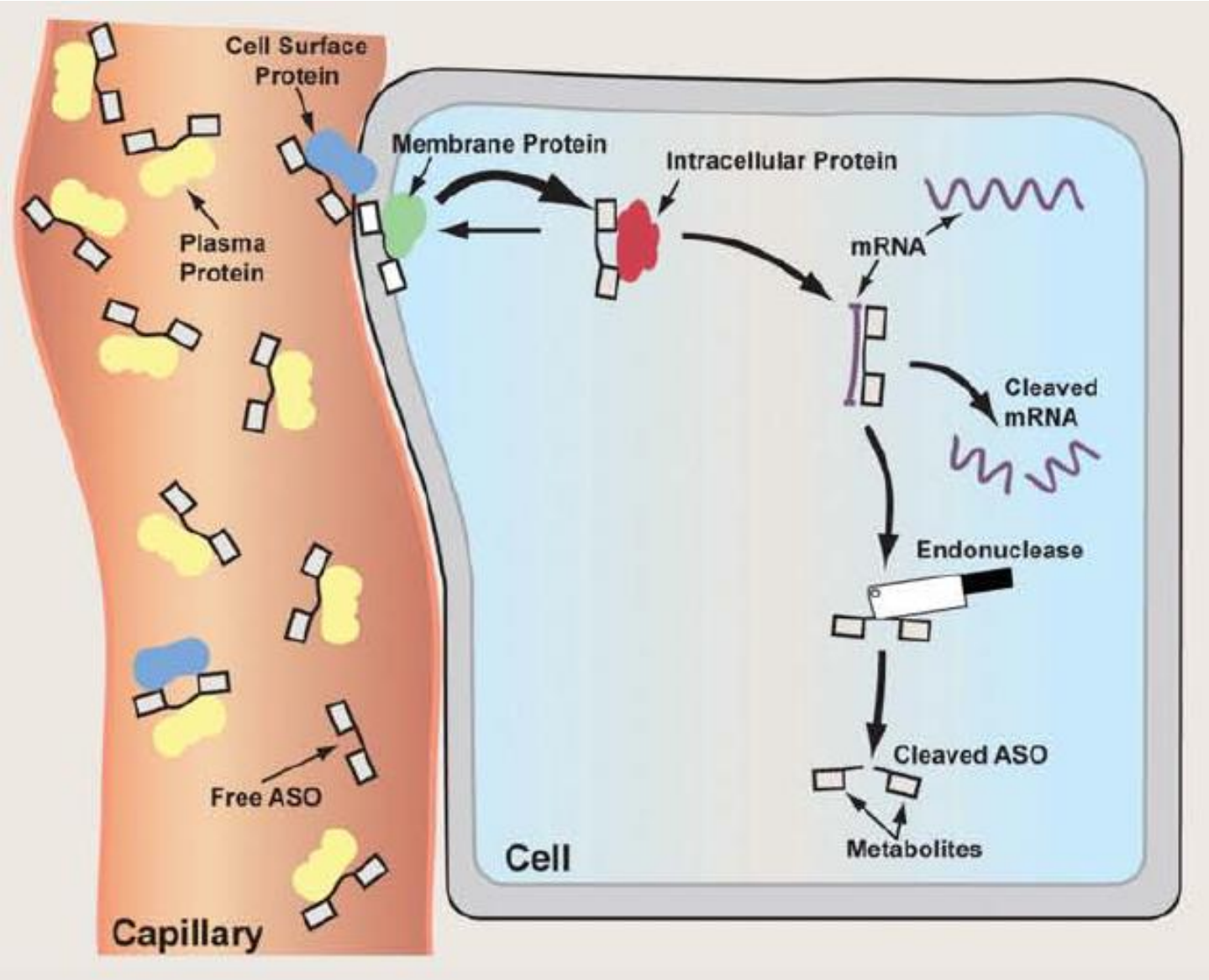
BSI and Antisense Technology targeting RNA



Klaus D. Linse, Ph.D.

Ref.: Crooke et al.; RNA-Targeted Therapeutics. Cell Metabolism 27, 2018.

Ionis Pharmaceuticals, Inc. Approach (2015)

Was ISIS Pharmaceuticals (1989)

Book: Antisense Drug Technology. CRC Press 2008

bioSYNTHESIS Since 1984

ISO 9001 ISO 13485

Why Antisense Technology

Natural nucleic acids (DNA & RNA) make poor drugs

- Low binding affinity for target RNA
- Drug clearance from the body is fast
- Natural products are used in medicinal chemistry
- Natural nucleic acids have structural limitations

ASOs

- Improve distribution to target tissues
- Increase stability of the drug in the body
- Improve potency (Decreases dose; oral delivery possible)
- Improve therapeutic index

ASOs

- Antisense oligonucleotides are **synthetic polymers** in which some or all of the natural nucleotide monomers of the oligonucleotide are chemically-modified deoxynucleotides (in DNA) or ribonucleotides (in RNA).
- **Watson-Crick base pair hybridization**, to a specific mRNA that can inhibit its expression and induce a blockade in the transfer of genetic information from DNA to protein.
- Target a **specific sense mRNA**.
- Antisense oligonucleotides can act by **blocking the upstream message** for receptor substrates, proteins over-expressed in pathological versus physiological states.
- Blocking the expression of selected proteins in signaling cascades may avoide unwanted side effects.

RNase H-dependent ASO (Wu et al., 2004), and

RNase H-independent (steric block) ASO (Baker et al., 1997).

ASOs & siRNAs

Antisense oligonucleotide (ASO)

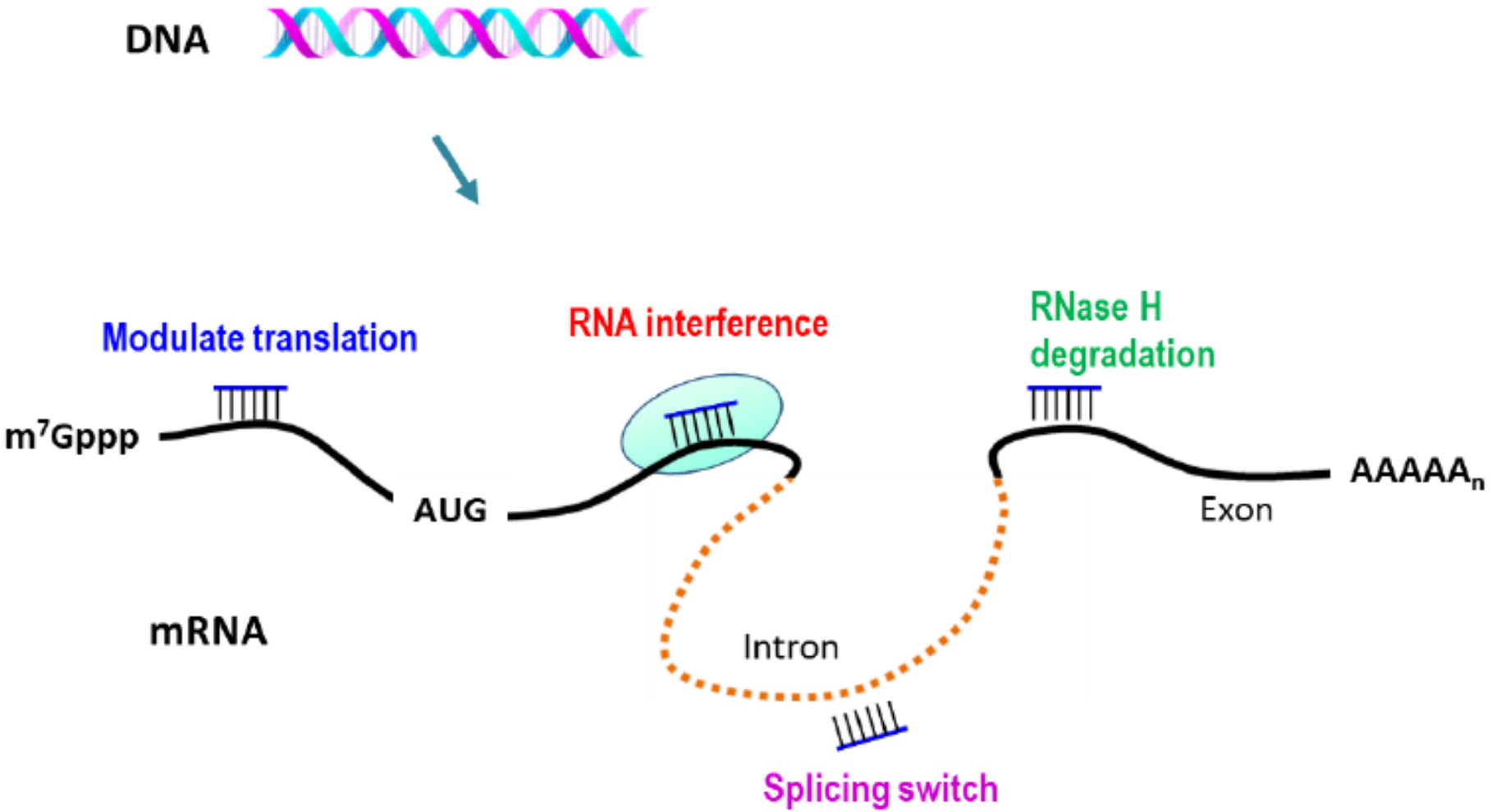
A short (12–24 nucleotides) oligonucleotide that can base-pair with complementary RNA and trigger different post hybridization mechanisms to modulate gene expression.

ASOs can be either single-stranded or double-stranded.

Small interfering RNAs

(siRNAs). A short (~21 base pairs) RNA duplex that can trigger degradation of RNAs containing homologous sequences through endo-nucleolytic cleavage by AGO proteins.

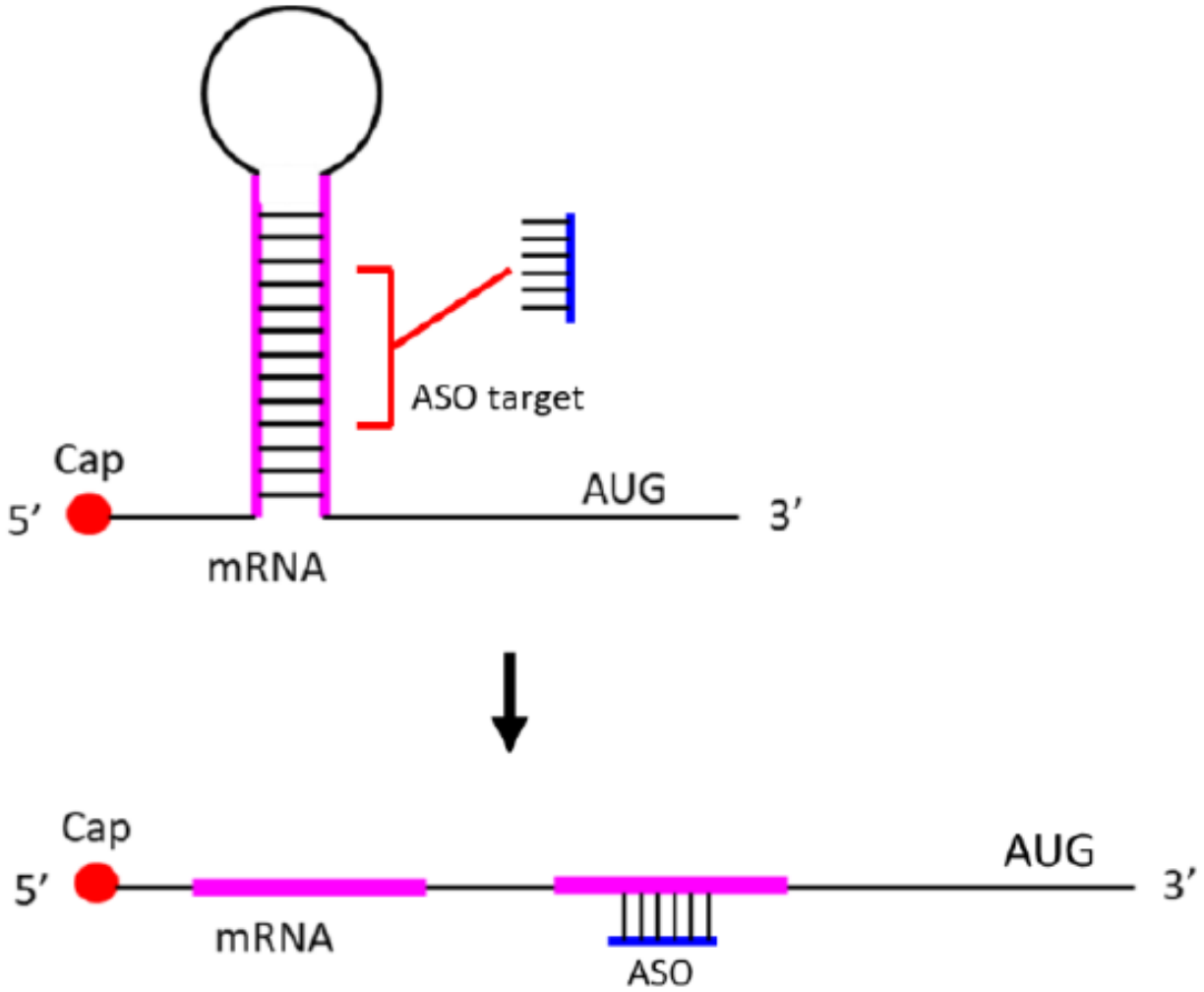
Therapeutic Strategies



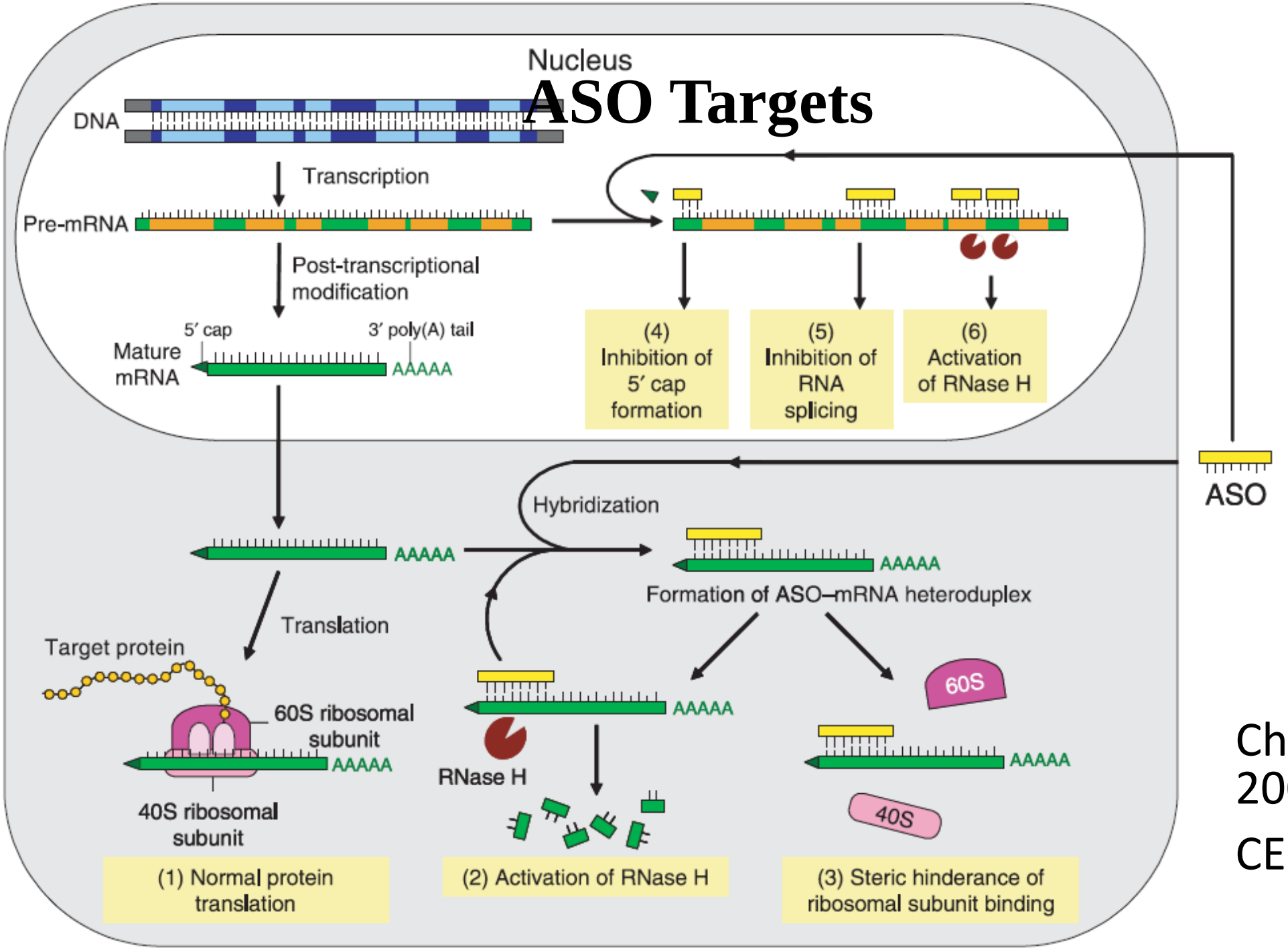
Block
Modulate
Interfere
Switch
Degrade

ASO Increases Translation

An **antisense oligonucleotide** resolves secondary structure to increase the translation of low-density lipoprotein receptor (LDLR) mRNA.



ASO Targets



Chan et al. 2006

CEPP 33, 533.

Antisense oligonucleotides can modulate gene expression through two different mechanisms based on post-hybridization events:

(1) Enzymatic RNA degradation and **2) Occupancy-only mechanisms**.

(1) **RNA degradation includes**

(A) DNA-like antisense oligonucleotides that trigger complementary RNA cleavage by RNase H1 and

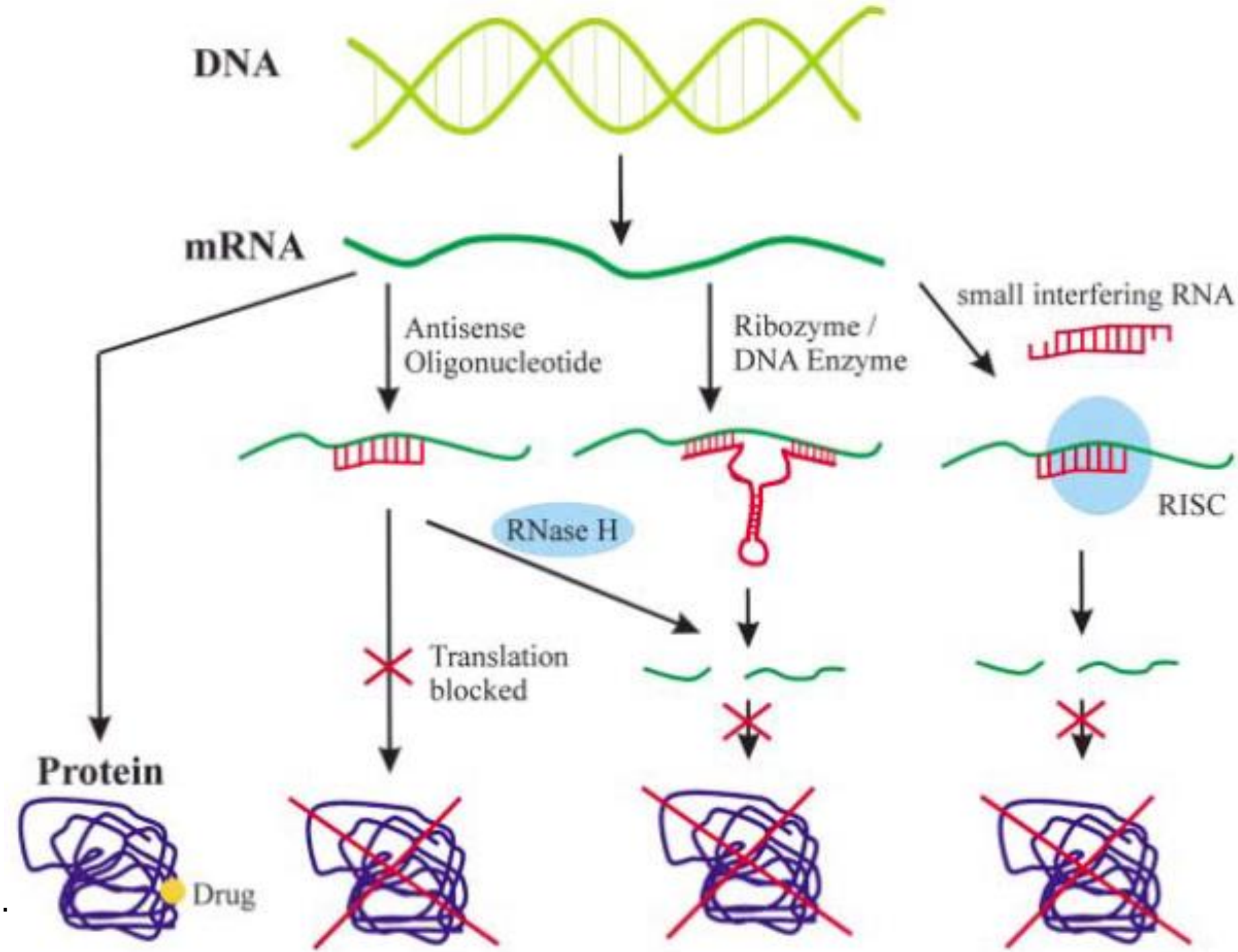
(B) siRNA-mediated reduction of complementary RNA via the Ago2 RISC pathway, whereas other mechanisms such as ribozymes have also been explored.

