OLIGONUCLEOTIDE THERAPEUTICS HANDBOOK

By Klaus D. Linse, Ph.D and Frank Hong, Ph.D., and Miguel Castro, Ph.D.

Lewisville, Texas, 2022

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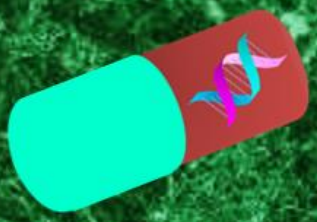

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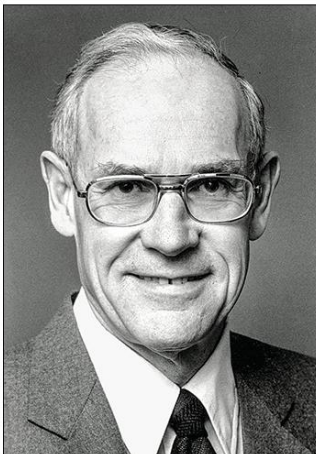
Pioneers of Oligonucleotide Synthesis



Robert L. Letsinger (1921 to 2014). Was an American biochemist and professor of chemistry at Northwestern University. Pioneer in the chemical synthesis of DNA. In the 1960s, Letsinger's laboratory developed methods for solid phase synthesis of oligonucleotides, including the phosphate triester method and the phosphite triester method.

1963: Multistep synthesis of biopolymers on solid polymer carriers for the synthesis of polynucleotides. {Robert L. Letsinger and Milton J. Kornet; **1963;** Letsinger and Lundsford **1976***}.*

Photo courtesy NIH U.S. National Library of Medicine (Source)

Har Gobind Khorana (1922 to 2011). Was an Indian American biochemist. Khorana shared the 1968 Nobel Prize for Physiology or Medicine with Marshall W. Nirenberg and Robert W. Holley for research of the cell's genetic code and function in protein synthesis. He led the effort for the total synthesis of the gene for Alanine Transfer yeast tRNA, a historical first. {H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana; **1963;** Brown EL, Belagaje R, Ryan MJ, Khorana HG. **1979,** K. L. AGARWAL, H. BÜCHI, M. H. CARUTHERS, N. GUPTA, H. G. KHORANA, K. KLEPPE, A. KUMAR, E. OHTSUKA, U. L. RAJBHANDARY, J. H. VAN DE SANDE, V. SGARAMELLA, H. WEBER & T. YAMADA Total Synthesis of the Gene for an Alanine Transfer Ribonucleic Acid from Yeast. *Nature* **227,** 27–34 (**1970**).}

Photo courtesy NIH U.S. National Library of Medicine (Source)

Marvin H. Caruthers (born February 11, 1940) is an American biochemist and a Distinguished Professor at the University of Colorado Boulder. He and his research group developed methods for the phosphoramidite synthesis of DNA. This laid the foundation for the efficient automated synthesis of gene fragments and the automation of solid-phase nucleic acid synthesis as well as for the commercial production of DNA synthesizers. {Matteucci and Caruthers, **1981**; Beaucage and Caruthers **1981**}

Photo courtesy NIH U.S. National Library of Medicine (Source)

1. INTRODUCTION

With the increased prevalence of diseases that have defied cures for ages such as cancer and neurodegenerative diseases as well as the resurgence of drug-resistant microbial or viral pathogens, there is an urgent need to develop innovative and efficacious therapies. One such modality is genetic therapy, which has acquired momentum following the identification of multiple genes, whose disruption predisposes to various human disorders. Though the exact mechanisms through which these alterations cause the disorders are still being deciphered, pharmaceutical industries have increasingly been focusing on molecular interactions or signaling initiated by the gene products for target-based drug discovery. This is also the case for virally induced immune disorders like AIDS, whose infectious agent HIV has been extensively characterized at the molecular level. Previously, to abrogate the propagation of cancer cells, modified nucleosides have been administered to interfere with DNA replication. Likewise, for HIV, nucleoside analogs have been used to inhibit reverse transcriptase essential for its replication. Nevertheless, the present inability to treat recurrent cancers or eliminate the viruses calls for the need to develop alternative strategies. Against this backdrop, the potential use of oligonucleotides as therapeutics is increasingly being explored.

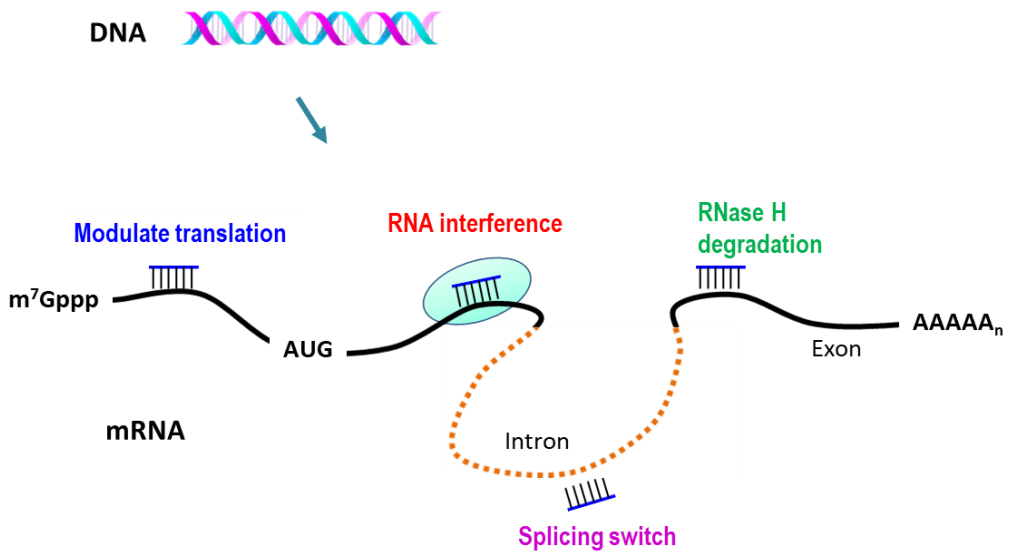


Figure 1. Therapeutic strategies utilizing oligonucleotides.

2. THE EFFECT OF OLIGONUCLEOTIDE: RNA HYBRID ON TRANSLATION

2.1 Antisense oligonucleotide forms hybrid to arrest translation

Within cells, antisense oligonucleotides may inhibit gene expression by triggering RNase-H mediated cleavage of targeted mRNAs or through hybrid-arrest of binding/scanning by mRNA translation complexes. The existence of the latter pathway was convincingly demonstrated by using antisense alpha-oligodeoxynucleotides, which do not elicit RNase-H activity. The stereoisomers alpha-oligodeoxynucleotides and beta-oligodeoxynucleotides differ in configuration at 1' carbon in the deoxyribose sugar of DNA.

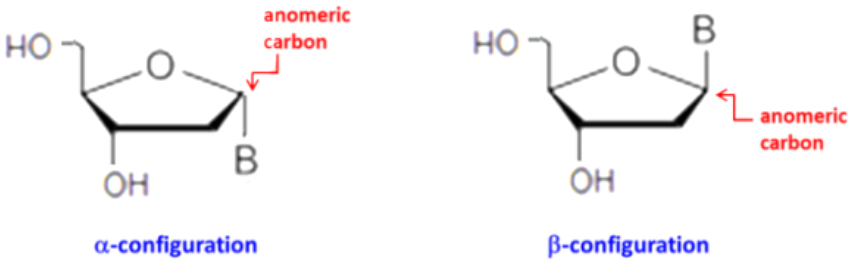


Figure 2. The **configuration** of alpha- and beta-stereoisomers of deoxynucleoside. (B = nucleobase)

The investigators at *Centre National de la Recherche Scientifique* (France) showed that alpha-oligomers prepared complementary to the region adjacent to the cap site or a region spanning the AUG initiation codon reduced the translation of rabbit beta-globin mRNA but left the target mRNA intact. Thus, antisense oligonucleotides can inhibit the translation of mRNA through the formation of oligonucleotide: RNA hybrids without triggering RNase-H activity.

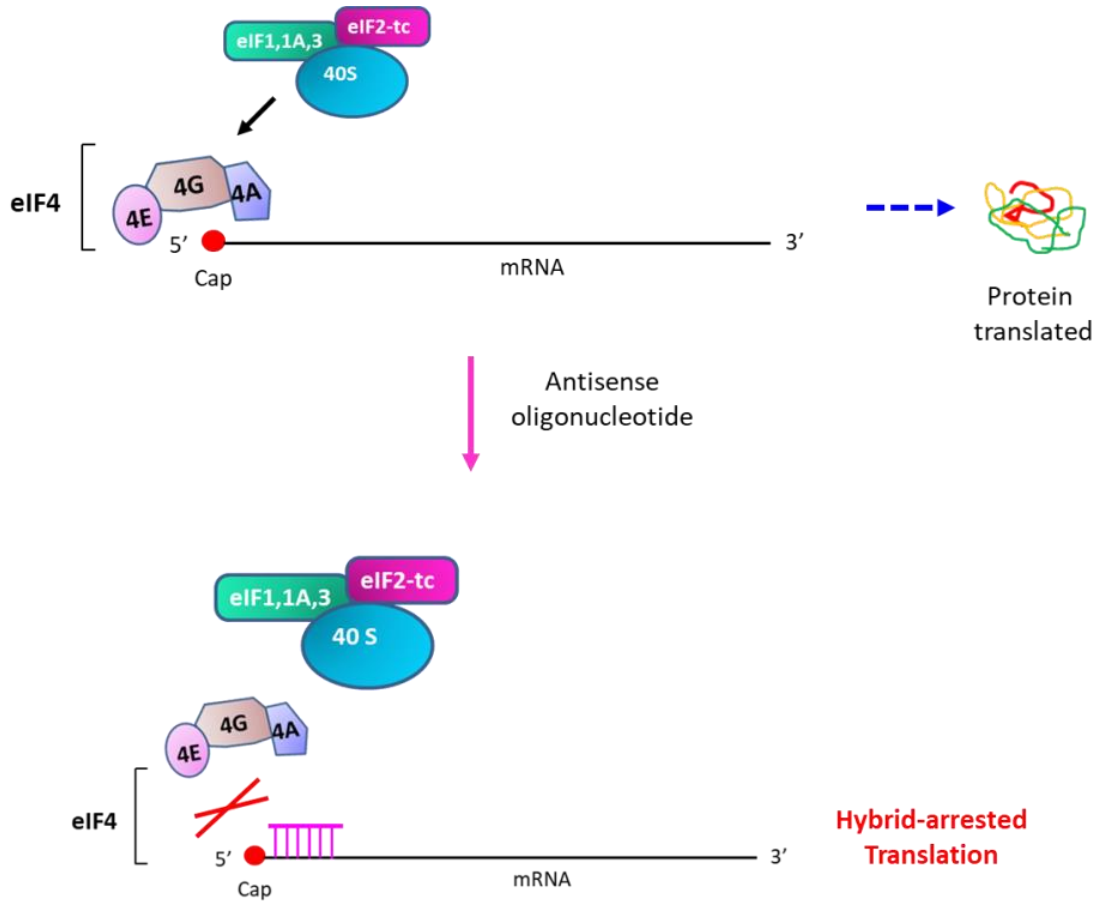


Figure 3. Hybrid arrest by an antisense oligonucleotide reduces the translation of rabbit beta-globin mRNA.

2.2 Antisense oligonucleotides enhance translation

Whereas most applications have been to reduce gene expression, antisense oligonucleotides also have the potential to enhance mRNA translation. This is noteworthy as there is an unmet need to address disorders caused by inadequate levels of protein synthesized. In eukaryotes, cap-dependent translation involves the binding of eIF4F complex (consisting of eIF4A, eIF4E, eIF4G) with the 5′ m^7G cap. Resolving secondary structures formed near the 5' terminus by eIF4E helicase allows 43S complex (comprised of 40S small ribosomal subunit, eIF2-GTP-$tRNA_i^{Met}$ ternary complex, and other factors) to interact, yielding 48S 'pre-initiation complex' to scan the mRNA. At the start codon AUG, 60S large subunit is recruited to form 80S ribosome to begin translation. Within the 5' UTR

(untranslated region), cis-active regulatory elements may exist to modulate translation, such as upstream open reading frames (uORFs) that decrease downstream ORF translation or IRES (internal ribosome entry sites) for cap-independent translation. A stem structure near the cap was suggested to inhibit the loading of pre-initiation complex to mRNA, and antisense oligonucleotides that resolve the structure augmented the translation of human ACP1, LDLR (low-density lipoprotein receptor), or RNASEH1 gene.

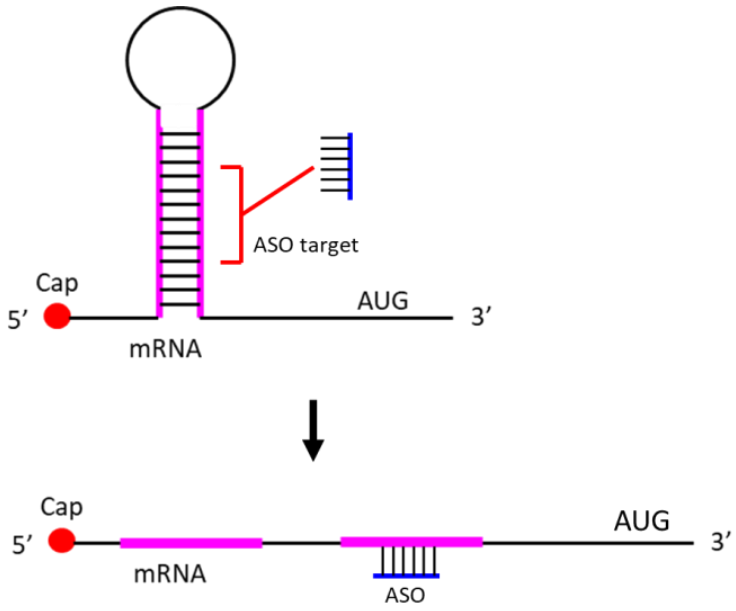


Figure 4. An **antisense oligonucleotide** resolves secondary structure to increase the translation of LDLR mRNA.

3. INDUCING RNASE H1-DEPENDENT DEGRADATION OF RNA

3.1 RNase H1-dependent antisense oligonucleotide

RNase H represents a family of endonucleases that catalyze the degradation of the RNA strand in an RNA/DNA duplex. Regarding its evolution, the RNase H family is broadly divided into H1 and H2 subtypes based on substrate preferences. Whereas RNase H1 is involved in RNA processing and mitochondrial DNA replication, RNase H2 is localized to DNA replication foci and functions in the removal of misincorporated ribonucleotides. The above properties have been exploited to develop antisense oligonucleotides that suppress gene expression by degrading complementary RNA in an RNAse H-dependent manner. The RNase H1-dependent degradation by antisense oligonucleotides occurs in both the cytoplasm and the nucleus.

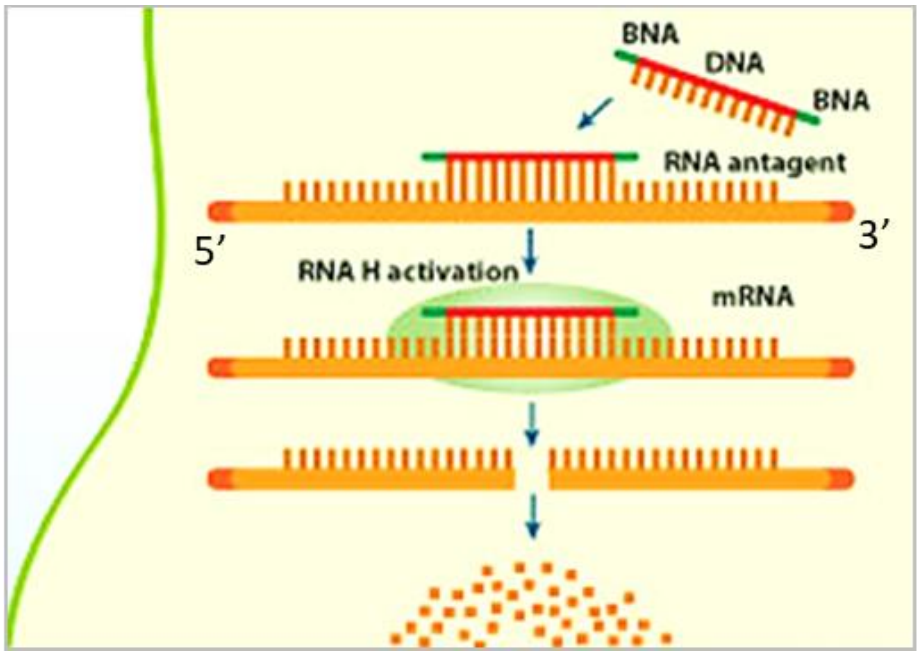


Figure 5. A **gapmer antisense oligonucleotide** targeting mRNA and its subsequent degradation by RNAse H1.

3.2 Development of gapmer oligonucleotides

'Gapmer' is the second-generation antisense oligonucleotide developed that greatly augments their efficacy. Its RNA-DNA-RNA-like configuration consists of 5'-wing followed by a gap of 8 to 12 deoxynucleic acid monomers with phosphorothioate internucleotide linkages, which is followed by a 3'-wing. Both wings contain ribonucleotides with 2'-O-modification or BNA (bridged nucleic acid) or bridged nucleic acids like LNA (locked nucleic acid). BNA^{NC} represents the third generation bridged nucleic acid with superior unequaled advantages in base stacking, binding affinity, aqueous solubility, and nuclease resistance. The degradation of off-target transcripts can be avoided by minimizing sequence complementarity to unintended RNAs and avoiding excessive binding affinities. For clinical use, various antisense oligonucleotide therapeutics have been approved by FDA (U. S. Food and Drug Administration), which includes several gapmers.

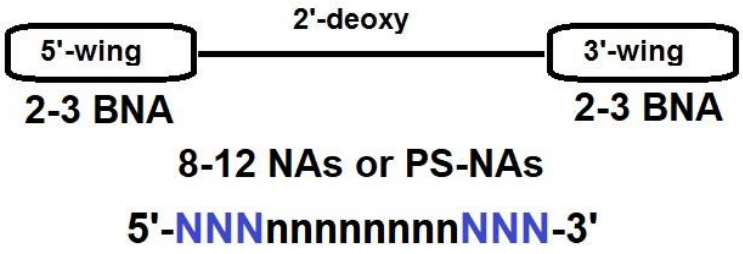


Figure 6. Gapmer Design Example. A gapmer is a chimeric antisense oligonucleotide that contains a central block of deoxynucleotide monomers sufficiently long to induce RNase H cleavage. The central block of a gapmer is flanked by blocks of 2'-O modified ribonucleotides or other artificially modified ribonucleotide monomers such as bridged nucleic acids (BNAs). In a gapmer these modified nucleic acids protect the internal block from nuclease degradation. Natural unmodified DNA, as well as modified DNA analogs such as phosphorothiote DNA analogs can be used to stabilize RNA molecules useful as gapmers for therapeutic approaches.

A gapmer oligonucleotide modified with bridged nucleic acids (BNAs) used as an antisense oligonucleotide (ASO) for the reduction of circulating LDL-cholesterol levels lowers the level of hepatic PCSK9.

In Hypercholesterolemia, high levels of cholesterol are present in the blood. In humans with this condition, during a blood test, elevated levels of cholesterol and lipoproteins are detected. The presence of high plasma cholesterol levels, together with normal plasma triglyceride levels, causes the rise of cholesterol and apolipoprotein B (apoB)-rich lipoproteins, called low-density lipoprotein (LDL) that characterize Hypercholesterolemia.

Cholesterol, a fatty molecule, occurs naturally in the human body since it is essential in cell walls. Cholesterol is also the primary molecule for the biosynthesis of hormones. The intestine absorbs eaten fat and cholesterol transports them to the liver through the blood. Two main types of cholesterol exist in blood: low-density lipoprotein (LDL) cholesterol (the "bad" cholesterol) and high-density lipoprotein (HDL) cholesterol (the "good" cholesterol).

