticles

Poly(lactic co-glycolic acid) (PLGA) is a commonly used polymer for drug delivery approved by the Food and Drug Administration (FDA) for multiple clinical applications.[30] It is readily taken up by cells and degrades into non-toxic metabolites, which makes it a good vehicle for *in vivo* drug delivery.[30] A common mechanism of uptake of PLGA nanoparticles across the cell membrane is by a combination of fluid phase pinocytosis and clathrin-mediated endocytosis.[31] PEG polymers can be added to the PLGA particle, conferring stealth characteristics allowing the particles to evade the reticulo-endothelial system and remain circulating in the blood for increased time.[30] Babar et al. showed that PLGA nanoparticles (NP) can be used to deliver antisense PNA for targeting miRNA and mRNA, and that cellular uptake is further enhanced when CPP penetratin is added to the NP surface.[11] Similarly, Gupta et al. encapsulated a chemically modified PNA (mini-PEG-γPNA) in PLGA NP to inhibit oncomiR-210, resulting in an anti-cancer effect.[32] Additionally, in an HIV model, PLGA NP efficiently delivered antisense γPNA targeting the mRNA of entry-associated chemokine receptor 5 (CCR5) membrane protein receptors in THP1 cells.[31] *In vitro* assays demonstrated reduced CCR5 expression when PLGA NP loaded with γPNA were administered. By preventing the interaction of HIV with CCR5 on the CD4+ T cell membrane, the virus is unable to enter and infect the cell and viral progression is halted.[31]

Tetrahedral DNA nanostructures

A majority of existing PNA cellular delivery methods are non-specific and inefficient when attempting to target a desired sequence of RNA. Utilizing vector technology serves as an additional novel mechanism of oligonucleotide transport.[33] Tetrahedral DNA nanostructures (TDNs) are a method of DNA nanotechnology that possess structural stability and rigidity, and are able to enter cells autonomously, in size-independent fashions.[34] TDNs are able to minimize electrostatic repulsion in the membrane and enter the cell without the assistance of transfection agents. Some studies have suggested that TDNs enter cells via a caveolin-dependent endocytosis pathway.[34] Recently, Zhang et al. developed an antisense PNA that was incorporated into a TDN, which was effectively taken up by cells.[33] The antisense PNA was designed to target and inhibit the filamenting temperature-sensitive mutant Z (ftsZ) gene in methicillin-resistant *Staphylococcus aureus* (MRSA). However, it was noted that the TDN-PNA structure was more susceptible to degradation versus naked TDN, indicating that additional study is warranted regarding how to improve stability of TDN cargo.

Endosomal escape

When ASOs cross the plasma membrane, they are endocytosed into target cells and must escape the vesicle in order to enter the cell's cytoplasm. Although there have been recent advancements in strategies allowing the delivery of ASOs across membranes and into target cells, endosomal entrapment still limits the availability of ASOs within target cells.[35] In order to investigate the intracellular trafficking and endocytic recycling of PNA, Malik et al. began by first developing two antigen-conjugated PNA complementary to miR-155 and miR-21, and subjecting these PNAs to temperature-dependent cellular uptake studies. Flow cytometry analyses yielded a 50% decrease in cellular uptake at low temperatures (4°C vs. 37°C), indicating endocytosis is the primary uptake mechanism for PNA. They also demonstrated that, within an HeLa cell model, PNA that is endocytosed is trafficked to the endocytic recycling compartment, where it is then sent back to the plasma membrane via multivesicular bodies. Rabs are GTP-binding proteins that regulate vesicle fusion and release from the plasma membrane. When rab11a and rab27b were knocked down, a higher accumulation of PNA occurred within the HeLa cells, which also yielded higher anti-miRNA activity.[35] This confirms the role rabs play in shuttling vesicles containing PNA back to the plasma membrane. This study established the need for further investigation into methods to enhance endosomal escape of PNA upon delivery to target cells.

ANTISENSE APPLICATIONS AS ANTI-PATHOGENIC AGENTS

Anti-microbial resistance (AMR) is explained by the World Health Organization (WHO) as the presence of resistance to anti-microbial

medications in infectious agents such as bacteria, fungi, and viruses.[36] In 2019, WHO had classified AMR as one of the top 10 global public health threats facing humanity, due to the accumulating number of organisms acquiring resistance and a decrease in the number of therapeutic agents available to combat such organisms and the infections they cause.[36] An increase in bacterial resistance and subsequent decrease in our ability to combat bacterial infections creates a compounded threat to effectively treat patients who have an increased risk of infection. This results in increased cost and duration of treatment for patients, thus representing a significant burden on patients and healthcare systems alike. The pathogens associated with the highest rate of death include *E. coli, Staphylococcus aureus, Klebsiella pneumonia, Streptococcus pneumonia, Acinetobacter baumannii*, and *Pseudomonas aeruginosa*.[37] Cumulatively, in 2019, these organisms were associated with 3.57 million deaths and were solely attributed to causing over 900,000 deaths.[37]

Current mechanisms of anti-microbial agents focus on targeting functional components of infectious organisms and inducing errors in their activity. Common mechanisms of anti-microbial agents include targeting and inhibiting cell wall formation, blocking bacterial support proteins, and inhibiting pathogen potentiation at the ribosome. However, resistance to existing therapeutics has been perpetuated by misuse and overuse, coupled with a decline in the development of novel anti-pathogenic mechanisms of action.[38] The ability to evade anti-microbial therapeutics can be acquired by infectious organisms via horizontal transfer of antibiotic resistance genes from one organism to the other, or it can arise via evolution or spontaneous generation within the genome.[37,38] Most notably, via conjugation, resistance genes can be transferred from one bacterium to another via direct physical contact. Additionally, adaptive resistance arises via environmental stressors triggering rapid and transient bacterial response and is most often caused by epigenetic alterations in genetic expression.[38,39]

Viruses possess unique differences when compared with bacteria, which influence the mechanisms of available anti-virals. Whereas bacteria can replicate autonomously, viruses depend on entrance into host cells in order to hijack the host's replication machinery and reproduce. It is for this reason that anti-viral therapies are more difficult to create. Targeting viruses directly would inherently target host cells; preventing viral replication would prevent host cell replications, resulting in toxic effects on the host. Therefore, most anti-viral therapies are limited in their mechanisms, and rely on the targeting of accessory, support, or regularory proteins essential to viral survival within the host.

PNA as an anti-pathogenic agent can target and modulate the activity of a large collection of support genes or genes inherent to the activity of an infectious agent (Table 1). By creating a PNA sequence able to target a bacterial or viral agent or necessary supporting proteins, inner cellular functions are disrupted, and the pathogen fails to replicate and survive within its host.

Anti-microbial agents

Many potential gene targets have been identified in pathogenic microbials, and the minimum inhibitory concentrations of CPP-PNA conjugates have been determined.[15] Thus far, the most effective anti-microbial PNAs were those designed to target mRNA segments in close proximity to the start codon.[15] In these scenarios, PNA antisense mechanisms work via steric hindrance to block translational activity, resulting in downregulation of proteins.

Targeting the mRNA of genes essential to bacterial survival has been recognized as a promising method of inhibiting bacterial cellular growth. Functions disrupted and their corresponding gene targets include DNA replication (DNA gyrase A [gyrA]), DNA transcription (sigma 70 [rpoD]), folate biosynthesis (dihydrofolate reductase [folA/P]), cell wall biogenesis (UDP-N-acetylglucosamine enolpyuvyl transferase [murA]), and fatty acid biosynthesis (acyl carrier protein [acpP]), among others.[15] One of the best-established targets of antisense PNA *in vivo* is the fatty acid biosynthesis ACP protein encoded by the acpP gene.[40] In bacteria, the ACP protein plays a critical role in supporting fatty acid biosynthesis via donating activated fatty acid groups. Thus, depleting acpP results in the inhibition of growth and normal cell functions ultimately resulting in death. Tan et al. investigated the ability of acpP targeting antisense PNA to inhibit intraperitoneal (i.p.) *E. coli* growth in a BALB/c mouse model. Of note, a mutant strain of *E. coli* was used (SM105/101), which possesses a membrane with low lipid A content, yielding a membrane with higher permeability to high molecular weight molecules such as PNA. Mice were injected intra-abdominally with the *E. coli* to induce peritonitis, and were administered PNA or PNA-$(KFF)_3K$. PNA and peptide-PNA conjugate significantly reduced bacterial load, decreased proinflammatory serum cytokine production (tumor necrosis factor alpha [TNF-α], interleukin [IL]-1β, IL-6, and IL-12), and prevented fatal infection in mice infected with *E. coli*, resulting in a 100% rescue effect 7 days after PNA treatment.[40]

Additionally, antisense PNAs target and regulate expression of rRNA. Ribosomal targets that result in decreased cell proliferation include the PTC, elongation factors, and specific mRNA binding sites located on rRNA.[15] Within *E. coli*, the 23S rRNA on the 50S subunit contains a hairpin loop, referred to as H69, which is a conserved and essential element involved in ribosomal functioning.[41] Using a plasmid vector model, Kulik et al. demonstrated that PNA targeted to H69 of *E. coli* 23S rRNA *in vitro* resulted in hairpin strand invasion, blocked translation, and inhibition of *E. coli* growth.[41] Importantly, it was noted that after conjugation of PNA with $(KFF)_3K$ (three repeating units of lysine and phenylalanine followed by another lysine amino acid), the half maximum inhibitory concentration (IC_{50}) of PNA required to exert significant effects had increased, likely due to the charge of the conjugated peptide and high molecular weight compared with free PNA.[41]

Various studies have investigated the synergy accomplished when PNA technologies are coupled with antibiotic administration. Many mechanisms of AMR arise due to adaptive bacterial mechanisms circumventing anti-microbial susceptibility.[39] Thus, targeting resistance genes and re-establishing susceptibility to existing antibiotics has proven to be an area of interest. MRSA arises due to mutations in penicillin-binding proteins (PBP2a), conferring resistance to

Table 1. Anti-pathogenic PNA targets and respective molecular/phenotypic effects

Organism	Target gene	PNA design	Mechanism of PNA action	Route of delivery	Model	Molecular/Phenotypic effect	Reference
E. coli	acpP (mRNA)	10-mer PNA, conjugated with $(KFF)_3K$	Translational arrest	i.p.	BALB/c mice	Decreased CFU, cytokine production Increased survival	Tan et al.[40]
E. coli	H69 of 23S ribosome (rRNA)	13-mer PNA, conjugated with $K(KFF)_3$	Translational arrest	Cellular transfection	*In vitro,* pGEM-Gal DNA plasmid	*E. coli* cell death	Kulik et al.[41]
MRSA	mecA (−7 to +3 bp)	10-mer PNA conjugated with peptides	Translational arrest	Cellular transfection	*In vitro* MRSA cell lines	Decreased mRNA Increased oxacillin sensitization	Goh et al.[42]
	ftsZ (−4 to +6 bp)	10-mer PNA, conjugated with $K(KFF)_3$				Decreased CFU, mecA mRNA levels, cell size, cellular integrity, cellular growth	
inh M. tuberculosis	inhA (−5 to +7 bp)	12-mer PNA, conjugated with $K(KFF)_3$	Translational arrest	Inter-macrophage injection	THP-1 monocyte cells	Decreased bacterial load Increased anti-bacterial efficacy	He et al., Cotta et al.[43,44]
inh M. smegmatis	inhA	10-mer PNA, conjugated with $K(KFF)_3$					
Duck HBV	Epsilon signal	15-mer PNA, conjugated with poly arginine (Arg_7) or NLS	Translational arrest	Added to culture medium	*In vitro*, PDH	Decreased reverse transcriptase activity, DHBV synthesis	Ndeboko et al., Robaczewska et al., Ndeboko et al.[45–47]
				i.v., i.p.	*In vivo*, Ducklings	Decreased viremia and intrahepatic replication	
HIV	TAR (mRNA)	16-mer PNA, conjugated with penetratin, TAT, transportan-27, transportan-21, transportan-22	Translational arrest	Cellular transfection	*In vitro*, CEM cell model	Decreased reverse transcriptase activity, HIV infectivity (virucidal)	Pandey et al., Tripathi et al.[48,49]
HIV-1	PTT (mRNA)	13-mer PNA, unconjugated	Translational block	Cellular transfection	*In vitro*, CMV	Decreased viral protein production	Tripathi et al., Boutimah-Hamoudi et al.[49,50]

methicillin, oxacillin, and other beta lactam antibiotics.[42] Within MRSA, mutations within the mecA gene code for mutated versions of PBP2a, which are most commonly attributed to cause resistance. Goh et al. found that an antisense PNA targeting the mecA gene restored sensitization to oxacillin and reduced mecA mRNA level *in vitro*. Various PNAs were used to target different regions around the start codon; the most successful for targeting mecA mRNA was determined to be a 10-mer PNA, which was investigated independently and conjugated to a carrier peptide $[(KFF)_3K]$. Additionally, Goh et al. used antisense PNA to target ftsZ mRNA, a gene essential for cell division and maintaining cell wall integrity in *S. aureus*. Multiple regions of RNA were targeted by different PNAs, with the most successful being −4 to +6 around the start codon. Results yielded cellular damage indicated by cellular swelling, growth inhibition, and restored oxacillin susceptibility.[42] This represents the *in vitro* proof of concept that this method can be used to reverse resistance against MRSA.