(2) Occupancy-only mechanisms utilize antisense oligonucleotides to base pair with target RNAs without triggering RNA degradation. These include but are not limited to:

(C) **splicing modulation** using non-DNA like antisense oligonucleotides to base pair with sequence elements in pre-mRNA to inhibit or enhance the utilization of splice sites;

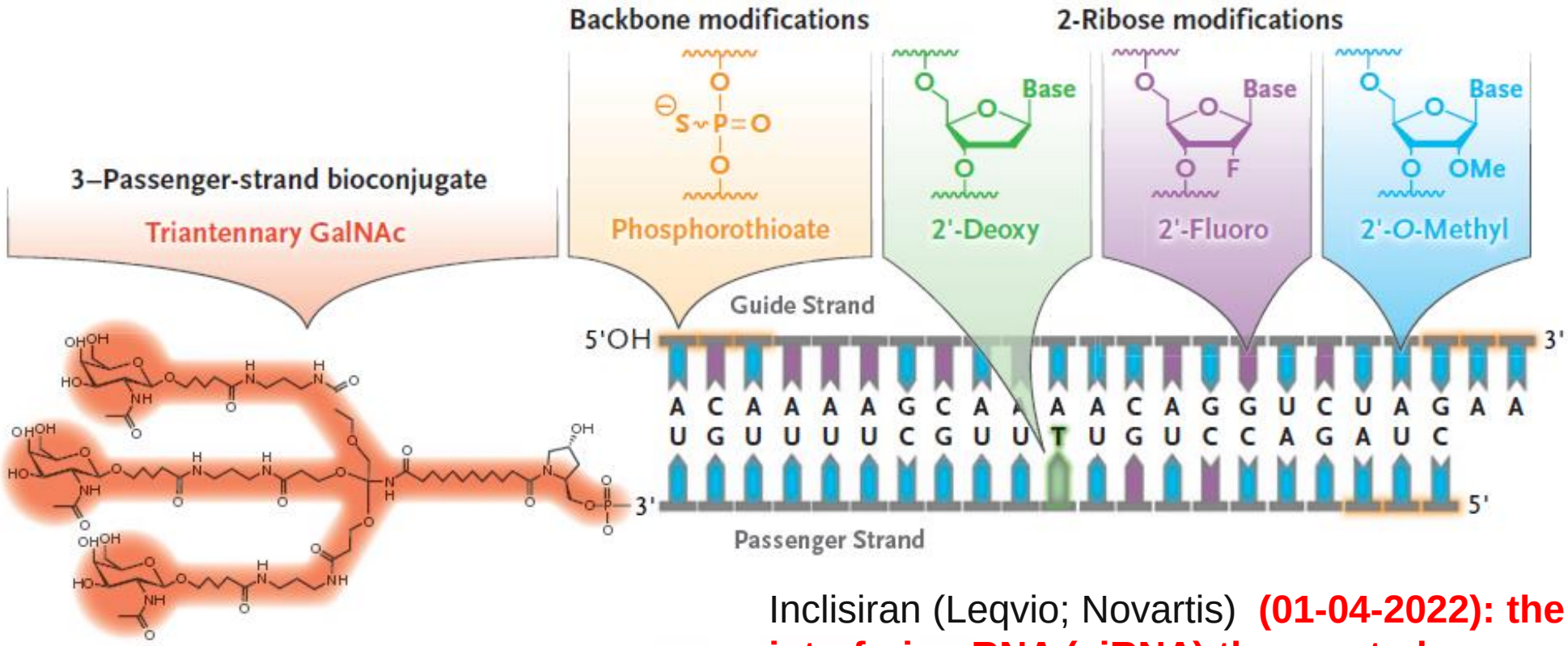
(D) **translation modulation** using non-DNA-like antisense oligonucleotides to base pair with mRNA, either to inhibit translation (steric blocking) or to activate translation through binding to inhibitory elements such as upstream open reading frames (uORF) or other Translation inhibitory elements (TIEs); and

(E) **miRNA modulation** either by base pairing with miRNA to inhibit the function of the miRNA or by base-pairing with miRNA-binding sites of a particular mRNA to eliminate the effect of a particular miRNA. uAUG, upstream AUG codon; pAUG, primary AUG codon.



Kurreck 2003.

Oligonucleotide Therapeutics



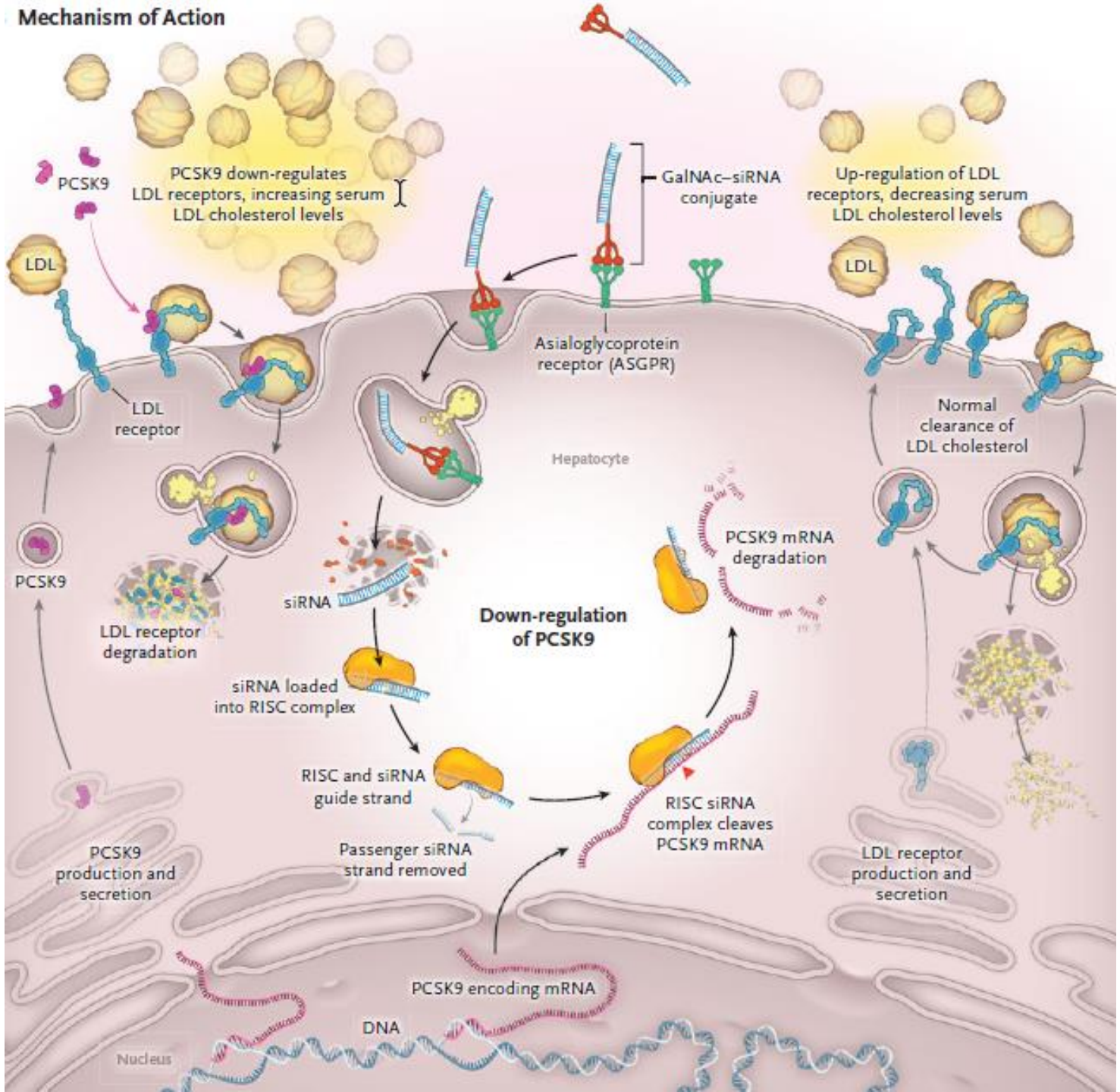
Inclisiran (Leqvio; Novartis) **(01-04-2022): the first small interfering RNA (siRNA) therapy to be approved by the FDA** to reduce low-density lipoprotein cholesterol (LDL-C) with 2 doses per year, following an initial dose and a dose at 3 months.

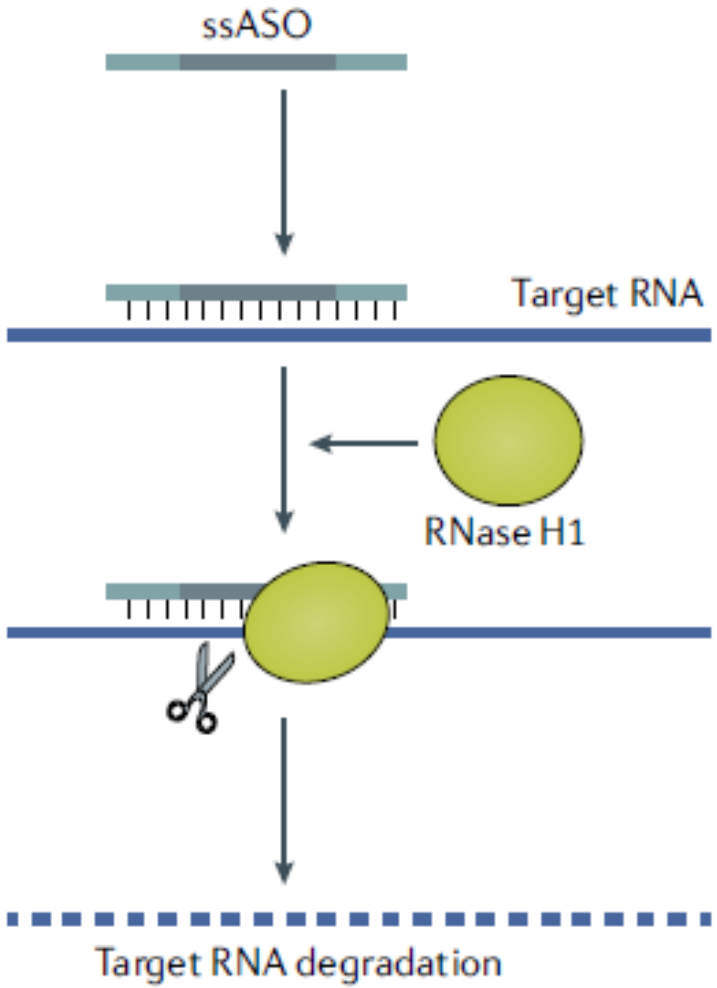
Khvorova, 2017, NEJM 276;1. "Novartis"
LEQVIO (**Inclisiran**) lowers low-density lipoprotein cholesterol (LDL-C).
Medication for the treatment of people with atherosclerotic cardiovascular disease (ASCVD), ASCVD risk equivalents and heterozygous familial hypercholesterolemia (HeFH).

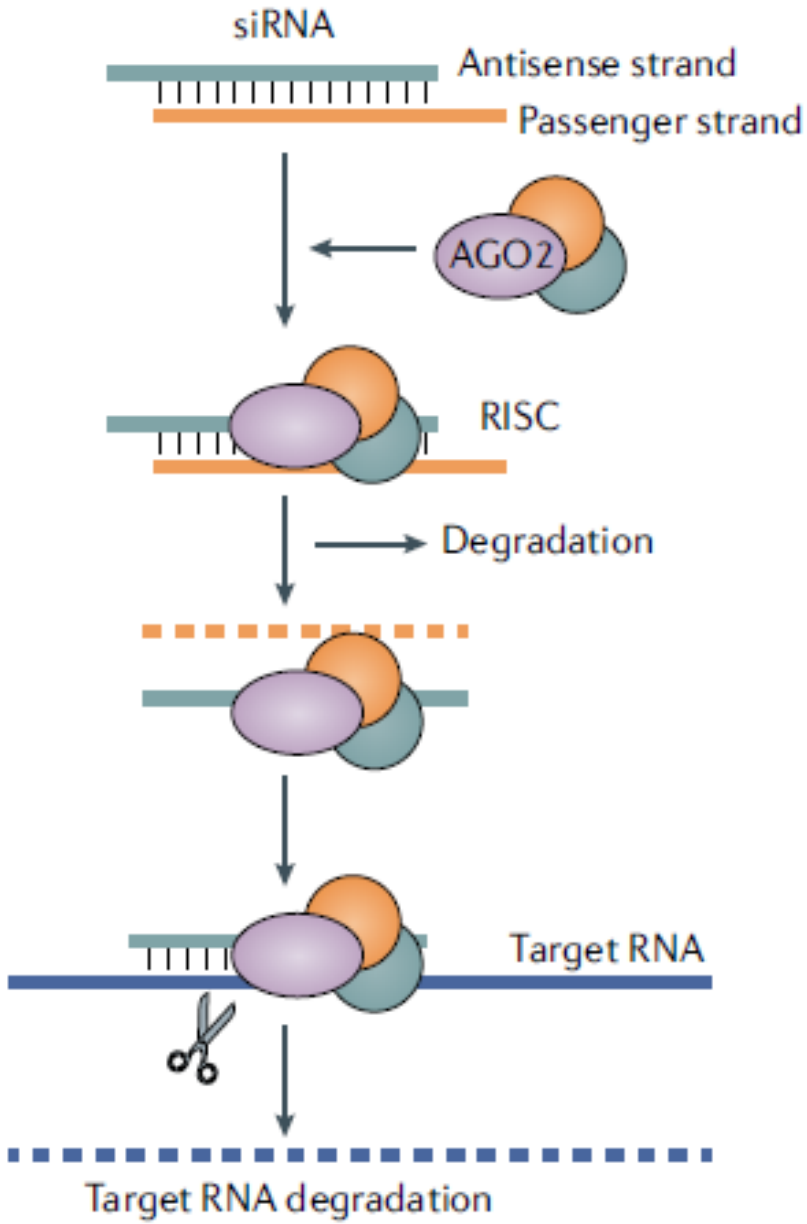
Oligonucleotide Therapeutics

The loaded RISC has a long half-life.
As few as 100 to 200 loaded RISC complexes per cell are sufficient to eliminate expression of the targeted gene.
A **single subcutaneous injection** can lower a patient's low density lipoprotein cholesterol level for **6 months.**
Injections needed every 2 to 4 weeks for monoclonal antibodies and every 6 to 12 months for siRNAs!

Khvorova, 2017, NEJM 276;1.



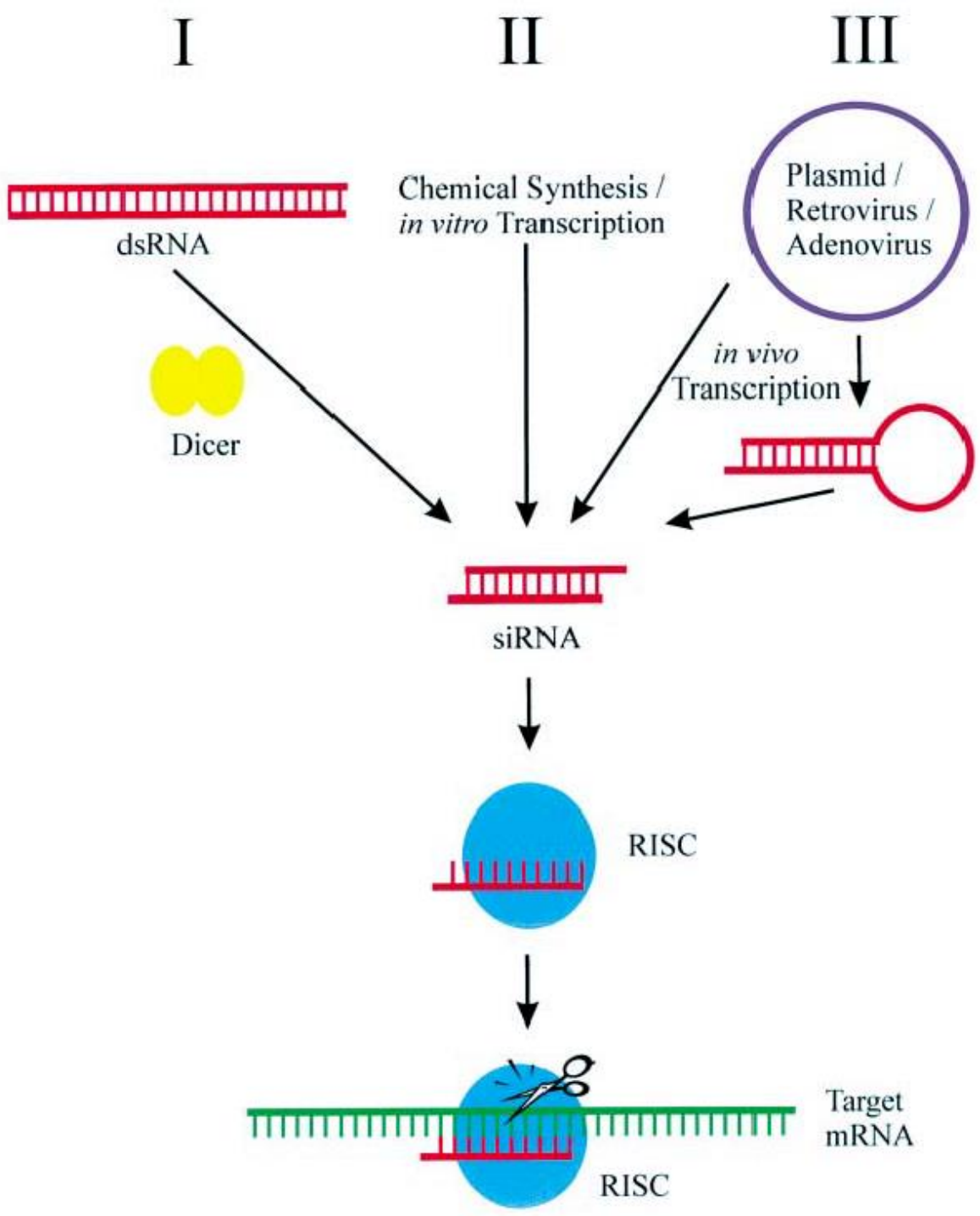




RNA degradation mediated by RNase H1 or
AGO2.

RNase H1 activation: a single-stranded antisense oligonucleotide (ssASO) containing deoxynucleotides (grey portion) hybridizes with the target RNA, generating
a DNA–RNA heteroduplex, which is recognized by RNase H1 to cleave the RNA strand in the heteroduplex.

siRNAs: the double-stranded RNA containing antisense and passage strands is bound by RNA-induced silencing complex (RISC) proteins including the endonuclease AGO2. The antisense strand is released by removal of the passenger strand, allowing the antisense strand that associates with AGO2 and potentially additional proteins to base-pair with the target RNA, leading to the **cleavage of the target RNA by**



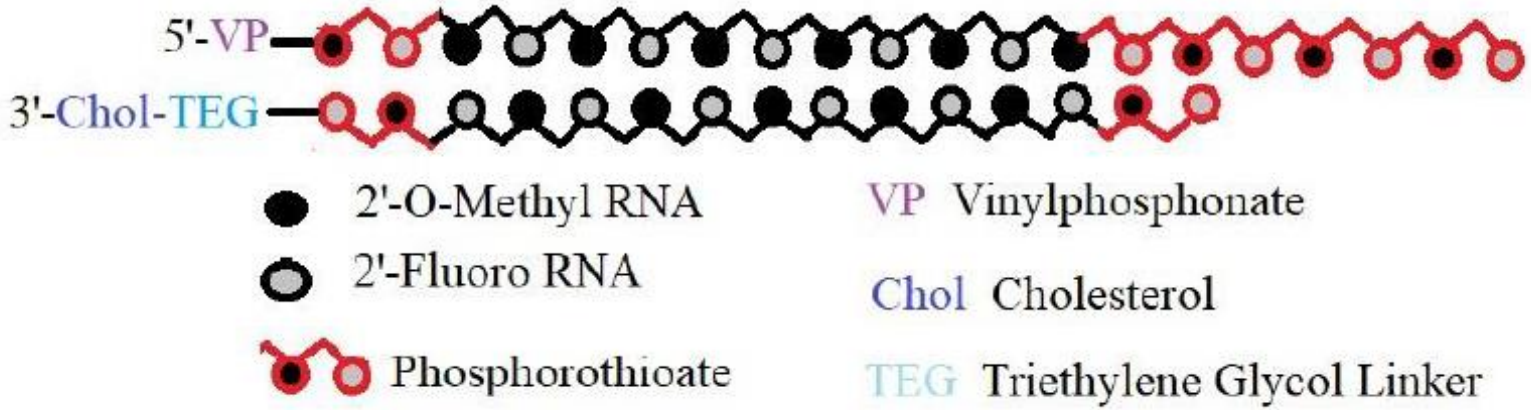
siRNA generation

(I) Long double-stranded RNAs are processed into siRNA by the Dicer enzyme.