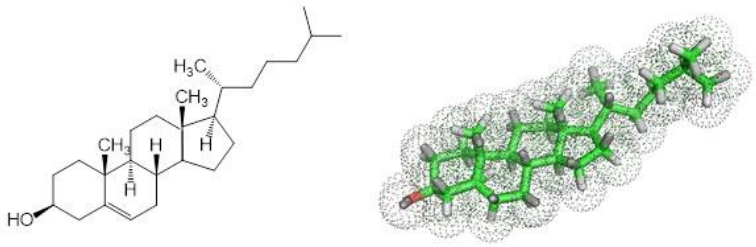


Figure 7. Chemical structure and mode of cholesterol.

The low-density lipoprotein (LDL) receptor binds and internalizes cholesterol-containing particles. Since cholesterol is insoluble in water and in body fluids, it must be transported by a water-soluble carrier. The LDL particle is a sphere 20 to 25 nm in diameter with an outer surface that is a monolayer membrane made up of phospholipids and cholesterol in which one molecule of the large protein called apo-B is embedded. In the nonpolar core, cholesterol is esterified through the single hydroxyl group to a long-chain fatty acid. After transport into the cell by a process called endocytosis, LDL particles are transported to lysosomes. In lysosomes, the apo-B protein is degraded to amino acids and the cholesterol esters are hydrolyzed to cholesterol and fatty acids. The cell incorporates cholesterol directly into the cell membranes or re-esterifies it and stores it as lipid droplets.

Familial Hypercholesterolemia is characterized by very high levels of cholesterol in the blood. This condition is inherited. The body of people that have familial Hypercholesterolemia is unable to excrete extra cholesterol, and therefore it builds up in the blood. Humans with Familial Hypercholesterolemia are at a high risk of developing a form of heart disease called coronary artery disease at a young age.
The proprotein convertase subtilisin/kexin type 9 (PCSK9) protein controls the number of low-density lipoprotein receptors. PCSK9 is a regulatory factor in Familial Hypercholesterolemia (FH). Hence it is an attractive therapeutic target for the reduction of low-density lipoprotein (LDL) cholesterol. PCSK9 is responsible for the degradation of hepatic low-density lipoprotein receptor (LDLR). Low levels of hepatic PCSK9 activities are associated with reduced levels of circulating LDL-cholesterol.

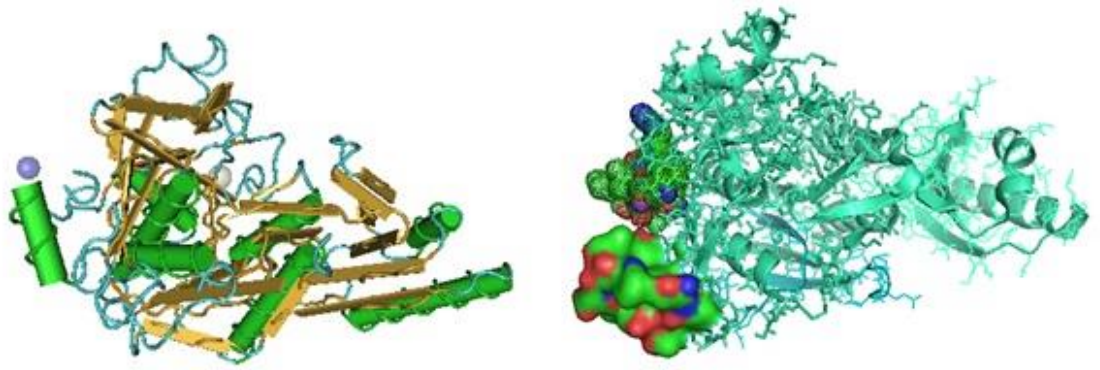


Figure 8. Structural model of Proprotein Convertase Subtilisin/kexin Type 9 protein in complex with 2 peptides. [PDB 5VLK].

In 2020, Gupta et al. reported the use of a gapmer oligonucleotide modified with bridged nucleic acids (BNAs) as an antisense oligonucleotide (ASO) for the reduction of circulating LDL-cholesterol levels by lowering the level of hepatic PCSK9. Gupta et al. designed an LNA antisense oligonucleotide (LNA ASO) complementary to the human and mouse PCSK9 mRNA. A 13-nucleotide long gapmer (Sequence: **GT**ctgtggaa**GCG**; **uppercase BNA/LNA**, lowercase DNA) modified with phosphorothioate internucleoside linkages.

Gupta et al. utilized Human hepatocytes derived cell lines HepG2 and HuH7 and a pancreatic mouse β-TC3 cell line known to express high endogenous levels of PCSK9 for the study. The study showed that the ASO efficiently reduced the mRNA and protein levels of PCSK9. Also, after transfection, levels of LDL-receptor (LDLR) increased. In vivo efficacy of the ASO was further investigated in mice by tail vein intravenous administration of BNA ASO in saline solution. The

level of PCSK9 mRNA was reduced by ~60 %, lasting more than 16 days. Hepatic LDLR protein levels were significantly up-regulated by 2.5 to 3 folds for at least eight days and ~two-fold for 16 days. Analysis of liver alanine aminotransferase (ALT) levels showed that the cells tolerated long term LNA ASO treatment. Also, the treatment did not cause hepatotoxicity.

A second study investigated two bridged nucleic acids (BNA/LNA) antisense oligonucleotides targeting PCSK9 for a lowering of low-density lipoprotein cholesterol levels in nonhuman primates (Lindholm et al. 2012). The study showed that antisense bridged nucleic acid gapmers reduced PCSK9 messenger RNA and serum PCSK9 protein by 85%, resulting in a 50% reduction in circulating low-density lipoprotein cholesterol. Also, the treatment reduced serum total cholesterol levels to the same extent as circulating low-density lipoprotein cholesterol with no change in high-density lipoprotein levels.

Gapmers used for the study were complementary to human (accession #NM174936) and Macaca fascicularis (cynomolgus monkey) PCSK9 mRNA with the sequences: **TGC**tacaaaac**CCA** and **GT**ctgtggaa**GCG**.

4. INDUCING RNA INTERFERENCE TO DEGRADE RNA

4.1 Molecular mechanism of RNA interference

'RNA interference' refers to a defense mechanism developed to counter the invading viruses at the RNA level. The post-transcriptional gene silencing mechanism was originally identified in plants and subsequently shown to be operational in various eukaryotes (ex. fungus, vertebrates). It involves processing a long double-stranded RNA (dsRNA) into short 21-25 bp RNA duplexes (with 2 bp overhang at 3' terminus and phosphorylated 5' terminus) called 'small interfering RNAs' (siRNA) by the enzyme Dicer. The siRNA duplex is presented by Dicer and TAR RNA binding protein (TRBP) to Argonaute to form the 'RNA-induced silencing complex' (RISC). After unwinding the siRNA duplex, one strand is selected (the other strand dissociates from RISC) to serve as a guide to recognize and cleave target complementary single-stranded RNA. In addition to degrading viral genomic RNA or suppressing the expression of mRNA (ex. translational repression by miRNA), RNA interference may function to increase gene expression as targeting a promoter can activate transcription.

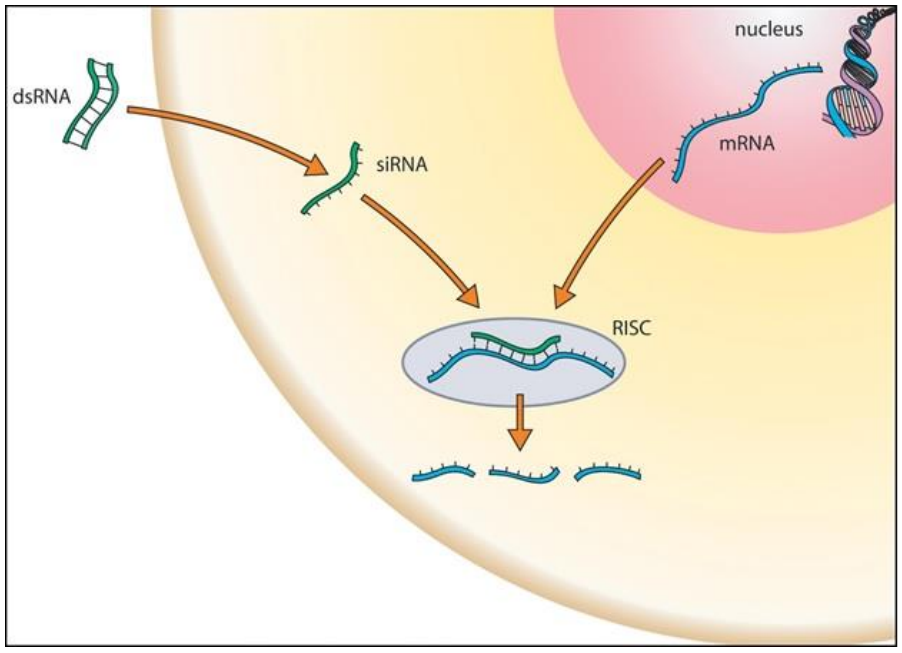


Figure 9. Molecular mechanism of **RNA interference (RNAi)**. The process in which RNA molecules activate the cellular response to destroy specific RNA molecules such as messenger RNAs (mRNAs). (Source: Wiki Commons).

4.2 Triggering RNA interference for therapy

The potential of developing RNA interference-based therapy for various infectious diseases or genetic disorders (ex. neurodegenerative disorders, cancer) is being explored. The siRNA technology involves introducing short dsRNA (~21 bp in length with 2 nucleotide 3'-overhang) into cells. Upon incorporation into the silencing complex RISC, the sense strand is removed typically. The remaining antisense strand guides RISC to target mRNA. Following the cleavage of the target mRNA at a discrete position, the resulting 5' fragment and 3' fragment are degraded by exosome and 5'-3' exoribonuclease 1 (XRN1), respectively. Its catalytic nature allows the degradation process to be repeated with additional mRNA targets. For pharmacological application, various issues such as stability, delivery, renal clearance, and immune response are being addressed.

4.3 Clinical application of siRNA therapeutics

RNA interference provides a unique opportunity to suppress gene expression without permanently inactivating the gene. The ability of siRNAs to suppress gene expression by repeatedly targeting mRNAs for degradation constitutes a distinct advantage pharmacologically. Several notable advances have been made in the last decade. In 2010, a phase I clinical trial showed that systemically delivered self-assembled nanoparticles containing siRNA targeting ribonucleotide reductase can induce RNA interference in melanoma patients. In 2013, a Phase I clinical trial of ALN-VSP, a lipid nanoparticle carrying dual siRNAs targeting vascular endothelial growth factor A (VEGF-A) and kinesin spindle protein KSP, in cancer patients was reported. Onpattro is the first siRNA drug to be approved by the FDA for treating polyneuropathy, a disorder of peripheral nerves, in 2018. Subsequently, the siRNA therapeutics Givlaari (acute hepatic porphyria) and Oxlumo (primary hyperoxaluria type 1) were also approved by FDA.

4.4 Methods for siRNA preparation

Multiple options are available for *in vitro* preparation or *in vivo* expression of siRNAs. For transfecting into cells (via lipofection, electroporation, etc), siRNAs can be prepared by chemical synthesis, digesting long dsRNA using Dicer or RNase III, or *in vitro* transcription. If the sequence is known, then *in vitro* transcribed siRNA could be used as it is less costly than chemical synthesis. If the sequence is unknown, siRNAs can be prepared by digesting long dsRNA. To identify targets for drug development, chemically synthesized siRNAs can be used to prepare libraries for the high throughput screening. For clinical applications that

require large quantities, highly pure siRNAs can be synthesized chemically. To express siRNAs using DNA-based constructs *in vivo*, plasmids or virus-based vectors are used. To bypass time-consuming construction steps, PCR-amplified siRNA expression cassettes can be utilized. For long-term knockdown *in vivo*, expression plasmids with selection markers could be employed.

Therapeutic biomolecules such as conjugated oligonucleotides or peptides biomolecules are manufactured under strict, quality control processes. Analytical HPLC and MS analyses are performed at every development cycle. Depending on the type of conjugation chemistry used, after buffer exchange (if necessary), conjugates undergo gel filtration or use of the centrifugal concentrator to remove excess cross-linking reagents and oligonucleotides. Then, by either size-exclusion chromatography (SEC) or reverse phase HPLC, they may also be used to either remove excess reagents or isolate and characterize the cross-linked product. Once the product has been purified, it may be subject to many different types of studies including spectroscopic (MALDI-TOF, ESI, LC-MS Fluorescence) and electrophoresis.

Quality control: QC (quality control) and QA (quality assurance) procedures are also followed independently to guarantee the highest quality of every conjugate made. The final quantity is systematically validated by UV absorbance at 260 nm.
siRNA are generally used as annealed duplexes. To ensure maximum stability modified RNA are transported or stored lyophilized in individual tubes. RNA oligonucleotides are highly susceptible to degradation by exogenous RNases introduced during handling. Therefore, it is crucial that all handling steps be conducted under sterile, RNase-free conditions. Upon receipt, dried RNA oligonucleotides may be safely stored in a freezer for up to 6 months at -20 Cº. Dilute each RNA oligonucleotide aliquot to a concentration of 50 µM. Combine 30 µl of each RNA oligonucleotide solution and 15 µl of 5x annealing buffer (see below). The final volume is 75 µl. The final concentration of the duplex is 20 µM.

To anneal siRNAs, incubate the solution for 1-2 minutes in a water bath at 90-95 Cº and allow to cool to room temperature on a workbench. Centrifuge the tube briefly to collect all liquid at the bottom of the tube. Generally, slow cooling should take about 45-60 minutes. Store on ice until ready to use. Once annealed, duplex siRNA is much more resistant to nuclease than single-stranded RNA and can be safely stored frozen at -20 Cº. The 5x annealing buffer can be freeze-thawed up to 5 times. 5x annealing buffer: 50 mM Tris, pH 7.5, 100 mM NaCl.

5. Modifications to increase stability

Modifications of the ribose unit in DNA and **RNA** oligonucleotides influence the biological properties and base-pairing ability of nucleic acids. In natural RNA, during RNA maturation, various enzymes can introduce chemical modifications into ribonucleotide residues. Chemical alteration can occur in the base, at the 2'-hydroxyl of the ribose, or both. Additional modifications can be introduced chemically in non-natural synthetic RNAs.

The modification of the ribofuranose in nucleic acids allows the manipulation of nucleic acid activities. The local conformation and chemical reactivity of the sugar is changes according to the modification type. Conformational restriction of the ribose units allows the increase in DNA and RNA affinity and enhances biological stability and antisense properties. Alterations in the sugar moiety conformation modify the local and potentially the global structure of nucleic acids. The properties of modified sugar units in oligonucleotides influence the recognition, binding of ligands, and enzymatic activity of proteins.

For example, 2'-modification often improves nuclease resistance, whereas the introduction of **bridged nucleic acids** (BNAs) significantly increases thermal stability.

Modern automated oligonucleotide synthesis methods enable technologies for the development of plasmid-based gene therapies, antisense, short interfering RNA (siRNA), short hairpin RNA (shRNA), aptamers, and other nucleic acid-based therapeutics. Since natural DNA or RNA oligonucleotides degrade easily, have low binding affinities and poor cellular uptake, chemical modifications overcome many of these limitations and allow the introduction of unique properties as well.
Many **chemical modifications** have been made to bases, the ribose sugar and the sugar-phosphodiester backbone with the goal to improve their properties for a whole range of applications.

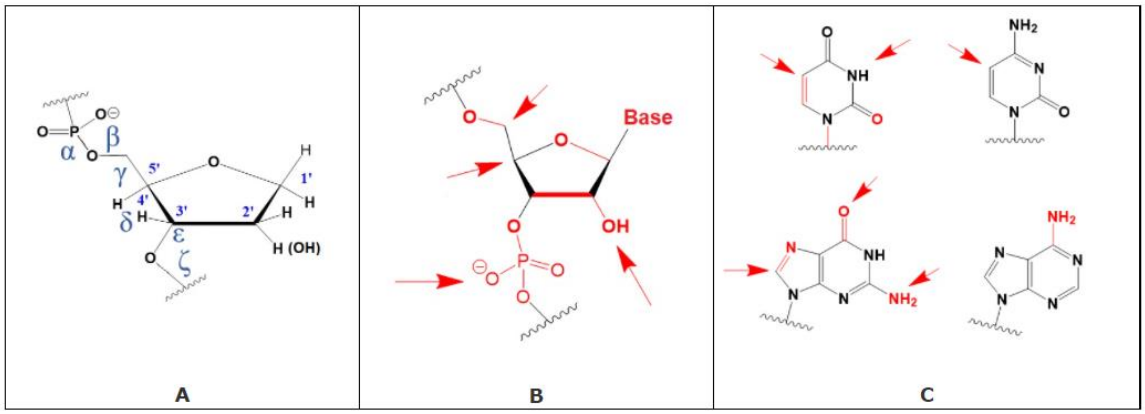
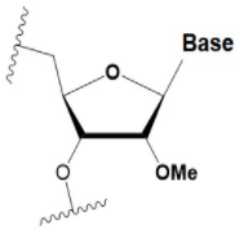
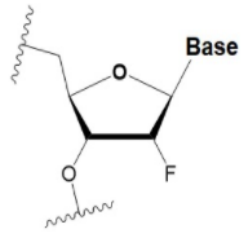
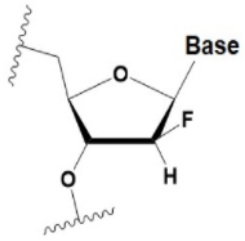
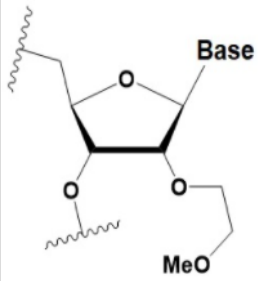
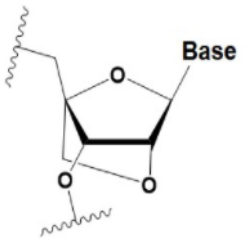
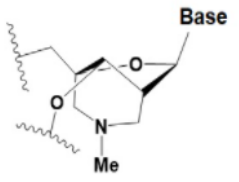
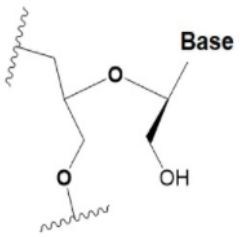
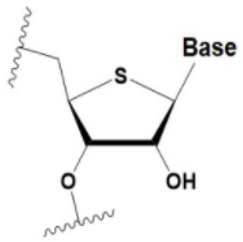
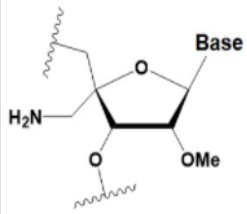
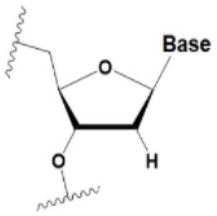
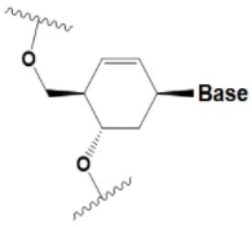
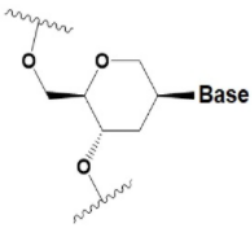


Figure 10. siRNA modification sites. A. Labeling nomenclature for ribose sugar unit and phosphate group. Sugar carbons are labeled as 1′-5′ and backbone torsion angles are described as follows: α=O3′(i−1)−P−O5′−C5′; β=P−O5′−C5′−C4′; γ=O5′−C5′−C4′−C3′; δ=C5′−C4′−C3′−O3′; ε=C4′−C3′−O3′−P(i+1); ζ=C3′−O3′−P(i+1)−O5′(i+1) B. Possible sites of introduction of chemical modifications in the sugar moiety and phosphate linkage are marked in red. C. Possible sites for chemical modifications in nucleic acid bases.