Additionally, antisense PNAs have shown promise alongside traditional therapies in combating tuberculosis by targeting the inhibin alpha (inhA) gene. The inhA gene codes for an enoyl acyl carrier protein reductase involved in the fatty acid synthesis pathway of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*.[43,44] Anti-inhA PNAs were developed to target sequences around the start codons in *M. tuberculosis* and *M. smegmatis*. Colony forming units (CFU) of *M. tuberculosis* were investigated in situations of monotherapy with anti-inhA PNA as well as when anti-inhA PNA was combined with either ethambutol, ceftazidime, or colistin. Ethambutol, ceftazidime, or colistin are permeabilizing drugs that increase cell wall permeability, and allow increased uptake of anti-inhA PNA at lower concentrations.[44] THP-1 monocyte cells were infected with *M. tuberculosis* or *M. smegmatis* before being treated with PNA, colistin, ceftazidime pentahydrate, ethambutol, or combinations of permeabilizing drug plus PNA. When combined with permeabilizing drugs, anti-inhA PNA resulted in enhanced anti-bacterial efficacy in

both *Mycobacterium* strains. Ethambutol yielded the highest effect on membrane permeability, resulting in reduction in bacterial load in both *M. tuberculosis* and *M. smegmatis*. This suggests the combination of anti-inhA PNA with current anti-tuberculosis medications could yield improved therapeutic effectiveness, especially against drug-resistant strains.[44]

Anti-viral agents

PNAs have shown promising anti-pathogenic effects by targeting viruses such as hepatitis B virus (HBV) and HIV. Although there is a prophylactic vaccine for HBV, the disease still poses a significant issue to public health and those infected are at increased risk of developing chronic, severe liver diseases. Additionally, current HBV therapies primarily induce a virostatic effect, leading to high rates of rebound infection after treatment is completed. Ndeboko et al. investigated the efficacy of antisense PNA targeting the epsilon signal in duck HBV (DHBV), which is the hepadnavirus encapsidation signal and is critical in viral reverse transcriptase activity.[45] Results were captured *in vitro* in primary duck hepatocytes (PDH) and *in vivo* in ducklings.[45] *In vitro*, anti-epsilon PNA induced a dose-dependent anti-viral effect, via decreasing HBV reverse transcriptase activity early in mechanism.[45–47] *In vivo* studies using primary duck hepatocyte cultures infected with HBV supported *in vitro* results, showing that anti-epsilon PNA decreased viral DNA by 30% when compared with untreated controls.[45] Additionally, the anti-viral activity of various standalone CPPs were investigated, and it was determined that $(D\text{-}Arg)_8$ and Decanoyl-$(D\text{-}Arg)_8$ were able to inhibit the replication of HDBV. This suggests that CPPs possess unique ability to augment the activity of antisense PNA, and the decision regarding which specific CPP to use may assist in specificity of inhibition.[45]

The standard of care for treating HIV consists of treatment mechanisms that target the enzymes protease and reverse transcriptase.[48] HIV is a virus prone to rapid genome variation leading to development of strains resistant to available treatments. Newer drugs being explored include those targeting accessory and regulatory proteins of HIV. An extensively investigated potential mechanism of anti-HIV therapy consists of antisense PNA targeting sequences of the viral genome that are essential for HIV cell function and replication. One established potential target for anti-HIV PNA is the HIV-1 transactivator response (TAR) regulatory region of the genome contained within the long terminal repeat region.[48] CEM-line cells were first infected with HIV-1 virions, which possessed a bioluminescence luciferase gene to enable detection of HIV-1. The anti-TAR PNA was incubated with the CEM cells, and HIV-1 levels were quantified via analyzing luciferase activity. Anti-TAR PNA effectively inhibited reverse transcription of TAR-RNA, decreasing HIV-1 replication.[48,49] When conjugated to carrier peptides to assist with cellular entry, the anti-TAR PNA exhibited potent virucidal properties, leaving HIV cells non-infectious.[48,49] None of the CPPs resulted in decreased affinity of PNA to target RNA.[49] The most effective CPP was penetratin, which exhibited the highest virucidal activity and lowest required dose.[49] Another target site within HIV-1 is the highly conserved polypurine tract (PPT) sequence.[48,50] Using streptolysin-O (SLO) permeabilized cells transformed with luciferase and GFP for visualization, anti-PPT PNA was incubated with cells to allow for hybridization to target sequences.[50] Anti-PPT PNA arrested *in vitro* translation elongation, resulting in truncated viral proteins that were unable to carry out crucial cellular functions.

ANTISENSE APPLICATIONS IN NEURODEGENERATIVE DISEASES

PNA can be used in neurodegenerative diseases to target mutant transcripts, reduce or restore protein expression, or modify proteins via the removal of disease-causing mutations.[51] Through these mechanisms, PNAs have been used to successfully target mutant genes associated with Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD) in preclinical study models (Table 2).

While strong preclinical data exist for the application of PNA in these neurodegenerative diseases, delivery to the central nervous system (CNS) poses a potential challenge. To enter the CNS, PNA needs to cross either the blood brain barrier (BBB) or blood-spinal cord barrier.[51] Given that these vascular barriers prevent most molecules from entering the CNS from peripheral circulation, delivery techniques beyond systemic administration must be considered. In addition to systemic administration, PNA can be delivered to the CNS via the intracerebroventricular (ICV), intrathecal (IT), IP, or intranasal (IN) route.[51]

Alzheimer's disease

AD is the most common form of dementia, characterized by intraneuronal neurofibrillary tangles and extracellular amyloid plaques.[51] Amyloid plaques are composed of amyloid-β (Aβ) peptides, which are proteolytic fragments of the transmembrane amyloid precursor protein (APP). A key event in the formation of amyloid plaques is cleavage of APP at the β-secretase site by the β-site APP-cleaving enzyme (BACE), which ultimately results in an overproduction of Aβ peptides.[51,52] Aβ(1–40) and Aβ(1–42) are the Aβ peptide forms that cause plaque formation, increased aggregation at elevated levels, and consequent neuronal death in AD.[52] Targeting of the APP and BACE genes has become a fundamental strategy for developing disease-modifying therapies for AD.

Ongoing studies by McMahon et al. have reported successful PNA-mediated targeting of both the APP and BACE genes in AD rats and mice.[52–54] McMahon et al. administered i.p. injections of unconjugated, antigene -6s APP PNA and observed a significant decrease in Aβ(1–42) brain levels by 40% in rats.[52] In transitioning to a more transgenic animal model, McMahon et al. administered the same PNA sequence to mice and observed significant reductions in Aβ(1–40) brain levels by 37%, Aβ(1–42) brain levels by 47%, and Aβ plasma levels by 30%.[53] While successful PNA-mediated targeting of APP was achieved in these preliminary studies via an antigene mechanism, more recent results demonstrate successful PNA-mediated targeting of BACE via an antisense mechanism. McMahon et al. administered i.p. injections of unconjugated, sense +29 BACE PNA and observed a significant decrease in Aβ plasma levels by 25%.[53] Additionally, intraventricular administration of an

Table 2. Anti-neurodegenerative disease PNA targets and respective molecular/phenotypic effects

Disease	Target	PNA design	Mechanism of PNA action	Route of administration	Model	Molecular/Phenotypic effect	Reference
AD	BACE gene	PNA, unconjugated	Translational arrest	i.p.	AD mice	Decreased Aβ levels	McMahon et al.[53]
				ICV		Decreased BACE mRNA levels	McMahon et al.[54]
ALS	p75NTR gene	11-mer PNA, unconjugated	Translational arrest	Cellular transfection	Schwann cells	Decreased p75NTR levels Increased cell viability	Rembach et al.[57]
				i.p.	Trangenic, SOD1 mutant mice	Decreased p75NTR levels, capsase-3 activity, onset of locomotor impairment, increased lifespan	
	GluR3 gene	12-mer PNA, unconjugated	Translational arrest	Cellular transfection	NSC-34 cells	Decreased GluR3 levels Increased cell viability	Turner et al.[56]
				i.p.	Transgenic, SOD1 mutant mice	Decreased onset of locomotor impairment Increased lifespan	
					HEK-293 cells	Decreased expression of 4R isoforms	
HD	HTT gene	19-mer PNA, conjugated with CPP at C terminus	Translational block	Cellular transfection	GM04281 patient-derived fibroblast cells	Decreased mutant HTT	Hu et al.[58]
		13-mer PNA/16-mer PNA/19-mer PNA, conjugated with CPP at C terminus				Increased mutant HTT inhibition with decreased PNA length	
		19-mer PNA, conjugated with CPP at N terminus				Increased mutant HTT inhibiton with PNA conjugates at N terminus	

unconjugated, sense -7s BACE PNA significantly reduced BACE mRNA levels via transcription inhibition.[54]

Amyotrophic lateral sclerosis

ALS is a progressive neurodegenerative disorder characterized by the degeneration of motor neurons in the brain and spinal cord.[51] An accumulation of superoxide radicals due to mutant superoxide dismutase 1 (SOD1) accounts for approximately 15–20% of the neuronal degeneration observed in familial ALS.[55] In healthy individuals, SOD1 codes for the superoxide dismutase enzyme, which binds to molecules of copper and zinc to break down superoxide radicals and prevent cell damage.[51] In ALS patients, the inability of mutant SOD1 to break down these toxic byproducts leads to superoxide-induced cell death and poor prognosis. Mutant SOD1 has also been linked to a simultaneous, abnormal upregulation in both the p75 neurotrophin receptor (p75NTR) and glutamate receptor subunit 3 (GluR3) genes.[56,57] The roles of p75NTR and GluR3 activation and mediated pathways in mutant SOD1 toxicity and ALS pathogenesis is not well understood. However, the accumulation of p75NTR and GluR3 genes has been linked to an increase in ALS symptom severity and disease progression, making them attractive therapeutic targets.[56,57]

Turner et al. co-transfected Schwann cells with 11-mer, antisense PNA and observed a significant dose-dependent reduction in p75NTR levels by 28–39%.[56] Further analysis revealed the ability of PNA to inhibit direct ligand-dependent p75NTR-mediated death-signaling by β-nerve growth factor (βNGF). βNGF treatment decreased cell viability to 74%, but subsequent PNA treatment restored p75NTR levels up to 92–100%. I.p. injections of the same PNA into SOD1 mutant mice resulted in a significant decrease in p75NTR levels in lumbar spinal cord and kidney levels. A direct reduction in the apoptotic marker, caspase-3, was also observed. Phenotypic effects of PNA treatment included a 10% delay in the onset of locomotor impairment and mortality.[56]

In a similar study, Rembach et al. co-transfected neuroblastoma spinal cord (NSC-34) cells with 12-mer, antisense PNA and observed an abolition of GluR3 levels at high concentrations.[57] Further analysis revealed the neuroprotective and inhibitory effects of PNA on (S)-5-fluorowillardine (FW)-induced excitotoxicit. A significant increase in cell viability was observed in cells exposed to (S)-5-FW and treated with PNA. I.p. injections of the same PNA into SOD1 mutant mice resulted in a significant preservation in the onset of locomotor impairment and approximate 10% extension in survival.[57]

Huntington's disease

HD is a polyglutamine (polyQ) disorder, characterized by intracellular protein aggregates that result in the degeneration of neurons

in the striatum and cerebral cortex.[51,58,59] The neuronal degeneration observed in HD is caused by an abnormal expansion of CAG repeats in the first exon of the huntingtin gene (HTT). Mutant HTT results in a translated protein that contains disease-causing expansions of polyQ, making it prone to misfold and aggregate.[58,59] While the overproduction of mutant HTT drives HD progression, HTT plays major roles in the regulation of several important cellular functions, such as neuronal and glial cell development.[51] In diseases such as HD, where the downregulation of HTT could have detrimental consequences, specific lowering of the mutation-containing protein is an ideal therapeutic approach.