(II) Chemically synthesized or in vitro transcribed siRNA duplexes can be transfected into cells.

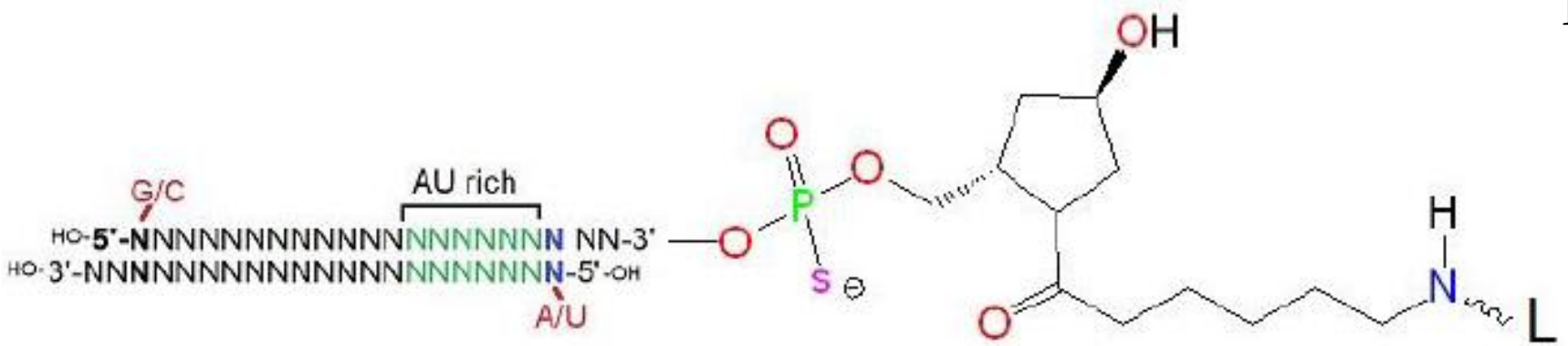
(III) siRNA molecules can be generated in vivo from plasmids, retroviral vectors or adenoviruses.

Conjugated siRNA –> Lipophilic siRNA



Lipids and Linkers

Cholesterol
Docosanic Acid
Docosahexaenoic Acid
Fatty Acids
Lithocholic Acid

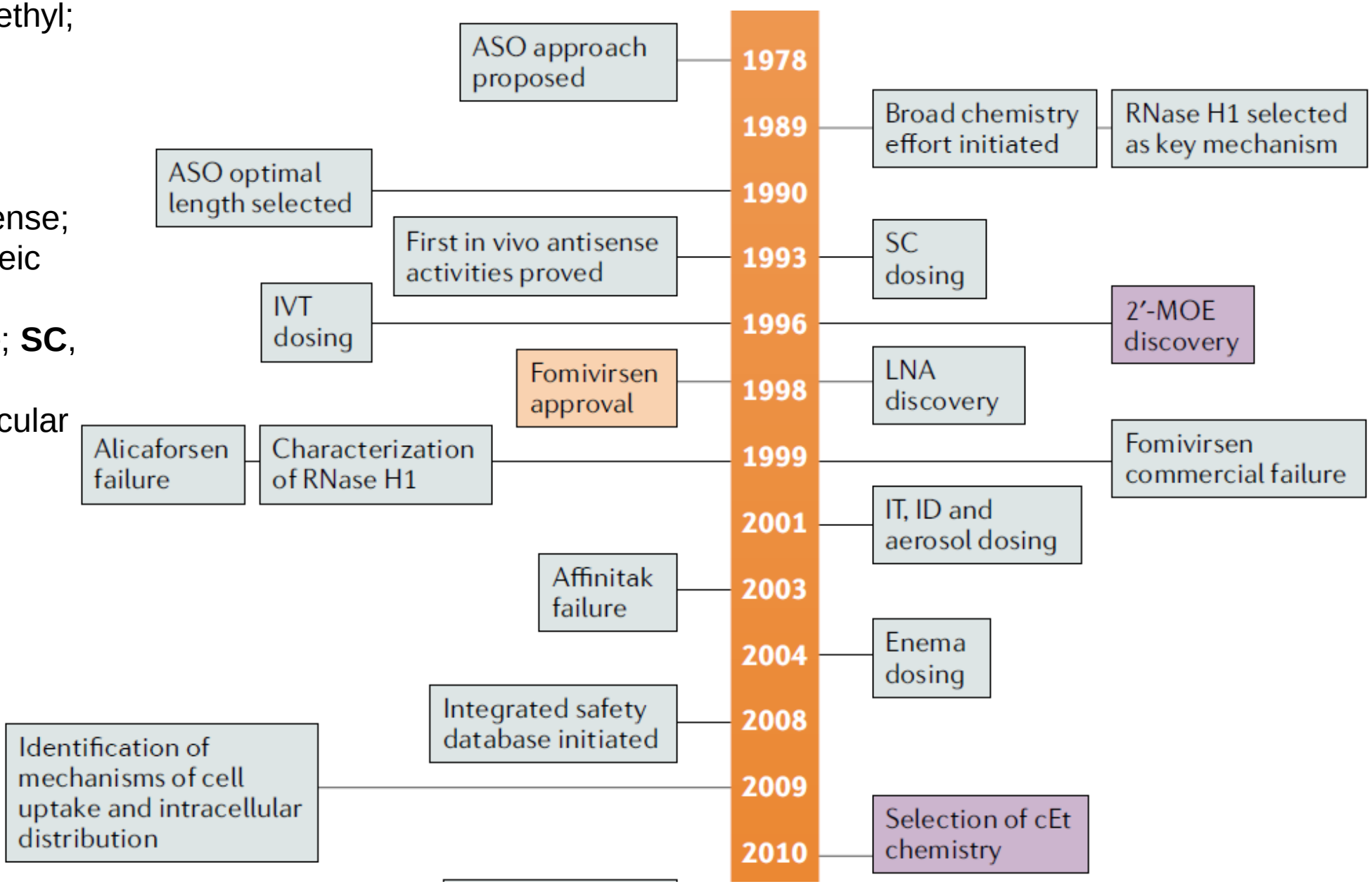


Approved ASOs

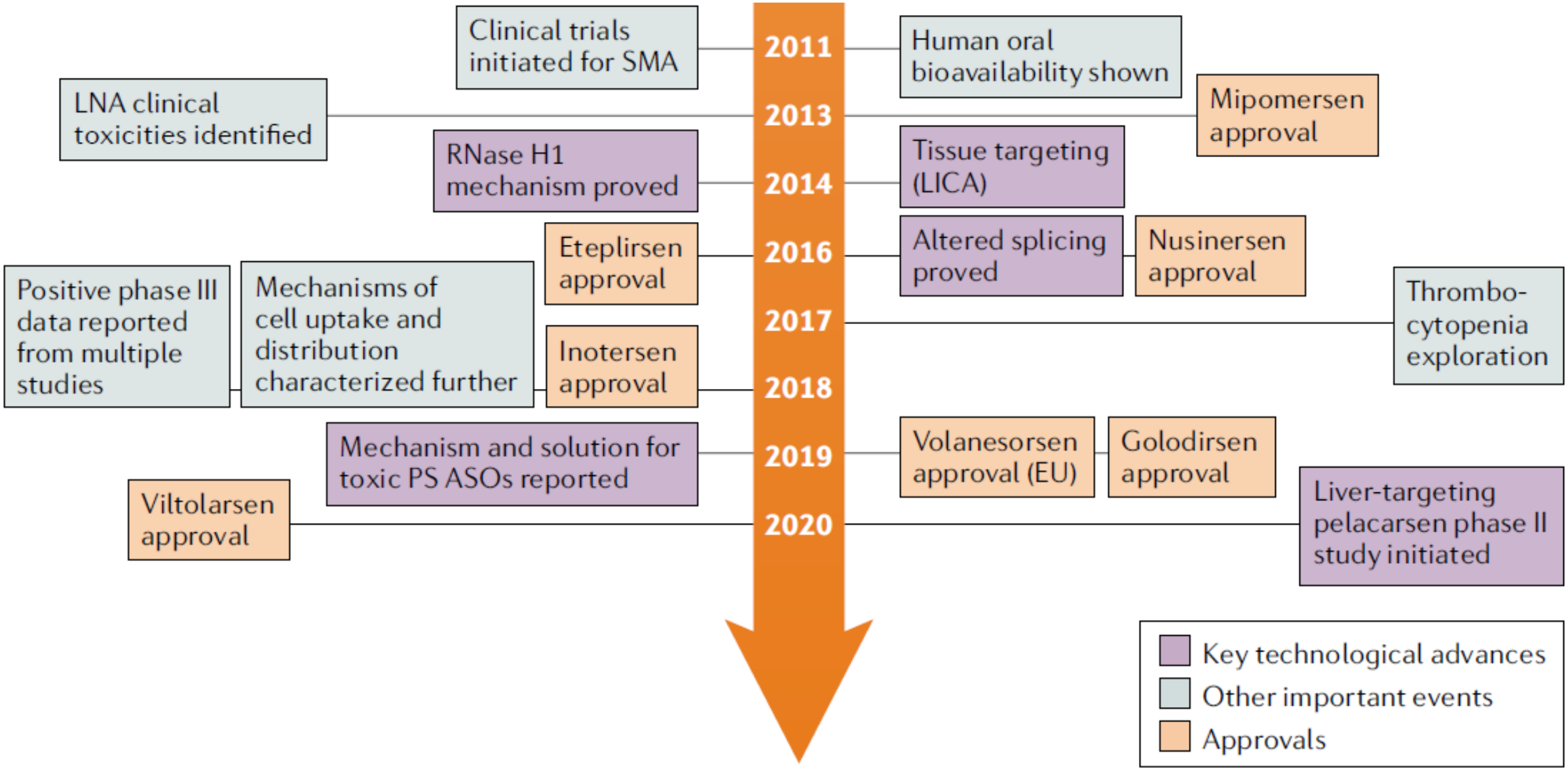
- **Nine (9) single-stranded antisense oligonucleotide (ASO) drugs** representing **four (4) chemical classes**, **two mechanisms of action and four routes of administration** have been **approved** for commercial use, including the **first RNA-targeted drug** to be a major commercial success, **nusinersen**.
- Although all the approved drugs are for use in patients with rare diseases, **many of the ASOs in late- and middle-stage clinical development** are intended to treat patients with very common diseases.
- **13 RNA-targeted oligonucleotide drugs** have received regulatory approval: **nine single-stranded ASOs** and **four siRNAs** — patisiran, givosiran, lumasiran and inclisiran.

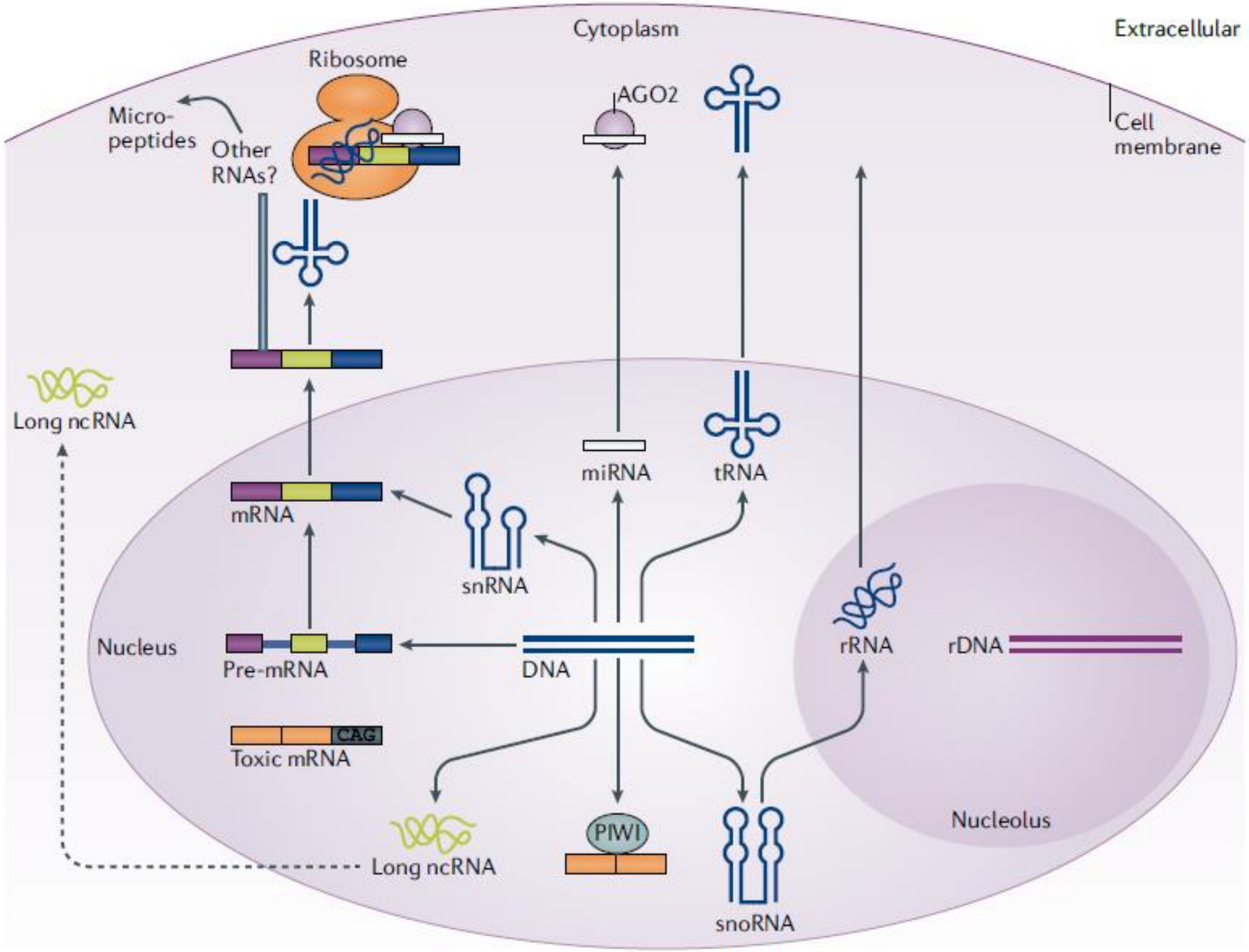
2′-MOE, 2′-*O*-methoxyethyl; **cE**t, constrained ethyl; **ID**, intradermal; **IT**, intrathecal; **IVT**, intravitreal; **LICA**, ligand-conjugated antisense; **LNA**, locked nucleic acid; **PS**, phosphorothioate; **SC**, subcutaneous; **SMA**, spinal muscular atrophy.

A Short History (Crooke S.T.)



A Short History



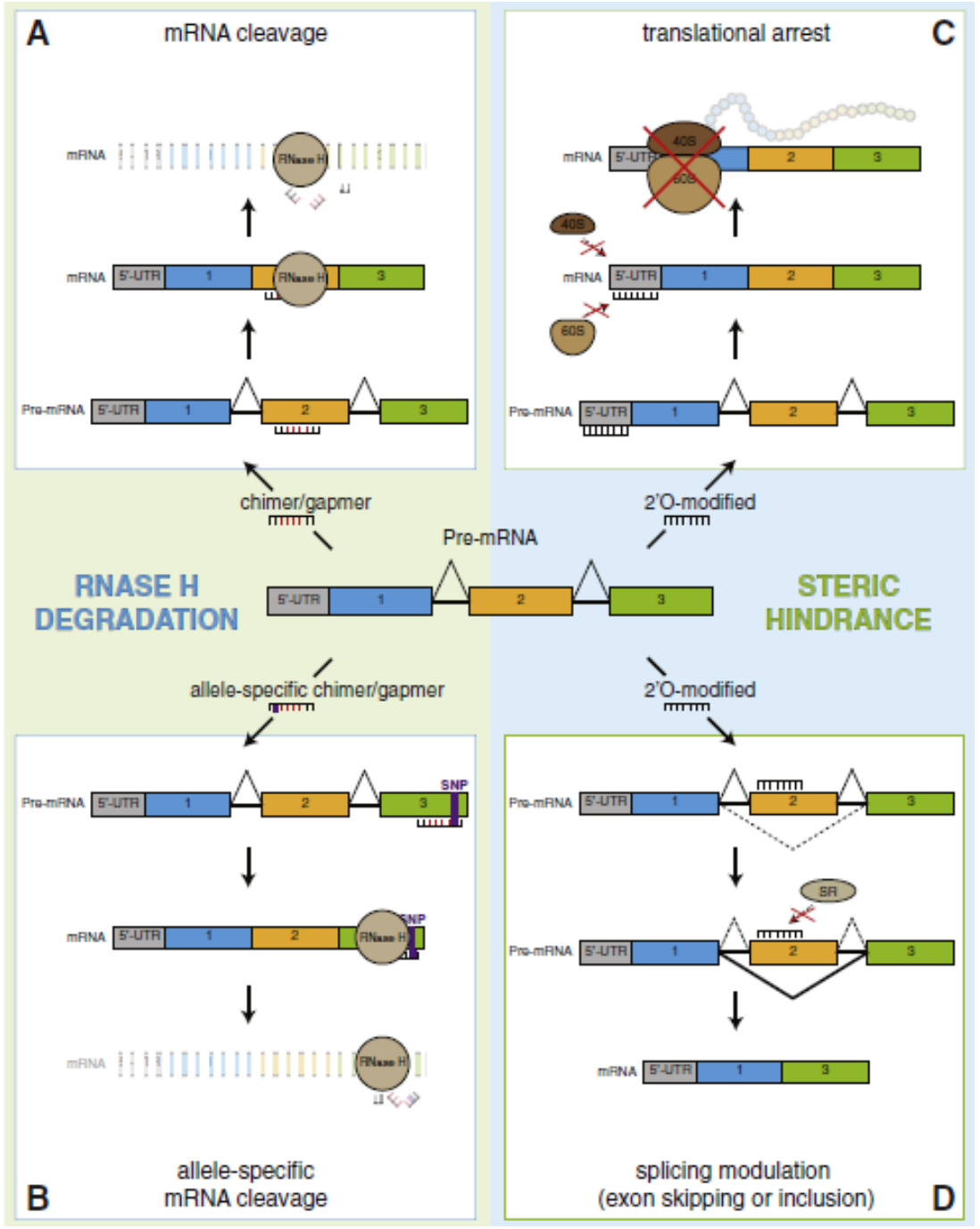


Legend:

mRNA, messenger RNA
rRNA, ribosomal RNA
tRNA, transfer RNA
=> **Translation**
sncRNA, small non-coding RNA
snRNA, small nuclear RNA
=> **Pre-mRNA splicing**
snoRNA, small nucleolar RNA
=> **pre-rRNA processing and modification**
miRNA, microRNA
piRNA, PIWI-interacting RNA
=> **modulating transposons**
Enhancer RNA
=> **affect transcription**

long ncRNA, long non-coding RNA
Toxic RNA, tri nucleotide repeats

Crooke et al. 2018



Evers et. Al. 2015:

Functional mechanisms of antisense oligonucleotide for central nervous system disorders.

A. Antisense oligonucleotide gapmers induce RNase H-mediated breakdown of **target RNA**.

B. RNase H-mediated breakdown by antisense oligonucleotide **gapmers** targeting **specific point mutations**, structural differences between wild-type and mutant mRNA or a single nucleotide polymorphism (SNP) that is unique to the mutant RNA.

C. **Suppressing RNA translation** by antisense oligonucleotides targeting the **RNA translation start site** or sterically **blocking the binding of RNA binding protein complexes**,
such as ribosomal subunits.