Table 1: RNAi modifications of Sugar Units

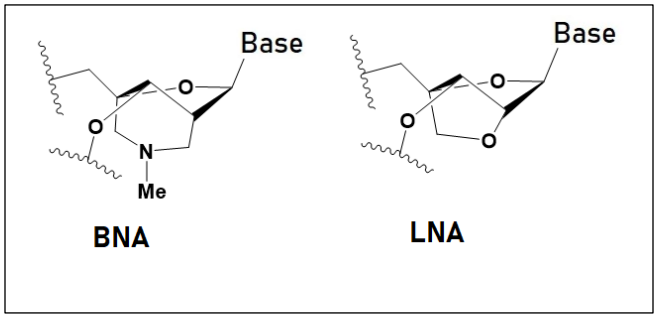
Modification	Structure	ΔTm duplex per modification	Impact on the efficiency of RNAi	Others
2′-O-methyl (2′O-Me)		+0.5–1.5◦C	Two or more consecutive 2′O-Me **inhibit RNAi.** Some siRNAs containing 75–82% 2′O-Me are biologically active.	**Stabilizes 3′ endo ribose conformation.** ≥5–30% of 2′O-Me **increase nuclease resistance in vitro** and **in vivo.** 2′O-Me analogs of A, G and U **reduce the immune response**.
2′-fluoro (2′F)		+1.5–4◦C	**2′F analogs** in all siRNA positions only **slightly reduces the activity of RNAi**.	**Stabilizes 3′ endo ribose conformation.** ≥50% 2′F **increase nuclease resistance in vitro** and **in vivo**. 2′F analogs of adenine (≥7%) **reduce the immune response in vitro**. >50% of the 2′F in siRNA **may cause toxicity**.

2 'F-arabinonucleic acid (2'FANA)		+1.2◦C	100% 2'FANA in the sense chain **reduce the efficiency of RNAi.** ≥30% 2 'FANA in the antisense chain **inhibit RNAi.**	**Stabilizes 2' endo ribose conformation.** ≥50% 2'FANA **increases nuclease resistance in vitro** more effectively than **2'F.** **Protect siRNA from the action of exoribonucleases**.
2 ' -O-methoxyethyl (2'O-MOE)		+0.9–1.7◦C	2' -MOE at the flanks of the sense strand and the central part (6–11) of the antisense strand are **tolerable for RNAi. Replacement of 9th or 10th nucleotides from the 5' end** to 2 'O-MOE analogs of nucleotide **increases the probability of entry in RISC**.	**Stabilizes 3' endo ribose conformation**. ≥15% 2'O-MOE at the ends of the siRNA sense chain increases nuclease resistance in vitro.
Locked nucleic acid (LNA)		+2–8◦C	**≥40% LNA in the sense chain inhibit RNAi by 5–20%.** **>20% LNA** in the antisense chain, or the first LNA nucleotide at the 5' end **completely inhibit RNA**. LNA can change thermal asymmetry of the duplex, increasing the efficiency of siRNA.	**Reduces the conformational flexibility of nucleotides by fixing the C3' endo conformation of the ribose**. ≥10–20% LNA in siRNAs **increase nuclease resistance in vitro** and **in vivo.**
Bridged nucleic acid (BNA)			**≥40% BNA in the sense chain inhibit RNAi by 8–25%.** **>20% BNA** in the antisense chain, or the first BNA nucleotide at the 5' end **completely inhibit RNA**. BNA changes thermal asymmetry of the duplex, increasing the efficiency of siRNA.	**Reduces the conformational flexibility of nucleotides by fixing the C3' endo conformation of the ribose**. ≥10–20% BNA in siRNAs **increase nuclease resistance in vitro** and **in vivo.**
Unlocked nucleic acid (UNA)		−5–8◦C	>15% UNA **inhibit RNAi.** UNA can change thermal asymmetry of the duplex, **increasing the efficiency of siRNA**.	**Increases conformational flexibility of nucleotides and reduces the melting point of the duplex.** UNA at the 3'ends of the duplex protect siRNA from 3' exoribonucleases in vitro and in vivo.

4′-thio-ribonucleosides (4′S)		+1 ◦C	**>7–15% 4′S** in the antisense strand **inhibit RNAi.**	**>10–15% 4′S at the ends** of the strands **increase the nuclease resistance in vitro.**
4′-C-aminomethyl-2′-O-methyl (4′CAm2′OMe)		−1 ◦C	**>2 analogs in the sense** or **>1 analog in the antisense strand inhibits RNAi**.	**≥2 modifications at the 3′ ends increase nuclease resistance in vitro.**
Deoxyribonucleotide (dNMP)		−0.5◦C	>50% dNMP inhibits RNAi. dNMP can change thermal asymmetry of the duplex, **increasing the efficiency** of siRNA in vitro.	Protects against exoribonucleases.
Cyclohexenyl nucleic acids (CeNA)		+1.5◦C	**5% CeNA** in siRNA are **tolerated** by RNAi. CeNA can change thermal asymmetry of the duplex, **increasing the efficiency of siRNA in vitro**.	**Stabilizes 3′ endo ribose conformation.** ≥25% CeNA analogs increase serum nuclease resistance.
Hexitol nucleic acids (HNA)		+0.85◦C	15% HNA in siRNA are tolerated by RNAi (Fisher et al., 2009). HNA can change thermal asymmetry of the duplex, increasing the efficiency of siRNA in vitro (Herdewijn and Juliano, 2007).	Slightly increases siRNA resistance to nucleases in serum (Fisher et al., 2009).

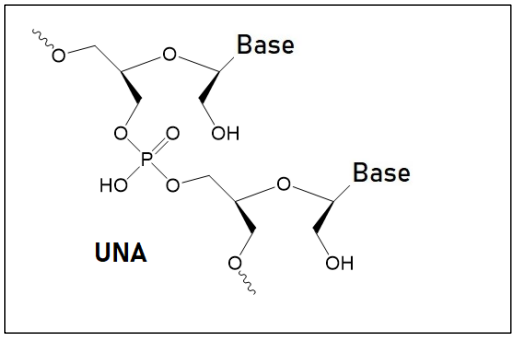
(Adapted from: Chernikov et al. 2019)

5.1. Bridged or Locked Nucleic Acids



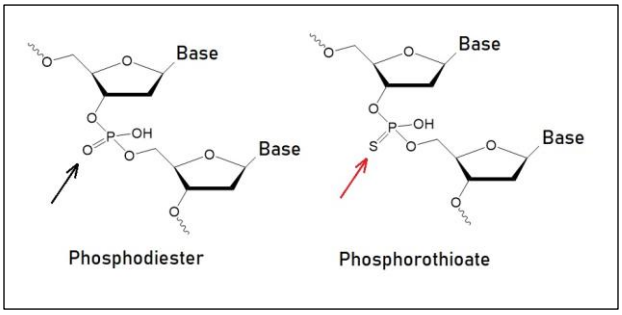
Bicyclic nucleic acids are compounds in which a ribonucleoside is linked between the 2'-oxygen and the 4'-carbon atoms with linking groups. The linking groups result in a constrained 3'-endo conformation resulting in increased duplex stability with highest thermal stability of all available modifications and increased nuclease resistance.

5.2 Unlocked Nucleic Acids (UNAs)



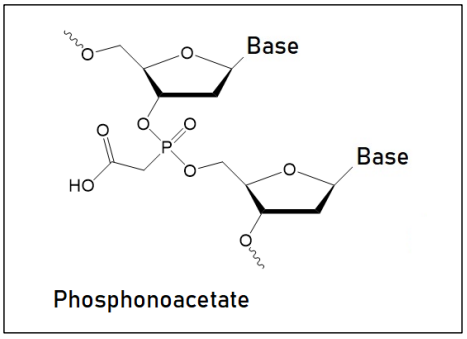
Here the C2' and C3' atoms of the sugar moiety have been cleaved. These compounds enable fine tuning of duplex thermodynamic stabilities and have resistance to nucleases as well.

5.3 Phosphorothioate



Here the phosphodiester bond is modified by replacing one of the non-bridging oxygens with a sulfur atom. Oligonucleotides with this modification hybridize to the target sequences with a lesser affinity than oligonucleotides with a phosphodiester backbone. Imparts resistance to nuclease degradation.

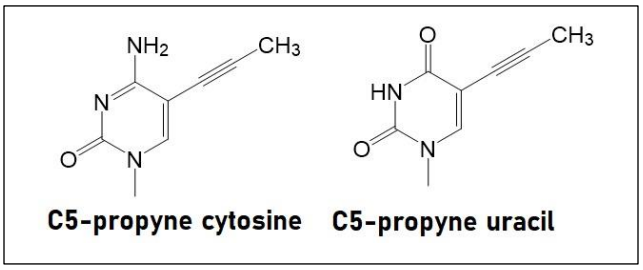
5.4 Phosphonacetate



Here the oxygen atom in the phosphate group has been replaced with a carboxylic acid. Oligonucleotides with this modification have increase duplex stability and are resistant to nucleases.

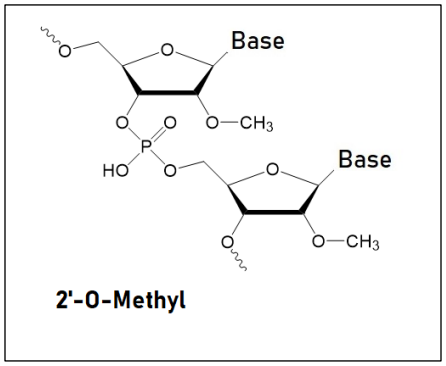
5.5. Propyne Analogs

C-5 propyne analogs of dC and dT are available with increased binding affinity to target mRNA, increased stability, and increased nuclease resistance



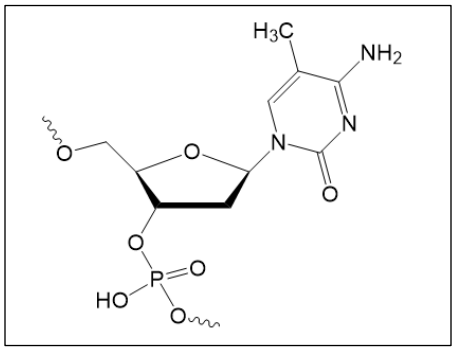
5.6. 2’-O-methyl RNA

This compound contains a 2'-O-methyl group located at the 2’ hydroxyl position which is a common type or RNA modification. It’s binding is similar to DNA but has increased resistance to nucleases.



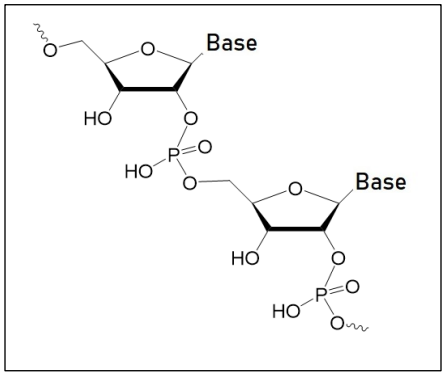
5.7. 5-Me-dC

Compared to non-methylated bases, C-5 methyl pyrimidine nucleotides stabilize duplexes. Oligonucleotides containing this modification have increased duplex stability but can be degraded by nucleases like DNA.



5.8. 2’-5’-linked RNA

Oligonucleotides with a 2'-5' linked phosphodiester linkage form stable complexes with poly rU with slightly lower melting temperatures (Tms) than natural nucleic acid-based oligonucleotides. However, oligonucleotides modified with these nucleic acids have increased binding efficiency to RNA and an increased resistance to nucleases.

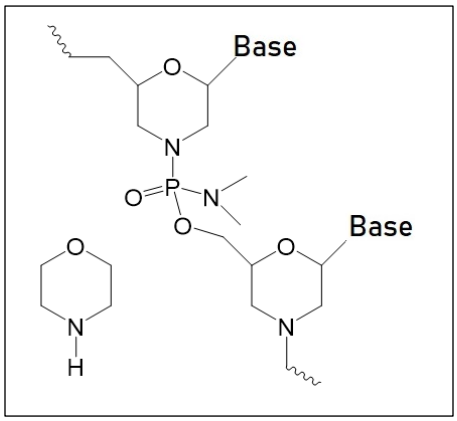


5.9 Chimeric Linkages

Oligonucleotides modified with a mix of phosphorothioate and phosphodiester linkages show increase duplex stability and resistance to nucleases.

5.10. Morpholinos

Replacement of sugar and diester linkages. Morpholinos replace the sugar part of the nucleotide for a morpholine ring (a 6 atom ring) instead of deoxyribose rings and are linked through phosphorodiamidate groups instead of phosphates. This change renders these molecules uncharged at physiological pH.



6. In Vivo Delivery of Therapeutic ASOs

6.1. Carrier Molecules for RNA delivery

The extremely short half-lives, poor chemical stability, and natural degradation of RNAs by nucleases resulted in the development of carrier molecules at the nanoscale for RNA delivery into cells. Nanoparticle-based delivery systems protect RNA molecules from enzymatic degradation and immune system attacks and enable the accumulation of RNA in tumors.

In general, nanoparticles range from 10 to 200 nanometers (nm), which enhances their tumor permeability and retention effect.

Currently, nanocarriers utilized for RNA delivery are

[1] lipid-based nano-systems,
[2] polymeric nanomaterials,
[3] inorganic nanoparticles, or
[4] Bio-inspired nano vehicles.

Table 2: Nanoparticle-based platforms for RNA delivery (Lin et al. 2020).

Nanocarriers	Classifications	Advantages	Disadvantages
Lipid-based nanostructures	Liposomes; solid lipid nanoparticles; lipid emulsions.	Easy preparation, with good biocompatibility and biodegradability.	Limited stability, easy leakage of payloads, and rapid clearance.
Polymer-based nanomaterials	Natural or naturally derived polymers: chitosan, poly-l-lysine, atelocollagen, etc. Synthetic polymers: PLGA, PEI, PVA, PLA, PEG, etc.	Good biocompatibility and biodegradability for natural or naturally derived polymers, low cost of production, stimulation of drug release, easy modification.	Nondegradable for some responsive polymers, dose-dependent toxicity.
Inorganic NPs	MSNs, CNTs, QDs, and metal nanoparticles (e.g., iron oxide and gold nanoparticles)	Easy surface modification, good reproducibility, and easy cell uptake.	Non-biodegradability, potential toxicity.
Bio-inspired nano-vehicles	DNA-based nanostructures, exosome-mimetic nanovesicles, red blood cell member-based ghosts.	Good biodegradability, low toxicity, strong targeting and low immune induction.	High cost, stability concern.

6.2. Efficient Delivery of Therapeutic RNA

The efficient delivery of RNA molecules into cells or tissue, such as siRNAs, is a necessity for the development of therapeutic RNAs. For many years, the difficulty encountered in efficient and safe *in vivo* delivery of RNAs prevented the widespread clinical use of siRNA-based therapies until recently.

The first RNA interference (RNAi) drug Onpattro for the treatment of polyneuropathies was approved by the FDA in 2018.

Already, siRNAs for the SARS coronavirus targeting the regions in the genome encoding spike protein and ORF1b (NSP12) have been designed as well, one targeting the 'Leader' sequence that could potentially suppress the replication of SARS coronaviruses.

To improve cellular uptake of small interfering RNAs (siRNAs), Osborn et al. studied the effect of lipid conjugated siRNAs on cellular endogenous lipid transport pathways to determine the biodistribution of conjugated siRNAs.

For the design and synthesis of lipid hydrophobically modified RNA conjugates (hsiRNAs), the researchers used a panel of biologically occurring, but structurally diverse lipids. A clinically validated, chemically altered siRNA scaffold is at the heart of the design. Earlier studies established that cholesterol-conjugated siRNAs have an 8- to 15-fold higher activity when pre-complexed into purified high-density lipoprotein (HDL) than when injected alone. Recent studies indicated that the asialoglycoprotein receptor (ASGPR) contributes to the uptake of unconjugated phosphorothioate-modified antisense oligonucleotides (PS ASOs). The most clinically advanced trivalent N-acetylgalactosamine (GalNac)-siRNA binds to ASGPR on hepatocytes with high selectivity triggering a potent and durable gene silencing in patients. Lipids, when conjugated to siRNA molecules, enhance circulation time, and promote local and systemic delivery and efficacy. For example, cholesterol-modified siRNA silence liver apolipoprotein B (ApoB) expression at high doses.

6.3. Design of hydrophobically modified siRNA (hsiRNA) conjugates

The scaffold used for the design of lipid-hsiRNA conjugates consists of a hydrophobically modified siRNA scaffold, called hsiRNA. The conjugate contains a 20-nucleotide (nt) antisense or guide strand and a 15-nt sense or passenger strand with alternating 2'-O-methyl and 2'-fluoro sugar modifications. The 5'-end of the antisense strand contains the stable phosphate mimic (E)-vinylphosphonate (VP). The addition of VP to the 5'-end of the guide strand increases siRNA tissue accumulation, extends silencing activity duration, and shields oligonucleotides from exonuclease-mediated degradation, thereby promoting cellular internalization. Because of the rapid degradation and clearance of unmodified oligonucleotides, these modifications stabilize siRNAs and prevent their degradation in cells and tissue. The 3'-end of the sense strand is an optimal position for the attachment of conjugates since it has a minimal effect on siRNA-RISC or intracellular RNA-induced silencing complex loading. The use of linkers, such as the TEG linker, allows attachment of lipid moieties.

Table 3: Lipids, Linkers and Modification for the design of hsiRNA conjugates

Lipids used:	Lithocholic acid (LCA), Docosahexaenoic acid (DHA), Docosanoic acid (DCA), Cholesterol (Chol).
Linker used:	Triethylene glycol (TEG).
Modifications used:	2'-Fluoro RNA, 2'-O-Methyl RNA, Cholesterol, Phosphorothioate, TEG linker, 5'-Vinylphosphonate.

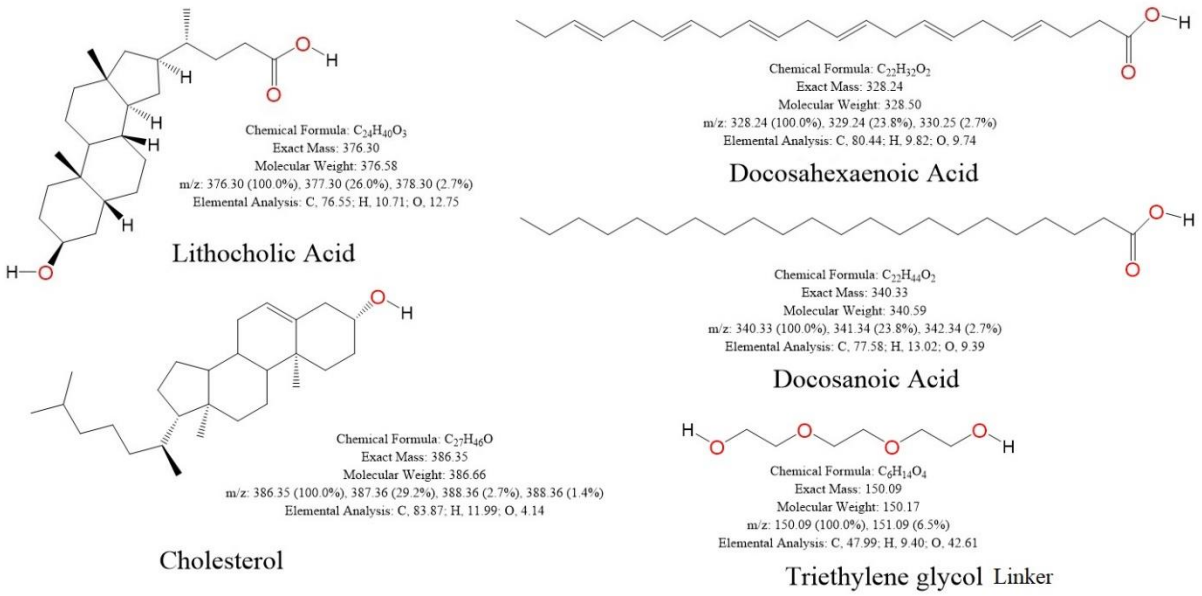


Figure 11. Chemical structures of lipids and linkers used for the conjugation of siRNAs.