To block the neurodegenerative effects of mutant HTT and preserve the normal biological function of the wild-type allele, a study by Hu et al. used single-stranded, antisense PNA (PNA REP19 and PNA 3J/HTT) to target the repeat expansion directly.[58] The PNAs were designed complementary to mRNA outside of the CAG repeat to enhance specificity for mutant HTT mRNA relative to other cellular mRNA.[58] Cellular transfections with PNA REP19 and PNA 3J/HTT resulted in an 80% allele-specific decrease in the expression of mutant HTT.[51] Inhibition was characterized by IC_{50} values 0.34 mM and 0.96 mM, as well as 3.5-fold and 5-fold selectivities (wild-type IC_{50}/mutant IC_{50}).[58] The allele-selectivity of these molecular effects was then assessed by testing these PNA on representative genes containing CAG repeats (TATA box binding protein [TBP], atrophin-1 [ATN1], forkhead box protein P2 [FOXP2], AP2 associated kinase 1 [AAK1], and POU class 3 homeobox 2 [POU3F2]).[58] At concentrations sufficient for selective inhibition of mutant HTT, the expression of these genes remained unchanged.

In an attempt to optimize selectivity, the effects of varying PNA length and peptide conjugation were assessed. The inhibitory effects of PNA peptide conjugates that were 13 and 16 bases in length were compared.[58] Results yielded greater inhibition by the 13-mer (IC_{50} value of 0.47 mM) than the 16-mer (IC_{50} value of 0.39 mM), suggesting that shorter PNA can achieve potent and selective inhibition. The conjugate design was then modified to attach the peptide domain at the N terminus (PNA REP19N) rather than the C terminus (PNA REP19). In comparing the conjugates, PNA REP19N showed greater selectivity than PNA REP19. While an IC_{50} value of 2.1 mM for inhibiting mutant HTT expression was observed, no inhibition of the wild-type HTT was observed at concentrations as high as 16 mM[58]

Overall, CAG repeat-specific targeting was successfully achieved, with results yielding significant reductions in CAG-containing transcripts in HD patient-derived fibroblasts.[51,58] Findings also suggest that shorter PNA with conjugates at the N terminus yield optimal selective inhibition of mutant HTT.[51]

ANTISENSE APPLICATIONS AS ANTI-CANCER AGENTS

Many ncRNAs play key roles in human pathologies, including cancer. In various human carcinomas, certain miRNAs and lncRNAs have been proposed as therapeutic targets due to either their abnormal up-regulation or involvement in tumor onset and progression. PNA as an anti-cancer agent provides tumor-specific targeting of these potential biomarkers. To date, existing literature supports the use of PNA in glioblastoma (GBM), colorectal cancer (CRC), ovarian cancer, breast cancer, lymphomas, cervical cancer, lung cancer, and many additional cancer types (Table 3).

While comprehensive analyses have been conducted to reveal the expression profile of these ncRNAs in certain cancer types, tumor tissues are highly heterogeneous with respect to their molecular and genetic features, supporting the concept that variability of these targets may exist among patients.[60] Thus, the therapeutic intervention of using PNA targeting ncRNA in patients may require an analysis of tumor tissues prior to administration to confirm their expression pattern, which can be done via liquid or surgery-based biopsies.[60] To avoid the invasive nature of surgery-based biopsies, only ncRNAs with fully established roles in tumorigenesis should be considered as PNA targets.

Further barriers to the practical application of PNA in cancer remain successful administration and distribution at the tissue site. Methods for optimizing the local delivery of PNA are discussed in this section but warrant further consideration and research.

Glioblastoma

Glioblastoma (GBM) is one of the most common and fatal forms of malignant brain cancer. Over 200 miRNAs have been found to be dysregulated in GBM, but miR-21 is one of the most extensively studied miRNAs in the context of cancer biology due to its reported overexpression and role in various cellular processes, such as proliferation, migration, and apoptosis.[12] Inhibition of miR-21 disrupts the migratory ability of glioma cells through its regulation of the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), transforming growth factor β (TGF-β), epidermal growth factor receptor (EGFR), matrix metalloproteinase (MMP), and p53 pathways. Of additional benefit, anti-miRNA mediated suppression of miR-21 is thought to affect the sensitivity of GBM cells to anti-cancer agents, such as temozolomide (TMZ). Despite these proposed benefits, the BBB prevents the transport of anti-miRNA into the brain and rapidly clears any small amounts that do cross. Hence, there is a need for a local delivery strategy to provide sufficient levels of anti-miR 21 in brain tumors that can be sustained over a longer therapeutic window.[12]

Seo et al. developed a surface-modified poly(lactic acid) (PLA) NP formulation of a distinct miR-21 inhibitor for optimal delivery and intracranial distribution. They encapsulated miR-21-inhibiting PNA in PLA-based NP formulations with different surface chemistries (PLA and hyperbranched polyglycerol [PLA-HPG] and PLA-HPG and aldehydes [PLA-HPG-CHO]) to influence cellular tropism and tumor uptake into the brain post-convection-enhanced delivery (CED).[12] CED consists of a slow, continuous infusion of a therapeutic agent through catheters placed at the tumor site for regional delivery over a controlled volume of tissue. While CED has been established as a safe and effective method for bypassing the BBB and allowing significant drug exposure to intracranial tumors, a majority of trials

Table 3. Anti-cancer PNA targets and respective molecular/phenotypic effects

Disease	Target	PNA design	Mechanism of action	Route of administration	Model	Molecular/Phenotypic effect	Reference
GBM	miR-21	PNA, encapsulated in PLA-HPG and PLA-HPG-CHO NPs	Decreased miRNA-mRNA interactions	Cellular transfection	U87	Decreased cell viability, increased PTEN mRNA, sensitivity to TMZ treatment	Seo et al.[12]
				CED	Tumor-bearing rats	Decreased miR-21 expression, increased PTEN mRNA levels, median survival	
	miR-10b,	8-mer, γPNA, encapsulated in PLA-HPG-CHO NPs			Orthotopic GBM tumor model (U87)	Decreased miR-10 and miR-21 expression, delayed tumor growth, increased survival	Wang et al.[61]
	miR-21				PDX mice (G22)	Decreased miR-10 and miR-21 expression, delayed tumor growth, increased survival	
CRC	miR-425-3p, miR-584-5p	PNA, unconjugated	Decreased miRNA-mRNA interactions	Cellular transfection	HT-29 cells, LoVo cells	Decreased miR-425-3p expression, miR-584-5p expression, cell growth, increased apoptotic cell rate	Gasparello et al.[63]
Ovarian and breast cancer	HOTAIR	PNA, conjugated with CPP	Decreased lnc-RNA-PRC2 interactions	Cellular transfection	Multiple ovarian and breast cancer cell lies	Decreased cell survival, cell invasion, NF-κB activity, IL-6 expression, MMP-9 expression, ALDH1A1 expression, Increased CDDP activity	Özeş et al.[13]
		PNA, conjugated with pHLIP		i.v.	BALB/c nu/nu ovarian tumor-bearing mice	Decreased tumor volume, IL-6 expression, MMP-9 expression, ALDH1A1 expression Increased survival, CDDP sensitivity	
Lymphoma	miR-155	PNA, conjugated with CPP, ANTP, encapsulated in PLGA NPs	Decreased miRNA-mRNA interactions	Cellular transfection	Toledo cells	Decreased cell viability	Babar et al.[11]
					$miR\text{-}155^{LSLtTA}$ mice cells	Decreased miR-155 expression Increased SHIP1 expression	
				Tail vein injection	$miR\text{-}155^{LSLtTA}$(miR 155 addicted mouse mode)	Decreased miR-155 expression, tumor growth	

(*Continued on next page*)

Table 3. *Continued*

Disease	Target	PNA design	Mechanism of action	Route of administration	Model	Molecular/Phenotypic effect	Reference
		PNA conjugated with pHLIP		Cellular transfection	KB cells	Decreased cell viability	Cheng et al.[22]
				i.v.	miR-155^{LSLtTA}(miR 155 addicted mice)	Decreased miR-155 expression, reduced tumor growth, improved survival,	
					Mice with subcutaneous lymphoma flank tumors	reduced metastasis	
		Tail clamp anti-miRNA-155 γPNA with arginine units		Cellular transfection	U2932, SUDHL-5, and SUDHL-2 cell lines	Decreased miR-155 expression, decreased cell viability	Dhuri et al.[64]
				Intratumoral	U2932 cell line-derived xenograft mice	Decreased miR-155 expression, reduced tumor growth	
	miR-21, miR-155	Anti-miR-21 PNA PLGA NPs, anti-miR-155 PNA PLGA NPs		Cellular transfection	U2932, SUDHL-5, and SUDHL-2 cell lines	Decreased miR-155 expression, decreased cell viability	Dhuri et al.[65]
Cervical cancer	miR-210	22-mer γPNA, encapsulated with PLGA NPs	Decreased miRNA-mRNA interaction	Intratumoral	HeLa tumor xenograft mice	Decreased miR-210 expression, decreased tumor growth, improved tumor morphology and histopathology	Gupta et al.[32]

utilizing CED to treat GBM have failed to demonstrate therapeutic efficacy. In this study, PNAs were encapsulated into NP with the objective of slow degradation, sustained release, and maintenance of the physicochemical properties that deem them suitable for CED.[12]

In the initial stages of the study, PNA NP provided miR-21 inhibition and PTEN upregulation *in vitro*.[12] U87 cells were treated with the PNA NP and qRT-PCR results showed a 40–60% knockdown of miR-21 after 48 h of incubation. Additionally, the effect of miR-21 suppression was evaluated by measuring the levels of PTEN (a predicted target of miR-21 and a tumor suppressor that is inactive in GBM) and results revealed a 2- to 3-fold increase in PTEN mRNA levels, as well as a 30–40% reduction in cell viability at the highest treatment dose. The effect of miR-21 inhibition on tumor cell sensitivity to TMZ was then tested by treating cells with PNA NP in addition to TMZ compared with TMZ monotherapy. Viability assay findings indicated a significantly reduced cell viability in the co-treatment group compared with TMZ alone. Further differences were observed between the PLA-HPG NP and PLA-HPG-CHO NP, with the PLA-HPG-CHO NP demonstrating stronger synergy with TMZ at lower treatment doses, suggesting the possibility of TMZ dose reductions via concomitant administration.[12]

Later phases of the study employed PNA NP *in vivo* via intracranial CED.[12] U87 tumors were implanted into immunodeficient rats 10 days before a micro-infusion pump was used to infuse the PNA NP. The PNA NPs were administered by CED to the tumor site and the brains were harvested 2 days later. PCR analysis indicated that treatment with PLA-HPG and PLA-HPG-CHO NPs resulted in 53% and 49% knockdown of miR-21 expression compared with untreated rats. Consistent with the *in vitro* findings, treatment with the PNA NP resulted in a 1.8- to 3.4-fold increase in PTEN expression in tumors. Last, the study assessed whether miR-21-inhibiting NP delivered by CED would provide the same therapeutic benefit with TMZ *in vivo*. TMZ was administered by i.p. injection 1 day after the PNA NPs were administered by CED. The median survival without treatment was 24 days, and in the groups treated with PLA-HPG and PLA-HPG-CHO NPs, the median survival was extended to 29 and 28 days, respectively. Combination treatment with PLA-HPG-CHO NPs and TMZ further improved survival, resulting in a median survival of 49 days (log rank $p = 0.0001$).[12]

In summary, this study provides a new approach for local therapy of GBM utilizing NP as a delivery platform for miR-21-inhibiting PNA.[12] The PNA NP synthesized in this study enabled intracellular delivery and intracranial distribution, decreased miR-21 expression and increased PTEN expression, and demonstrated synergistic benefits with TMZ *in vitro* and *in vivo*. Although this work serves as a framework for the application of PNA in GBM, further research is needed to assess the potential off-target effects of this localized therapeutic approach.[12]

Similar methodology was employed by Wang et al. using short, chemically modified PNA substituted at the gamma (γ) position of the PNA backbone with serine amino acid (sγPNA) and entrapped in PLA-HPG-CHO (sγPNA/bioadhesive NP [BNP]).[61] The sγPNA/BNPs were designed to target the seed regions of miR-10b and miR-21 in a series of GBM cell lines (including patient-derived xenograft [PDX] cells G22 and human GBM cells U87). Results yielded improved survival in both an orthotopic GBM tumor model and PDX mice model. In mice bearing U87-derived intracranial gliomas receiving sγPNA/BNPs, median survival was increased to 53 days compared with 45 days in the untreated control group, demonstrating delayed tumor growth.[61] Further, combination treatment of sγPNA/BNP and TMZ greatly improved survival until the study endpoint of 120 days. The relative levels of miR-10b and miR-21 were also measured at the end of the tumor survival study and an *in vivo* knockdown of both oncomiRNAs was observed (∼72% decrease of miR-10b and 95% decrease of miR-21), indicating successful inhibition by combination treatment of sγPNA/BNP and TMZ. Additionally, toxicity assessments were promising, revealing no alterations in blood components, liver enzymes, or renal function markers.[61]

The same level of efficacy and safety was reported in the PDX mice model. As anticipated, combination treatment of sγPNA/BNP and TMZ greatly prolonged survival, with 80% of mice surviving to the study endpoint of 120 days and exhibiting normal physical activity compared with the untreated control group.[61] In alignment with the orthotopic GBM tumor model results, the relative levels of miR-10b and miR-21 were found to be low and comparable to healthy mice brains at the end of the survival study. Last, no differences were detected upon toxicity evaluation. Together, these results confirm the efficacy and safety of sγPNA/BNP in combination with TMZ. Additionally, this study supports PNA as a promising strategy to improve existing GBM treatment and provide a personalized treatment approach based on tumor-specific oncomiRNAs.