D. **Antisense oligonucleotides** binding to **targeting splice sites** or exonic/intronic inclusion signals that will result in skipping or inclusion of the targeted exon.
SR, pre-mRNA splicing machinery.

Novel analytical chemistry was required for product characterization and pharmacokinetic analysis, and innovative process chemistry and manufacturing systems were needed so that the cost of synthesis could be dramatically reduced and scale dramatically increased!

Many RNAs contain more than one identical attractive ASO receptor sequence.

It is essential to understand the potential interactions between cognate sites present in a target RNA and how potency varied as a function of the number of cognate receptors for the ASO.

Rapid-throughput screening systems to identify optimal sites in RNAs for ASOs to bind are now established, as well as methods to evaluate pharmacokinetic behavior and potential toxicities.

The **causes of failure** need to be understood so that lessons can be applied to advance the performances of ASOs.

Approved single-stranded antisense oligonucleotide drugs 1

Drug	Chemistry	Target	Dose	Indication	Efficacy & Safety
Fomivirsen	2′-H	CMVIE2 (eye)	330 μg per eye once every 4 weeks (ITV)	CMV retinitis (FDA, 1988; EMA, 1999)	**Inhibited the progression of CMV retinitis** in patients with AIDS. Mild ocular inflammation.
Mipomersen	2′-MOE	ApoB-100 (liver)	200 mg once weekly (SC)	HoFH (FDA, 2013)	**Reduced apoB-100, LDL-C and VLDL.** ALT increases, ISRs.
Eteplirsen	PMO	Dystrophin exon 51 (muscle)	30 mg kg−1 once weekly (IV)	DMD (FDA, 2016)	**Increased dystrophin production in skeletal muscle**; the interpretation of the clinical significance of this effect is controversial. Generally, well tolerated.
Nusinersen	2′-MOE, fully modified	*SMN2* intron 7 (CNS)	12 mg once every 4 months (IT)	SMA (FDA, 2016; EMA, 2017)	**Corrected the *SMN2* splicing defect**; the two-phase III studies were stopped early owing to demonstration of significant clinical benefit. No drug-related adverse events.
Inotersen	2′-MOE	TTR (liver)	300 mg once weekly (SC)	Hereditary ATTR (FDA, 2018; EMA, 2018)	**Reduced progression of neuropathic disease** and improved quality of life. Decreased platelets, ISRs, renal dysfunction.

Approved single-stranded antisense oligonucleotide drugs 2

Drug	Chemistry	Target	Dose	Indication	Efficacy & Safety
Golodirsen	PMO	Dystrophin exon 53 (muscle)	30 mg kg−1 once weekly (IV)	DMD (FDA, 2019)	**Increased dystrophin production in skeletal muscle.** Hypersensitivity reactions and renal toxicity.
Volanesorsen	2′-MOE	ApoC-III (liver)	300 mg once weekly (SC)	FCS (EMA, 2019)	**Reduction of triglycerides** (mean reduction of 1,712 mg dl−1), reduced abdominal pain and pancreatitis. Decreased platelets, ISRs.
Viltolarsen	PMO	Dystrophin exon 53 (muscle)	80 mg kg−1 once weekly (IV)	DMD (MHLW, 2020; FDA, 2020)	**Increased dystrophin production** in skeletal muscle and clinical improvement in the 6-minute walk test. Generally, well tolerated.
Casimersen	PMO	Dystrophin exon 45 (muscle)	30 mg kg−1 once weekly (IV)	DMD (FDA, 2021)	**Increased dystrophin production**. Generally, well tolerated.

Most **modifications have focused on increasing the affinity per nucleotide unit for the cognate sequence** and/or on **enhancing resistance to nucleases**, the enzymes that degrade these drugs (Bennett and Swayze, 2010, Levin et al., 2008, Swayze and Bhat, 2008).

This effort has resulted in several chemical classes that have proven to be clinically effective and progressively more potent and better tolerated.

More recently, research has focused on **enhancing productive delivery to specific cell types by conjugation of moieties that take advantage of high-capacity receptors** such as the **asialoglycoprotein receptor expressed by hepatocytes** (Nair et al., 2014, Prakash et al., 2014, Prakash et al., 2016b).

(A) Thousands of nucleic acid analogs of RNA and DNA have been made and **tested** in antisense oligonucleotides. These consist of **base modifications**, **sugar modifications**, **inter-nucleoside linkage modifications**, and **conjugates** of small and large molecules to essentially every position of a dinucleotide.

(B) **Key formulations and conjugate strategies** used in later stage clinical trials to enhance delivery.

(C) **Single- and double-stranded** oligonucleotide structures.

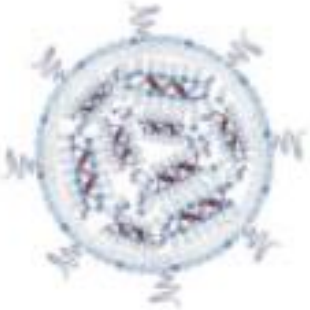
Structure Matters

PS ASO – ss: Amphibatic.
Distribute broadly.
Bind to proteins.

B

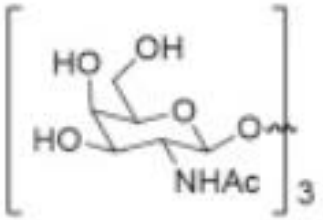
Lipid nanoparticles

- *Protect unmodified nucleic acids*
- *Delivery mostly to hepatocytes, efforts to target other cells underway*
- *Immunostimulatory*



N-Acetyl galactosamine (GalNAc) clusters

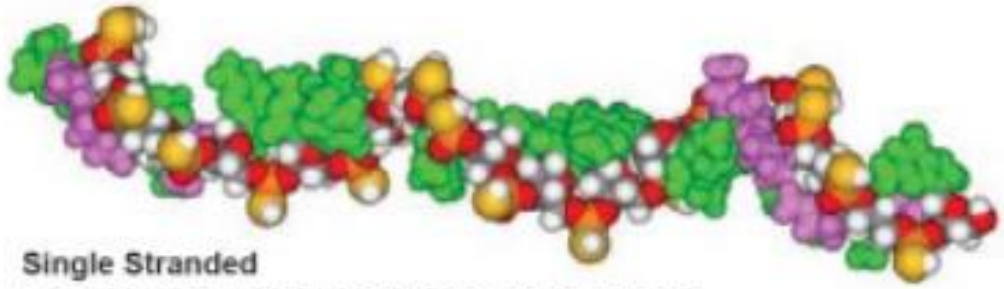
- *Specifically targets hepatocytes with large improvement in potency*
- *Useful for all mechanisms and double stranded and single stranded oligonucleotides*



Fatty acids and lipids

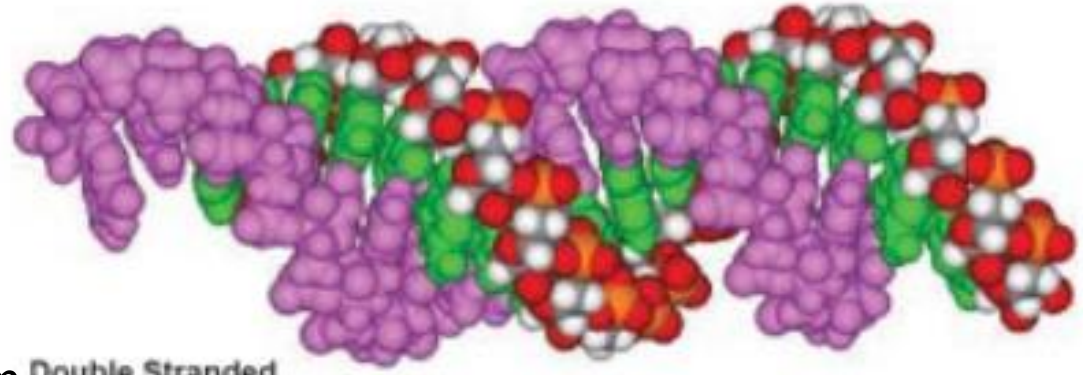
- *Increases plasma protein binding*
- *Improves distribution to secondary tissues*
- *Not broadly used to date*

C



Single Stranded

- *Amphipathic, with hydrophobic groups exposed*
- *With phosphorothioate, has good protein binding and distribution to tissues*



Double Stranded

- *Hydrophilic, with little exposed hydrophobicity*
- *Poor distribution to tissues absent formulations or conjugates*

siRNA – ds: Do not bind to serum proteins.
Need formylation, e.g. conjugation.

Green = nucleobases; **red** = oxygen; **yellow** = sulfur; **orange** = phosphorus; **gray** = sugar residues; **purple** = 2'-MOE modifications

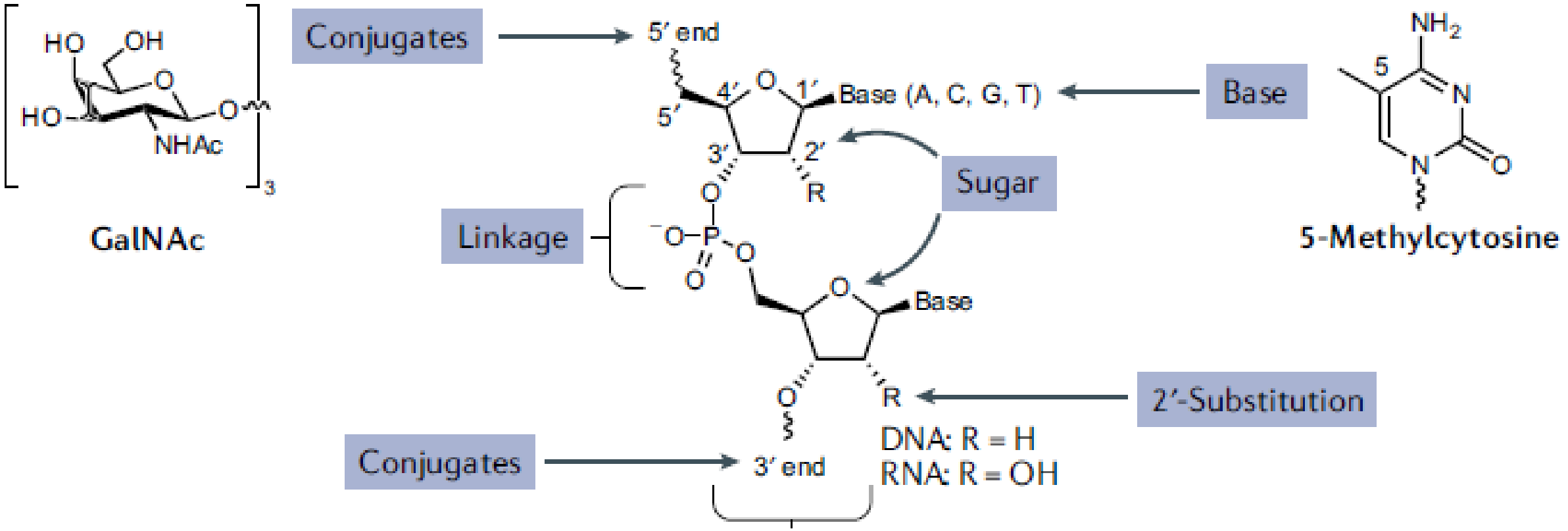
Chemistry Matters

Because individual RNA targeting oligonucleotides within a specific chemical class differ only in sequence, the **members of each class have similar physico-chemical characteristics and thus common pharmacokinetic and biological properties**.

However, every chemical class differs, and even **subtle modifications** that appear to scientists not experienced in the field as chemically similar—e.g., 2′-*O*-methoxyethyl (2′-MOE) versus 2′-methoxy—**can result in substantial changes in potency**, pharmacokinetics, and generic chemical class effects (Bennett and Swayze, 2010).

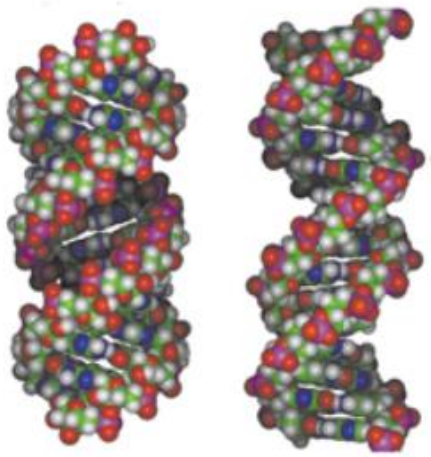
It is essential to precisely **define the chemistry of RNA-targeted oligonucleotides**.

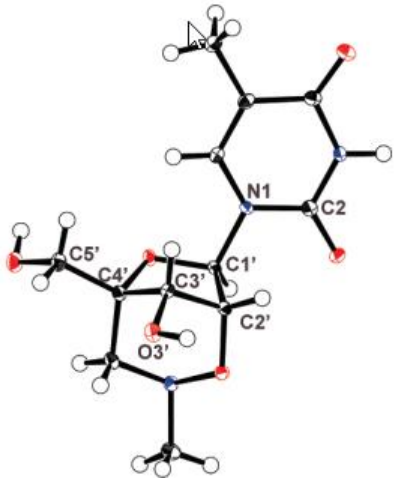
Modifications

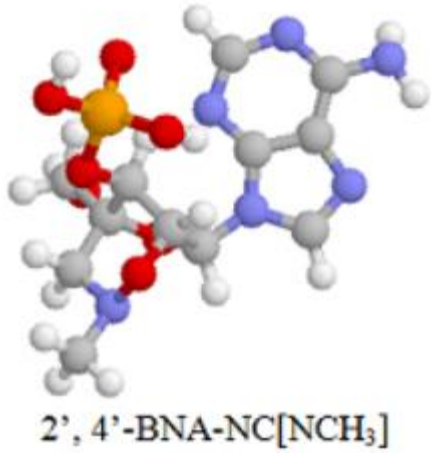


BNA ASOs

Control of conformation



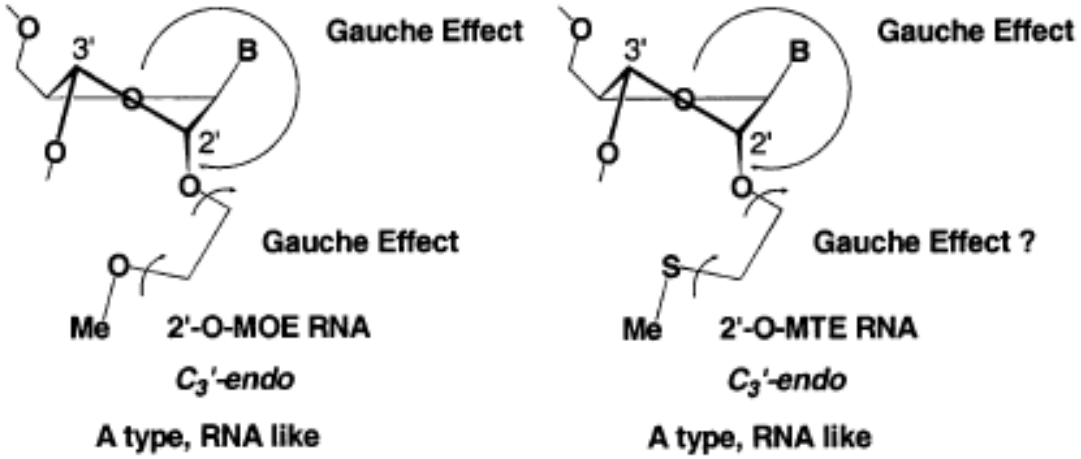




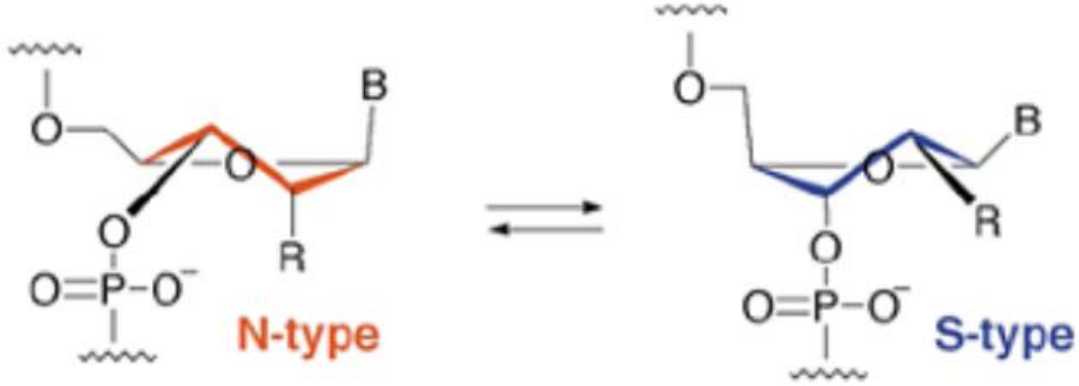
Rahman et al. 2008

2′,4′-BNA^{NC} :
(i) [LNA] equal or **higher binding affinity against an RNA**
(ii) Better **RNA selectivity**.
(iii) Stronger and more sequence selective **triplex-forming**.
(iv) Higher **nuclease resistance**.

A- and B- DNA

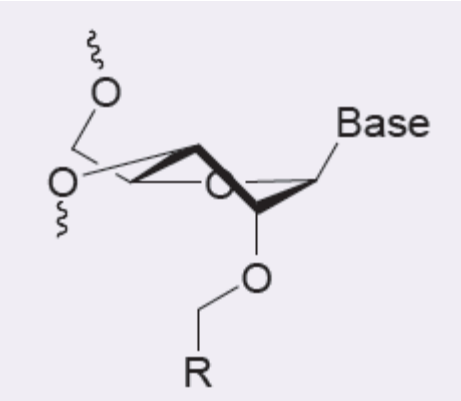


Prakash 2002



N- and S-type sugar puckering. The N-type exists predominantly in A-RNA and the S-type in duplexes with B-DNA helical structure.