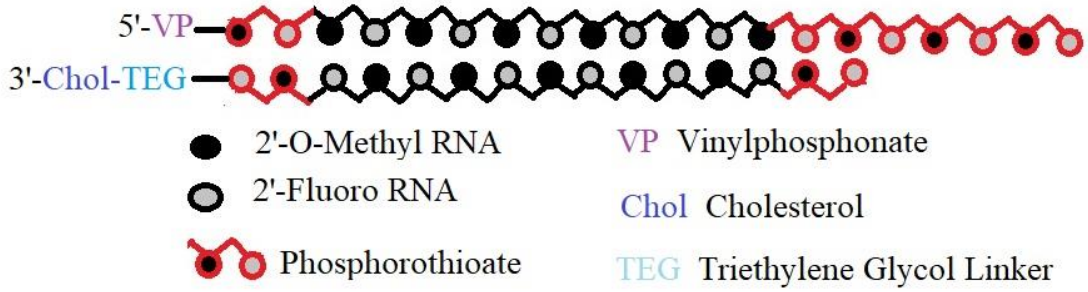


Figure 12. Modification pattern of a lipid-hsiRNA.

6.4. In vivo delivery of lipophilic siRNA

RNA interference (RNAi) is a conserved biological process for specific silencing of gene expression. Small interfering RNAs (siRNAs) are important tools for the control of gene expression, such as post-transcriptional gene silencing in mammals, including humans. However, to function as desired, they need to be taken up by cells or tissues and delivered to selected targets. For *in vivo* applications of siRNAs, delivery across plasma membranes remains the most significant obstacle.

Synthetic oligonucleotides can be modified via conjugation to lipophilic functional groups to allow efficient uptake of siRNAs by cells and tissues. Lipophilic groups are often attached to the 3′-end of the sense strand via oligomethylene linkers of various lengths. Alternatively, various commercially available lipophilic phosphoramidites also allow conjugation to oligonucleotides.

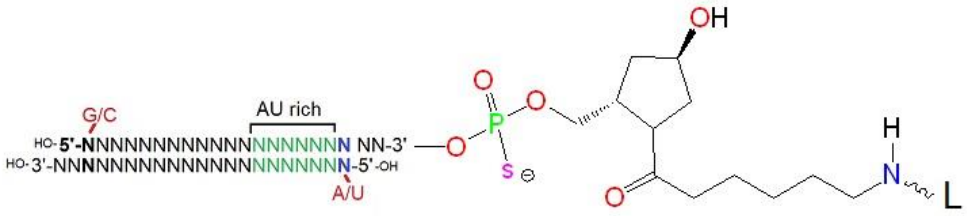


Figure 13. Lipophilic siRNA conjugate structure. siRNAs when conjugated to different lipids have different in vivo activities. The desired lipophile (L) is conjugated to the 3′-end of the sense strand of the desired siRNA, here via a trans-4-hydroxyprolinol linker (Modified after Wolfrum et al. 2007).

Covalent siRNA cholesterol conjugates are known to increase import into cellular where they induce RNAi resulting in the silencing of endogenous genes *in vivo*. Wolfram et al. in 2007 reported the preparation of a variety of lipophilic carrier molecules useful for optimized cell delivery. Long-chain fatty acid conjugates are helpful for cell delivery. However, for all these lipophilic molecules, efficient and selective cellular uptake depends on their interaction with lipoprotein particles, lipoprotein receptors, and transmembrane proteins. As Wolfrum et al. reported, the efficiency of siRNA delivery into cells depends on the combined length of the linker and the lipophilic group.

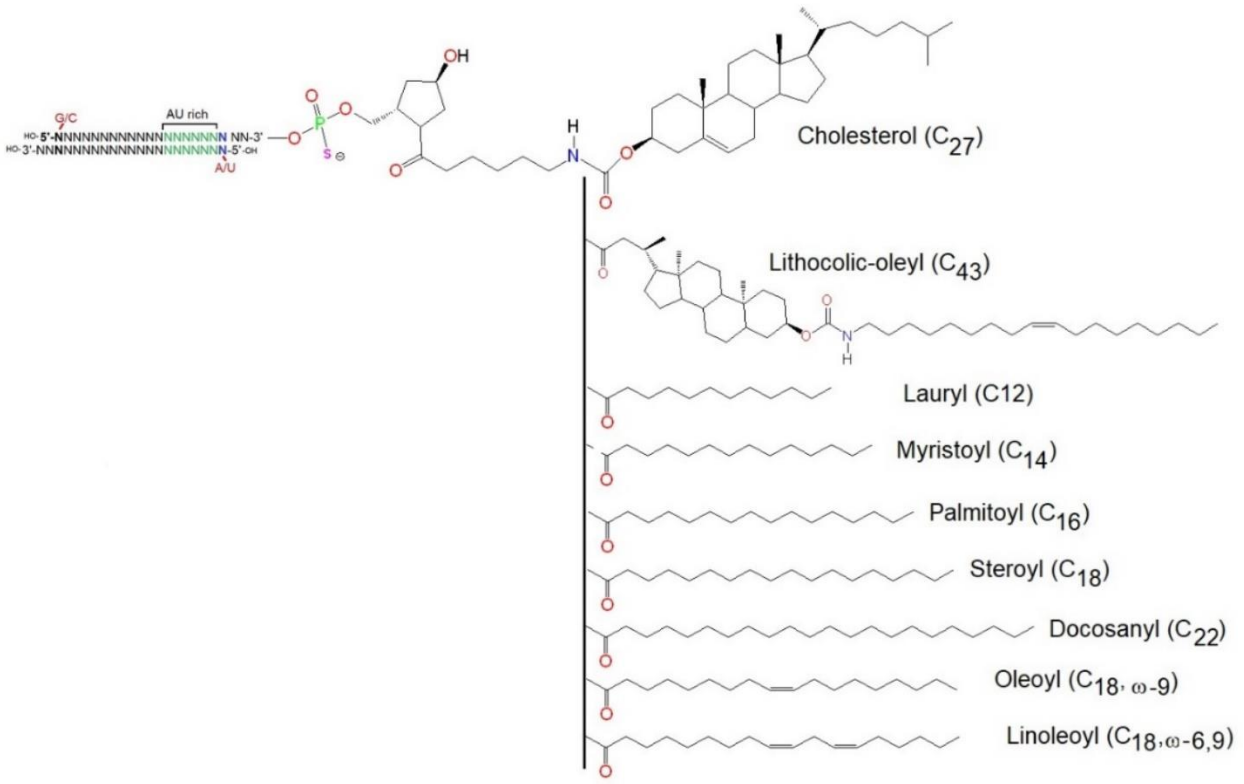


Figure 14. Structures of lipophilic siRNAs with various lipids conjugated to the core structure of the siRNA conjugate.

6.5. Cell-penetrating Peptides as Vehicles for Cargo Delivery

Also, a wide range of cell-penetrating peptides (CPPs) can be used as a vehicle for intracellular delivery of macromolecules. Conjugation of CPPs to oligonucleotides increases the oligonucleotide's intracellular delivery efficiency. Similarly, conjugation of siRNAs to CPPs enhances their intracellular delivery.

Cell permeable peptides (CPPs) are carrier molecule with small peptide domains that can freely cross cell membranes. They have been mainly used as carriers of proteins and nucleic acids into the cell. The first CPP was discovered independently by two laboratories in 1988 when it was found that the trans-activating transcriptional activator (Tat) from Human Immunodeficiency Virus 1 (HIV-1) could be efficiently taken up from the surrounding media by numerous cell types in culture.

Typically, CPPs have an amino acid composition containing either a high relative abundance of positively charged, cationic amino acids such as lysine or arginine,

or have sequences that contain an alternating pattern of polar/charged amino acids and non-polar, hydrophobic amino acids. Some examples include the TAT peptide (YGRKKRRQRRR), the lipid membrane translocating peptide (KKAAAVLLPVLLAAP) and the Antennapedia leader peptide (KKWKMRRNQFWVKVQRG). CPPs belong to a wide variety of peptide families.

CPPs can enter the cell with their carrier by three mechanisms: (i) Direct delivery involving energy independent entry of the CPPs into the cell, (ii) endocytosis where the cells take up the CPPs by engulfing the peptides with their cell membranes, and (iii) translocation through the formation of transient structures.

CPPs have found numerous applications in medicine as drug delivery agents in the treatment of different diseases including cancer, virus inhibitors, and contrast agents for cell labeling.

6.6. Therapeutic siRNAs for SARS-CoV-2 (COVID19)

The outbreak of the novel coronavirus Severe-Acute-Respiratory-Syndrome-2 (SARS-CoV-2, COVID-19) pandemic in December 2019, started the testing of many drugs for the treatment of the disease. Candidates for testing are the antiviral drugs remdesivir, favipiravir, lopinavir, ritonavir, and arbidol. Other candidates for clinal trials are the antimalarial drug hydroxychloroquine, and anticancer agents interferon-alpha 2b. However, the efficacy of these drugs against SARS-CoV-2 has yet to be proven. In that light, siRNA-based treatments may also provide a therapeutic solution. A few studies have already demonstrated that selected siRNA candidates have the potential to be effective against the outbreak of SARS and the Middle-East Respiratory Syndrome (MERS). Several siRNA-related patents already were also issued: CN1548054, WO2005019410, CN101173275, CN101113158, CN1010085986, and US865352.

Earlier studies showed that siRNAs targeting sequences coding for several viral genes of various SARS viruses decreased the viral load between 50 to 95 %. siRNAs targeting the N protein gene appeared to work the best. Zhang et al., in 2004, demonstrated that the expression of SARS-CoV spike protein is silenced in cultured cells using RNA interference. For expression the spike protein in cultured cells, the research group used an expression vector with a cytomegalovirus (CMV) promoter. Tagging the spike protein with hemagglutinin allowed the monitoring of its expression. Vero E6 cells were used for the propagation of the SARS-CoV

strain investigated. A vector containing a U6 promotor enabled the construction of a 22 base pair hairpin siRNAs using the sequence UUCAAGAGA for the hairpin loop.

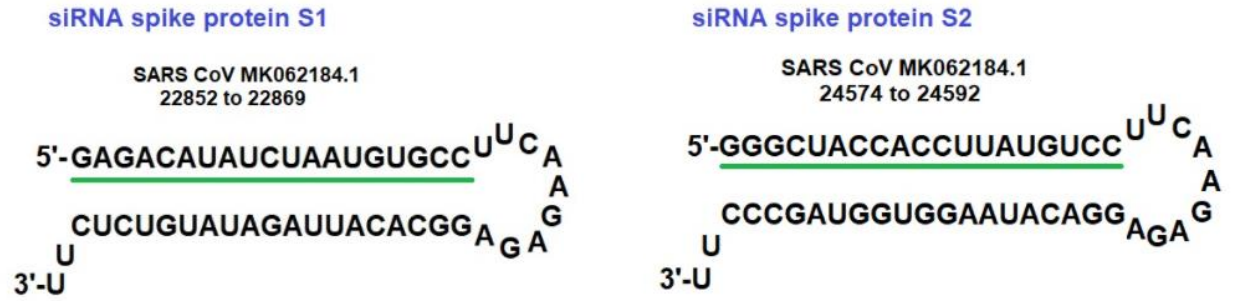


Figure 15. Diagram of predicted siRNA structures (Zhang et al. 2004).

The following year Li et al. reported the design of siRNAs targeting the leader sequence of SARS-CoV to inhibit virus replication. The results showed that siRNAs inhibit the replication of the SARS-CoV virus in infected Vero E6 cells. The scientists identified a putative 5′ leader sequence like the conserved coronavirus core leader sequence, 5′-CUAAAC-3′, at the 5′-end of the genome. Furthermore, manual alignment of sequences upstream of potential methionine codons with the leader sequence region allowed the identification of the putative conserved transcription-regulating sequence (TRS), AAACGAAAC. Next, the research group cloned the transcripts of the four genes from SARS-CoV BJ01 and confirmed the SARS-CoV Leader sequence as

CCAGGAAAAGCCAACCAACCTCGATCTCTTGTAGATCTGTTCTCTAAACGAAC.

This sequence is present in the SARS CoV HKU-39849 genome. Li et al. showed that a siRNA targeting the leader sequence of SARS-CoV inhibited gene expression and replication of the virus in cultured cells. siRNAs can also be chemically synthesized and modified such that they can enter cells with high efficiency allowing optimal delivery into cells. For example, modifying synthetic oligonucleotides with lipophilic groups allows for efficient uptake of siRNAs by cells.

SARS CoV HKU-39849 - JQ316196

```
       1 ATATTAGGTT TTTACCTACC CAGGAAAAGC CAACCAACCT CGATCTCTTG TAGATCTGTT
      61 CTCTAAACGA ACTTTAAAAT CTGTGTAGCT GTCGCTCGGC TGCATGCCTA GTGCACCTAC
     121 GCAGTATAAA CAATAATAAA TTTTACTGTC GTTGACAAGA AACGAGTAAC TCGTCCCTCT

SiRNA     5′-GCCAACCAACCUCGAUCUC-UUCAAGAGA
         3′-UUCGGUUGGUUGGAGCUAGAG
```

Figure 16. Sequence information for siRNA design for SARS-CoV. The leader sequence as reported by Li et al. is underlined and highlighted in turquoise. The sequence region used for an siRNA is highlighted in green. The sequence UUCAAGAGA was used for the hairpin loop. In a similar fashion, siRNAs can be designed for silencing the leader sequence of SARS-CoV-2 (COVID-19).

SARS-CoV-2 MT601295.1

```
       1 ATTAAAGGTT TATACCTTCC CAGGTAACAA ACCAACCAAC TTTCGATCTC TTGTAGATCT
      61 GTTCTCTAAA CGAACTTTAA AATCTGTGTG GCTGTCACTC GGCTGCATGC TTAGTGCACT
     121 CACGCAGTAT AATTAATAAC TAATTACTGT CGTTGACAGG ACACGAGTAA CTCGTCTATC

SiRNA     5′-CCAACCAACUUUCGAUCUCUU-UUCAAGAGA
         3′-UUGGUUGGUUGAAAGCUAGAGAA
```

Figure 17. Example of siRNA design for SARS-CoV-2.

In summary, Li et al. demonstrated that siRNAs significantly inhibit Spike protein expression in transfected cells as well as in SARS-CoV infected cells. The observed inhibition of S gene mRNA accumulation suggests that siRNAs may inhibit SARS-CoV replication in cells. Therefore, completely knocking out or silencing one of the viral genes should block replication of the virus. However, since one siRNA will only knock down the expression of the targeted gene, several siRNAs may be needed for complete inhibition of viral replication.

Tang et al., in 2008, studied the effect of siRNA duplexes as SARS CoV inhibitors in vitro and evaluated in vivo efficacy and safety in a rhesus macaque SARS model. Delivery of siRNAs occurred using intranasal administration with a clinically viable delivery carrier in three dosing regimens. The use of microscopy allowed the inspection of SARS CoV-induced SARS-like symptoms in the animal's respiratory tract and lungs. Viral RNA testing and immunohistochemistry

sections from 21 tested macaques consistently demonstrated siRNA-mediated anti-SARS CoV activity. This study illustrated that siRNA enables a reduction in development time for novel siRNA based targeted therapeutic agents.

Sutou et al., in 2009, reported the design of siRNAs for knocking down SARS-CoV genes. The researchers used siRNA-expression vectors and synthetic double-stranded RNA (dsRNA) as a model for siRNA Design. The study found that 19-mer to 21-mer dsRNAs with a matched 2-nt overhang at the 3'- end of the antisense strand exhibited the highest activity. Since the thermodynamic stability of siRNAs at terminal ends had little effect the 2-nt overhang appears to be more critical than thermodynamic stability for the selection of the antisense strand to the RNA-induced silencing complex (RISC).

Nur et al., in 2015, used computational methods for the identification and design of siRNAs predicted to silence MERS-CoV genes. The study designed four miRNAs and five siRNAs for silencing nine different MERS-CoV strains. The use of ORF1ab sequences allowed the prediction of siRNA and miRNA molecules.

7. Other Applications

7.1 Splice Switching Oligonucleotides

Splice-switching oligonucleotides are short, synthetic, oligonucleotides that act as antisense oligonucleotides. Oligonucleotides containing modified nucleic acids, for example bridged nucleic acids (BNAs), base-pair with pre-mRNA with high affinity and disrupt normal splicing of transcripts by blocking the RNA–RNA base-pairing or protein–RNA binding interactions that occur between components of the splicing machinery and pre-mRNAs. Splice-switching antisense oligonucleotides containing a backbone modified with 2'-O-methyl-phosphorothioate groups and approximately 60% BNAs, as well as two BNA-modified nucleotides at the 3'-end and one BNA-modified nucleotide at the 5'-end appear to work well for controlling and modulating the expression of specific exons hence they can act as antisense oligonucleotides enabling modulation and regulation of splicing events.

Exon skipping oligonucleotides can be designed rationally. Design approaches involve the selection of an antisense sequence targeting the exon that is supposed to be skipped. Design parameters to be taken into account include the activity of the splice-switching oligonucleotide, the melting temperature, the guanine-cytosine content, the length of the oligonucleotide, as well as the secondary structure or sequence motif corresponding to a splicing signal of the target RNA that influence the activity of the splice-switching oligonucleotide. Since the synthesis of modified oligonucleotides is now routinely done in an automated fashion, splice-switching oligonucleotides, containing modified or unmodified nucleic acid, are now commercially available.

Splicing explained: According to the NCI Dictionary of Genetics Terms "splicing" is defined as the "process by which introns, the noncoding regions of genes, are excised out of the primary messenger RNA transcript, and the exons (i.e., coding regions) are joined together to generate mature messenger RNA. Mature mRNA then serves as the template for synthesis of a specific protein." Proper expression of most protein-coding genes requires the splicing of pre-mRNA. In other words, splicing refers to the editing of newly transcribed pre-messenger RNA (pre-mRNA), the removal of introns and the joining or ligation of exons. Genes encoded in the nucleus are spliced within the nucleus either during or after transcription.

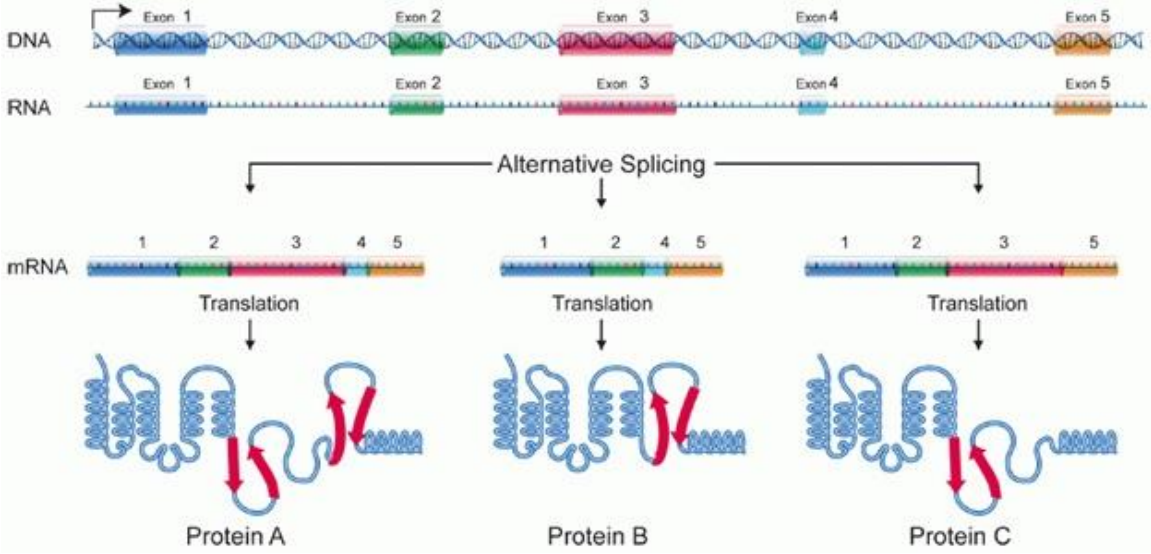


Figure 18. Illustration explaining the process of "Alternative, or Differential Splicing." As a result of splicing a single gene can code for multiple proteins. During alternative splicing a specific exons of a gene may be included within or excluded from the final messenger RNA (mRNA) produced from that gene.