Colorectal cancer

Colorectal cancer (CRC) is the third most common cancer with respect to incidence and the second most common cancer with respect to mortality worldwide, as of 2020.[62] Analyses of the distinctive miRNA signature in the blood of early-stage CRC patients have demonstrated the presence of dysregulated miRNA in CRC onset and progression, specifically miR-584-5p and miR-425-3p.[63] Gasparello et al. used anti-miRNA PNA to evaluate the biological effects of targeting miR-584-5p and miR-425-3p and inhibiting the activation of the pro-apoptotic pathway in CRC cellular models.[63]

In this study, R8-PNAs were synthesized to target select miRNA: R8-PNA-a425 for miR-425-3p targeting and R8-PNA-a584 for miR-584-5p targeting, among others of smaller focus.[63] Once the expression of these miRNAs was confirmed in the colon cancer cell lines (HT-29 and LoVo cells), selective targeting with the R8-PNA was quantified and a selective downregulation was observed. Treatment with the R8-PNA also resulted in a slight inhibition of HT-29 cell growth, suggesting that inhibition of miR-425-3p and miR-584-5p in colon cancer cells is associated with anti-tumor activity *in vitro*.[63]

Although only a weak increase in the apoptotic cell rate was observed when the R8-PNAs were administered singularly, apparent synergism was noted when used in combination with other well-known

pro-apoptotic compounds, such as sulforaphane.[63] The synergism was quantified using the Chou-Talalay method with the following combination index (CI) ratings: a CI < 1 indicating synergism, a CI = 1 indicating additivity, and a CI > 1 indicating antagonism. The triple-treatment of R8-PNA-a584, R8-PNA-a425, and separately studied R8-PNA-a15b yielded a CI score of 0.69, while co-treatment of R8-PNA-a584 and R8-PNA-a425 with sulforaphane yielded CI scores of 0.44 and 0.40. Additionally, data demonstrated that treatment with each R8-PNA led to a very high proportion of apoptotic HT-29 cells when combined with sulforaphane (over 75% for both R8-PNA-a584 and R8-PNA-a425). These synergistic findings support the use of PNAs that target multiple miRNAs and the co-treatment of anti-miRNA PNA with anti-tumor agents as promising strategies to increase efficacy and potentially mitigate side effects.[63]

While these findings support the therapeutic benefits of PNA-based anti-miRNA molecules in CRC, it is important to recognize the high level of intratumor heterogeneity that exists with respect to the expression of cancer-related biomarkers, including the cancer-associated miRNAs proposed in this study.[63] As referenced in the literature, when considering patient-to-patient variability of miRNA upregulation, the personalized approach of using anti-miRNA PNA should be primarily employed to those with confirmed, differential target expression.

Ovarian and breast cancer

Aberrant lncRNA expression correlates with multiple carcinomas, including ovarian and breast cancer.[13] HOX antisense intergenic RNA (HOTAIR) is an lncRNA that is overexpressed in solid tumors and contributes to disease progression chemoresistance and poor patient prognosis. Mechanistically, lncRNA HOTAIR transcriptionally activates nuclear factor kappa B (NF-κB) during DNA damage and increases IL-6 and matrix metalloproteinase 9 (MMP-9) in platinum-resistant ovarian cancer. As discussed previously, PNA can alter gene expression by inhibiting the binding interactions between the subunits of PRC2, such as EZH2, and PRC2-interacting lncRNAs, such as HOTAIR. Özeş et al. used PNA to inhibit HOTAIR-EZH2 activity and resensitize resistant ovarian tumors to platinum, as well as decrease the invasion and increase the chemotherapy sensitivity of HOTAIR-overexpressing ovarian and breast cancer cell lines. Further effects of pHLIP-PNA on reduced tumor formation and improved survival were observed *in vivo* via the treatment of mice harboring platinum-resistant ovarian tumor xenografts.[13]

To confirm the effect of inhibiting HOTAIR and EZH2 on platinum sensitivity and cell behaviors, ovarian and breast cancer cell lines were treated with antisense PNA (PNA3) alone and in combination with a chemotherapeutic (cisplatin [CDDP] or etoposide). Following cell studies, BALB/c-nu/nu mice were injected with CDDP-resistant A2780_CR5 cells, followed by pHLIP-PNA3 and CDDP treatments biweekly for 2 weeks. When pHLIP-PNA and CDDP were co-administered, tumor volume was reduced and survival was increased by 64%.[13] Additional *in vivo* findings revealed an anticipated and positive correlation between tumor growth and IL-6 upregulation. In ovarian tumor-bearing mice, CDDP monotherapy increased blood IL-6 levels, while concomitant pHLIP-PNA3 and CDDP treatment resulted in reduced levels. Additionally, pHLIP-PNA3 monotherapy decreased the tumor expression of IL-6, MMP-9, and aldehyde dehydrogenase (ALDH1A1). ALDH1A1 induction by IL-6 is one of the hypothesized mechanisms behind chemoresistance in cancer stem cells. Similar to the effects of PNA3 observed *in vitro*, a reduction in HOTAIR targets and increase in both CDDP sensitivity and overall survival was observed *in vivo*. Despite no changes in weight among treatment and control groups and the ability of PNA to mitigate CDDP-induced adverse effects (e.g., increased liver and spleen size), data on the toxicity of PNA *in vivo* are still lacking.[13]

Lymphoma

Lymphoma is a cancer of the lymphoid system, which includes the lymph nodes, spleen, thymus gland, and bone marrow. miR-155 is an oncogenic miRNA that is overexpressed in lymphoid tissues and induced in several lymphomas, including diffuse large B cell lymphomas, Hodgkin lymphomas, and subsets of Burkitt lymphomas.[11] Further, miR-155 regulates several pathways involved in cell division and immunoregulation, and its induction in hematopoietic cells has been linked to a myeloproliferative presentation in lymphoid tissues. From a molecular perspective, miR-155 targets and suppresses SH2-containing inositol phosphatase (SHIP1), which is a negative regulator of myeloid cell proliferation and survival and positive regulator of apoptosis. Thus, compelling evidence exists for the involvement of miR-155 in lymphoproliferative diseases. A study by Babar et al. characterizes the role of miR-155 in the survival of these cancers using Cre-loxP and tetracycline-controlled transactivator (tTA) technologies. With respect to their application *in vivo*, Cre-loxP allows for control over the location and timing of gene expression, while tTa enables the ability to produce inducible and reversible gene expression. In this study, an miR-155 Cre-loxP tetracycline-controlled knockin mouse model (miR-155^{LSLtTA}) was designed to contain a tTA element that deactivated miR-155 induction upon exposure to doxycycline (DOX) for miR-155 expression control.[11]

Later phases of this study confirmed the delivery of anti-miRNA PNA to lymphocytes and subsequent effect of treatment on miR-155-addicted tumors.[11] To enhance delivery, antimiR-155 PNAs were encapsulated into PLGA polymer NP that were then conjugated to the CPP penetratin (ANTP). AntimiR-155 PNA delivery by ANTP-NP into human Toledo B cells decreased cell viability with an IC_{50} of 6.1 μM. Additionally, when SHIP1 was knocked down prior to treatment, efficacy was decreased further by 10-fold (to an IC_{50} of 90.1 μM). In addition to using Toledo cells for a model of human B cell lymphoma, the study isolated cells from the miR-155^{LSLtTA} mice and found that treatment with antimiR-155 PNA-containing ANTP-NP reduced functional miR-155 levels by 65%, as well as an expected decrease in the suppression of SHIP1 (confirmed by elevated SHIP1 levels). Together, these findings demonstrate the involvement of miR-155 in regulating apoptosis and the relevance of SHIP1 as a potential pro-apoptotic target of miR-155.[11]

Last, the study investigated the anti-tumor potency of antimiR-155 PNA *in vivo* by measuring tumor growth post-treatment with

antimiR-155 PNA-containing ANTP-NP.[11] Tail vein injections of 1.5 mg/kg for two treatments over the course of 3 days significantly delayed tumor growth and there was a 50% decrease in tumor growth after 5 days. Additionally, an anticipated decrease in miR-155 tumor levels was observed post-treatment. Overall, these studies propose a promising delivery system for the inhibition of oncomiRNA in lymphoma and show that a systemic dose of antimiR-155 PNA treatment is sufficient to achieve a significant therapeutic response.[11]

Cheng et al. demonstrated highly effective delivery of a pHLIP-PNA to target oncogenic miR-155 in cell culture as well as in two different mouse models derived from miR-155^{LSLtTA} mice.[22] AntimiR-155 pHLIP-PNA derepressed luciferase in miR-155-overexpressing KB cells that stably expressed an miR-155-targeted dual luciferase sensor. Further inhibition of miR-155 by pHLIP-PNA reduced viability of KB cells. Intravenous administration of antimiR-155 pHLIP-PNA antagonized miR-155 in tumors, delayed tumor growth, and suppressed the metastatic spread of neoplastic lymphocytes to other organs. This highly effective antimiR-155 pHLIP-PNA approach resulted in targeted anti-cancer silencing effects with no observed toxicities with substantial regression of the oncomiRNA-addicted lymphomas.[22]

Dhuri et al. tested the anti-cancer activity of a PNA that contains modified bases at the gamma position of the PNA backbone (γPNA) and the design of the PNA allows it to bind to the target site via triplex formation (tail clamp PNA). The bases modified at the gamma position of the PNA backbone contained serine amino acid residues. Tail clamp γPNA was used to target miR-155 in U2932, SUDHL-5, and SUDHL-2 lymphoma cell lines and in a U2932 cell line-derived xenograft mouse model.[64] Tail clamp antimiR-155 γPNA contained homopurine stretches to facilitate triplex formation with the target miR-155 and arginine units for cellular uptake and tumor uptake. Lymphoma cell lines treated with Tail clamp antimiR-155 γPNA showed 90–95% reduction in levels of miR-155, 1.5–3.0-fold effect on the downstream targets, dose-dependent (0–4 μmol/L of dose) decrease in cell viability and 4.5-fold increase in apoptosis. Intratumoral injection of 1 mg kg^{-1} per dose (total three doses at a week apart of an interval) of tail clamp antimiR-155 γPNA decreased expression of miR-155 *in vivo* by 75–80% and reduced tumor growth.[64]

In another study, Dhuri et al. used a 22-mer PNA loaded in PLGA NPs to observe the effect of simultaneously targeting miR-21 and miR-155 in SUDHL-2 and SUDHL-5 cell lines.[65] In comparison with individual targeting to a single miRNA, simultaneous targeting of miR-21 and miR-155 showed greater reduction in levels of miRNA and in cell viability. In the SUDHL-2 cell line, PLGA NPs containing only antimiR-21 PNA NPs or antimiR-155 PNA NPs reduced the cell viability by 20%. However, concurrent treatment with antimiR-21 PNA and antimiR-155 NPs, resulted in ∼35% decrease in the cell viability. Likewise, antimiR-21 PNA NPs or antimiR-155 PNA NPs reduced the cell viability by 50% and 60%, respectively. Further, simultaneous treatment with antimiR-21 PNA and antimiR-155 NPs showed a decrease in cell viability by ∼75%. Thus, overall, these results suggest that simultaneous targeting of two different miRNAs results in decreased cell proliferation of lymphoma cells.[65]

Cervical cancer

Cervical cancer is a common, malignant tumor of the lowermost part of the uterus and has been associated with an upregulation of miR-210.[32] In addition to cervical cancer, miR-210 has been detected in almost all solid tumors and is thought to contribute to tumor cell survival and proliferation under hypoxic conditions. Given its numerous roles in DNA repair, mitochondrial oxidative metabolism, and cellular survival, a number of miR-210 targets have been identified. Importantly, one of the predicted effects of miR-210 inhibition is decreased suppression of the iron-sulfur cluster assembly protein (ISCU). ISCU is a downstream target of miR-210, and ISCU suppression has been linked to increased miR-210 expression under hypoxic conditions. A chemically modified γPNA encapsulated in PLGA NP has been developed to antagonize the activity of miR-210. The bases modified at the gamma position of the PNA backbone contained mini-PEG (polyethylene glycol) residues. The effect of antimiR-210 γPNA was studied in a xenograft mouse model created by growing HeLa tumors in immune-deficient nude mice. Intratumoral injections of antimiR-210 γPNA NPs decreased miR-210 levels, significantly diminished tumor growth, and improved tumor morphology.[32]

In addition, histopathological analyses of tumors treated with antimiR-210 γPNA NPs showed regions of central coagulative necrosis, dense fibrosis, increased levels of apoptosis (via caspase staining), and decreased proliferation (via Ki-67 staining).[32] In summary, treatment with antimiR-210 γPNA NPs served as an effective miR-210 inhibition strategy with observed anti-cancer effects in the absence of notable toxicities.