Chemistry Improves Affinity & Potency

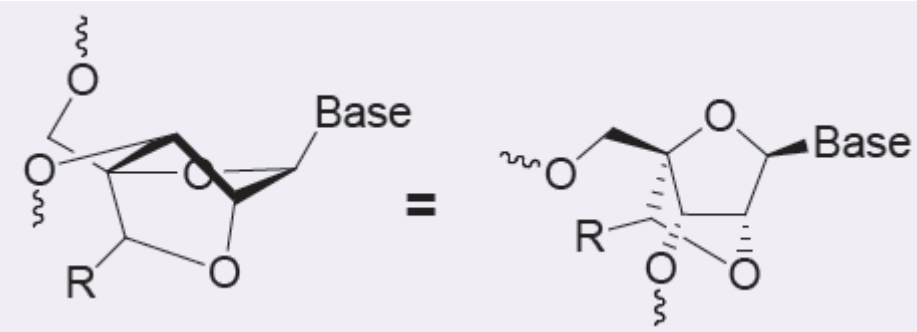


2’-modified nucleic acids

- R = H, 2’-O-methyl
- R = CH_3, 2’-O-ethyl
- R = CH_2OCH_3, MOE

Goals

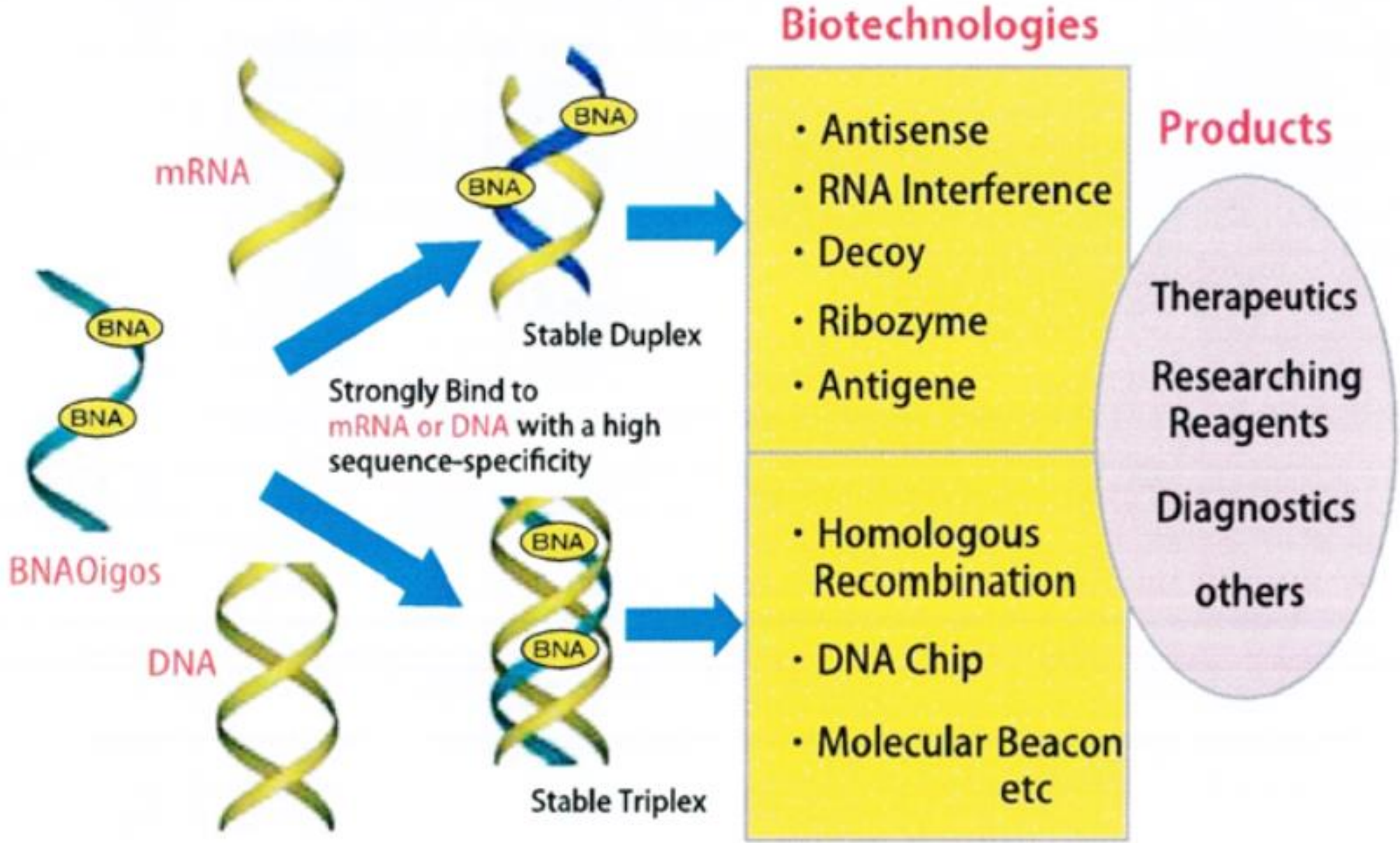
- Increase Potency
- Increase Convenience
  - Lower dose
  - Oral dosing
- Expand the breath of targets



Tested by Ionis

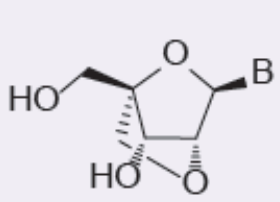
2’-4’ Constraint

- R = H, BNAs (LNA)
- R = CH_3, cET BNA
- R = CH_2OCH_3, cMOE BNA

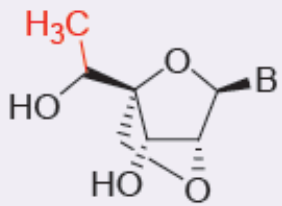

Biotechnologies
mRNA
BNA
BNA
BNA
BNA
BNAOigos
Stable Duplex
Strongly Bind to
mRNA or DNA with a high
sequence-specificity
DNA
BNA
BNA
Stable Triplex
• Antisense
• RNA Interference
• Decoy
• Ribozyme
• Antigene
• Homologous Recombination
• DNA Chip
• Molecular Beacon
etc
Products
Therapeutics
Researching Reagents
Diagnostics
others

Constrained Nucleosides Tested by ISIS (Ionis)

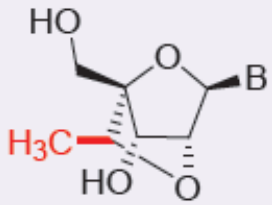
BNA nucleosides



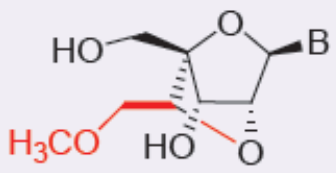
LNA



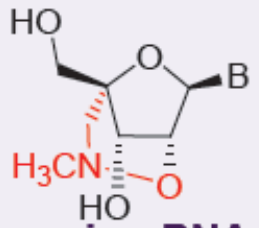
5'-Me-BNA



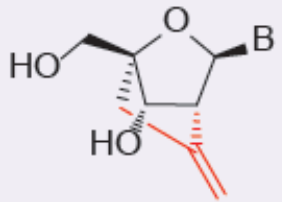
cEt BNA



cMOE BNA

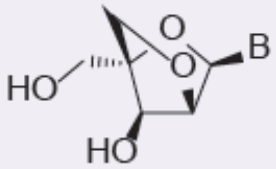


oxyamino-BNA

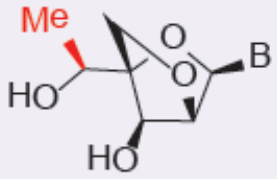


vinyl-carbo-BNA

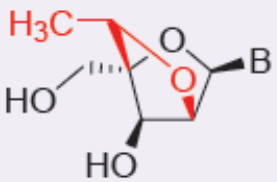
α-L-LNA nucleosides



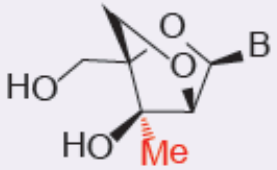
α-L-LNA



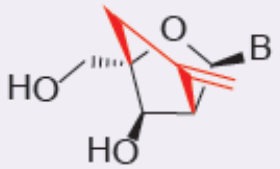
S-5'Me-α-L-LNA



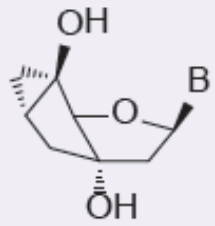
R-α-L-cEt



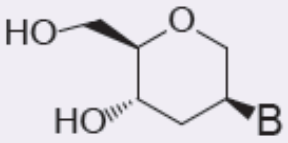
3'Me-α-L-LNA



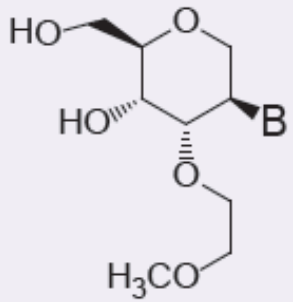
α-L-carbo-BNA



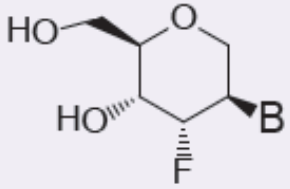
Tricyclo-DNA



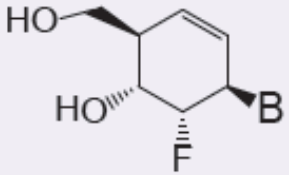
HNA



MOE-ANA



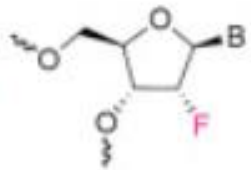
F-HNA



F-CeNA

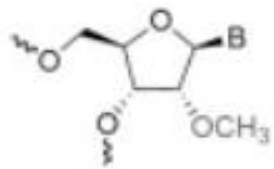
A

- *Improves binding affinity to target RNA*
- *Does not improve stability or PK*
- *Useful in RISC mechanisms*
- *Nucleoside metabolites incorporated into host DNA and RNA*
- *Can cause degradation of paraspeckle proteins*



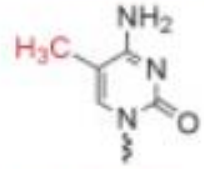
2'-Fluoro

- *Improves PK and stability*
- *Only a modest improvement in potency*
- *Modest reduction in immune stimulation*



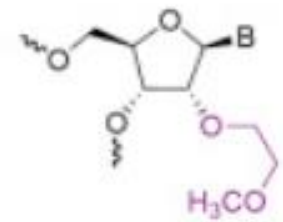
2'-O-methyl

- *Improves binding affinity to target RNA*
- *Reduces immune stimulation*
- *The only broadly useful heterocycle modification*



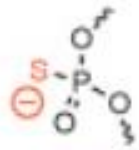
5-methylcytosine

- *Improves PK, elimination half life 2-4 weeks*
- *Improves binding affinity to target RNA, increases potency*
- *Broad reduction in class toxicities due to nonspecific protein binding, e.g. immune stimulation*
- *ED_{50} for liver targets in man ~150 mg/week*

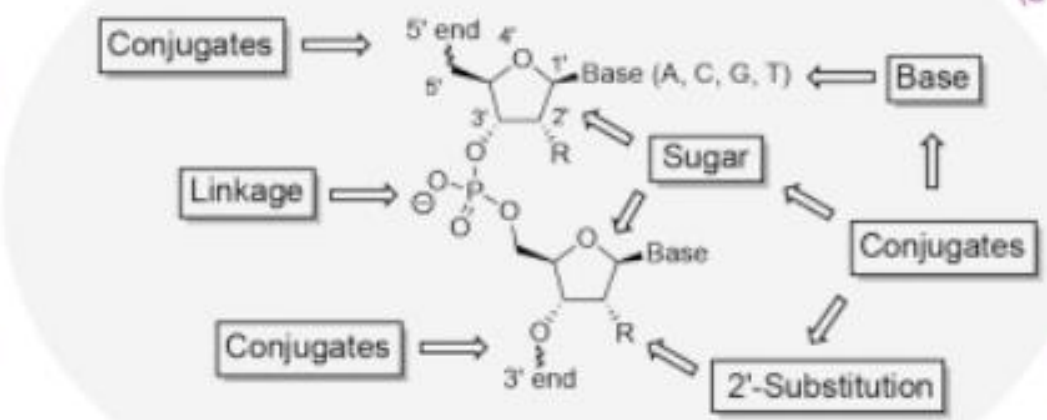


2'-MOE
(2'-O-methoxyethyl)

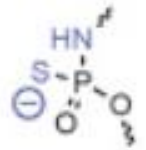
- *The most useful modification to date*
- *Supports multiple mechanisms*
- *Improves PK, elimination half life 1-3 days*
- *Optimal protein binding*
- *Useful in GalNAc siRNAs*



PS
(phosphorothioate)

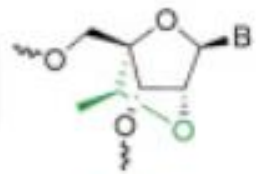


- *Only suitable for occupancy only mechanisms*
- *Not broadly used*



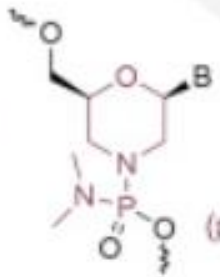
thiophosphoroamidate

- *Improves PK, elimination half life 2-4 weeks*
- *Greatly improves binding affinity, improves potency*
- *Increased sequence dependent toxicity risk*
- *Demonstrates activity in human cancers*



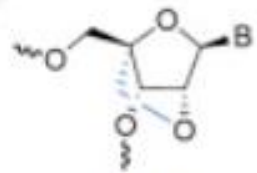
cEt
((*S*)-constrained ethyl)

- *Chemistry of eteplirsen*
- *Useful for occupancy only mechanisms*
- *High stability*
- *Low protein binding results in poor PK*
- *Limited potency (30 mg/kg/wk)*
- *Excellent safety*



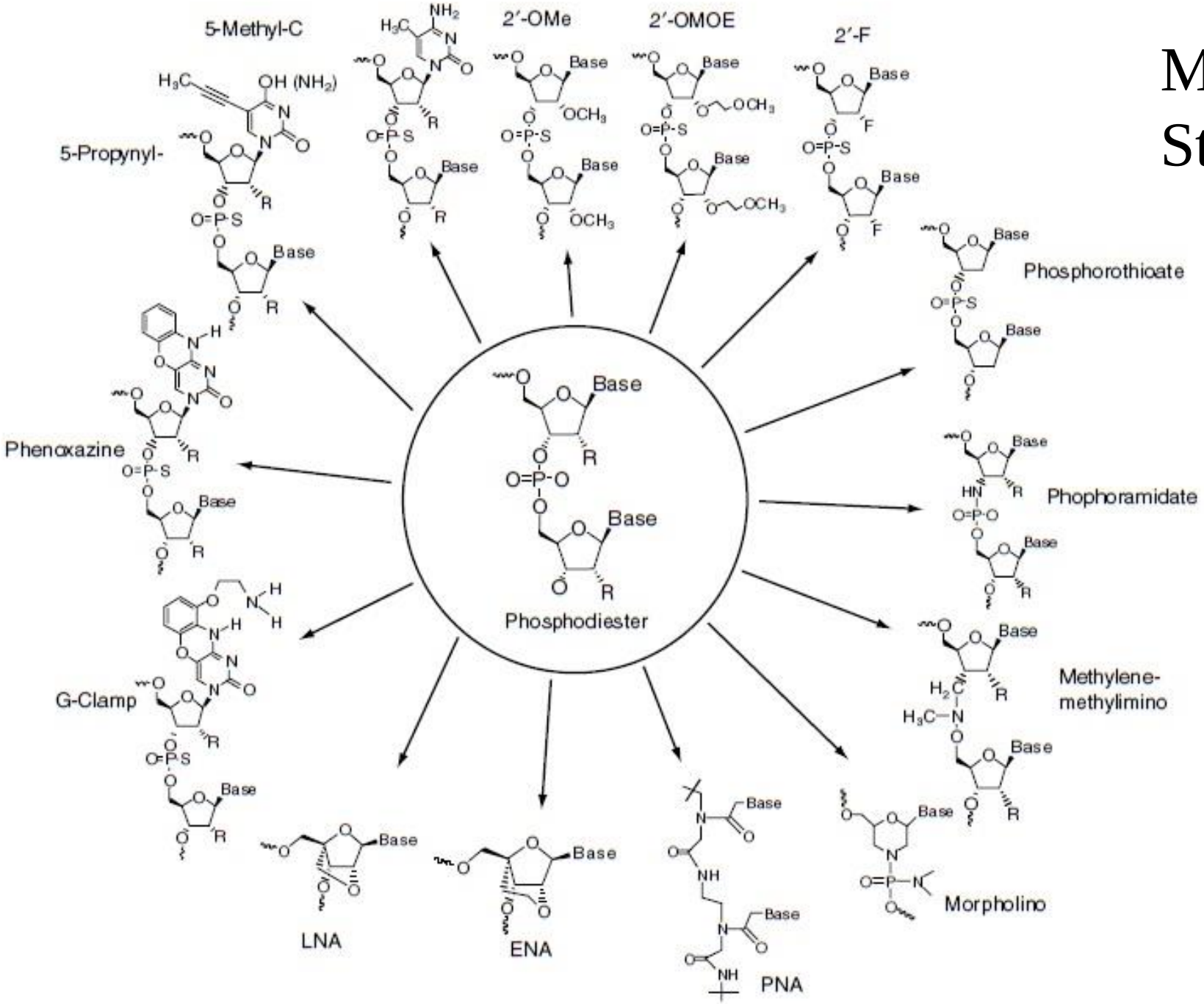
PMO
(phosphorodiamidate morpholino)

- *Greatly improves binding affinity, improves potency*
- *Increased sequence dependent toxicity risk*
- *Hepatotoxicity and renal toxicity observed in clinical trials*



LNA
(locked nucleic acid)

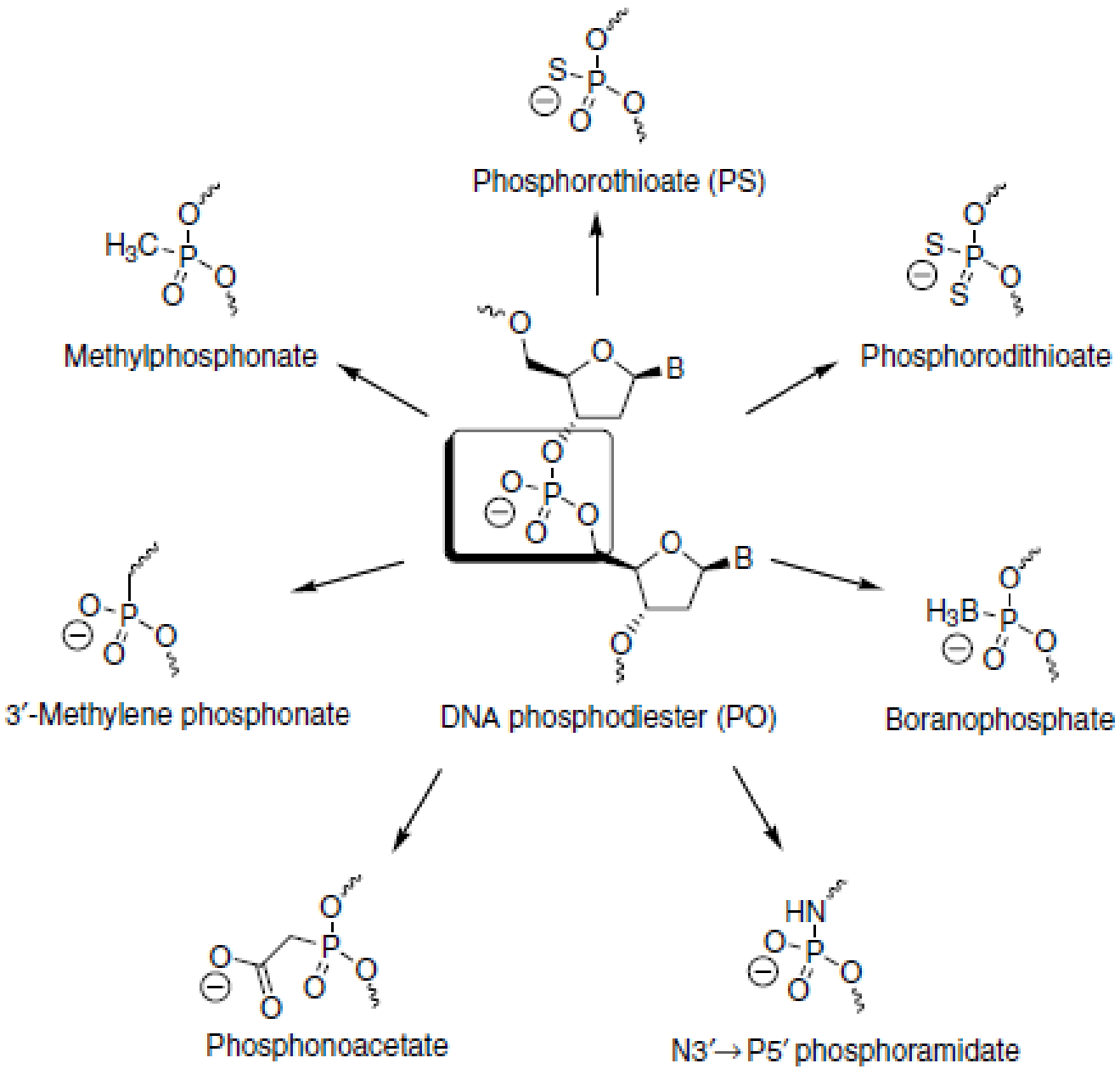
Modifications Studied

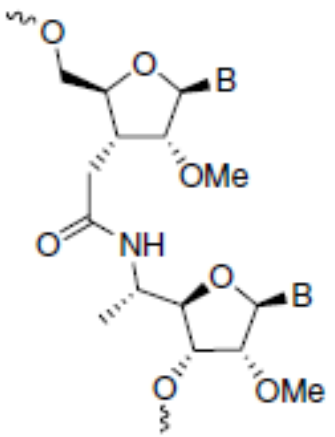


Backbone Modification

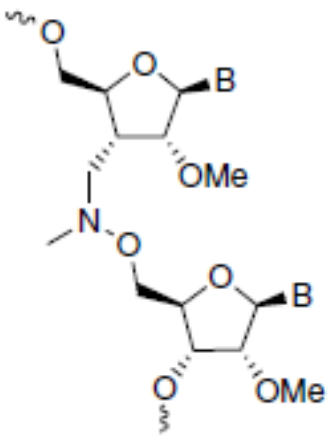
<- Furanose-containing phosphate backbones

Furanose-containing nonphosphate backbones !