In 2017, Manning et al. showed that BNA^{NC} gapmers revert splicing and reduce RNA foci with low toxicity in Myotonic Dystrophy Cells.

Myotonic dystrophy type 1 (DM1) is a disease caused by an expanded CTG repeat in the 3'-UTR of the dystrophia myotonica protein kinase (DMPK) gene. The research group utilized short DNA-based antisense oligonucleotides known as gapmers for the degradation of the toxic CUG expanded repeat (CUG_{exp}) RNA.

The study demonstrated that the 2',4'-BNA^{NC}[NMe] (BNA^{NC}) modification is a promising nucleoside analog with high potency similar to 2',4'-LNA (LNA). The utilized BNA^{NC} gapmers targeting a nonrepetitive region of the DMPK 3' UTR resulted in an allele-specific knockdown of CUG_{exp} RNA and reverted characteristic DM1 molecular defects including mis-splicing and the accumulation of RNA foci. Also, the BNA^{NC} gapmers tested did not induce caspase activation, in contrast to a sequence matched LNA gapmer.

7.2. Antisense Oligonucleotides in Neuroscience

Antisense oligonucleotides slow the progression of the pediatric neurodegenerative disorder Batten's disease by correcting mRNA splicing, paving the way for personalized medicine.

Neuronal ceroid lipofuscinoses (NCLs) refer to a group of neurodegenerative disorders, which negatively impacts vision, behavior, learning, speech, etc., causing seizure, impaired motor skill, reduced intellectual capacity and early death. The NCLs have been categorized based on the age of onset for symptoms (i.e. infantile, late infantile, juvenile, and adult) and the identity of the predisposing gene. 'Batten's disease' selectively refers to juvenile NCL (JNCL) form; however, some have used the term to refer to all NCL forms collectively. The neurodegenerative disorder, which is progressive and can be fatal, typically occurs during childhood and there is no cure presently. Approximately 2-4 per 100,000 children are affected in the U. S. while the frequency may be higher (~1/12,500 people) in some populations.

To date, 14 genetically distinct NCL forms have been identified. 'Infantile neuronal ceroid' (INCL) is associated with CLN gene encoding PPT1 (palmitoyl-protein thioesterase 1) that functions as a lysosomal enzyme. Late infantile NCL (LINCL) with the life expectancy of 8-12 yrs is linked to CLN2 gene, which encodes the lysosomal enzyme TPP1 (tripeptidyl-peptidase 1). Variant of the late infantile NCL is caused by the disruption of CLN6 gene, which encodes the transmembrane protein of endoplasmic reticulum CLN6 (linclin). Another variant of late infantile NCL is associated with the CLN7 gene encoding MFSD8 ('major facilitator superfamily domain containing eight'), a lysosomal transmembrane protein. 'Juvenile NCL' (JNCL) occurs due to the inactivation of CLN3 gene, which encodes a lysosomal transmembrane protein similar to SLC (solute carrier) transporters. Accurate diagnosis of NCL requires the determination of the mutated gene and the resultant dysfunctional protein (for genetic counseling).

Most NCLs exhibit autosomal recessive mode of inheritance. The disease is manifested if both alleles of the predisposing gene inherited from parents are defective. Though a carrier may not develop symptoms, the individual is capable of passing the defective allele to a descendent. An exception may be found in Kufs disease, a distinct form of NCL, whose symptoms appear around age 30 with a limited survival of less than 15 years after the onset. Its type A is linked to PPT1 and CLN6 genes, while type B (autosomal dominant) is associated with CTSF (cathepsin F) and DNAJC5 ('DnaJ homolog subfamily C member 5') genes. Cathepsin is involved in proteolytic degradation in lysosomes.

Batten's disease belongs to a group of disorders known as 'lysosomal storage disease', which is comprised of ca. 50 distinct autosomal recessively inherited genetic disorders. The disease occurs due to the dysfunctional lysosomes, resulting in the intracellular accumulation of excess amount of undegraded substrates. The

dysfunction is caused by a defect or insufficient level of the lysosomal enzymes (ex. cathepsin, alpha-glucosidase, acid phosphatase) for the degradation of carbohydrates, lipids, proteins, nucleic acids, etc. The failure to breakdown into smaller components could lead to the death of the affected cells (ex. neurons) eventually. Lysosomes are also involved in autophagy (wherein autophagosomes fuse with lysosomes to generate autolysosomes to degrade defective intracellular proteins, damaged organelles, pathogenic microbes, etc.), which has been associated with aging-associated disorders, heart disease, cancer, etc.

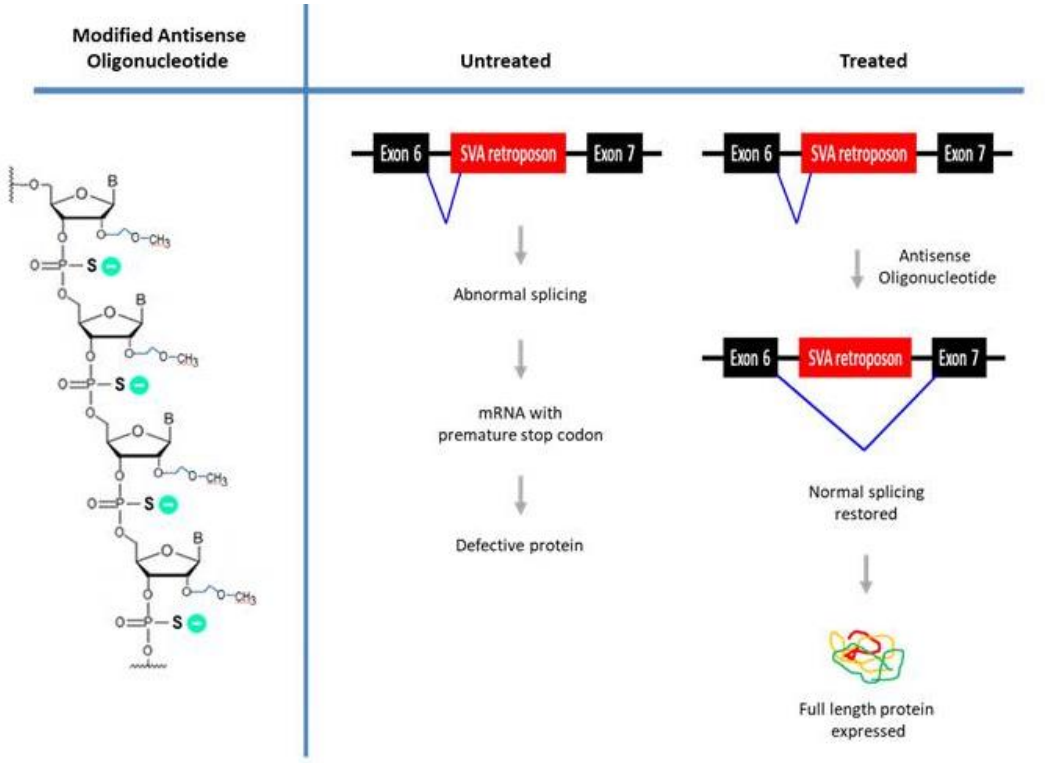


Figure 19. Illustration explaining the process of restoring normal splicing.

Batten's disease remains a terminal disease with limited treatment options. In 2017, FDA approved the enzyme replacement therapy, Brineura, which involves intraventricularly administering recombinant human TPP1, the missing enzyme for NCL patients with defective CLN2 gene. More recently, an mRNA-modulating treatment utilizing antisense oligonucleotides was reported though applicable for only a single patient. A 6-year-old girl with worsening symptoms, which included seizures, ataxia, developmental regression, deteriorating vision with mild cerebral/cerebellar atrophy, was diagnosed with Batten's disease. Genetic analysis

revealed that she was heterozygous for a pathogenic mutation in the MFSD8 (CLN7) gene. The whole genome sequencing showed that the other MFSD8 allele (both the patient and mother) contains the SVA (a composite of SINE-VNTR-Alu) retroposon inserted in intron 6. The retroposon caused exon 6 to mis-splice into the cryptic splice-acceptor site, 'i6.SA', located upstream of the retroposon, resulting in a faulty transcript with premature translational termination.

To correct the altered splicing, investigators at the Harvard University-affiliated Boston Children's Hospital designed Milasen, a 22 bp antisense oligonucleotide, to block the aberrant 'i6.SA' splice acceptor site or *exonic splicing enhancer* (ESE) elements nearby. To increase stability, the oligonucleotide consisted of 2′-O-methoxyethyl (MOE) ribonucleotides with phosphorothioate internucleotide linkages. How the modified oligonucleotides were uptaken by neural cells is not clear though phsophorothioate-modified oligonucleotide is known to interact with several receptors (ex. stabilin receptor in the liver). Milasen increased the ratio of normal-to-mutant splicing 2.5 to 3-fold in patient derived fibroblasts. The investigational drug Milasen was administered intrathecally to distribute to the brain via cerebrospinal fluid. During the treatment (~1 year), no serious adverse events occurred according to the authors. Though it did not cure, a notable reduction in the intensity and frequency of seizures was reported.

7.3. BNAs for the treatment of triple negative breast cancer

Cancer continues to pose a problem as approximately ~1500 are expected to die daily from cancer in the U.S. alone. Breast cancer is the leading type of cancer for women and is more common with higher survival rates in developed world. In addition to conventional therapies, treatments include hormone blocking therapy and targeted therapy, ex. trastuzumab inhibiting human epidermal growth factor receptor 2 (Her2) receptor.

For therapy, breast cancers are often classified according to their 'triple status'. Triple status refers to the expression level of human epidermal growth factor receptor 2 receptor (Her2), estrogen receptor (ER), and progesterone receptor (PR). Triple negative breast cancer lacks the expression of these receptors, undermining the efficacy of drugs targeting these receptors. Given their poor survivability, an urgent need exists to develop novel therapies.

Oncogenic signaling pathways driven by protein kinase B (AKT) or mitogen activated protein kinase (MAPK) play a critical role in regulating the growth of

various human cancers including breast cancer. The activity of these kinases is negatively regulated by phosphatases.

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression by targeting mRNAs for degradation or suppressing mRNA translation. The microRNA miR-21 was shown to be upregulated in human breast cancer cells. As the targets of miR-21 include tumor suppressors phosphatase and tensin homolog (PTEN) (Meng *et al.*, 2007) and human MutS protein homolog 2 (hMSH2), suppressing the activity of miR-21 represents an attractive approach for treating triple negative breast cancer.

A promising therapy is to devise an oligonucleotide-based blocking agent that hybridizes to miR-21 in the RNA-induced silencing complex (RISC) to free target mRNAs from degradation. The potency of the miR-21 blocker could be greatly improved by using aminomethyl bridged nucleic acid (BNA) as it allows better base-pair stacking and a high stability, elevates Tm, and lowers toxicity. Thus, the therapeutic efficacy of the miR-21 blocker could be increased through the BNA technology.

7.4. Therapeutic Editing of Mutated RNA (RNA Editing)

The conversion of adenosines to inosines by ADARs is known as RNA editing.

RNA is the blueprint for protein production in cells. Adenosine deaminases acting on RNA (ADAR) present in cells can catalyze single-base changes in RNAs. Adding a guide RNA to ADAR allows RNA editing of complementary strands. Compared to DNA editing, RNA editing is reversible since cells constantly metabolize new RNA copies. A transcript's sequence is altered during RNA-editing by insertion, deletion, or modification of nucleotides. The result is a change in information content from the original encoded genomic transcript.

Adenosine deaminases acting on RNA (ADARs) enzymes convert adenosine to inosine in duplex RNA via a deamination reaction of adenosine. Inosine functions similarly to guanosine in many cellular processes including during splicing, translation, and reverse transcription by base pairing with cytidine.

Adenosine-to-inosine editing can form alternative splice variants, alter microRNA processing and targeting, change codon sequences and suppress the activation of the innate immune system by endogenous double-stranded RNAs.

Dysregulated RNA editing is linked to neurological disorders such as epilepsy, seizures, and Amyotrophic Lateral Sclerosis (ALS) involving ADAR2. Apparently, mutations in the ADAR1 gene also cause the autoimmune disease Aicardi-Goutieres Syndrome and Dyschromatosis Symmetrica Hereditaria.

Adenosine-to-inosine (A-to-I) editing enables the treatment of guanosine-to-adenosine (G-to-A) mutations. A-to-I editing involves the hydrolytic deamination of adenosine to an inosine base. The protein family of RNA-specific deaminases called "adenosine deaminases acting on RNA (ADARs) mediate this reaction by acting on different types of RNA. However, editing events in coding regions of mRNAs are of particular interest since every A-to-I change is read as an A-to-G change during translation. Utilizing this reaction allows the recoding of RNA sequences to correct genetic mutations within mRNAs. One major challenge is re-directing ADAR's activity towards A's that are not naturally edited.

In 1986, Benne et al. reported that the mitochondrial cytochrome oxidase (cox) subunit II gene from trypanosomes contains a frameshift at amino acid 170. Since no second version of the coxII gene was detected, the research group concluded that the extra nucleotides are added during or after transcription of the frameshift gene by an RNA-editing process.

In 1995, Woolf et al. pointed out that treating genetic diseases caused by specific base substitutions is possible by rationally designed RNA editing of mutated RNA sequences. To demonstrate that therapeutic RNA editing is possible, the research group used a synthetic complementary RNA oligonucleotide to direct the correction of a premature stop codon mutation in dystrophin RNA.

The researchers employed complementary RNA oligonucleotides in conjugation with cellular double-stranded RNA adenosine deaminase (dsRAD). Directed RNA editing involves hybridization of the complementary RNA oligonucleotide to a premature stop codon followed by treatment with nuclear extracts containing the cellular enzyme double-stranded RNA adenosine deaminase. This experiment resulted in a dramatic increase in the expression of a downstream luciferase coding region. The analysis of the cDNA sequence revealed the deamination of the adenosine in the UAG stop codon to inosine by double-stranded RNA adenosine deaminase. As a proof of principle, the injection of oligonucleotide-mRNA hybrids into Xenopus embryos also increased luciferase expression.

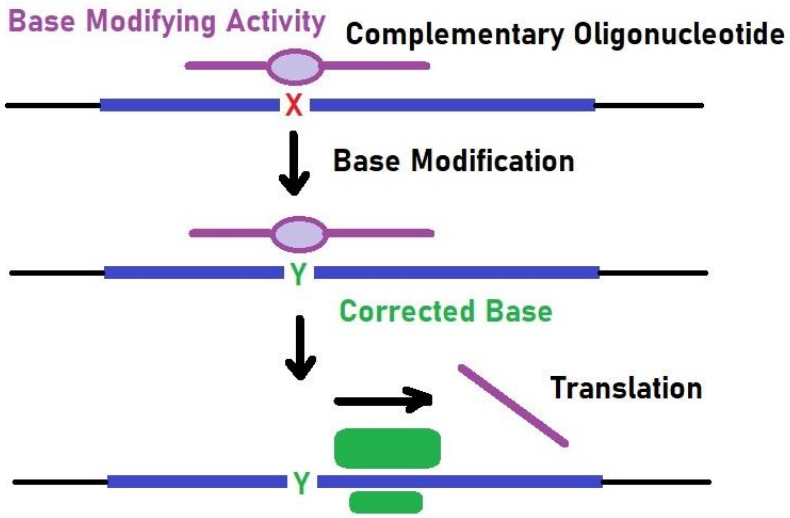


Figure 20. Schematic representation of general therapeutic RNA editing. X represents a mutated nucleotide and Y represents a corrected nucleotide. The hybrid formed by the RNA oligonucleotide and the targeted region containing the mutated base forms a substrate for double-stranded RNA adenosine deaminase (dsRAD) (Adapted from Woolf et al. 1995).

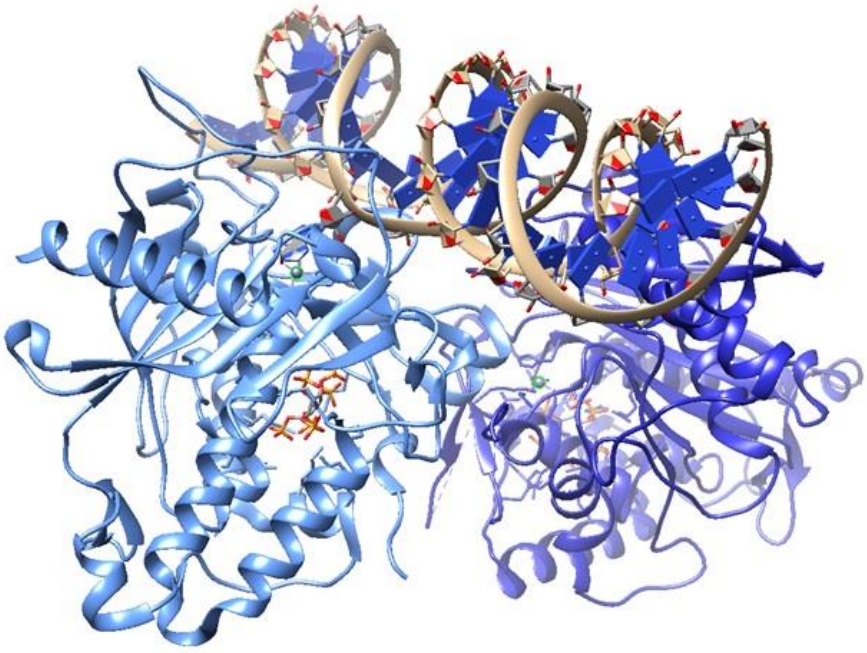


Figure 21. Adenosine Deaminase Acting on dsRNA mutant E488Q in complex with a dsRNA from the human GLI1 gene sequence [PDB ID 6VFF].

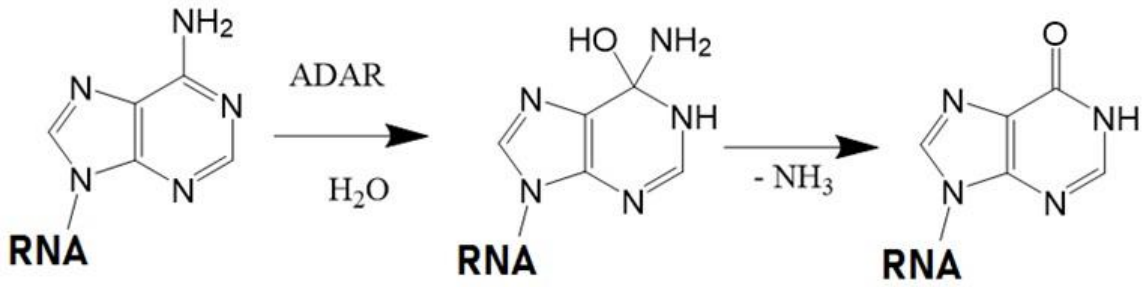


Figure 22. Reaction mechanism of ADAR2 including showing the intermediate.

ADARs contain two main structural motifs. The first motif contains a double-stranded RNA binding Domain (dsRBD). The second motif contains a Deaminase Domain (DD) carrying out the catalytic activity. ADARs use a flipping mechanism to move the adenosine out of the A-form RNA helix into the enzyme's catalytic pocket where the A-to-I conversion occurs.

Kim et al. recently used an exon-skipping antisense oligonucleotide (ASO) strategy in cultured human bronchial cells expressing the mutation to achieve gene-specific NMD evasion in the hope to cure the disorder. A mixture of two antisense oligonucleotides (ASOs) was utilized that promotes the skipping of exon 23 of the CFTR-W1282X mRNA resulting in NMD resistance thereby preserving the reading frame. The hope is to develop a therapeutic approach allowing the treatment of CF in affected people.

Cystic fibrosis (CF) is an inherited disorder of the lungs, digestive system, and other organs of the human body. Cystic fibrosis affects the cells producing mucus, sweat and digestive juices. In people with CF, a gene defect causes these secretions to become sticky and thick. These thick secretions plug up tubes, ducts, and passageways, especially in lungs and pancreas. Presently, there is no treatment available for CF caused by the CFTR-W1282X mutation located on CFTR exon 23. Nonsense-mediated messenger RNA (mRNA) decay (NMD) degrades the CFTR-W1282X mRNA. The result is a low level of functional CFTR protein.