ANTISENSE APPLICATIONS AS DIAGNOSTIC AGENTS

Advancements of PNA within the therapeutic domain have lagged, primarily due to aforementioned issues with specificity and poor cellular uptake at target tissues. However, there have been numerous applications of antisense PNA as research and diagnostic agents via complementarily binding to mRNAs, rRNAs, or microRNAs of interest.[3,7] PNAs have proven applicability as diagnostic agents across many applications, including biosensors, microarrays, *in situ* analyses, and fluorescence *in situ* hybridization (FISH).[3] Table 4 and the following sections will investigate four diagnostic tools (biosensors, microarrays, *in situ* assays, and FISH) by analyzing two novel mechanisms of PNA implementation within each of the technologies.

Biosensors

A biosensor device signals the presence of an analyte during chemical reactions in a concentration-dependent manner.[66] Biosensors are used in many applications including disease monitoring, drug discovery, detection of pollutants and disease-causing pathogens, and as markers indicating disease in bodily fluids.[66] Biosensors offer a minimally invasive platform for point-of-care diagnostics that are cost effective and high throughput.[67] PNA biosensors have the potential to identify various analytes using antisense technologies, such as by detecting the presence of mRNA, rRNA, or miRNA. Compared with conventional DNA complementary sequences, using PNA sequences yields more specific and sensitive results due to PNA having higher thermal stability, increased ability to detect imperfect matches, lower ionic

Table 4. Diagnostic applications of PNA

Diagnostic tool	Detecting, disease	PNA design	Model	Assay (colorimetric, fluorescence, electrical)	Results	Reference
Biosensor	miR-21 extracellularly, Cancers	15-mer acpcPNA	Blood plasma	Electrochemical	Detection of miR-21 in plasma Limit of detection: 0.20 fM Time to detection: 5 min	Kangkamano et al.[70]
	miR-150-5p extracellularly, Preterm birth	7-mer PNA + butane thiol or coumarin	Blood plasma	Fluorogenic (streptavidin)	Detection of miR-150-5p in plasma Limit of detection: 9 nM Time to detection: 10 min	Pavagada et al.[71]
Microarray	T790M-mutated EGFR protein mRNA	10-mer PNA conjugated with FTIC	Human lung carcinoma cells (mutant NCI-HI975, wild type A549)	Fluorogenic (fluorescein isothiocyanate)	Detection of cells containing EGFR mutant mRNA Limit of detection: detects 5–20% of mutated cells using 1×10^6–5×10^6 cells Time to detection: 1 h (incl. cell preparation time)	Shigeto et al.[72]
	miR-122	22-mer PNA,	Blood plasma	Fluorogenic (Cy5)	Detection of miR-122 in plasma Limit of detection: 0.043 nM Sensitivity: 1750 nM^{-1}	Forte et al.[73]
In situ analyses	miR-18, intracellularly	23-mer PNA	*In vitro* (HepG2 cells)	Fluorogenic (Cy5)	Detection and dynamic monitoring of intracellular miR-18a 48.7% downregulation of cells when incubated with miR-18a inhibitor (PNA)	Liao et al.[74]
	rRNA from *G. vaginalis* and *Lactobacillus* spp.	Gard162: 15-mer PNA	*In situ* (HeLa cells)	Fluorogenic (Alexa Fluor 488)	Detected *G. vaginalis* and *Lactobacillus* spp with no cross detection of other cells Gard162: 100% sensitivity, 100% specificity Lac663: 100% sensitivity, 98% specificity	Machado et al.[75]
		Lac663: 15-mer PNA		Fluorogenic (Alexa Fluor 594)		
FISH	*Legionella* 16S rRNA	15-mer PNA	Water samples	Fluorogenic (Alexa Flour 488)	Detection of *Legionella* within water samples above 10^1 CFU Time to detection: 3–4 h Sensitivity: 100% Sepecificity: 100%	Huang et al.[76]
	Eubacterial 16S rRNA	15-mer PNA	Blood plasma	Fluorogenic (Alexaflour 488)	Detection of *E. coli, K. pneumoniae,* and *P. aeruginosa* within water samples Time to detection: 5–6 h for *E. coli* and *K. pneumoniae,* and 10 h for *P. aeruginosa*	Nácher-Vázquez et al.[77]

strength of PNA required, and increased resistance to nuclease and protease digestion.[68] Detection can occur via optical, electrochemical, or gravimetric techniques, often relying on a tagging method to identify target sequences; fluorescence optical tagging is typically used due to high sensitivity.[68]

Pyrrolidinyl PNA polypyrrole/silver nanofoam electrode biosensor for detection of circulating miRNA-21

Electrochemical miRNA biosensors function by transducing a molecular recognition event, most frequently the hybridization of PNA to target miRNA, into a detectable signal via charge accumulation.[69] Due to the neutrality of PNA, hybridization between PNA-RNA produces an accumulation of negative charge, which can be amplified via addition of a reporter unit such as gold/iron oxide/silver nanoparticles or metal/organometallic compounds.[69] Kangkamano et al. demonstrated development of a pyrrolidinyl PNA polypyrrole (acpcPNA)/silver nanofoam (AgNF) electrode biosensor to detect miR-21, which is abnormally expressed in most cancers.[70] Their designed biosensor could detect electrochemical changes in a current to identify the presence of unlabeled miR-21 in samples of blood plasma within a short analysis time.

Differential nucleotide length pyrrolidinyl PNAs were synthesized to possess a conformationally rigid D-prolyl-2-aminocyclopentanecar boxylic acid (ACPC) backbone.[70] As depicted in Figure 4A, AgNF was electrodeposited on a base Au electrode, after which pyrrole was electropolymerized onto the sensor. Target miRNAs were then drop-casted onto the PNA-immobilized electrode and allowed time

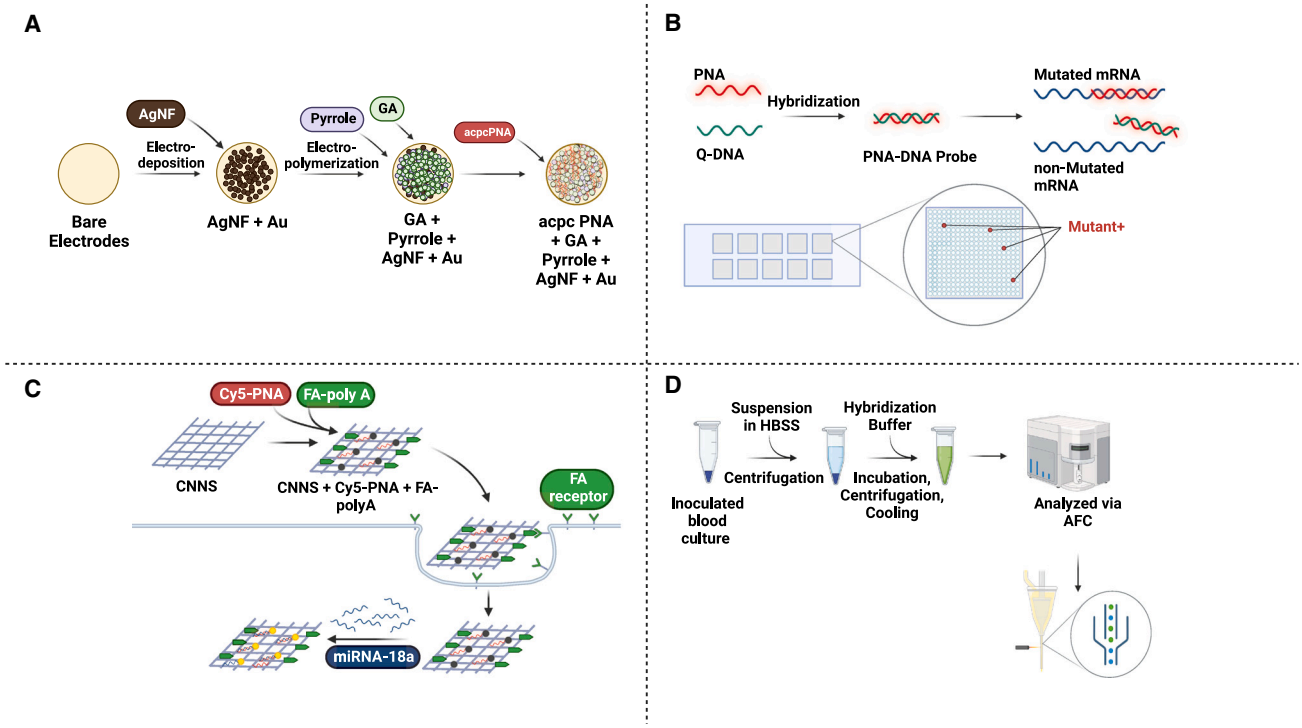

Figure 4. Applications of PNA as diagnostic agents

(A) Development of a pyrrolidinyl PNA polypyrrole/silver nanofoam electrode biosensor. (B) Detection of mRNA for T790M-mutated EGFR protein. (C) f-CNNS preparation, cellular endocytosis, and miRNA detection. (D) Hybridization and analysis of pure bacterial culture samples using acoustic flow cytometry.

to hybridize. The signal detected was the change in current of the AgNF redox reaction before and after hybridization of acpcPNA to target miRNA via cyclic voltammetry. The ideal PNA probe length was established to be the apcPNA-15 mer probe, and a higher concentration of probe was associated with a decreased limit of detection. The biosensor was able to discriminate between complementary single, double, and non-complementary targets, and the amount of signal detected was found to be proportional to miR-21 concentrations. Within a human model, blood serum samples were collected and diluted to denote differential concentrations of miR-21. miR-21 could be detected in samples above a limit of 20 fentometers.[70]

In summary, samples containing miR-21 could be detected in as little as 5 min with a very low limit of detection of 0.20 fentometers.[70] These results indicate the applicability of PNA-based biosensors in clinical applications for the diagnosis and prognosis of cancers, as the system can directly detect miR-21 and does not rely on extraction or purification of samples.

PNA lateral flow assay for the detection of circulating miR-150-5p

Preterm birth is the largest cause of death in children under 5 years old worldwide and it is very difficult to predict, as most cases occur in women with no identifiable risk factors.[71] The current standard predictors of preterm birth include assessing for cervical shortening and fetal fibronectin, both of which are conducted later in pregnancies and are not suitable for longitudinal assessment of risk throughout the progression of a pregnancy. Thus, a low-cost, transportable, and non-invasive test for detection of preterm birth risk has the potential to save many lives.

miR-150-5p has been identified as a predictive biomarker for preterm birth and is present within blood serum as a circulating free nucleic acid. Within 500 pregnant women's blood samples, miR-150-5p was associated with a 100% true positive rate and 50% false positive rate when assessed for risk of preterm birth at 12 weeks into a pregnancy.[71]

Recently, Pavafada et al. developed the first example of a fluorogenic, oligonucleotide templated reaction using a paper, nitrocellulose lateral flow assay (LFA) for the detection of circulating miR-150-5p using PNA.[69,71] The system consists of two 7-mer PNA: (1) a capture probe, which includes PNA with butane thiol and biotin, and (2) a sensing probe consisting of PNA with coumarin.[71] The capture probe was immobilized on the streptavidin-coated LFA test line, and the sensing probe was added to the sample loading pad with the target miRNA. Upon wicking, the miRNA of interest hybridized with the capture PNA on the test line, while also hybridizing to the sensing PNA, resulting in fluorescence.[72]

To assess the sensitivity of the LFA biosensor, increasing concentrations of miR-150-5p were loaded to the strips; this yielded a *limit of detection* of 9 nM. To investigate the LFA biosensor's ability to detect the miRNA of interest, 18 total miRNA extracts from plasma samples used for the biomarker discovery study from women between weeks

11 and 19 of pregnancy were tested through the LFA system.[71] Ten of the samples had low levels of miR-150-5p, and were from women who delivered at term, whereas eight of the samples that had abnormally high levels of miR-150-5p were from women who delivered preterm. Samples from women who delivered preterm gave a statistically higher rate of fluorescence than women who delivered at term.[71]

Overall, this miR-150-5p targeted LFA is a low-cost, paper-based blood test successful in identifying blood samples with a threshold concentration of miR-150-5p, allowing it to distinguish term and preterm patients.[71] Additionally, time to detection was within 10 min, samples can be withdrawn from whole blood, and it does not require miRNA amplification, allowing simple integration within healthcare providers' offices.