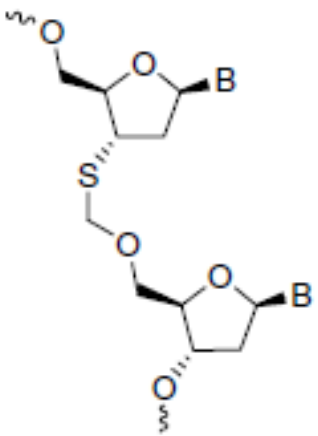




5′-(*S*)-methyl amide-3

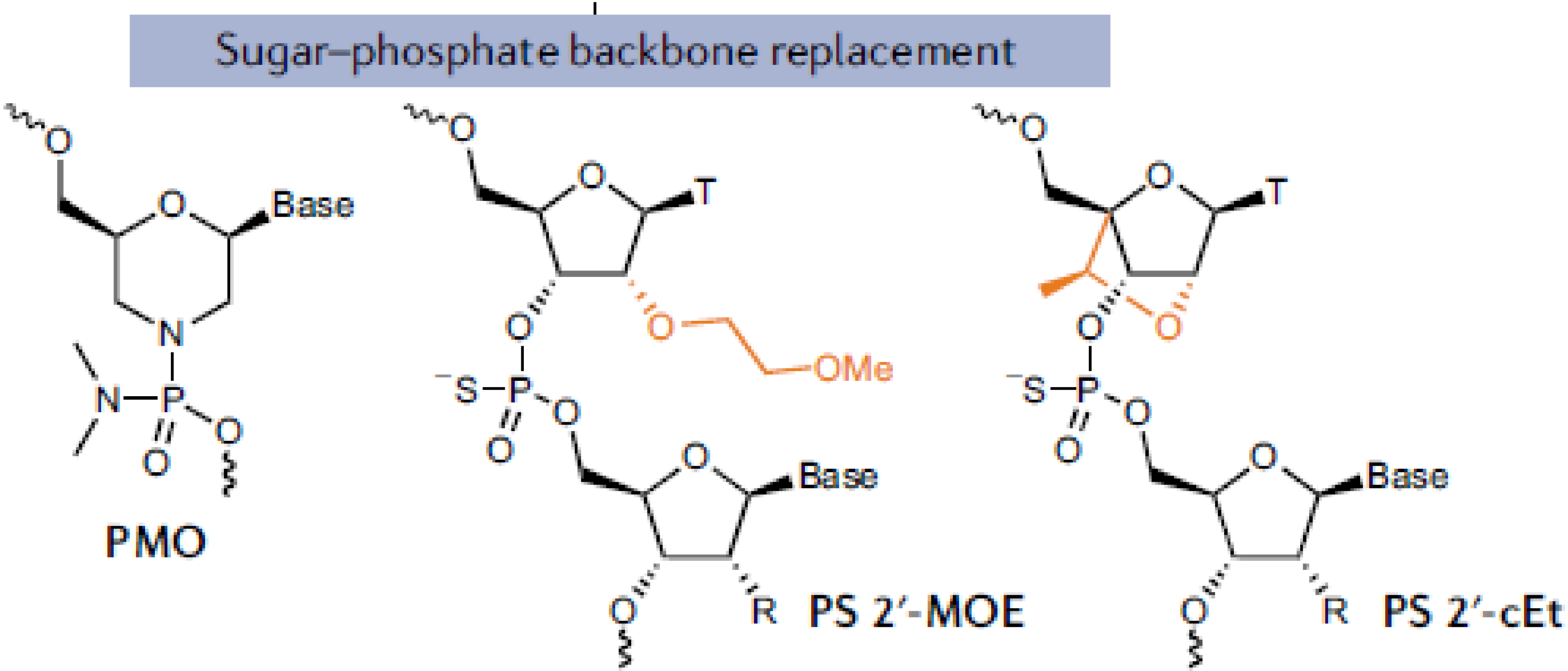


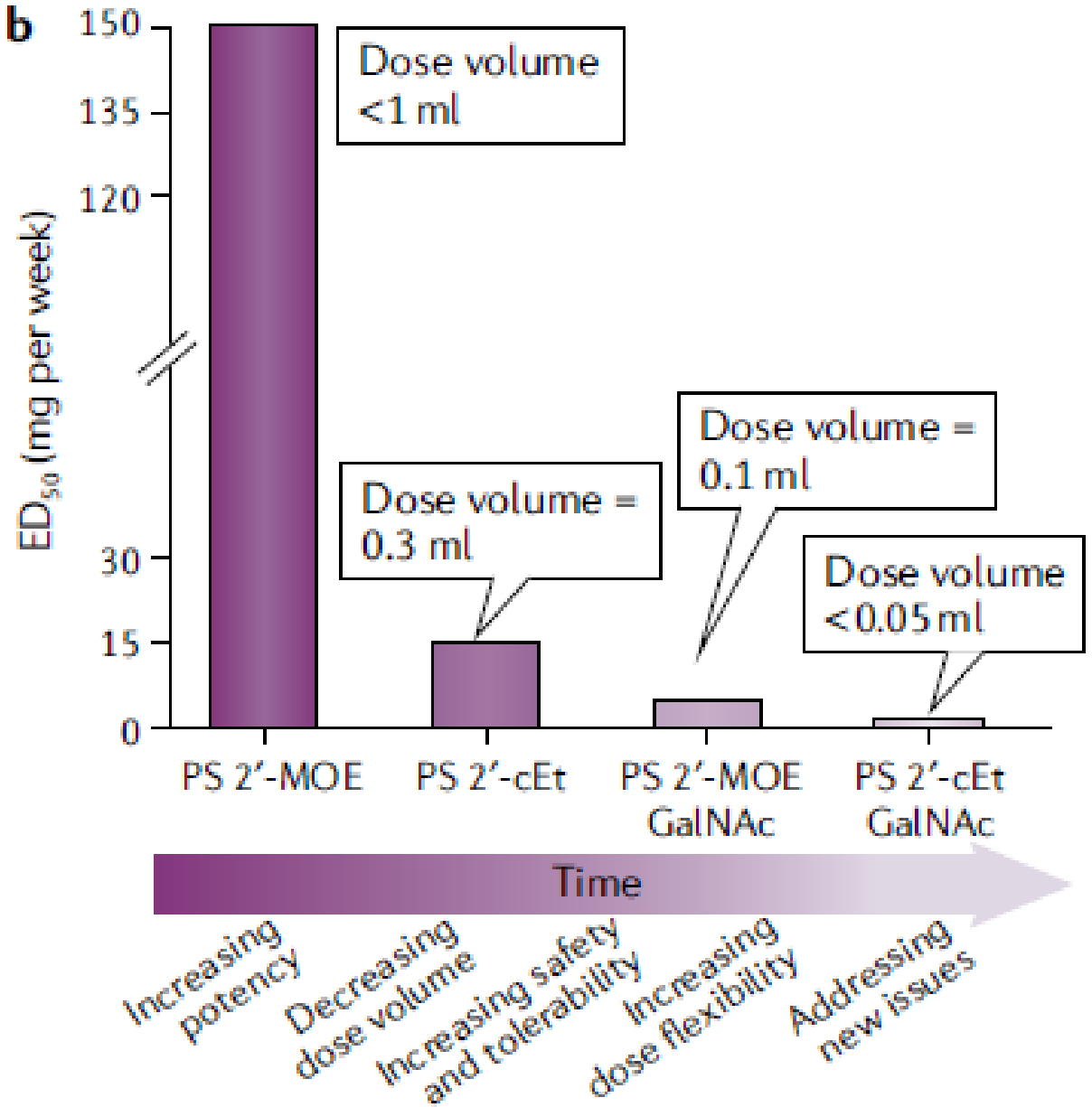
MMI



Thioformacetal

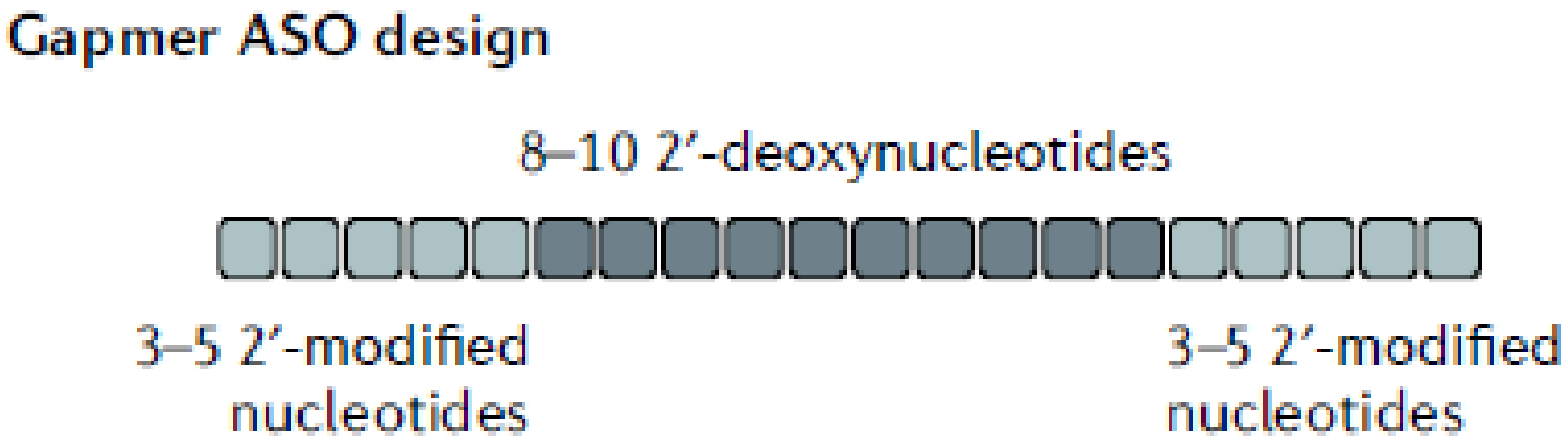
Modifications influencing drug performance



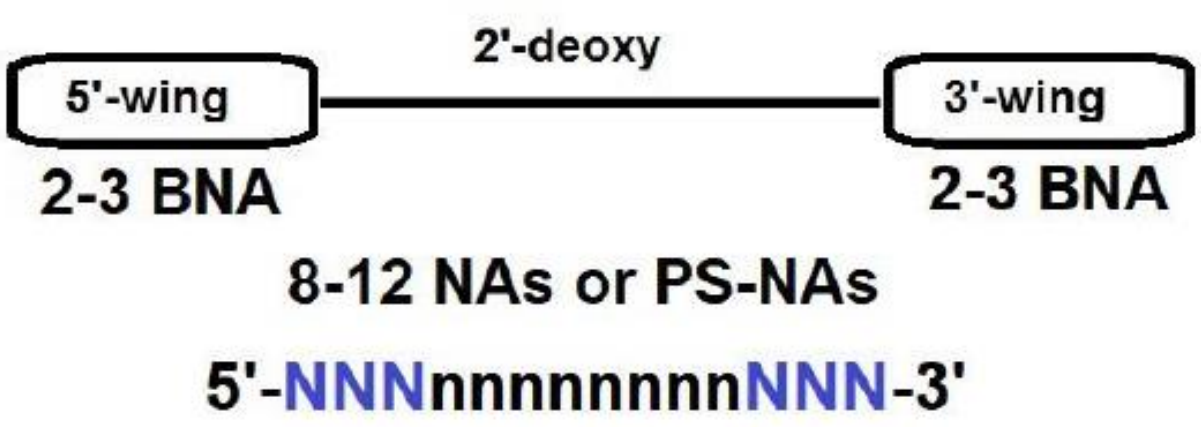


Improvements in the clinical performance of PS ASOs. The most accurate way to compare improvements in potency is to **compare the median effective dose** (ED50) of various chemical classes to reduce the level of targets in hepatocytes in controlled normal volunteer trials.

Gapmer ASO


Gapmer ASO design
8–10 2′-deoxynucleotides
3–5 2′-modified nucleotides
3–5 2′-modified nucleotides
Occupancy-only ASO design
15–30 2′-modified nucleotides

BNA Gapmer ASO Design



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).

Chirally pure phosphorothioate oligonucleotides

Phosphorothioate (**Ps**) antisense oligonucleotides (**ASOs**): the replacement of a non-bridging oxygen in a phosphate group with a sulfur creates a **chiral center at every Ps linkage** (referred to as sp and rp).

This could affect ASO conformation and performance.

Efforts to develop **stereochemical synthetic schemes for Ps ASOs** and to understand the effect of chirality on their performance.

Rp and Sp stereoisomers may have different sensitivity to nucleases.

The **Sp isomer is more nuclease resistant** than the Rp isomer.

A Sp chirally pure Ps ASO is more stable to nucleases than the corresponding Rp chirally pure ASO in biological systems.

Chiral purity or chiral purity at individual Ps units provides no meaningful improvement in the overall performance of Ps ASOs in cells and in mice, and clinical results so far appear to reflect this, as discussed in the main text.

Post-hybridization mechanisms of antisense oligonucleotides

Overview of available mechanisms from Ionis.

Occupancy-mediated degradation

Downregulation
1. RNase-H1-mediated degradation 2. AGO2-mediated degradation 3. Nonsense-mediated decay 4. No-go decay

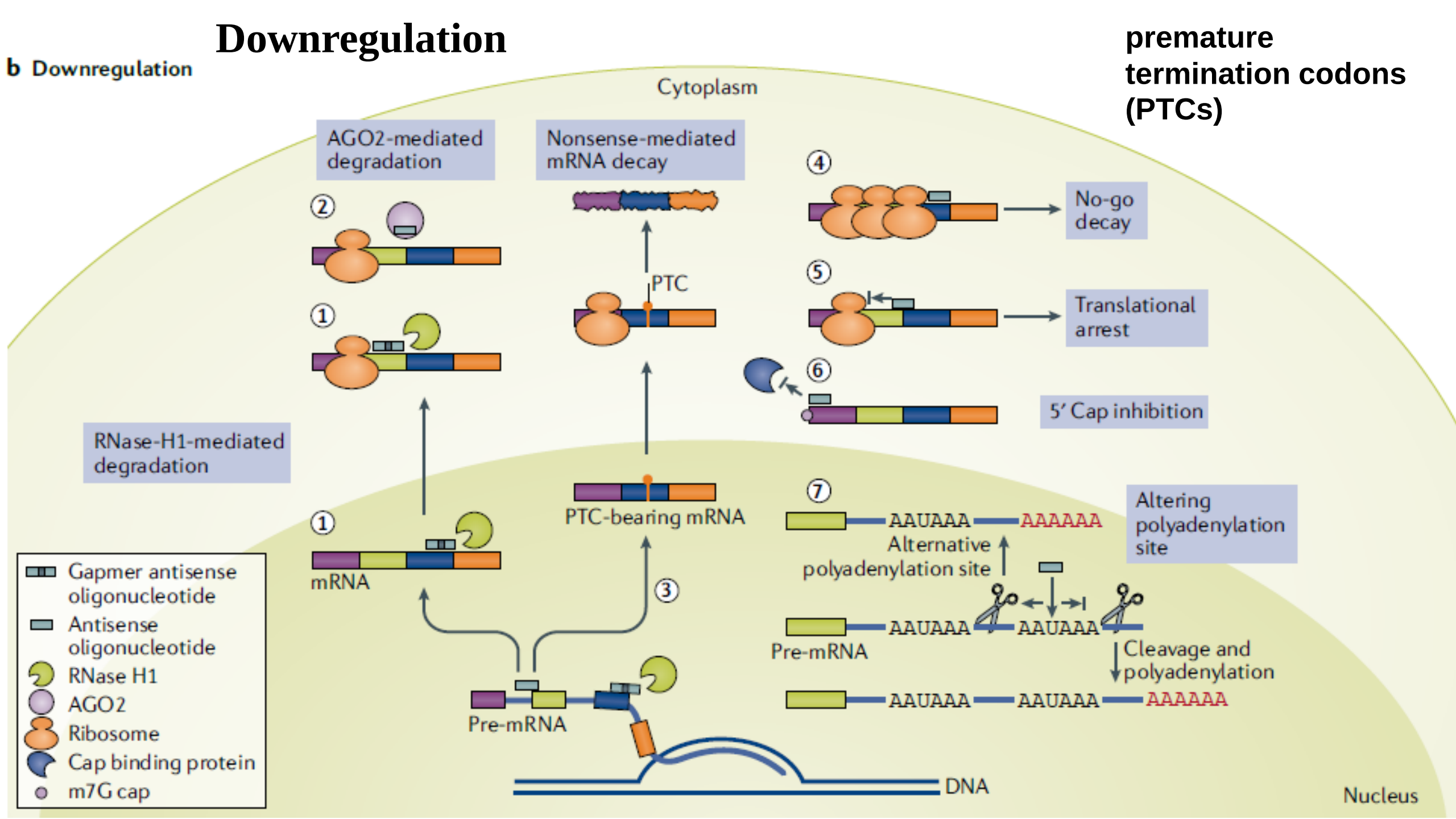
Occupancy only

Downregulation	Upregulation
5. Translational arrest 6. 5′ Cap inhibition 7. Altering polyadenylation site	1. Splicing modulation 2. Inhibition of miRNA function 3. Modulating uORF and TIE utilization 4. Inhibition of nonsense-mediated decay 5. RNA activation

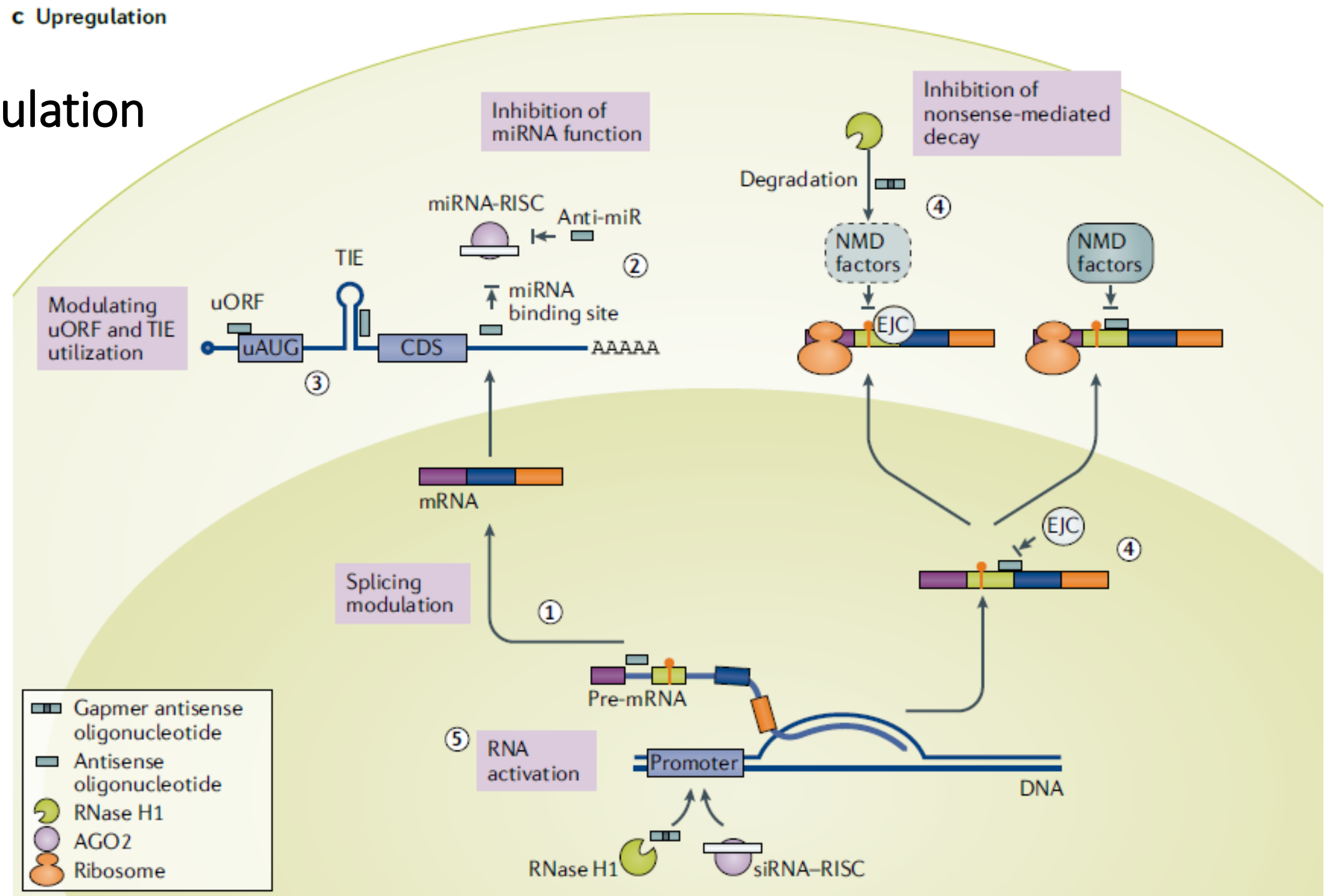
Translation inhibitory elements (**TIE**s)
Upstream open reading frames (**uORF**s)