7.5. Oligonucleotides and the Blood Brain Barrier

Like other drugs, oligonucleotide drugs will have to pass through multiple barriers before reaching their cellular targets in the human body. For oligonucleotide-based therapies to work in the central nervous system, therapeutic antisense oligonucleotides (ASOs), interfering RNAs (RNAi), or silencing RNA (siRNA), need to cross the blood-brain barrier (BBB).

The term blood-brain barrier (BBB) describes the unique properties of the microvasculature of the central nervous system (See Daneman and Prat for a review). The blood-brain barrier consists of a system of blood vessels of the central nervous system (CNS) with unique properties that allow these vessels to tightly regulate the movement of ions, molecules, and cells between the blood and the brain. The blood-brain barrier maintains brain homeostasis and prevents foreign molecules from entering the brain.

Oligonucleotide-based molecules potentially allow the design and synthesis of therapeutics for the treatment or cure of many diseases. Conjugation to hydrophobic molecules such as cholesterol and tocopherol and modifications of nucleic acids promise to enhance their delivery into cells or tissue and increase the stability of therapeutic oligonucleotides.

Most medicines approved by the US Food and Drug Administration (FDA), European Medicines Agency (EMA), and other regulatory authorities do not significally accumulate in the brain since these medicines do not cross the blood-brain barrier.

Potentially, oligonucleotide-based agents can treat or cure almost any disease and promise to be critical therapeutic drug classes of the future. Bio-conjugated oligonucleotides are now emerging from basic research and are successfully translated to the clinic.

In 2015, Nishina et al. developed a system for the delivery of short interfering RNA (siRNA) to the liver by using α-tocopherol conjugation. Nishina et al. identified a new BNA-DNA gapmer structure in which drug delivery system molecules are bound to ASOs with unlocked nucleic acid (UNA) sequences. The study found an α-tocopherol–conjugated siRNA is effective and safe for RNA interference–mediated gene silencing in vivo.

The research group extended the 5′-end of an ASO sequence by using 5′-α-tocopherol–conjugated to 4- to 7-mer UNAs for improved performance. In the

liver of mice, the intravenous injection with the α-tocopherol–conjugated chimeric ASO achieved a more potent silencing than ASO alone. The UNA wing was cleaved or degraded inside the cells resulting in the release of α-tocopherol from the 13-mer gapmer ASO. This reaction resulted in activation of the gapmer. The α-tocopherol–conjugated chimeric ASO showed high efficacy, with hepatic tropism in vivo. In 2021, Nagata et al. showed that DNA/RNA heteroduplex oligonucleotides (HDOs) conjugated to cholesterol or α-tocopherol at the 5' end of the RNA strand reach the CNS in mice and rats after subcutaneous or intravenous administration. After administration, the HDOs distribute throughout the brain, spinal cord, and peripheral tissues. The specifically selected HDOs suppressed the expression of four target genes by up to 90% in the CNS. In contrast, single-stranded ASOs conjugated to cholesterol showed limited activity. The research group observed gene knockout in major CNS cell types with the most significant effect in neurons and microglial cells. Subcutaneous delivery or dividing intravenous injections limited side effects. This study showed that cholesterol-conjugated HDOs crossed the blood-brain barrier more effectively making them candidates for future CNS drugs.

How does cholesterol help transport molecules such as oligonucleotides between cells? Cholesterol transport occurs among organelles. Cholesterol is delivered to lysosomes by low-density plasma lipoprotein (LDL) via receptor-mediated endocytosis. Each LDL particle contains approximately 500 molecules of free cholesterol and about 1,500 molecules of esterified cholesterol. Acid lipase hydrolyzes esterified cholesterol in the lumen of the lysosome. The free cholesterol exits the lysosomal compartment to reach the plasma membrane and the endoplasmic reticulum (ER) to perform its role. The exit of cholesterol from lysosomes requires two proteins, membrane-bound Niemann-Pick C1 (NPC1) and soluble NPC2. NPC2 binds cholesterol with its iso-octyl sidechain buried and its 3ß-hydroxyl exposed.

In 2009, Kwon et al. solved the structure of the N-terminal domain of NPC1. The two proteins, NTD of NPC1, were thought to interact either with the NPC1's membrane domain or with the membrane domain of a neighboring NPC1 molecule. Several years later, Chu et al., in 2015, reported a dynamic membrane contact between peroxisomes and lysosomes. Lysosomal Synaptotagmin VII binding to the lipid PI(4,5)P2 on the peroxisomal membrane mediates the interaction. The presence of LDL-cholesterol enhances these contacts. As a result, lysosomes transport cholesterol to peroxisomes. These findings established the role of peroxisomes in intracellular cholesterol transport.

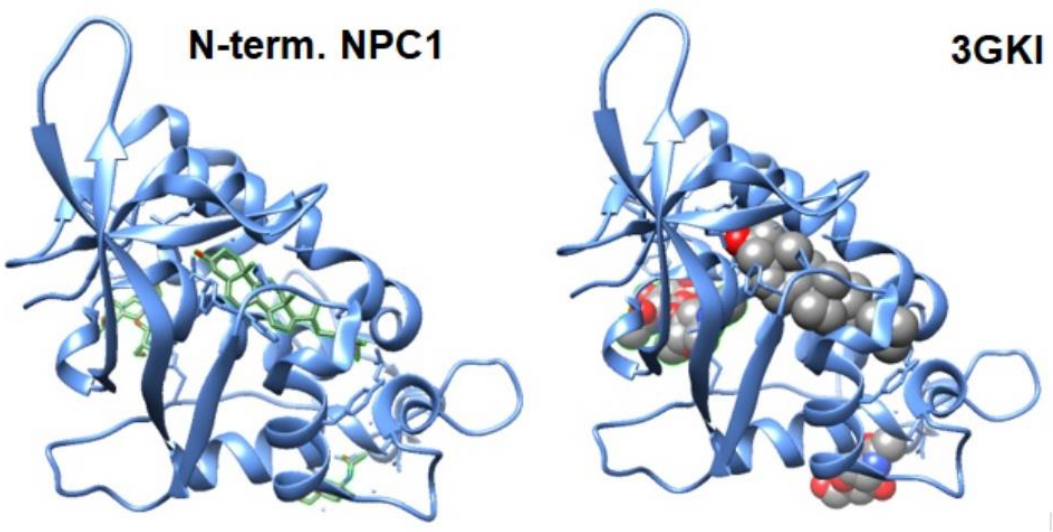
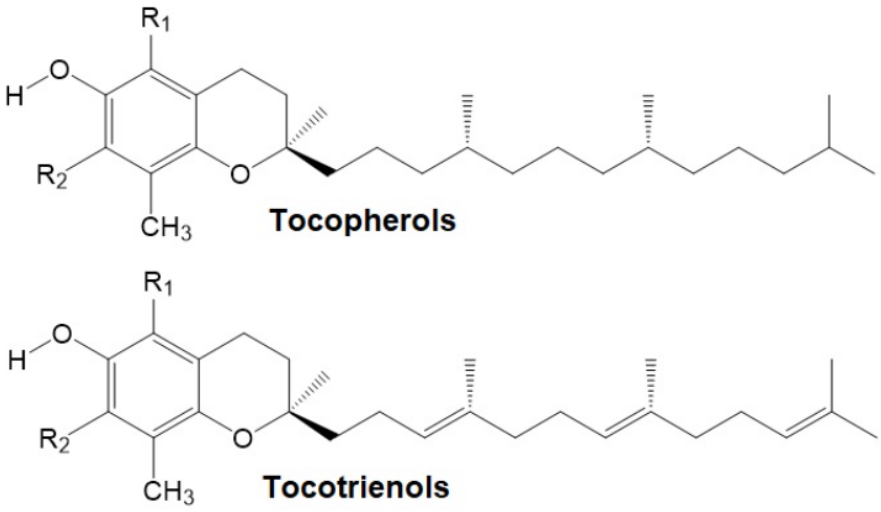


Figure 23. The structure of N-terminal domain of NPC1 shows the distinct subdomains for binding and transfer of cholesterol (3GKI. Kwon et al. 2009).

Vitamins are essential for the growth, reproduction, and functioning of the human body. Intracellular carrier proteins solubilize and transport the fat-soluble vitamins A, D, E, and K. Vitamin E comprises a chromanol ring and an aliphatic side chain. In liver cells, the α-Tocopherol transfer protein (α-TTP) recognizes tocopherol by the three methyl groups on the chromanol ring, specifically, the methyl group at position 5, the hydroxyl group on the chromanol ring, and the orientation of the phytyl side chain. The protein α-TTP transfers α-tocopherol between membranes through direct protein-membrane interactions.

The interaction of α-TTP with phosphoinositides plays a critical role in the intracellular transport of α-tocopherol. Defects of α-TTP cause vitamin E deficiency and neurological disorders in humans.



Isomer	R1	R2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H

Figure 24. Vitamin E homologs. Tocopherols and tocotrienols, each have four isomers (α, β, γ and δ).

The lipophilic α-Tocopherol-TEG, vitamin E, can be chemically incorporated at any position within an oligonucleotide. To penetrate cell membranes and improve cellular uptake, it is often necessary to conjugate therapeutic oligonucleotides with non-toxic, lipophilic molecules. Cholesteryl TEG, alpha-tocopherol, GalNAc, and stearyl labelling of oligonucleotides have proven to be useful in delivering oligonucleotides into cells.

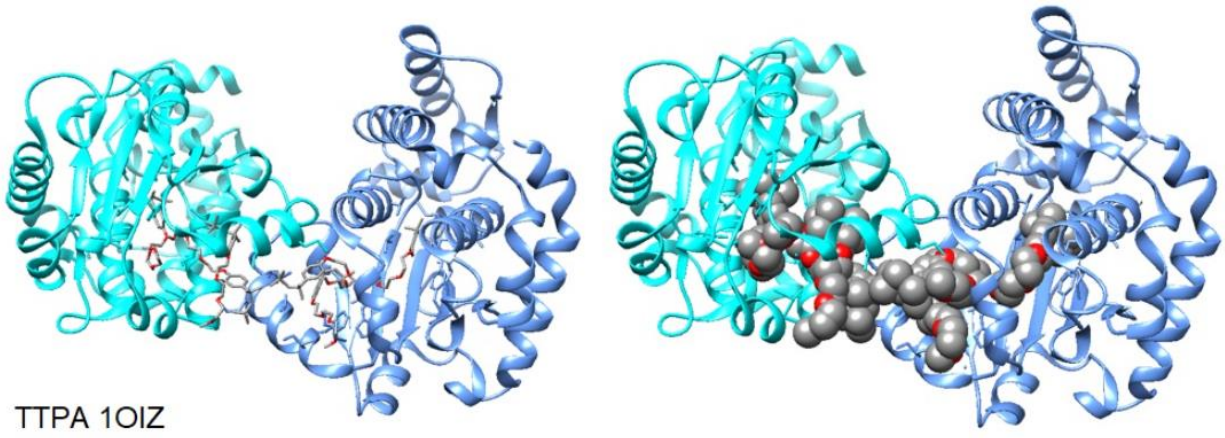


Figure 25. Structure Of Human Alpha-Tocopherol Transfer Protein in complex with a fragment of Triton X-100 [PDB ID 1OIZ].

In humans, the liver protein α-Tocopherol transfer protein (α-TTP) selectively retains tocopherols and tocotrienols from dietary vitamin E. It mediates their

transfer to nascent plasma very-low-density lipoprotein (VLFL). The crystal structure of alpha-TTP revealed two conformations. Tocopherol is bound such that a mobile helical surface segment hides the hydrophobic binding pocket. However, detergents are bound in an open conformation. This conformation may represent the membrane-bound form (Meier et al. 2003).

Bioconjugation chemistry is crucial for the design and synthesis of many therapeutic oligonucleotides. These molecules enable mechanistic studies at the molecular interphase of biological targets to develop agents for molecular medicine. An example for a conjugated oligonucleotide useful for photoregulatory studies is shown in the next figure.

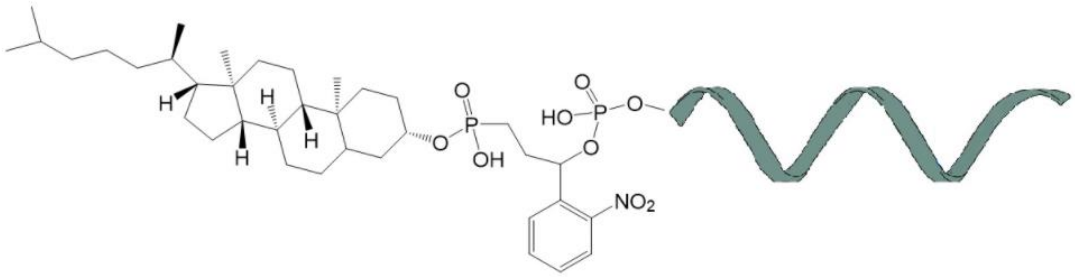


Figure 26. Example of a photocleavable cholesterol-conjugated oligonucleotide. This molecule type can be used for the design and synthesis of caged siRNA useful for photoregulation approaches.

7.6 RNA vaccines

Vaccination prevents many millions of illnesses and saves many lives every year. The smallpox virus vaccine is an excellent example of a successful vaccination approach.

Messenger RNA (mRNA) vaccines are a promising vaccine approach for high potency, rapid development, and potential low-cost manufacture and safe administration. mRNA is the intermediate between transcription and translation of a gene. A single strand of DNA is decoded by RNA polymerase during transcription, resulting in the biosynthesis of mRNA. mRNA transfers genetic information encoded in DNA to the ribosomal translational machinery in the cytoplasm to produce proteins.

Figure 27. Structure of a mature messenger RNA.

Until recently, instability and inefficient *in vivo* delivery of mRNA restricted the application of mRNA vaccines. Recent technological advances help to overcome these issues. Multiple platforms against infectious diseases and several types of cancer no exist.

Classical vaccines that protect against many dangerous diseases include living attenuated and inactivated pathogens and subunit vaccines. However, for newly emerging viral infections, such as COVID-19, more rapid development and large-scale deployment is needed.

Nucleic acid-based therapies are promising alternatives to conventional approaches. Recently, mRNA became a promising therapeutic tool in vaccine development and protein replacement therapy.

Beneficial features for using mRNA as a vaccination platform are:

Safety. mRNAs are non-infectious, non-integrating, with no potential risk of infection or insertional mutagenesis. Also, normal cellular processes degrade mRNA. Using a variety of modifications and delivery methods allow regulating the in vivo half-life of mRNA. For increased safety, inherited immunogenicity of mRNA needs to be downregulated.

Efficacy. A variety of modifications allow increasing mRNA stability and translatability to produce a desired and satisfactory mRNA product. To ensure improved *in vivo* delivery, formulating mRNA into carrier molecules allows rapid uptake and expression in the cytoplasm. The use of mRNA-based vaccines avoids anti-vector immunity and enables repeated administration.

Productions. Messenger RNA vaccines potentially allow rapid, inexpensive, and scalable manufacturing due to the high yields of in vitro transcription reactions.

Recent results suggest that mRNA vaccines potentially solve many challenges in vaccine development for infectious diseases and cancer.

mRNA and Innate Immunity: How innate immune sensing works is not well understood. IVT mRNA show an immunostimulatory profile that can be shaped by purification, the introduction of modified oligonucleotides, and the complexing of mRNA with a variety of carrier molecules. Enzymatically synthesized mRNA contains double-stranded (dsRNA) contaminations. Pattern recognition receptors present in multiple cellular compartments sense dsRNA as a potent pathogen-associated molecular pattern (PAMP). IVT mRNA that contains dsRNA results in robust type I interferon production. Type I interferon upregulates the expression and activation of protein kinase R (PKR or EIF2AK2) and 2'-5'-oligoadenylate synthetase (OAS), resulting in inhibited translation, and degradation of cellular mRNA and ribosomal RNA. However, chromatographic purification methods allow efficient removal of contaminated dsRNA. Both reversed-phase fast protein liquid chromatography (FPLC) or high-performance liquid chromatography (HPLC) remove dsRNA to achieve a highly pure product. The result is an increased protein production in primary human dendritic cells from IVT mRNAs.

Externally delivered single-stranded RNAs are also PAMPs. Endosomal Toll-like receptors 7 (TLR7) and TLR8 sense degradation products of RNAs resulting in type I interferon production. Incorporation of naturally occurring chemically modified nucleosides, including pseudouridine and 1-methylpseudouridine as well as others, prevents RNA sensing by TLR7, TLR8, and other innate immune sensors, resulting in a reduction of type I interferon signaling. Nucleoside modification also partially suppress the recognition of dsRNA species.

Recent studies showed increased in vitro translation of nucleoside-modified mRNA compared to unmodified mRNA, which is also the case in vivo in mice. High levels of protein production can be achieved in DCs using purified and nucleoside-modified mRNA.

These findings advanced our understanding of innate immune sensing and how to avoid the adverse effects of innate immunity.

Lipid-encapsulated or naked forms of sequence-optimized mRNA vaccines can potentially produce potent vaccines against a variety of viruses such as SARS-CoV-2, influenza virus, Zika virus, rabies virus, and many others.

Different types of mRNA vaccines are cancer vaccines, dendritic cell vaccines, and various kinds of directly injectable mRNA vaccines.

However, for therapeutic considerations, good manufacturing practice (GMP) production must be established to guarantee the safety and increased efficacy of mRNA vaccines.

7.7. Incorporation of modified nucleotides into DNA or RNA-based vaccines

In trying to find an immediate intervention strategy to counter the COVID-19 pandemic, researchers have been focusing on developing antiviral vaccines. Various designs that have been to put forward for anti-COVID-19 vaccines have been described, which include recombinant virus (ex. adenovirus expressing COVID-19 protein) and inactivated virus (of COVID-19 itself). In the past, live attenuated viruses have been used successfully to control poliovirus and other viruses due to its ability to incite strong humoral (B-cell based) or cellular (T-cell based) immunity against the infectious agent. Nonetheless, its manufacturing can be time-consuming as it entails preparing a large scale of highly infectious virus, safeguarding against its contamination and/or its release, the need to stockpile, the potential for failure due to the acquiring of mutations by the virus, etc. Recombinant viruses carry the risk of inciting immune reaction against the virus used as the delivery vector, which may provide a potential reason for the unexpected observation that recently developed adenovirus delivered COVID-19 vaccine against spike protein (Oxford University, England) was more effective at low dose than high dose.

In this regard, nucleic acid-based vaccines could be ideal as they can be prepared in a short period of time without requiring an extensive knowledge regarding the biology of the virus. There are two types of the vaccines: DNA or mRNA--with the former being easier to prepare with less concern about the stability/degradation and lower costs. Upon internalization, the DNA vaccine must travel to the nucleus to be transcribed to mRNA (a process which may pose the risk of potential integration into the genomic DNA), which must then be exported to the cytoplasm to express antigen. Thus far, a limited success has been achieved with DNA vaccines in clinical trials presumably due to the insufficient 'strength' of the immunity induced.

In contrast, mRNA vaccines only need to translocate across the cell membrane to allow the translation of the encoded antigen in the cytoplasm. The issue with low stability may be resolved through lyophilization as the lyophylized mRNA vaccines was reported to stay undegraded for a considerable duration at 25-40^{o}C. As for the cost, mRNA vaccines could be made available at prices comparable to

other vaccines based on protein, DNA, peptide, etc. The genetic target of mRNA vaccine could be switched from one to another easily and its production could be adapted to meet the GMP manufacturing standard.