Microarrays

A microarray is similar to a biosensor in that they both yield detection of a target sequence by exploiting complementary binding to a manufactured nucleic acid sequence.[68] However, whereas biosensors allow for a single analysis, facilitating microarray technology permits the analysis of many targets in parallel. PNA-based microarray procedures rely on immobilizing a PNA sequence complementary to a target sequence of interest on an electrode surface or nanosheet.

Single-cell microarray chip for the detection of cellular mRNA for T790M-mutated EGFR protein

Many forms of cancer are capable of developing resistance to therapeutic agents, further limiting treatment options. One such example is lung cancer cells exhibiting the T790M-mutated EGFR protein, which creates a phenotype of EGFR that is capable of evading the tyrosine kinase inhibitor gefitinib, thereby developing resistance.[72] Due to the potential of resistance, an analysis of the number of T790M-mutated cancer cells is needed prior to initiation of treatment, to ensure effective treatment of lung cancer. Current methods of profiling mutation ratios, such as next-generation sequencing and real-time PCR-based analyzing systems, are only effective if there is a high enough percentage of mutated cancer cells within the cell sample; a higher amount of mutated cells correlates with later-stage disease.[72]

Shigeto et al. developed a microarray detection system for cancer cells that have a single-nucleotide mutation, and have proven its efficacy within a lung cancer cell system.[72] The polystyrene microarray chip consists of 6,241 microchambers capable of accommodating cells. A 10-mer PNA probe conjugated with fluorescein isothiocyanate (FITC) was designed to target the mRNA of T790M-mutated EGFR. As depicted in Figure 4B, initially, the FITC-conjugated PNA was hybridized to a quencher DNA (Q-DNA). The cell suspensions containing various concentrations of NCI-H1975 (mutated) and A549 (non-mutated) lung cells were dispersed onto the microarray chip and incubated to allow cells to settle into the microchambers. Following successful PNA-mRNA hybridization, the probe exhibited increased fluorescence intensity due to the Q-DNA dissociating from the FITC-PNA. The fluorescence intensity of the T790M-mutated cells was nearly double that of the non-mutated cells validating the detection method.[72] Conventional methods of mutated cell detection such as PCR requires that at least 20% of the cells are mutated and take 3 to 5 h, typically. This single-cell microarray chip was able to detect the signal when 5–20% of the cells were mutated and took 1 h, including cellular preparation time.[71] Many cancer types can be separated on the cellular level, allowing this microarray to have broad applications within diagnosis and therapeutic monitoring.[72] This system designates a promising detection method for small numbers of single-nucleotide mutated, drug-resistant cancer cells, with the potential in the future to detect mutated, drug-resistant cells from cancer tissue or liquid biopsy samples.

PNA-microarray system for detection of miR-122 in plasma

There are numerous conditions stemming from pathologies within the liver, such as acute liver injury, and hepatitis B and hepatitis C virus. miR-122 is expressed specifically within hepatocytes, with negligible expression in other tissues; further, the presence of miR-122 within patients with acute liver injury is increased 100–1,000-fold versus healthy controls.[73] For this reason, detection of circulating miR-122 has been recognized as a potential method for detection of liver injury.

Forte et al. described the development of a PNA microarray composed of agarose film on silicone support and mirror layers (Al-Si-Cu) with optical signal enhancement via Cy5 chromophore emission, and a 22-mer PNA probe specific for miR-122.[73] To assess the ability of the microarray to recognize miR-122, reactions were performed at various concentrations of miR-122 yielding a limit of detection of 0.043. Additionally, probe immobilization efficiency was confirmed via calculation of the hybridized-site density value using fluorescence signals at the various labeled probe amounts.

To investigate the ability of the PNA-microarray system to detect target miR-122 in human serum samples,the serum samples were spiked with differing concentrations of miR-122.[73] The hybridization signals for positive control and negative control were 5,998 ± 436 a.u. and 258 ± 329 a.u. For the miRNA-specific probes at concentrations of 10 nM and 50 nM, the hybridization signals were 2,151 ± 252 a.u. and 3,460 ± 472 a.u., respectively. These results demonstrate that the PNA microarray was successful at detecting the miR-122 of interest within human serum samples, confirming broad applicability of facilitating PNA as a diagnostic tool to detect circulating miRNA.

In situ analyses

As mentioned, miRNA and mRNA are detectable within blood serum and other bodily fluids, thereby making them appropriate targets for *in situ* diagnostics. *In situ* diagnostic assays have many useful applications in routine clinical testing and within biomedical research and development, as they allow for the hisopathological examination of biomarker status. Applications of PNA within *in situ* diagnostics represents a utilization that is easily performed following collection of patients' serum.

PNA functionalized carbon nitride nanosheet for in situ *monitoring of intracellular miRNA*

Existing techniques of detecting miRNA such as by PCR, northern blotting, and classical microarrays rely on a miRNA isolation step for

sample preparation, due to miRNA's presence intracellularly.[74] An additional but more elusive mechanism for detecting miRNA would be to detect the presence of an miRNA of interest within living cells.

In a novel approach, Liao et al. demonstrated a functionalized carbon nitride nanosheet (f-CNNS) for the recognition of intracellular miR-18a.[74] As illustrated in Figure 4C, the f-CNNS probe was created by affixing Cy5-labeled PNA (Cy5-PNA) and folate-Poly A onto the f-CNNS; Cy5 fluorescence was quenched when on the f-CNNS, and folate assisted with cell-target-specific delivery of the CNNS, as cancer overexpresses folate receptors.[74] The f-CNNS was transfected into HepG2 cells that contained the miRNA of interest (miR-18a), and hybridization between Cy5-PNA and the complementary target miRNA yielded the release of Cy5-PNA from the f-CNNS, leading to fluorescence.[74]

The specificity of the Cy5-PNA to the miR-18a of interest was confirmed by investigating the fluorescence intensity of reactions using the f-CNNS probe with different strand sequences. The following sequences were investigated: target sequence, single base mismatch, three base mismatch, and non-complementary RNA. Compared with 100% fluorescence intensity achieved with the target sequence, single base mismatch fluorescence intensity was 21.8%, three base mismatch was 18.2%, and non-complementary RNA was 0.782%.[74]

In order for the f-CNNS probe to be effective within the cells, it would need to pass the cellular membrane and escape the endosomal packaging that occurs upon cellular transfection. To verify that the probe was able to cross the cellular membrane, HepG2 cells were incubated with the f-CNNS probe and Hoechst 333342 (nuclear tracker), which yielded strong Cy5 fluorescence when in the cytoplasm. To confirm endosomal escape, cells were additionally incubated with f-CNNS and LysoTracker Green DND-26 (endosomal/lysosomal tracker) to confirm there was no colocalization between Cy5 fluorescence and endocytic vesicles within the cytoplasm. This indicates that the f-CNNS was successful in escaping endocytic vesicles, allowing for the potential to encounter miR-18a within the cytoplasm.

Further, HepG2 cells were incubated with an miRNA inhibitor or miR-18a mimic to induce differential levels of miRNA, and then incubated with f-CNNS probe. The HepG2 cells containing miR-18a mimic exhibited a strong fluorescence intensity of 135.5% compared with cells containing a normal expression of miR-18a, indicating high levels of miR-18a in HepG2 cells. The HepG2 cells containing the inhibitor showed 46.2% downregulation of expression.[74] This attests to the ability of the f-CNNS probe to monitor ongoing, dynamic changes of intracellular miRNA. More generally, this technique demonstrates high specificity and selectivity toward target miRNA with low background signals, proving its application in identifying target miRNA within living cells and potential within cancer diagnostics.

In situ *hybridization*

Antisense PNA probes have proven useful as a diagnostic tool to detect bacterial pathogens, and have received FDA clearance for application in microbacterial clinical analyses for the detection of *Candida albicans* in blood cultures.[75]

Machado et al. developed a quick and accurate diagnostic test for bacterial vaginosis (BV), whereby PNA probes target the rRNA of *Lactobacillus* and *Gardnerella vaginalis*, two bacterial groups frequently encountered in the vagina.[75] HeLa cells were incubated with different concentrations of 22 strains of *G. vaginalis* and 32 strains of *Lactobacillis* to create an *in vitro* design mimicking the shift from healthy vaginal flora to BV. Two different PNA probes were synthesized: one was designed to target *Gardenella* genus (Gard162), and the other was designed to target *Lactobacillus* species (Lac663).[75] Cultured aliquots of HeLa epithelial cells were seeded into 24-well tissue culture plates, and cell culture media with known concentrations of a *Lactobacillus* strain and *G. vaginalis* strain were added to each well of the 24-well culture plate. The glass slides containing the bacteria and HeLa cells were hybridized with both of the PNA probes, and were then visualized using a microscope.

It was determined that Lac663 and Gard162 probes were indeed selectively bound to their respective *Lactobacillus* and *G. vaginalis* strains, and the fluorescence signal was easily observable with no cross hybridization with other species detected.[75] The specificity and sensitivity of the PNA probes were found to be 98% and 100% for *Lactobacillus* species, and 100% and 100% for *G. vaginalis*.[75]

Compared with current methods of detecting *Lactobacillus* and *G. vaginalis* strains, the investigated Lac663 and Gard162 allowed for increased specificity and sensitivity in detecting the bacteria of interest at a reduced detection time of 3 h and exhibited no cross detection in the presence of human epithelial cells.[75] These results signify the viability of utilizing PNA probes to detect the presence of bacteria within mixed samples, allowing for efficient and rapid detection of pathogens.

Fluorescence *in situ* hybridization

The applicability of facilitating PNA within FISH techniques has been well validated across numerous situations via conjugation of PNA to fluorescent probes. FISH detection permits identification of pathogenic targets via short oligonucleotide probes targeted to rRNA. Current culture-based methods of pathogenic detection take 12–48 h, which correlates with delayed ability to positively impact treatment decisions and oftentimes selecting incorrect antibiotics to treat certain microbial infections. PNA-conjugated FISH probes have offered enhanced detection capabilities within shorter time frames.

PNA-FISH-acoustic flow cytometry

The detection of organisms causing sepsis-associated bacteremia, which can be mono- or polymicrobial, can be a time-intensive process lasting roughly 12–48 h. The process is exacerbated by intensive laboratory culturing procedures and bacterial growth rates that extend for as long as 5 days.[76] Increased culture characterization times correlates with a delay in targeted anti-microbial treatment, which is associated with unfavorable outcomes.

PNA-FISH-acoustic flow cytometry (PNA-FISH-AFC), as described by Huang et al. is a process whereby the detection of bacteria in blood cultures is achieved via PNA-FISH targeted to eubacterial 16s rRNA, enhanced with acoustic flow cytometry.[76] Flow cytometry has historically been used for analyzing blood cells, and the acoustic-enhanced version of the flow cytometer instrument focuses cells into a single stream using ultrasound waves, allowing for the measurement of physical and chemical properties of cells of interest. Development of the eubacterial 16S rRNA-targeted PNA-FISH probe yielded a 15-mer PNA probe conjugated to Alexa Fluor 488, to enable visualization. Assays were developed by simulating blood cultures of *Klebsiella pneumoniae, P. aeruginosa,* and *E. coli*.[76]

The PNA-FISH-AFC technique yielded results in a much shorter time to detection period for *E. coli, K. pneumoniae*, and *P. aeruginosa* when compared with other standard methods such as MALDI-TOF and FilmArray.[76] The time to detection for *E. coli* and *K. pneumonia* was 5–6 h, and for *P. aeruginosa* it was observed after 10 h, which correlates with a bacterial concentration of 10^3–104 CFU/mL. Under current clinical situations using the BacTEC system, it takes a minimum of 13–14 h for *E. coli* and *K. pneumonia* and 19–21 h for *P. aeruginosa*, corresponding with a much higher required bacterial concentration (107–109 CFU/mL). In relation to current standards of bacterial identification, this PNA-FISH-AFC system can be applied directly to positive blood cultures at an earlier time point and without culture preparation. This accelerated bacterial identification equates to the ability to more quickly administer the correct antimicrobials, potentially reducing adverse effects, AMR, and rates of death due to pathogenic infection.