Downregulation

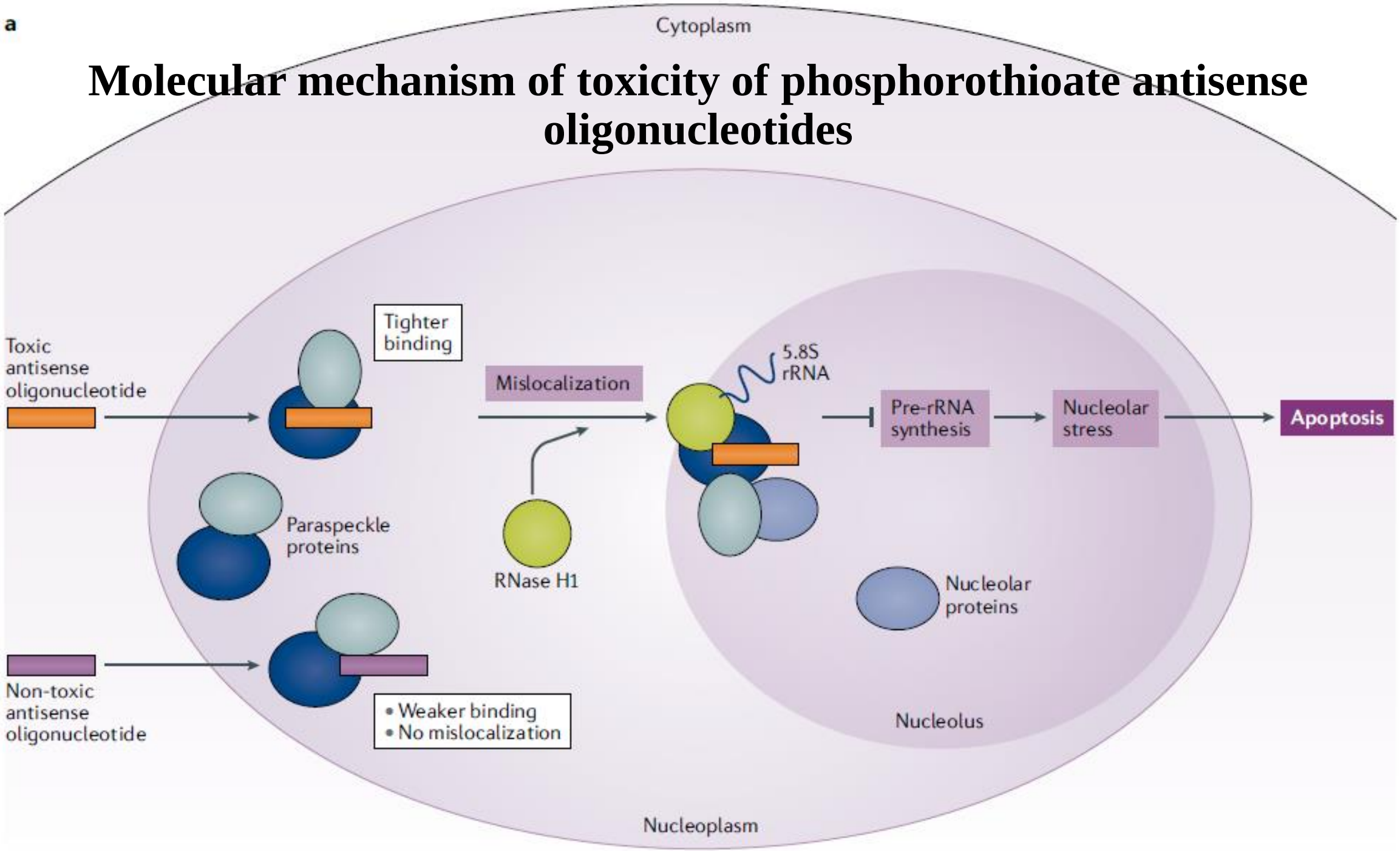
premature termination codons (PTCs)



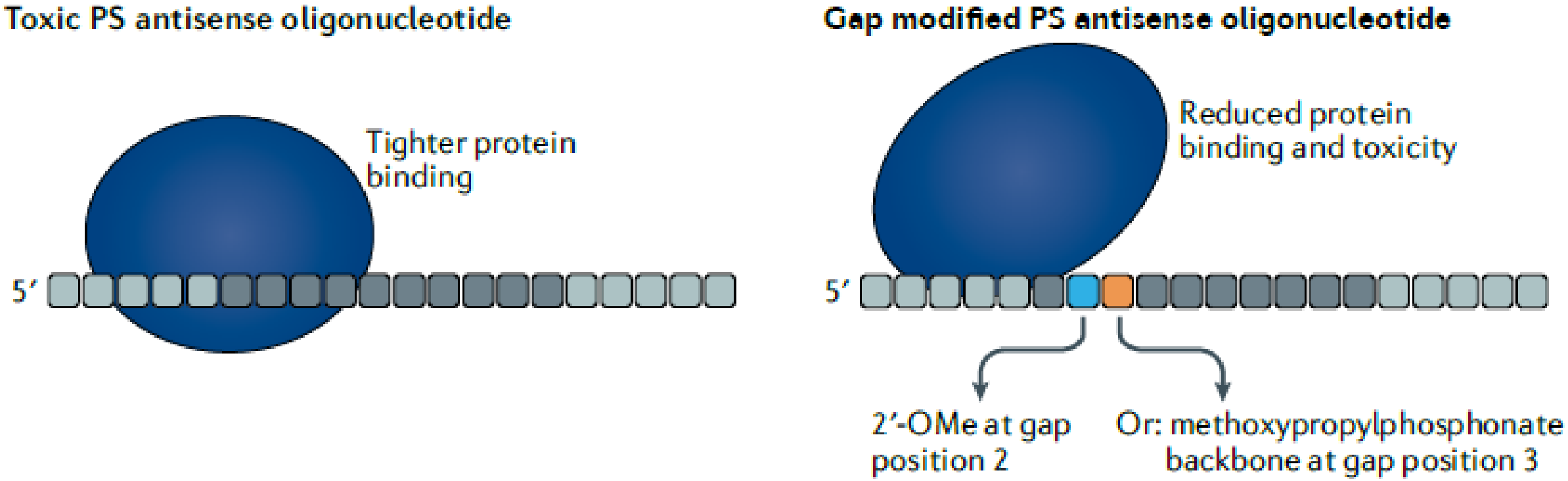
Upregulation



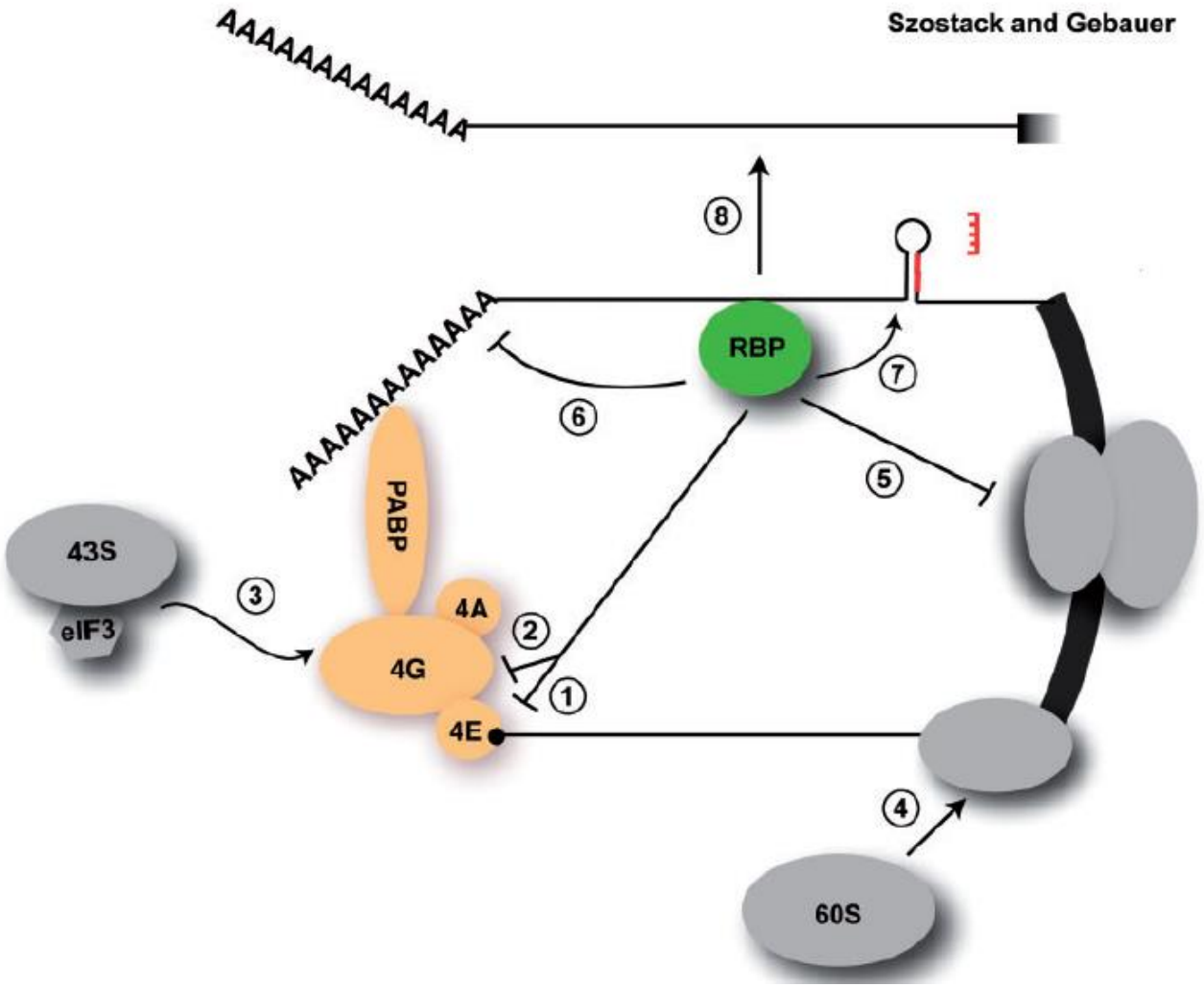
Molecular mechanism of toxicity of phosphorothioate antisense oligonucleotides



Molecular mechanism of toxicity of phosphorothioate antisense oligonucleotides
“Reducing Toxicity”



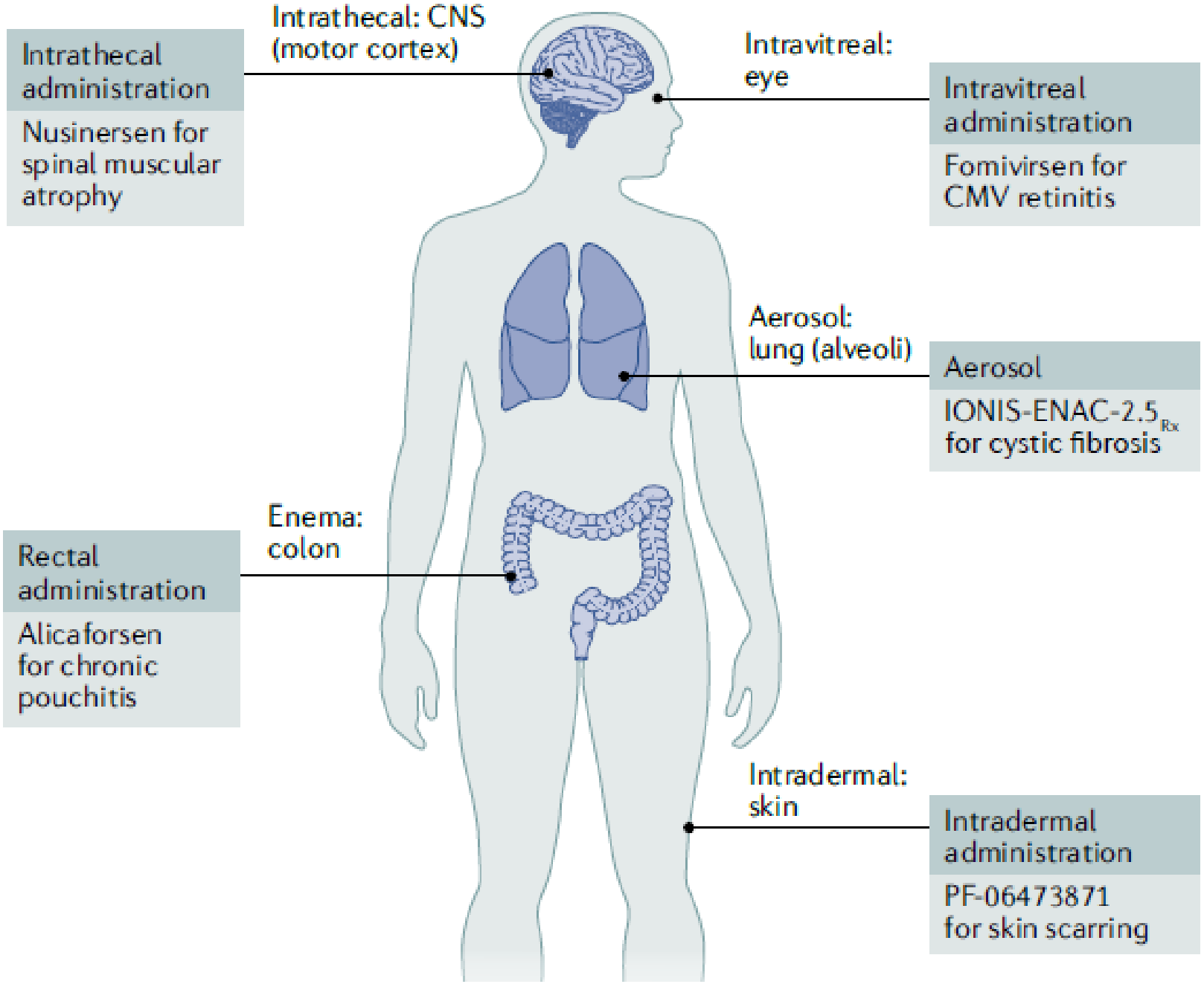
Mechanisms of translational regulation by 3-UTR-binding proteins.



Numbers indicate the different modes that RBPs use to modulate translation.
Large ovals depict the small (43S) and large (60S) ribosomal complexes. The initiation factors that participate in closed-loop formation are indicated.

A miRNA, as well as its binding site on the target mRNA, is also highlighted.

Szostack & Gebauer, 2012.



Local delivery of phosphorothioate antisense oligonucleotides.

Local routes of administration broadened the utility of phosphorothioate (PS) ASOs.

Fomiversen administered **intravitreally** to treat cytomegalovirus (CMV) retinitis.

Nusinersen administered **intrathecally** to the central nervous system (CNS) to treat spinal muscular atrophy.

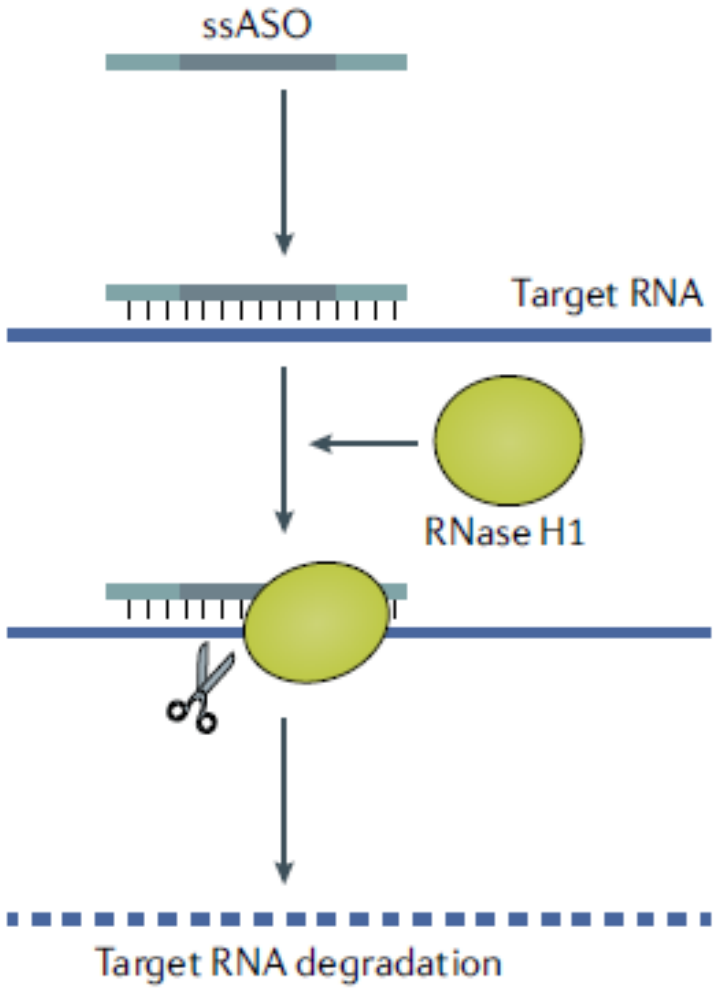
Alicaforsen administered by **enema** to treat chronic pouchitis.

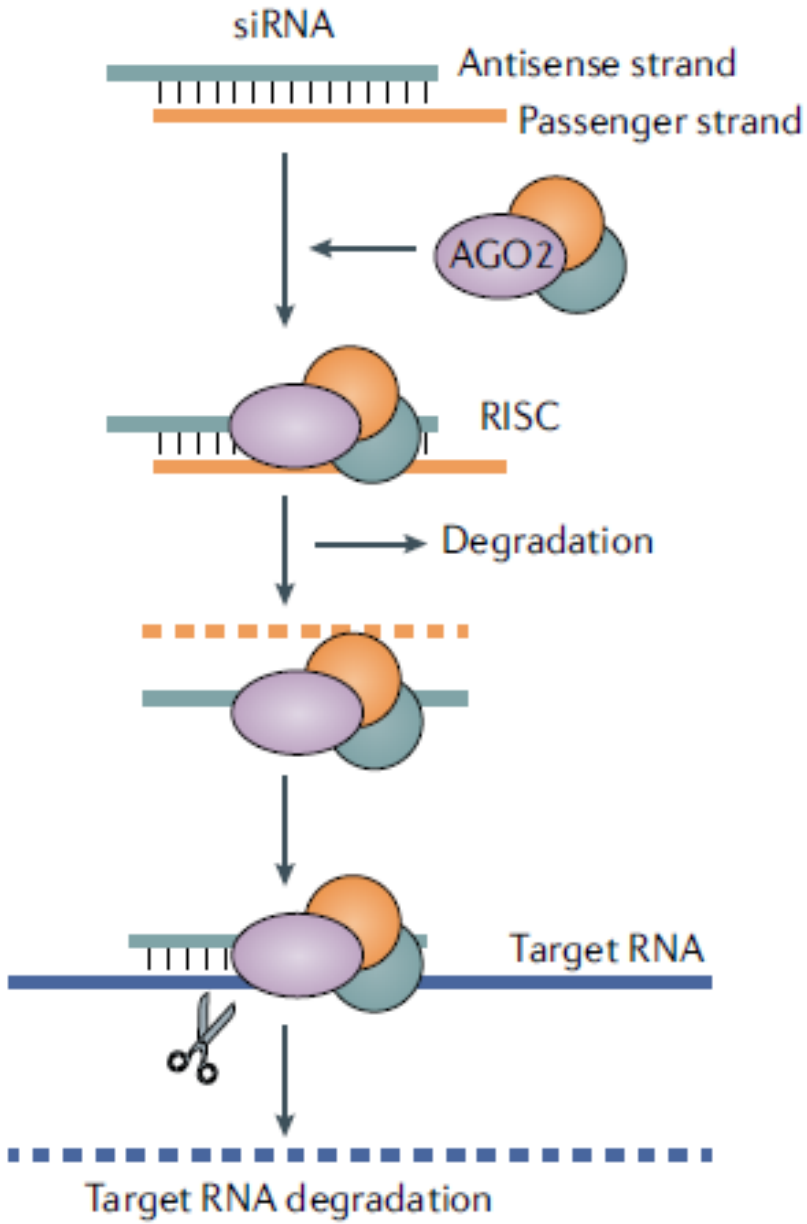
IONIS-ENaC-2.5Rx administered by **aerosol** to treat cystic fibrosis.

PF-06473871 administered **intradermally** to treat wound scarring.

Table 2 | **Validated routes of administration for phosphorothioate antisense oligonucleotides**

Route		Exemplary PS ASO	Indication	Chemistry	Trial/refs
Systemic	Intravenous	Alicaforsen	Crohn's disease	PS oligodeoxynucleotide	102
	Subcutaneous	Mipomersen	HoFH	PS 2′-MOE	122,123,184,186
		Pelacarsen	Apo(a)-related cardiovascular events	GalNAc PS-2′-MOE	89
Local	Intravitreal	Fomivirsen	CMV retinitis	PS oligodeoxynucleotide	181–183
		QR-1123/ ION357	Autosomal dominant retinitis pigmentosa	PS 2′-cEt, MOE	NCT04123626
	Intrathecal	Nusinersen	Spinal muscular atrophy	PS 2′-MOE	58,97–99
	Aerosol	IONIS-ENAC-2.5L$_{Rx}$	Cystic fibrosis, chronic bronchitis	PS 2′-cEt	NCT03647228
	Rectal	Alicaforsen	Chronic pouchitis	PS oligodeoxynucleotide	103





RNA degradation mediated by RNase H1 or
AGO2.