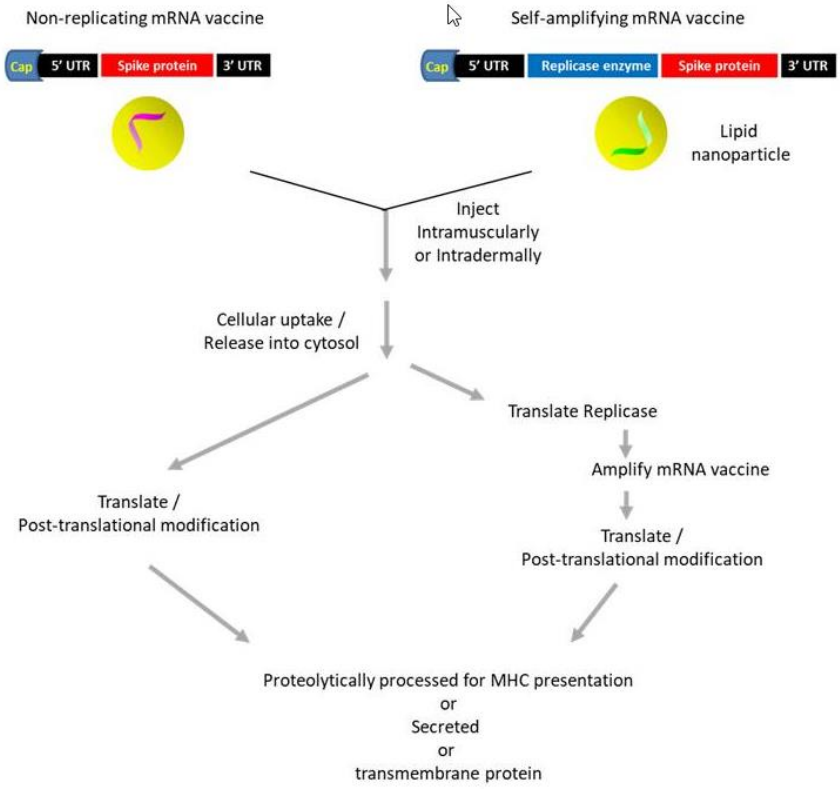


Figure 28. Processing of messenger RNA-based vaccines.

Earlier works demonstrated that direct injection of DNA or RNA expression vectors into muscle could allow protein expression in mice. This has inspired the development of vaccines comprised of mRNA-encoded antigens that could be delivered *ex vivo* or injected intradermally (underneath the skin). The 'ex vivo' approach refers to the process of excising tissues, transfecting with a vaccine construct (ex. via electroporation) outside the body, and then returning it to the individual. The initial trial involved treating advanced-stage melanoma patients with dendritic cells electroporated with mRNA vaccines encoding MAAs (melanoma associated antigens), i.e. MAGE-A3, tyrosinase, MAGE-C2, gp100.

Critical to this undertaking was the recognition of the role of dendritic cells in antigen presentation, the ability to expand dendritic cells *in vitro*, and the identification of tumor-associated antigens. The commonly used approach is to characterize the 'mutanome' (mutated gene sequences) via deep sequencing to

identify mutated sequences that are highly immunogenic as mRNA vaccine. The antigen encoded could be chimeric as adding a signal peptide to N-terminus or an endosomal trafficking signal to C-terminus could improve antigenic presentation by MHC molecules. Further, the maturation status of dendritic cells could affect the uptake efficiency of mRNA vaccines.

Several types of mRNA vaccines currently exist. The 'non-replicating mRNA' vaccine consists of the coding sequence (of the antigen) that is 5'-capped, 5' and 3' untranslated regions, and poly-A sequence. The use of T7 polymerase to generate mRNA transcript using linearized plasmid may generate double stranded RNAs due to self-priming. The mRNA designed by Moderna Therapeutics (USA) incorporated modified nucleotides to avoid activating interferon-associated genes and also added 2 proline residues to stabilize the spike protein. While a lipid-based nanoparticle was used to protect mRNAs, delivery vector remains a significant issue. A phase I clinical trial (doses from 25 ug to 250 ug) showed that it induced antigen-binding and neutralizing antibodies (plus activating T cells) while causing side effects (ex. chills, fatigue, headache, muscle pain) which augmented after the second dose. Phase III study (to determine if it can protect against COVID-19) is still ongoing. In contrast, the 'self-amplifying mRNA' vaccine is generated using alphavirus whose structural gene is replaced with an antigen of interest while retaining the genes mediating viral replication. Being a positive stranded RNA virus, it generates double stranded RNA as intermediates, which could potentially trigger innate immunity (ex. cytokine release). Alternatively, the construct may contain another cistron expressing replicase to amplify the mRNA vaccine

7.8. Incorporation of N1-methyl pseudouridine into mRNA vaccines

The incorporation of N1-methyl pseudouridine by T7 bacteriophage derived RNA polymerase was instrumental for COVID-19 mRNA vaccine production. For the mRNA vaccines targeting the spike protein of the COVID-19 coronavirus were introduced in a timely manner to curtail its progression. This preventive measure relies on the ability of the injected mRNA to mount both the humoral and cellular immunity to preempt the oncoming infection. The administration of the mRNA vaccines, which began in the early months of 2021 led to a significant decline in the rate of ensuing COVID-19 infections (though it tends to decline during warmer months). Nevertheless, targeting a single molecular entity (spike protein) of a virus comprised of nearly 29 proteins could be challenging, especially, in lieu of the higher mutation rates of RNA viruses. Hence, the ability of the vaccine to counter the emerging COVID-19 variants is continually being assessed.

Supporting the therapeutic value of the mRNA vaccines is the finding that the rate of infection /hospitalization was significantly reduced amongst the vaccinated.

For a considerable period, the ability to synthesize nucleic acids has played a pivotal role in biological and medical research. It began with the ability to synthesize short oligonucleotides as they could be used in multiple biological applications such as mutagenesis, molecular cloning, DNA sequencing, etc. that involve hybridization. Equally significant is the ability to utilize oligonucleotides for various medical applications including PCR-based assays for diagnosing the disease onset, testing for genetic counseling, monitoring altered gene expression via microarrays, etc. For translational medicine, the potential use of oligonucleotides for therapeutic purposes is increasingly being recognized. This has resulted in numerous ongoing clinical trials and the FDA approval of multiple siRNAs, antisense oligonucleotides, and gapmers. Continuing with the advance is the application of oligonucleotides for gene synthesis as part of the genome construction endeavor at the DNA level.

At the RNA level, the therapeutic potential of the synthetic nucleic acids was extended to mRNAs, which are significantly longer than oligonucleotides. The earlier attempts were to intravenously or intradermally inject melanoma patients with dendritic cells, which have been electroporated with mRNA vaccine encoding cancer immunogens. In 2021, the medical utility of the mRNA vaccine technology was tested against the newly emerged COVID-19 coronavirus. To target the spike protein of COVID-19, it required synthesizing mRNAs of >4 kilobases in length. To achieve this length, biopharmaceutical industries resorted to the *in vitro* transcription technology.

The general design of the template used to produce mRNA vaccines utilized the following format: 5'-cap (to increase translation, stability; to decrease immunogenicity, degradation), 5'-UTR (to promote interaction with ribosome), codon-optimized spike protein coding sequence (to augment translation), 3'-UTR (to enhance stability), and poly-A tail (to increase stability, translation). The mRNA was engineered to incorporate modified nucleic acids to avoid immunogenicity or degradation, ex. N1-methyl pseudouridine. As such, critical to its development was the employing of RNA polymerase that could accommodate nucleotides with non-natural bases.

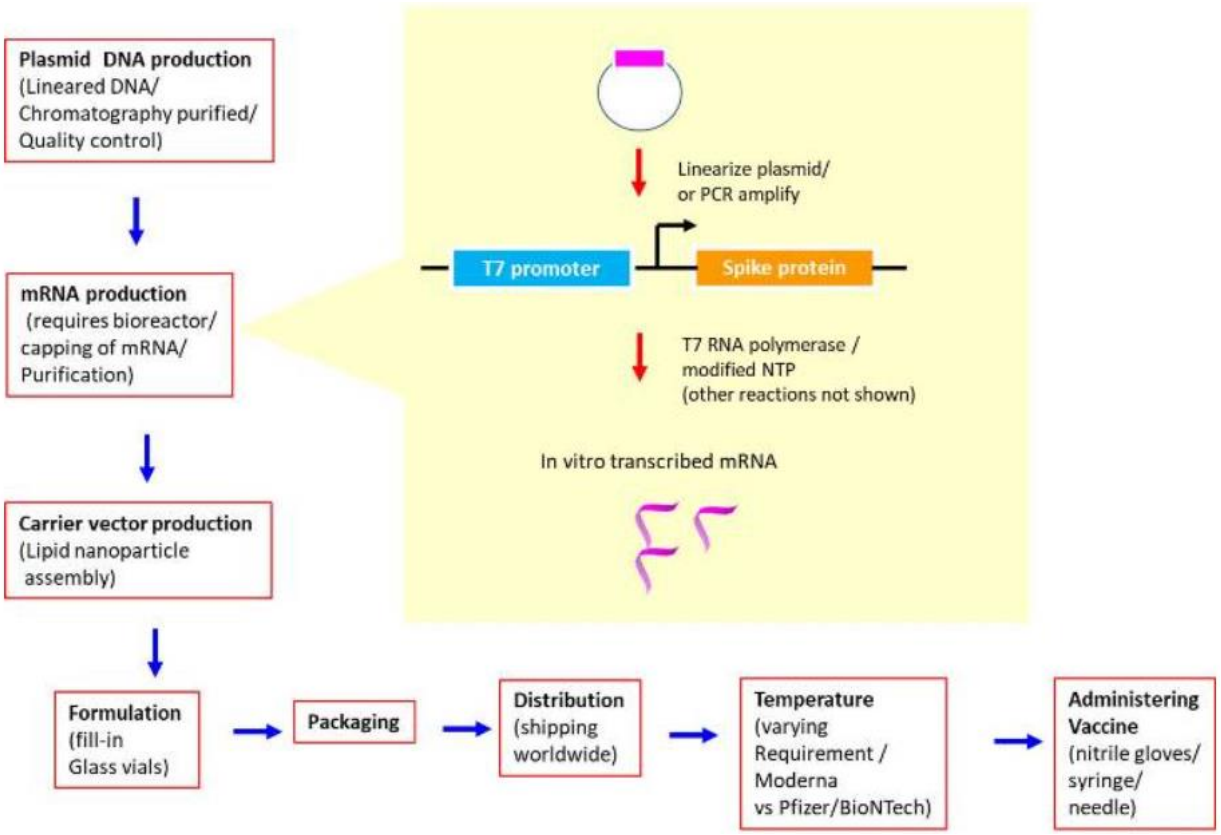


Figure 29. The Logistics of mRNA Vaccine Production.

Generally, there are 3 strategies to produce RNA with modified nucleotides. The first approach is to exploit the catalysis of RNA polymerase to incorporate modified NTPs (as in the case of T7 polymerase). The second method is to modify post-transcriptionally using enzymes (ex. using methyl transferase) after the RNA synthesis. Third option is through chemical modification of the RNA using bioothogonal (ex. click) chemistry.

The ability of RNA polymerase to incorporate modified nucleotides was recognized as early as 1963, when the ability of HeLa cell derived RNA polymerase to incorporate pseudouridine (incorporated adjacent to purine nucleotides preferentially) was observed. Later, the ability of T7 polymerase to incorporate modified NTPs was described, so long as it does not perturb base-pairing. Subsequently, it was found that T7 RNA polymerase permits a wide variety of modifications to nucleotides including biotin (affinity labeling), alkyne/azido groups (click chemistry), 5-vinylU (for further chemical modification), 5-iodoU (for cross-coupling modification), amino acid-like side

chain (aptamer selection), diazirin (crosslinking), and fluorophore. T7 RNA polymerase could also tolerate sugar modifications (ex. 2'-O-carbamoyl uridine).

Several advances preceded the production of the mRNA vaccine for COVID-19. The bulk synthesis of modified mRNA relied on the cloning/sequencing of T7 bacteriophage gene encoding RNA polymerase by W. Studier and colleagues (Brookhaven National Laboratory, USA) in 1983. Further attempts to mutagenize T7 RNA polymerase to obtain variants that could incorporate modified nucleotides (ex. 2'-C-branched uridine) also took place. Critical was the finding by Kariko and colleagues (University of Pennsylvania, USA) in 2005 that nucleotide modifications such as pseudouridine, thiouridine, and 5-methylcytidine decrease immunostimulation by TLRs (toll-like receptors), as it provided foundation for applying mRNA for anti-COVID-19 vaccine (or stem cell technology, cancer therapy potentially). The topics of further interest include the mRNA design (ex. the effect of ubiquitous incorporation of N1-methyl pseudouridine on 5'-UTR or 3'-UTR functionality), the impact on diagnosis (Rt-PCR), and recombination.

7.9. BNA RNAi and their Design

RNAi technologies utilize short double-stranded RNA (dsRNA) of approximately 21 base pair length with a two nucleotide (nt) 3’-overhang for the silencing of genes. These dsRNAs are generally called small interfering RNA (siRNA). siRNA 12 to 22 nucleotides in length are the active agent in RNAi. The siRNA duplex serves as a guide for mRNA degradation. Upon siRNA incorporation into the RNA-induced silencing complex (RISC) the complex interacts with a specific mRNA and ultimately suppresses the mRNA signal. The sense strand or passenger strand of siRNA is typically cleaved at the 9th nucleotide downstream from the 5’-end of the sense strand by Argonaute 2 (Ago2) endonuclease. The activated RISC complex containing the antisense strand or guide strand binds to the target mRNA through Watson-Crick base pairing causing degradation or translational blocking of the targeted RNA.

However, the in-vivo use of RNAi or siRNA as a drug has remained difficult due to obstacles encountered such as low biostability and unacceptable toxicity possibly caused by off-target effects. Various types of chemical modifications to improve the pharmacokinetics and to overcome bio-instability problems have been investigated over the years to improve the stability and specificity of the RNAi duplexes. In some cases, the chemical modification in siRNAs has improved the serum stability of siRNAs. Often RNAi activity was lost, but the careful placement of some specific modified residues enables enhanced siRNA biostability without loss of siRNA potency. Some of these modifications have reduced siRNA side

effects, such as the induction of recipient immune responses and inherent off-targeting effects and have even enhanced siRNA potency. Various chemically modified siRNAs have been investigated, among them were bridged nucleic acids such as 2',4'-methylene bridged nucleic acid 2',4'-BNAs, also known as locked nucleic acid or LNA. Some of these modified siRNAs showed promising effects.

Rahman et al., in 2010, investigated the effect of both 2',4'-BNA and 2',4'-BNA-NC modifications in gene silencing experiments using RNAi technology. The scientists observed that 2',4'-BNAs (LNAs) and 2',4'-BNA-NCs are equally effective in RNAi activity if incorporated appropriately, especially in the sense strand of siRNA.

Design examples of BNA nucleic acid analogs useful for gene silencing by RNAi to inhibit the expression of firefly luciferase in CHO-luc cells. Modifications of the sense strand consecutively up to five BNA residues might be tolerable.

For the identification of the best modification, Rahman et al. investigated a range of BNAs for their ability to inhibit firefly luciferase expression in CHO-luc cells. Rahman et al. found that the introduction of 2',4'-BNA modifications at 3'-overhangs in the sense and antisense strands of the siRNA completely retained the natural RNAi property of natural RNA.

Design Rules: The design rules or guidelines for the design of highly effective RNAi indicate that dsRNA used for RNAi should follow the following four design rules at the same time:

(i) Place A/U at the 5'-end of the antisense strand,

(ii) Place G/C at the 5'-end of the sense strand,

(iii) AU-richness in the 5'-terminal third of the antisense strand, and

(iv) Absence of any GC stretch over 9 base pairs in length. (See figure 30).

(v) Incorporate BNAs at strategic positions.

These guidelines appear related to the molecular mechanism of RISC assembly as reported by Ui-Tei et al. in 2004. The first three guidelines together with adding BNAs to the sense strand may allow the 5'-end of the antisense strand of siRNA to be situated at or near the thermodynamically less stable siRNA duplex end.

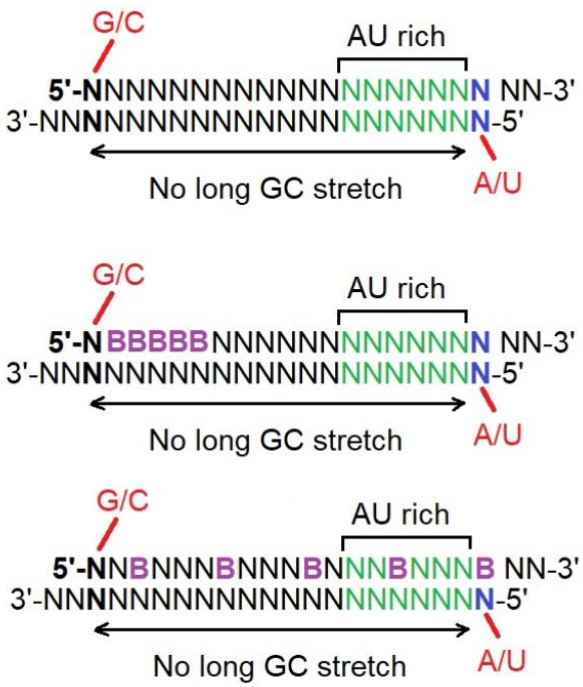


Figure 30. Structures of highly efficient siRNAs.

8. Conclusion

To improve low solubility, insufficient pharmacokinetic properties, and poor cellular uptake of oligonucleotide-based drugs such as ASOs and siRNAs, the covalent attachment of various ligands can be used to influence their biodistribution, cellular uptake, and advance therapeutic applications. Ligands such as aptamers, carbohydrates, cholesterol, DNA, folic acid, peptides, proteins, RNA, small molecules, as well as tocopherol can be attached chemically to therapeutic drug candidates. Available ligand subclasses are mainly divided by their biological actions.

These carrier molecules may include aptamers, cell-penetrating peptides, immunostimulatory oligonucleotides, miRNA, siRNA, splice-switching oligonucleotides, and others.

Stearyl C18 lipids are a favorable lipophilic carrier in siRNA and antisense therapeutic studies. Modification can be placed at the 5' end of an oligonucleotide. Antisense oligonucleotides, siRNA, aptamers, and miRNA have been used in various therapeutic developments. However, the challenge facing these developments has been how to deliver the oligos effectively to the desired cellular

targets. Strategies of using lipophilic and non-toxic carrier such as Stearyl C18 lipid, cholesteryl-TEG, α-Tocopherol-TEG, GalNAc have been used. Related carrier molecule are: Spermine, GalNac C3, Cholesteryl TEG, and α-Tocopherol-TEG.

Side effects continue to undermine the well-being of cancer patients undergoing therapy. To reduce side effects associated with current therapeutics, antibodies have been utilized as the delivery vector for previously isolated cytotoxic drugs. The ability of the antibodies to bind to molecules expressed on the surface of tumor cells provided a means to deliver drugs selectively to tumor cells. Using special linkers (cleavable or non-cleavable type), various cytotoxic drugs have been conjugated to 'tumor-specific antibodies'. To date, U. S. FDA has approved ca. 11 such antibody-drug conjugates. These include Enhertu (anti-Her2 antibody linked topoisomerase I inhibitor; AstraZeneca-Daiichi Sankyo) for urothelial cancer, Kadcyla (anti-Her2 antibody linked to antimicrotubule drug; Genentech-Roche) for metastatic breast cancer, Lumoxiti (anti-CD22 antibody linked to the bacterial toxin; AstraZeneca) for relapsed hairy cell leukemia, etc.

Nevertheless, the resultant antibody-drug conjugates continued to exhibit side effects. In the case of Enhertu, side effects include nausea, diarrhea, anemia, decreased clotting, etc. Kadcyla is associated with thrombocytopenia (low platelet count), hepatotoxicity, heart damage, neuropathy, etc. Several of these symptoms also occurred following the Lumoxiti treatment--i.e. in addition to the swelling of limbs, hypotension, breathing difficulty, abnormal red blood cell destruction, etc.

To explain the off-target effects of antibody-drug conjugates, potential uptake of monoclonal antibodies lacking terminal galactose on the Fc domain by mannose receptors expressed in various immune cell types, leading to their depletion, has been suggested (Gorovits et al., 2012). Further, the binding of antibodies to reticuloendothelial system may cause toxicity to the liver, bone marrow, and spleen. Other limitations of using antibodies include failure to reach the brain through blood-brain barrier, poor penetration of solid tumors due to high molecular weight (~160 kD), and premature release of drugs while in circulation. The difficulty of their preparation along with the exorbitant cost for large-scale production represents other disadvantages (Le Joncour et al., 2017).