FISH-PNA probe LEG22

Legionalla contamination of natural and manmade water systems contributes to the development of Legionnaires' disease and Pontiac fever.[77] Current methods of contamination detection rely on guidelines outlined within the International Organization for Standardization 11731:2017, which recommends culturing techniques that are time-consuming and of low sensitivity.[77]

Nácher-Vázquez et al. characterized a novel, culture-independent PNA-FISH method designed for the detection of the genus *Legionella* within contaminated tap water samples.[77] The PNA probe was developed by aligning the highly conserved 16S rRNA gene sequences of *Legionella* and comparing with other non-target strains of bacteria that may also be present in water. The selected FISH-PNA probe, named LEG22, specifically targets the 16S rRNA between positions 634 and 648 of the *Legionella pneumonophila* subspecies, which is estimated to cause 97% of all infectious outbreaks. Within artificially inoculated tap water samples contaminated with *Legionella* and non-*Legionella* bacterial species, the LEG22 probe yielded detection for all *Legionella* strains and no hybridization for other species, which correlates with 100% specificity and 100% sensitivity.[77] Detection of bacteria following filtration was observed at concentrations of at least 10^1 CFU/L; detection of LEG22 within less concentrated samples was not possible, as it was limited by the detection limit of FISH using microscope equipment.

Investigators also sought to test LEG22 detection of *Legionella* within cooling tower-derived samples. However, although the samples were positive for culture, LEG22 was unable to detect organisms.[77] It was hypothesized that the presence of autofluorescent interference hindered the detection of cells' fluorescent signals. Nevertheless, LEG22, with a procedure time of 3–4 h and high specificity and sensitivity for target organisms, has demonstrated encouraging potential to advance monitoring capabilities for *Legionella* within aquatic environments.[77]

Conclusion

PNAs possess numerous advantageous characteristics that permit their applications in diagnostics and as novel therapeutic agents. Multiple studies have established that PNAs possess numerous advantages like neutral backbone, no stimulation of immune response, and higher binding affinity than negatively charged oligonucleotides. Still, hydrophobicity and the cellular and organ delivery of PNAs remain challenging for the broader applications. Various delivery platforms, from solid nanoparticle formulations to peptide-based delivery, have successfully delivered PNAs. Next-generation PNA modifications have been developed to increase its binding properties and physicochemical features like solubility. Especially, gamma PNAs have made strides as it has already been well established that gamma PNAs possess high binding affinity and can target the genomic DNA and several coding and ncRNAs in various studies. Gamma PNAs have been used tremendously for pathogen detection, and antisense-based therapeutics. It is not easy to parallelly compare different studies on PNA and modified gamma PNA as several prior studies were centered on only *in vitro* analysis, and few studies have performed the *in vivo* analysis. In addition, variability of results due to differences in the PNA design, cell lines, and disease models further limits the parallel comparative studies. Thus, there is a need for a systemic study where different PNAs and modified gamma PNAs can be evaluated in parallel. While this review summarizes the compelling evidence supporting the efficacy of antisense PNA in various disease states, systematic and comprehensive studies are required for testing the cellular delivery and fate of various generation gamma PNAs that can correlate with its dose-dependent efficacy. Optimization of different nucleic acid chemistries and and targeted delivery platforms could provide a solid foundation for discovery and development of next-generation antisense PNAs.

ACKNOWLEDGMENTS

We acknowledge funding support by University of Saint Joseph (USJ) student research grants awarded to V.M. and M.K.; New Investigator Award (NIA) by American Association of Colleges of Pharmacy (AACP) & Teaching and Learning Center Grant by USJ, awarded to A.G. to support the Gupta lab. All figures were created using Biorender.com.

AUTHOR CONTRIBUTIONS

V.M. and M.K. conducted literature reviews, prepared the manuscript draft, and edited the manuscript. A.G. oversaw the project, provided regular feedback, and performed final manuscript review.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Miller, P.S. (1996). Development of Antisense and Antigene Oligonucleotide Analogs. In Progress in Nucleic Acid Research and Molecular Biology, *52*, W.E. Cohn and K. Moldave, eds. (Academic Press), pp. 261–291.
2. Chery, J. (2016). RNA therapeutics: RNAi and antisense mechanisms and clinical applications. Postdoc J. *4*, 35–50.
3. Singh, K.R., Sridevi, P., and Singh, R.P. (2020). Potential applications of peptide nucleic acid in biomedical domain. Eng. Rep. *2*, e12238.
4. Montazersaheb, S., Hejazi, M.S., and Nozad Charoudeh, H. (2018). Potential of Peptide Nucleic Acids in Future Therapeutic Applications. Adv. Pharmaceut. Bull. *8*, 551–563.
5. Dhuri, K., Bechtold, C., Quijano, E., Pham, H., Gupta, A., Vikram, A., and Bahal, R. (2020). Antisense Oligonucleotides: An Emerging Area in Drug Discovery and Development. J. Clin. Med. *9*, 2004–2024.
6. Pradeep, S.P., Malik, S., Slack, F.J., and Bahal, R. (2023). Unlocking the potential of chemically modified peptide nucleic acids for RNA-based therapeutics. RNA *29*, 434–445.
7. Brodyagin, N., Katkevics, M., Kotikam, V., Ryan, C.A., and Rozners, E. (2021). Chemical approaches to discover the full potential of peptide nucleic acids in biomedical applications. Beilstein J. Org. Chem. *17*, 1641–1688.
8. Winkle, M., El-Daly, S.M., Fabbri, M., and Calin, G.A. (2021). Noncoding RNA therapeutics — challenges and potential solutions. Nat. Rev. Drug Discov. *20*, 629–651.
9. Gambari, R., Gasparello, J., Fabbri, E., Borgatti, M., Tamanini, A., and Finotti, A. (2020). Peptide Nucleic Acids for MicroRNA Targeting. Methods Mol. Biol. *2105*, 199–215.
10. Cambronne, X.A., Shen, R., Auer, P.L., and Goodman, R.H. (2012). Capturing microRNA targets using an RNA-induced silencing complex (RISC)-trap approach. Proc. Natl. Acad. Sci. USA *109*, 20473–20478.
11. Babar, I.A., Cheng, C.J., Booth, C.J., Liang, X., Weidhaas, J.B., Saltzman, W.M., and Slack, F.J. (2012). Nanoparticle-based therapy in an in vivo microRNA-155 (miR-155)-dependent mouse model of lymphoma. Proc. Natl. Acad. Sci. USA *109*, E1695–E1704.
12. Seo, Y.E., Suh, H.W., Bahal, R., Josowitz, A., Zhang, J., Song, E., Cui, J., Noorbakhsh, S., Jackson, C., Bu, T., et al. (2019). Nanoparticle-mediated intratumoral inhibition of miR-21 for improved survival in glioblastoma. Biomaterials *201*, 87–98.
13. Özeş, A.R., Wang, Y., Zong, X., Fang, F., Pilrose, J., and Nephew, K.P. (2017). Therapeutic targeting using tumor specific peptides inhibits long non-coding RNA HOTAIR activity in ovarian and breast cancer. Sci. Rep. *7*, 894.
14. Wu, L., Murat, P., Matak-Vinkovic, D., Murrell, A., and Balasubramanian, S. (2013). Binding Interactions between Long Noncoding RNA HOTAIR and PRC2 Proteins. Biochemistry *52*, 9519–9527.
15. Wojciechowska, M., Równicki, M., Mieczkowski, A., Miszkiewicz, J., and Trylska, J. (2020). Antibacterial Peptide Nucleic Acids—Facts and Perspectives. Molecules *25*, 559.
16. Gc, K., To, D., Jayalath, K., and Abeysirigunawardena, S. (2019). Discovery of a novel small molecular peptide that disrupts helix 34 of bacterial ribosomal RNA. RSC Adv. *9*, 40268–40276.
17. Good, L., and Nielsen, P.E. (1998). Inhibition of translation and bacterial growth by peptide nucleic acid targeted to ribosomal RNA. Proc. Natl. Acad. Sci. USA *95*, 2073–2076.
18. Roberts, T.C., Langer, R., and Wood, M.J.A. (2020). Advances in oligonucleotide drug delivery. Nat. Rev. Drug Discov. *19*, 673–694.
19. Madani, F., Lindberg, S., Langel, U., Futaki, S., and Gräslund, A. (2011). Mechanisms of cellular uptake of cell-penetrating peptides. J. Biophys. *2011*, 414729.
20. Kaplan, A.R., Pham, H., Liu, Y., Oyaghire, S., Bahal, R., Engelman, D.M., and Glazer, P.M. (2020). Ku80-Targeted pH-Sensitive Peptide-PNA Conjugates Are Tumor Selective and Sensitize Cancer Cells to Ionizing Radiation. Mol. Cancer Res. *18*, 873–882.
21. Svoronos, A.A., Bahal, R., Pereira, M.C., Barrera, F.N., Deacon, J.C., Bosenberg, M., DiMaio, D., Glazer, P.M., and Engelman, D.M. (2020). Tumor-Targeted, Cytoplasmic Delivery of Large, Polar Molecules Using a pH-Low Insertion Peptide. Mol. Pharm. *17*, 461–471.
22. Cheng, C.J., Bahal, R., Babar, I.A., Pincus, Z., Barrera, F., Liu, C., Svoronos, A., Braddock, D.T., Glazer, P.M., Engelman, D.M., et al. (2015). MicroRNA silencing for cancer therapy targeted to the tumour microenvironment. Nature *518*, 107–110.
23. Wright, D.G., Zhang, Y., and Murphy, J.R. (2008). Effective delivery of antisense peptide nucleic acid oligomers into cells by anthrax protective antigen. Biochem. Biophys. Res. Commun. *376*, 200–205.
24. Good, L., Awasthi, S.K., Dryselius, R., Larsson, O., and Nielsen, P.E. (2001). Bactericidal antisense effects of peptide-PNA conjugates. Nat. Biotechnol. *19*, 360–364.
25. .https://www.alnylam.com/our-science/sirna-delivery-platforms.
26. Kumar, V., Wahane, A., Gupta, A., Manautou, J.E., and Bahal, R. (2023). Multivalent Lactobionic Acid and N-Acetylgalactosamine-Conjugated Peptide Nucleic Acids for Efficient In Vivo Targeting of Hepatocytes. Adv. Healthcare Mater. *12*, e2202859.
27. Pieńko, T., Czarnecki, J., Równicki, M., Wojciechowska, M., Wierzba, A.J., Gryko, D., Bartosik, D., and Trylska, J. (2021). Vitamin B(12)-peptide nucleic acids use the BtuB receptor to pass through the Escherichia coli outer membrane. Biophys. J. *120*, 725–737.
28. Równicki, M., Wojciechowska, M., Wierzba, A.J., Czarnecki, J., Bartosik, D., Gryko, D., and Trylska, J. (2017). Vitamin B(12) as a carrier of peptide nucleic acid (PNA) into bacterial cells. Sci. Rep. *7*, 7644.
29. Avitabile, C., Accardo, A., Ringhieri, P., Morelli, G., Saviano, M., Montagner, G., Fabbri, E., Gallerani, E., Gambari, R., and Romanelli, A. (2015). Incorporation of Naked Peptide Nucleic Acids into Liposomes Leads to Fast and Efficient Delivery. Bioconjugate Chem. *26*, 1533–1541.
30. Swider, E., Koshkina, O., Tel, J., Cruz, L.J., de Vries, I.J.M., and Srinivas, M. (2018). Customizing poly(lactic-co-glycolic acid) particles for biomedical applications. Acta Biomater. *73*, 38–51.
31. Gupta, A., Bahal, R., Gupta, M., Glazer, P.M., and Saltzman, W.M. (2016). Nanotechnology for delivery of peptide nucleic acids (PNAs). J. Contr. Release *240*, 302–311.
32. Gupta, A., Quijano, E., Liu, Y., Bahal, R., Scanlon, S.E., Song, E., Hsieh, W.C., Braddock, D.E., Ly, D.H., Saltzman, W.M., and Glazer, P.M. (2017). Anti-tumor Activity of miniPEG-γ-Modified PNAs to Inhibit MicroRNA-210 for Cancer Therapy. Mol. Ther. Nucleic Acids *9*, 111–119.
33. Zhang, Y., Ma, W., Zhu, Y., Shi, S., Li, Q., Mao, C., Zhao, D., Zhan, Y., Shi, J., Li, W., et al. (2018). Inhibiting Methicillin-Resistant Staphylococcus aureus by Tetrahedral DNA Nanostructure-Enabled Antisense Peptide Nucleic Acid Delivery. Nano Lett. *18*, 5652–5659.
34. Hong, S., Jiang, W., Ding, Q., Lin, K., Zhao, C., and Wang, X. (2023). The Current Progress of Tetrahedral DNA Nanostructure for Antibacterial Application and Bone Tissue Regeneration. Int. J. Nanomed. *18*, 3761–3780.
35. Malik, S., Saltzman, W.M., and Bahal, R. (2021). Extracellular vesicles mediated exocytosis of antisense peptide nucleic acids. Mol. Ther. Nucleic Acids *25*, 302–315.
36. TEAM, W. (2022). Global antimicrobial resistance and use surveillance system (GLASS) report.
37. Antimicrobial Resistance Collaborators (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet *399*, 629–655.
38. Christaki, E., Marcou, M., and Tofarides, A. (2020). Antimicrobial Resistance in Bacteria: Mechanisms, Evolution, and Persistence. J. Mol. Evol. *88*, 26–40.
39. Salimiyan Rizi, K., Ghazvini, K., and Noghondar, M.k. (2018). Adaptive Antibiotic Resistance: Overview and Perspectives. J. Infect. Dis. Ther. *06*, 363.
40. Tan, X.X., Actor, J.K., and Chen, Y. (2005). Peptide nucleic acid antisense oligomer as a therapeutic strategy against bacterial infection: proof of principle using mouse intraperitoneal infection. Antimicrob. Agents Chemother. *49*, 3203–3207.
41. Kulik, M., Markowska-Zagrajek, A., Wojciechowska, M., Grzela, R., Wituła, T., and Trylska, J. (2017). Helix 69 of Escherichia coli 23S ribosomal RNA as a peptide nucleic acid target. Biochimie *138*, 32–42.

42. Goh, S., Loeffler, A., Lloyd, D.H., Nair, S.P., and Good, L. (2015). Oxacillin sensitization of methicillin-resistant Staphylococcus aureus and methicillin-resistant Staphylococcus pseudintermedius by antisense peptide nucleic acids in vitro. BMC Microbiol. *15*, 262.

43. He, X., Alian, A., and Ortiz de Montellano, P.R. (2007). Inhibition of the Mycobacterium tuberculosis enoyl acyl carrier protein reductase InhA by arylamides. Bioorg. Med. Chem. *15*, 6649–6658.

44. Cotta, K.B., Ghosh, S., and Mehra, S. (2022). Potentiating the Anti-Tuberculosis Efficacy of Peptide Nucleic Acids through Combinations with Permeabilizing Drugs. Microbiol. Spectr. *10*, e201221.

45. Ndeboko, B., Omouessi, S.T., Ongali, B., and Mouinga-Ondémé, A. (2020). Cell Penetrating Peptides Used in Delivery of Therapeutic Oligonucleotides Targeting Hepatitis B Virus. Pharmaceuticals *13*, 483.

46. Robaczewska, M., Narayan, R., Seigneres, B., Schorr, O., Thermet, A., Podhajska, A.J., Trepo, C., Zoulim, F., Nielsen, P.E., and Cova, L. (2005). Sequence-specific inhibition of duck hepatitis B virus reverse transcription by peptide nucleic acids (PNA). J. Hepatol. *42*, 180–187.

47. Ndeboko, B., Ramamurthy, N., Lemamy, G.J., Jamard, C., Nielsen, P.E., and Cova, L. (2017). Role of Cell-Penetrating Peptides in Intracellular Delivery of Peptide Nucleic Acids Targeting Hepadnaviral Replication. Mol. Ther. Nucleic Acids *9*, 162–169.

48. Pandey, V.N., Upadhyay, A., and Chaubey, B. (2009). Prospects for antisense peptide nucleic acid (PNA) therapies for HIV. Expet Opin. Biol. Ther. *9*, 975–989.

49. Tripathi, S., Chaubey, B., Ganguly, S., Harris, D., Casale, R.A., and Pandey, V.N. (2005). Anti-HIV-1 activity of anti-TAR polyamide nucleic acid conjugated with various membrane transducing peptides. Nucleic Acids Res. *33*, 4345–4356.

50. Boutimah-Hamoudi, F., Leforestier, E., Sénamaud-Beaufort, C., Nielsen, P.E., Giovannangeli, C., and Saison-Behmoaras, T.E. (2007). Cellular antisense activity of peptide nucleic acid (PNAs) targeted to HIV-1 polypurine tract (PPT) containing RNA. Nucleic Acids Res. *35*, 3907–3917.

51. Evers, M.M., Toonen, L.J.A., and van Roon-Mom, W.M.C. (2015). Antisense oligonucleotides in therapy for neurodegenerative disorders. Adv. Drug Deliv. Rev. *87*, 90–103.

52. McMahon, B.M., Stewart, J.A., Bitner, M.D., Fauq, A., McCormick, D.J., and Richelson, E. (2002). Peptide nucleic acids specifically cause antigene effects in vivo by systemic injection. Life Sci. *71*, 325–337.

53. McMahon, B.M., Stewart, J., Fauq, A., Younkin, S., Younkin, L., and Richelson, E. (2002). Using peptide nucleic acids as gene-expression modifiers to reduce beta-amyloid levels. J. Mol. Neurosci. *19*, 71–76.

54. McMahon, B.M., Stewart, J., Fauq, A., Younkin, S., Younkin, L., and Richelson, E. (2003). Peptide nucleic acids targeted to the amyloid precursor protein. J. Mol. Neurosci. *20*, 261–265.

55. Valdmanis, P.N., Belzil, V.V., Lee, J., Dion, P.A., St-Onge, J., Hince, P., Funalot, B., Couratier, P., Clavelou, P., Camu, W., and Rouleau, G.A. (2009). A mutation that creates a pseudoexon in SOD1 causes familial ALS. Ann. Hum. Genet. *73*, 652–657.

56. Turner, B.J., Cheah, I.K., Macfarlane, K.J., Lopes, E.C., Petratos, S., Langford, S.J., and Cheema, S.S. (2003). Antisense peptide nucleic acid-mediated knockdown of the p75 neurotrophin receptor delays motor neuron disease in mutant SOD1 transgenic mice. J. Neurochem. *87*, 752–763.

57. Rembach, A., Turner, B.J., Bruce, S., Cheah, I.K., Scott, R.L., Lopes, E.C., Zagami, C.J., Beart, P.M., Cheung, N.S., Langford, S.J., and Cheema, S.S. (2004). Antisense peptide nucleic acid targeting GluR3 delays disease onset and progression in the SOD1 G93A mouse model of familial ALS. J. Neurosci. Res. *77*, 573–582.

58. Hu, J., Matsui, M., Gagnon, K.T., Schwartz, J.C., Gabillet, S., Arar, K., Wu, J., Bezprozvanny, I., and Corey, D.R. (2009). Allele-specific silencing of mutant huntingtin and ataxin-3 genes by targeting expanded CAG repeats in mRNAs. Nat. Biotechnol. *27*, 478–484.

59. Iennaco, R., Formenti, G., Trovesi, C., Rossi, R.L., Zuccato, C., Lischetti, T., Bocchi, V.D., Scolz, A., Martínez-Labarga, C., Rickards, O., et al. (2022). The evolutionary history of the polyQ tract in huntingtin sheds light on its functional pro-neural activities. Cell Death Differ. *29*, 293–305.

60. Gambari, R., Gasparello, J., and Finotti, A. (2019). Peptide nucleic acid-based targeting of microRNAs: possible therapeutic applications for glioblastoma. J. Cancer Metastasis Treat. *2019*, 55.

61. Wang, Y., Malik, S., Suh, H.W., Xiao, Y., Deng, Y., Fan, R., Huttner, A., Bindra, R.S., Singh, V., Saltzman, W.M., and Bahal, R. (2023). Anti-seed PNAs targeting multiple oncomiRs for brain tumor therapy. Sci. Adv. *9*, eabq7459.

62. Bando, H., Ohtsu, A., and Yoshino, T. (2023). Therapeutic landscape and future direction of metastatic colorectal cancer. Nat. Rev. Gastroenterol. Hepatol. *20*, 306–322.

63. Gasparello, J., Papi, C., Zurlo, M., Gambari, L., Manicardi, A., Rozzi, A., Ferrarini, M., Corradini, R., Gambari, R., and Finotti, A. (2022). MicroRNAs miR-584-5p and miR-425-3p Are Up-Regulated in Plasma of Colorectal Cancer (CRC) Patients: Targeting with Inhibitor Peptide Nucleic Acids Is Associated with Induction of Apoptosis in Colon Cancer Cell Lines. Cancers *15*, 128.

64. Dhuri, K., Gaddam, R.R., Vikram, A., Slack, F.J., and Bahal, R. (2021). Therapeutic Potential of Chemically Modified, Synthetic, Triplex Peptide Nucleic Acid-Based Oncomir Inhibitors for Cancer Therapy. Cancer Res. *81*, 5613–5624.

65. Dhuri, K., Pradeep, S.P., Shi, J., Anastasiadou, E., Slack, F.J., Gupta, A., Zhong, X.B., and Bahal, R. (2022). Simultaneous Targeting of Multiple oncomiRs with Phosphorothioate or PNA-Based Anti-miRs in Lymphoma Cell Lines. Pharm. Res. (N. Y.) *39*, 2709–2720.

66. Bhalla, N., Jolly, P., Formisano, N., and Estrela, P. (2016). Introduction to biosensors. Essays Biochem. *60*, 1–8.

67. D'Agata, R., Giuffrida, M.C., and Spoto, G. (2017). Peptide Nucleic Acid-Based Biosensors for Cancer Diagnosis. Molecules *22*, 1951.

68. Sassolas, A., Leca-Bouvier, B.D., and Blum, L.J. (2008). DNA Biosensors and Microarrays. Chem. Rev. *108*, 109–139.

69. Cadoni, E., Manicardi, A., and Madder, A. (2020). PNA-Based MicroRNA Detection Methodologies. Molecules *25*, 1296.

70. Kangkamano, T., Numnuam, A., Limbut, W., Kanatharana, P., Vilaivan, T., and Thavarungkul, P. (2018). Pyrrolidinyl PNA polypyrrole/silver nanofoam electrode as a novel label-free electrochemical miRNA-21 biosensor. Biosens. Bioelectron. *102*, 217–225.

71. Pavagada, S., Channon, R.B., Chang, J.Y.H., Kim, S.H., MacIntyre, D., Bennett, P.R., Terzidou, V., and Ladame, S. (2019). Oligonucleotide-templated lateral flow assays for amplification-free sensing of circulating microRNAs. Chem. Commun. *55*, 12451–12454.

72. Shigeto, H., Yamada, E., Kitamatsu, M., Ohtsuki, T., Iizuka, A., Akiyama, Y., and Yamamura, S. (2020). Analysis of Single Nucleotide-Mutated Single-Cancer Cells Using the Combined Technologies of Single-Cell Microarray Chips and Peptide Nucleic Acid-DNA Probes. Micromachines *11*, 628.

73. Forte, G., Ventimiglia, G., Pesaturo, M., and Petralia, S. (2022). A highly sensitive PNA-microarray system for miRNA122 recognition. Biotechnol. J. *17*, e2100587.

74. Liao, X., Wang, Q., and Ju, H. (2015). A peptide nucleic acid-functionalized carbon nitride nanosheet as a probe for in situ monitoring of intracellular microRNA. Analyst *140*, 4245–4252.

75. Machado, A., Almeida, C., Salgueiro, D., Henriques, A., Vaneechoutte, M., Haesebrouck, F., Vieira, M.J., Rodrigues, L., Azevedo, N.F., and Cerca, N. (2013). Fluorescence in situ Hybridization method using Peptide Nucleic Acid probes for rapid detection of Lactobacillus and Gardnerella spp. BMC Microbiol. *13*, 82.

76. Huang, X.X., Urosevic, N., and Inglis, T.J.J. (2019). Accelerated bacterial detection in blood culture by enhanced acoustic flow cytometry (AFC) following peptide nucleic acid fluorescence in situ hybridization (PNA-FISH). PLoS One *14*, e0201332.

77. Nácher-Vázquez, M., Barbosa, A., Armelim, I., Azevedo, A.S., Almeida, G.N., Pizarro, C., Azevedo, N.F., Almeida, C., and Cerqueira, L. (2022). Development of a Novel Peptide Nucleic Acid Probe for the Detection of Legionella spp. in Water Samples. Microorganisms *10*, 1409.