To address these shortcomings, peptides are increasingly being tapped for targeted drug delivery. Peptides (as short as 7 to 12 mer) can adopt myriad conformations by altering their sequences. Previously, these properties have been exploited to isolate 'homing peptides' (Joliot et al., 2004). Due to their minuscule molecular weight (1 to 2 kD), peptides allow deeper penetration into tissues--as has been

demonstrated for solid tumors (Hong et al., 2000). Additionally, peptide-conjugated drugs exhibit excellent tolerability, lesser immunogenicity, are easier to produce in large quantities, simpler purification scheme, etc. Plus, developing peptide-drug conjugate using previously FDA-approved drugs is far less costly than discovering novel drugs, and the conjugation process is generally less complex and rapid (Vrettos et al., 2018).

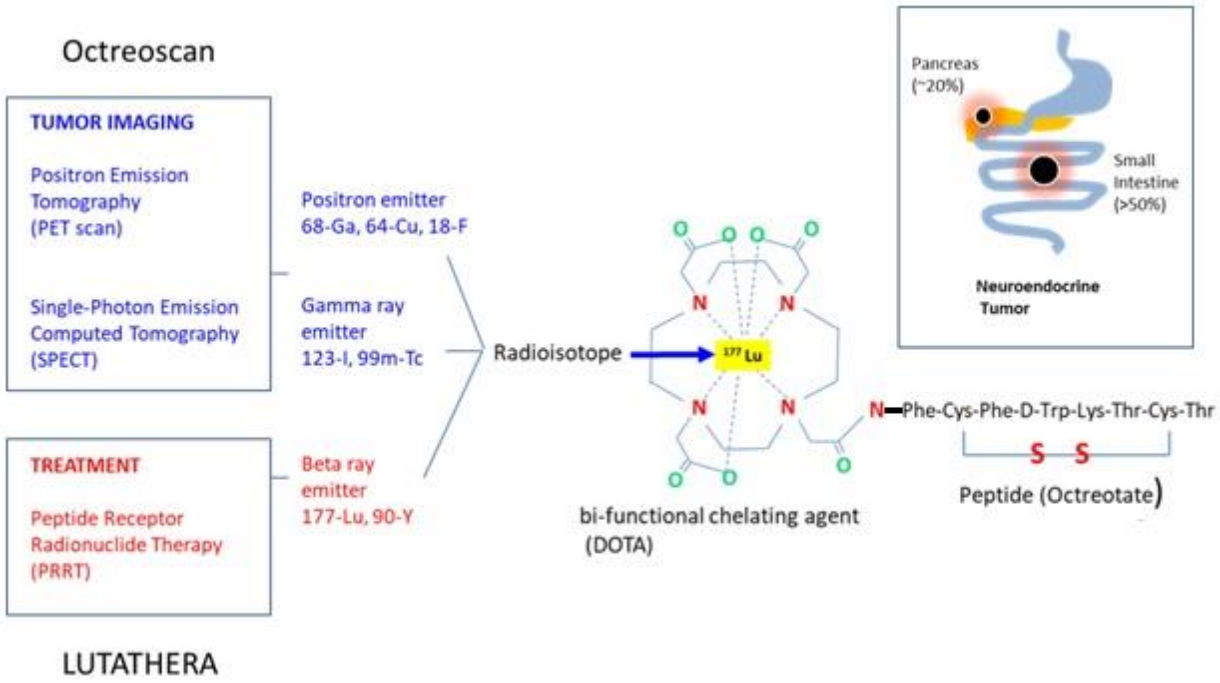


Figure 31. Peptide conjugates for radioisotope-based theranostic of tumors.

During the last several decades, significant efforts have been made by the biopharmaceutical industries to improve their application.

First, to isolate 'homing peptides', bacteriophage-based random peptide-display technology has been used extensively. The externally displayed random peptides as part of the coat protein of M13 bacteriophages served as the main 'work horse' to isolate peptides targeting various tumors or normal tissues (Brown, 2010). Other peptides have been derived from naturally occurring sources like the neurotransmitters (ex. octreotide) or matrix proteins (ex. RGD). These include peptides targeting cancer cells directly (ex. LyP-1, HN-1, TGN peptide), tumor-associated vasculature (ex. RGD, NGR), tumor-associated macrophages, tumor lymphatics, etc. (Arap et al., 1998; Gray et al., 2014). For other types of disorders, peptides that target various normal tissues (ex. immune cells, cardiac cells (ex. CTP peptide), muscle cells, kidney cells, liver cells) have been isolated (Gray et

al., 2014). The majority of previously isolated peptides bind to cell surface molecules albeit a minority retains the potential to internalize.

Second, peptides have been engineered to improve their pharmacokinetic (traffic) or pharmacodynamic (mechanism) properties.

[1] To increase binding affinity, peptides may be cyclized via 'stapling' (to lock their secondary structures into desired conformations).

[2] To increase half-life, the N- or C-terminus may be chemically modified (to avoid degradation by exoproteases). Further, specific amino acids may be replaced with D-configuration (potentially alternate residues) or unnatural amino acids (Le Joncour et al., 2018).

[3] To avoid renal clearance, higher molecular weight entities such as PEG, fatty acid, branched amylopectin, polysialic acid, hydroxyethyl starch, etc. may be linked.

[4] To improve oral bioavailability, it may be coated with the acid-stable coating (ex. citric acid) in the stomach, which breaks apart when it reaches higher pH of the intestine (Cooper et al., 2020).

Nevertheless, some of these modifications may run the risk of attenuating/losing the binding properties.

Third, to conjugate peptides to drugs, both the cleavable and non-cleavable types of the linker are available (Hoppenz et al., 2020). The cleavable types include disulfide (S-S) linkers that become cleaved upon reduction by glutathione intracellularly, pH-sensitive linkers (ex. hydrazine, acetals, imines) that are hydrolyzed at acidic pH in endosomes, etc. Cleavable linkers also include peptides that are cleaved by matrix-metalloproteinases (MMPs) extracellularly and others (ex. Valine-Alanine or Valine-Citrulline) that are cleaved by cathepsin B inside the endosome (albeit the latter becomes cleaved in the mouse plasma) (Cooper et al., 2021). Commonly used linkers containing 'enzyme hydrolyzable unit' may consist of carboxylic ester or an amide bond (Vrettos et al., 2018). The 'self-immolative' (self-destructive) linker PABC (para-amino benzyl alcohol) can be used to connect peptide with drug as it could be cleaved at the enzyme hydrolyzable unit to release the peptide, and then undergo 1,6-elimination to release the unmodified drug. Alternatively, aryl sulfate linkers that undergo 1,6-elimination to release unmodified drugs have been utilized. The non-cleavable type, which is more stable in circulation, may be used when the peptide is intended to undergo degradation upon internalization, releasing the drug-linker complex into the cytosol.

Recently, U. S. FDA issued the first approval of the peptide-drug conjugate to treat gastroenteropancreatic neuroendocrine tumors. Somatostatin is a neurotransmitter and there are 5 subtypes of somatostatin receptors (SSTR); among them, SSTR2 and SSTR5 are highly expressed in neuroendocrine tumors. Neuroendocrine tumors are derived from neuroendocrine cells and may occur in various tissue types. Somatostatin analogs have been used as probes to image tumors in the 1980s-1990s. Octreotide labeled with radioisotope 111In (gamma emitter; Octreoscan, Mallinckrodt) was useful for tumor imaging (albeit less effective in therapy), and was FDA approved for imaging in 1994 (ex. for PET scan with positron emitting isotope or SPECT with gamma ray emitter). Subsequently, the FDA approved (in 2018) LUTATHERA (77Lu-DOTA-octreotate; Novartis), representing 77Lu (beta emitter) conjugated to Octreotate using the bi-functional chelating agent DOTA, was able to achieve longer progression-free survival with a higher therapeutic response rate in advanced midgut neuroendocrine tumors (Hennrich et al., 2019).

One of the most effective ways to deliver cytotoxic drugs is by conjugation, where a relatively small molecular weight drug is covalently linked to usually a protein, which recognizes a specific cellular receptor that is expressed de novo or over-expressed by the targeted cells. In fact, some of the most useful proteins are the monoclonal antibodies (mAbs), which can be selected to recognize a single specific epitope on the cell surface. Yet, proteins like transferrin, hemoglobin, albumin and others are also being used to prepare these conjugates. Because of the recognition site on a protein, the essential pharmacophores on the drug responsible for its pharmacological activity, preparation of a conjugate needs to address preserving the binding capacity of the protein and most likely the release of the drug once it has entered the cell so that it can find its therapeutic target.

Hence, the design of a drug conjugate would require identifying functional groups in the protein that are far from its binding site, so binding of the drug does sterically hinders that site. If the protein is produced by recombinant DNA technology, the appropriate groups like thiol group can be introduced in the protein amino acid sequence; otherwise, there will be a need to introduce new functional groups by selective modification of certain groups in the protein. For instance, thiolation of amino groups using either N-acetyl homocysteine thiolactone (SATA) or S-acetylmercaptosuccinic anhydride (SAMSA) is a common approach modifying proteins.

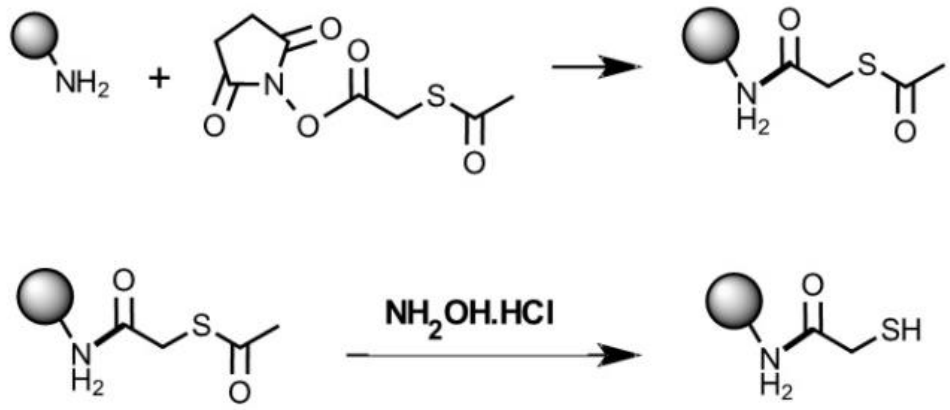


Figure 32. Modification of amino groups with SATA.

The success of the modifications depends on their selectivity and if the reactive groups are far from the protein's binding site. In some cases, if the ligand is a carbohydrate or other non-peptide compound, it would be possible to saturate the binding site before modification to protect the active site. Once the modification has taken place, the ligand can be removed from the binding site by extensive dialysis or similar procedures. An alternative method would be the reaction of the terminal amino group of a protein with an aldehyde carrying bifunctional linker. This method takes advantage of the lower pKa of the terminal α-amino group (pKa 7.6-8) than that of the lysine ε-amino groups (pKa ~ 9.4). Thus by selecting the pH of the reaction, the aldehyde group will form an imine with the terminal α-amino group, which may be reduced to a stable secondary amine with sodium cyanoborohydride. Evidently, this method would be useful only in proteins where the terminal amino group is not present at the binding site. While modifications of the carboxyl groups are possible, its selectivity would be less than the above-described methods and may lead to cross-linking with neighboring proteins; unless there is evidence that no carboxyl groups are present at the binding site.

In the case of conjugates with mAbs, one of the most used methods is to reduce one of the two disulfide bonds linking together the two IgG heavy chains to form two thiol groups. Under these conditions, the formed thiol groups are present in the Fc region and away from the IgG recognition sites formed by the heavy and light chains. These two thiols can them react with a maleimide of an iodoacetyl group to form a stable thioether; using this approach it is possible to introduce one or two drug molecules per IgG molecule. A complete reduction of the two disulfide bonds may deliver two half-IgG molecules, which can be linked to a drug molecule. However, after reduction of both disulfide bonds, in some cases the two half IgGs would not separate, which may interfere with the conjugate formation. While the

reduction of the disulfide bond is quite effective for IgGs, it is doubtful that it may be used with other proteins, a disulfide bond usually stabilizes the three-dimensional structure of a protein.

Because the conjugated drug usually needs to interact with a cellular target after intracellular delivery, it would need to be released from the protein to which it is linked. To achieve this goal, the drug is covalently linked to the protein via a cleavable linker, i.e. a chemical structure that upon exposure to an enzyme, low pH, excess of reducing thiol groups, will split leaving the drug free and able to interact with its target. There are several types of cleavable linkers, and some of the most frequently used are those having a hydrazone group, which is cleaved at acid pH, < 5, in the endolysosome compartment, after uptake of the conjugate by endocytosis. Another type of cleavable linker is the peptide pro-drug linker (self immolatory) having a Val-Cit-PAB-PNP sequence. This type of linker has two cleavage sites; site "a" specific for the enzyme cathepsin B cleavage, and site "b" that is the (1,6)-fragmentation site of the self-immolative link. To protect the drug, the self-immolatory spacer is located between the site of enzymatic cleavage and the drug; subsequent to the enzymatic cleavage, there is an extensive chemical rearrangement, a 1,6 elimination, that results in the drug release. Self-immolatory linkers are cleaved at the lysosome by proteases, after uptake of the conjugate by endocytosis. Although some cleavable linkers containing disulfide bonds have been described, they are not very stable and may be reduced in circulation by compounds like cysteine, glutathione, and other thiols.

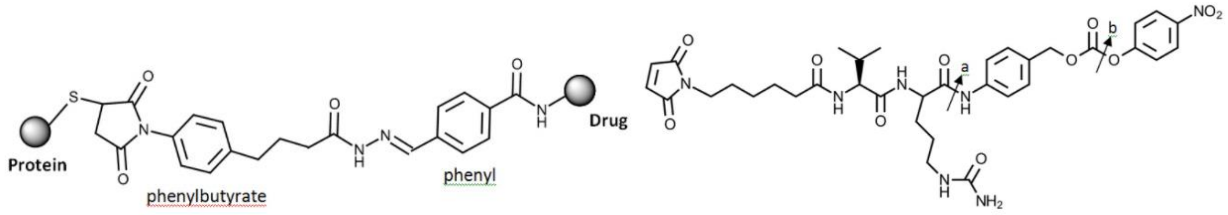


Figure 33. (Left) Cyclohexyl-aryl hydrazone cleavable linker. (Right) Peptide pro-drug linker: Mal-Val-Cit-PAB-PNP.

The other crucial component of these protein-conjugates, is the drug; which usually is linked by one of its functional groups that is not an essential for its pharmacological activity. Special care should be taken to avoid any significant structural changes that may alter the interactions of the drug with its target. Because the activity would be dependent on the amount of drug, it is important to

determine the composition of the conjugate and to have a relatively homogeneous conjugate population.

Lastly, the great challenge of applying peptide-oligonucleotide conjugates to gene therapy is highlighted in a seminal review by D. Marciani and M. Castro (Castro & Marciani, 2012). An antisense DNA-peptide conjugate could be administered to assuage disease symptoms caused by aberrant gene expression. The potentially treatable disorders include diabetes mellitus, neuropathies, neuromuscular diseases, infectious diseases, neurodegenerative disorders (ex. Alzheimer's disease), cancer, and various hereditary conditions. A major contributing factor to the above has been the discovery of peptides for cell transduction (ex. cell penetrating peptides) or for targeted drug delivery.

For conjugation, non-cleavable heterobifunctional crosslinkers such as SMBP, SMCC and SMPH or cleavable linkers such as SPDP could be utilized. Commonly used reactive moieties include N-hydroxysuccinimide (NHS) ester that reacts with primary amino groups and maleimide for the reaction with thiols. The suitable functional groups on peptides include amino groups (lysine), thiol groups (cysteine), hydroxyl groups (serine, threonine), and carboxyl groups (glutamate, aspartate). Side groups critical for peptides' function are protected during the conjugation and subsequently deprotected. Alternatively, the chemical synthesis of peptides can be adjusted to include a terminal spacer containing functional groups (ex. cysteamine, aminohexanoate) to enable conjugation.

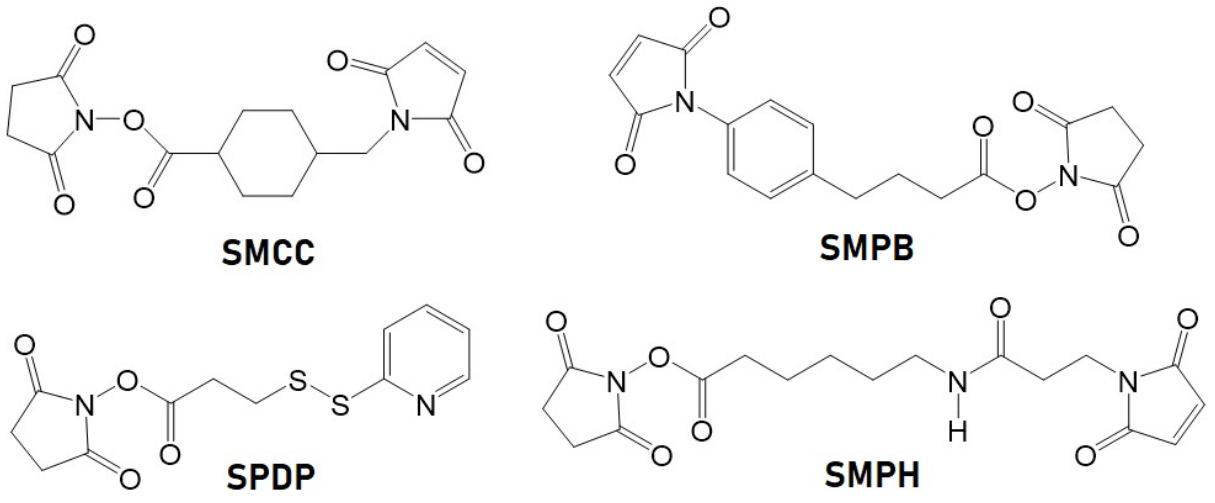


Figure 34. Examples of heterobifunctional crosslinker molecules. SMCC, SMPB, and SMPH are non-cleavable. SPDP can be cleaved with thiol reagents.

To crosslink oligonucleotides, the terminal 5'-phosphate group, hydroxyl groups on the sugar moiety, or the nucleotide bases could be modified. For instance, phosphitylation of 5' hydroxyl group of an oligonucleotide with 2-cyanoethyl-N,N,N',N' tetraisopropyl phosphoramidite will yield an 5' phosphoramidite intermediate, which could then be used to react with thiol or hydroxyl group of a linker. A spacer of appropriate length (>6 carbon) may be added to separate peptide from oligonucleotide in the conjugate to preserve their functionality. A further advantage of chemical synthesis lies in its ability to modify oligonucleotides through incorporating nucleic acid analogs such as locked nucleic acids (LNA) or bridged nucleic acid (BNA; consisting of a bridge linking 2' and 4' carbons of the sugar moiety) to provide high affinity and specificity.

The recently introduced synthesis of oligonucleotides using TdT terminal transferase provides an alternate enzymatic method of coupling nucleotides. While its utility is still being evaluated, uncertainties remain regarding its clinical application. These include its precision or the error rate resulting from the lack of proofreading capacity by the TdT enzyme. Further, the complex modification of nucleic acids, which has been critical for the clinical advancement of siRNA or mRNA-based therapeutics, may not be readily feasible with the TdT based synthesis. The need to prepare highly pure GMP-quality oligonucleotides in bulk (thousands of kilograms) quantities using TdT synthesis also awaits demonstration. Against this backdrop, the significance of chemical synthesis for oligonucleotide-based therapeutics is likely to resonate for the foreseeable future.

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Disclaimer: Please be aware that there may be IP associated with particular compounds and methodologies described in this write-up; our aim in this handbook is entirely educational/informative; we kindly suggest to, before any commercial application, to check for all IP that may be associated with compounds as mentioned above and methodologies.

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