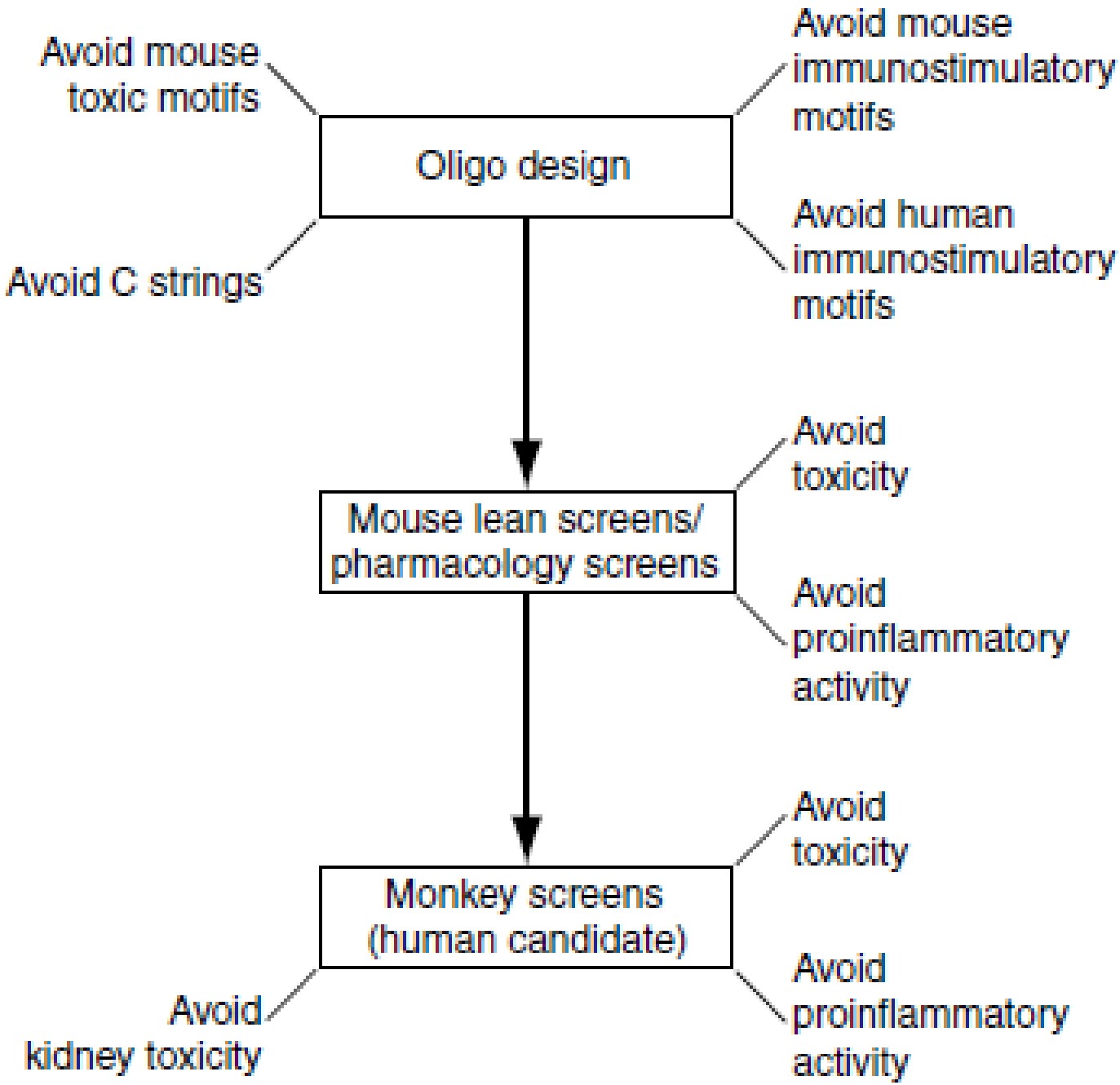
RNase H1 activation: a single-stranded antisense oligonucleotide (ssASO) containing deoxynucleotides (grey portion) hybridizes with the target RNA, generating
a DNA–RNA heteroduplex, which is recognized by RNase H1 to cleave the RNA strand in the heteroduplex.

siRNAs: the double-stranded RNA containing antisense and passage strands is bound by RNA-induced silencing complex (RISC) proteins including the endonuclease AGO2. The antisense strand is released by removal of the passenger strand, allowing the antisense strand that associates with AGO2 and potentially additional proteins to base-pair with the target RNA, leading to the **cleavage of the target RNA by**

Target selection for antisense oligonucleotide drugs

Drug discovery platform needed that takes advantage of the wealth of genomic data.

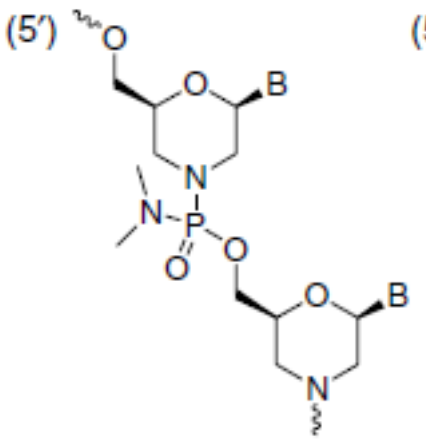
- **How does one identify and prioritize therapeutic targets?**
- There may be **no single method** used by those developing RNA-targeted drugs.
- Ionis has advanced many drugs into the clinic.
- Ionis begins with the **detailed knowledge of the pharmacodynamic and pharmacokinetic properties** of phosphorothioate (Ps) antisense oligonucleotides (ASOs) and leverages those data to prioritize targets.
- Detailed sub-organ pharmacokinetic studies that provide information on the levels of Ps ASOs in each major cell type and the concentrations and doses needed to achieve pharmacological effects are particularly important.
- This **process has been performed for the liver, kidney, central nervous system, pancreas and lung antisense.**



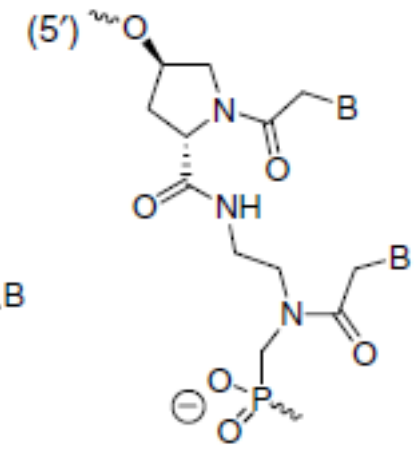
ASO Discovery Screening

- Results of an initial screen of 2-MOE chimeric antisense inhibitors designed to inhibit superoxide dismutase 1.
- Each inhibitor was transfected into A549 cells at 100 nM and the effects on SOD1 RNA levels evaluated 24 h after treatment of the cells.

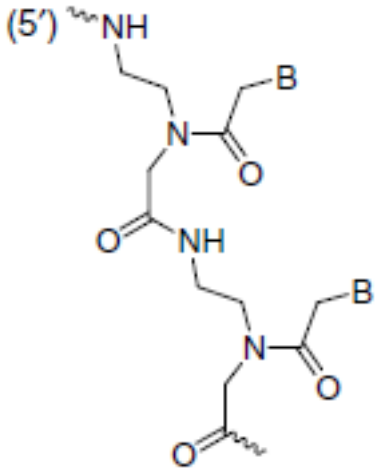
Modifications used in earlier studies to increase affinity



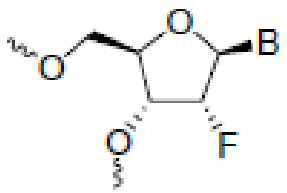
Morpholino



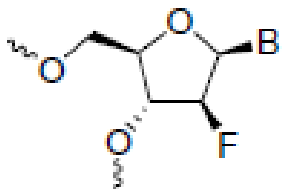
HypNA–pPNA



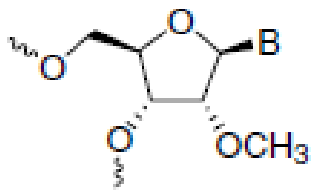
PNA
(peptide nucleic acid)



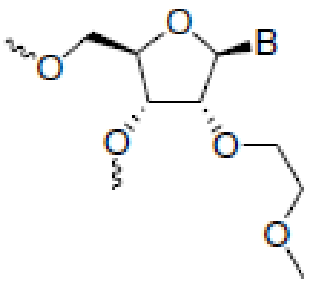
2′-Fluoro



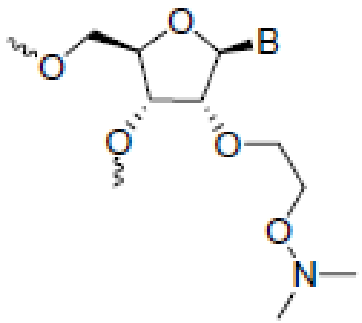
FANA



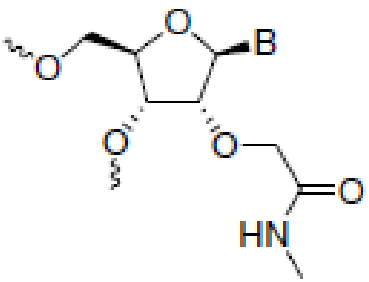
2′-*O*-methyl



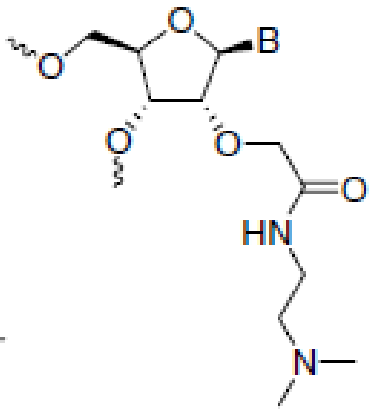
MOE
(2′-*O*-methoxyethyl)



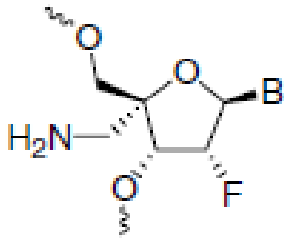
DMAOE



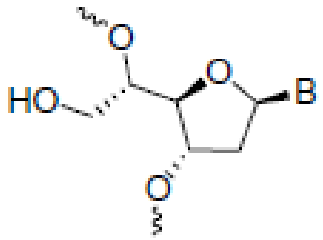
NMAc



DMAEAc

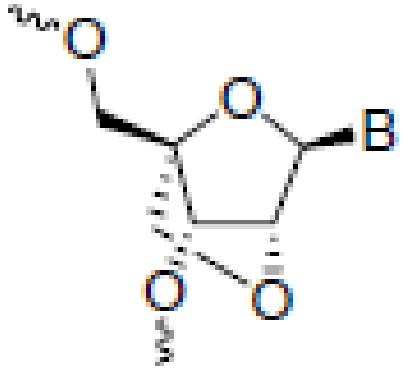


4′-*C*-aminomethyl
(with 2′-Fluoro)

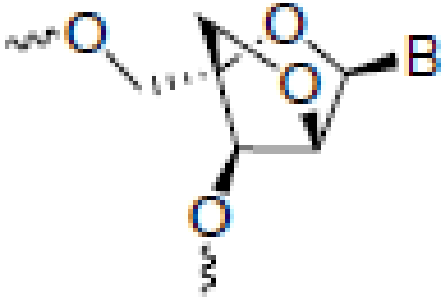


5′-(*S*)-hydroxymethyl

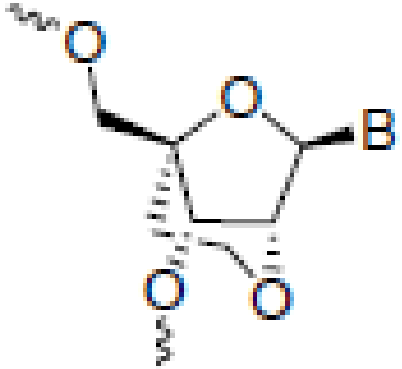
BNAs [Bicyclic Sugars]



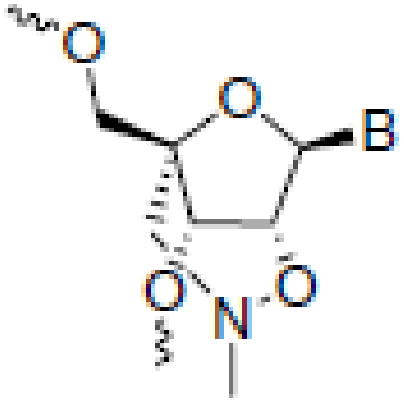
LNA (2′,4′-BNA)



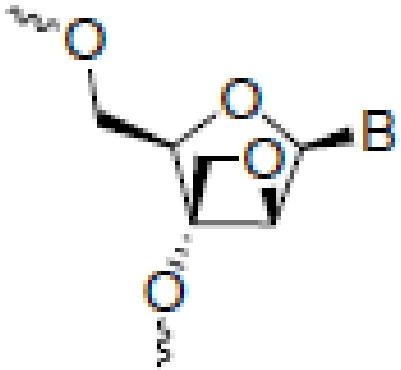
α-L- LNA



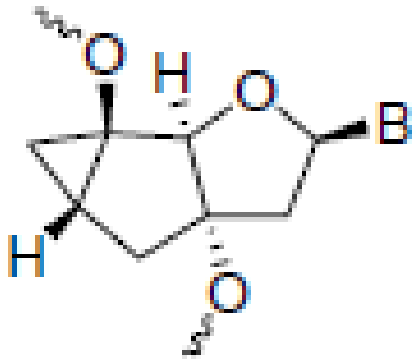
ENA



2′,4′-BNA^{NC}

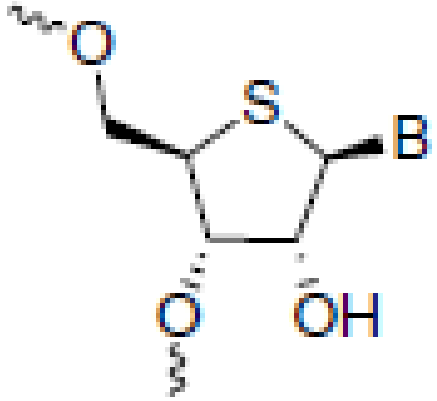


Oxetane 2′,3′-BNA

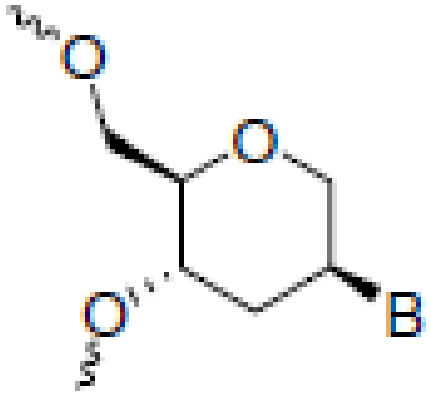


Tricyclo-DNA

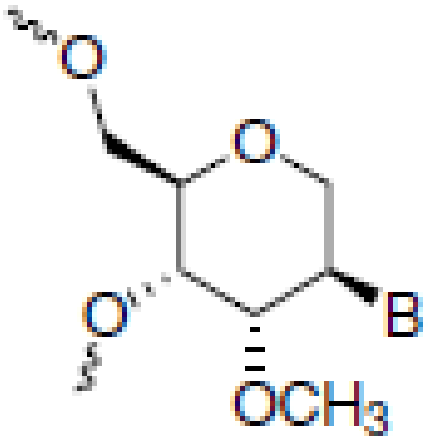
Sugar Substitutions



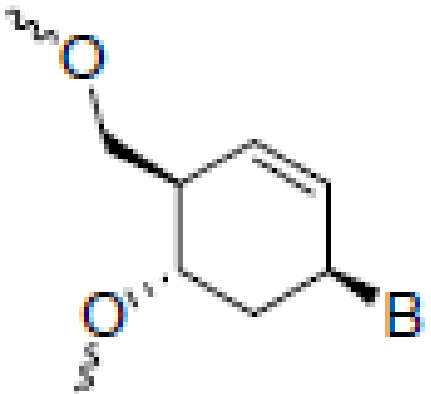
4′-Thioribose



HNA

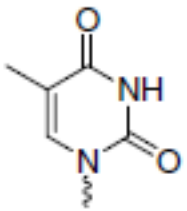


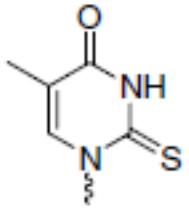
2′-*O*-methyl ANA

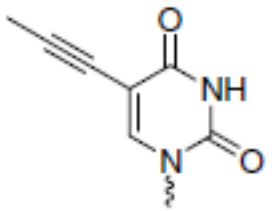


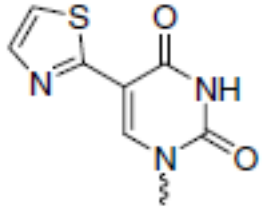
CeNA

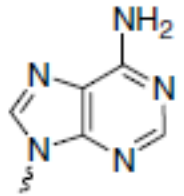
Modifications of DNA Bases

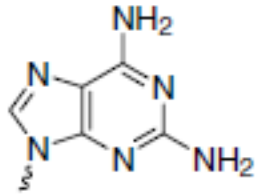

Thymine

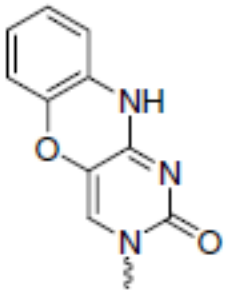

2-Thiothymine

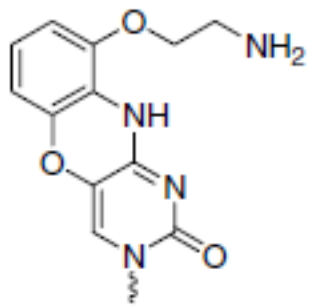

5-Propynyluracil

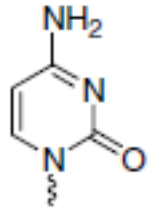

5-Thiazolyluracil

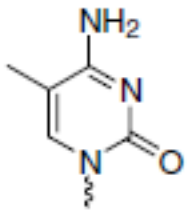

Adenine

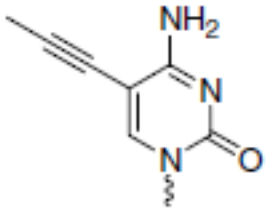

Diaminopurine

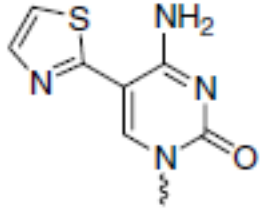

Phenoxazine

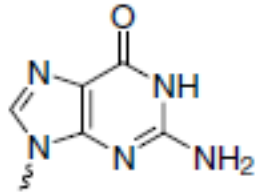

G-Clamp

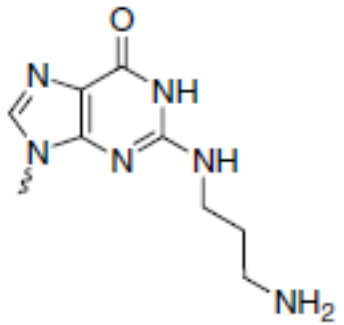

Cytosine


5-Methylcytosine

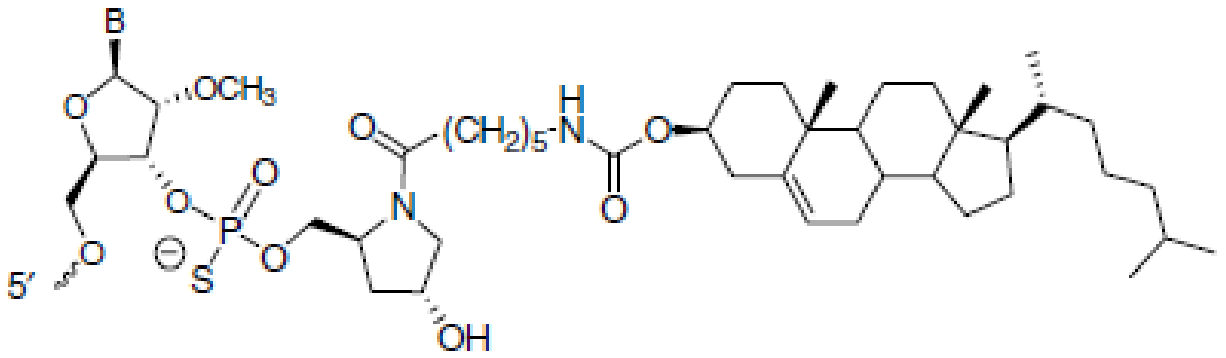

5-Propynylcytosine


5-Thiazolylcytosine

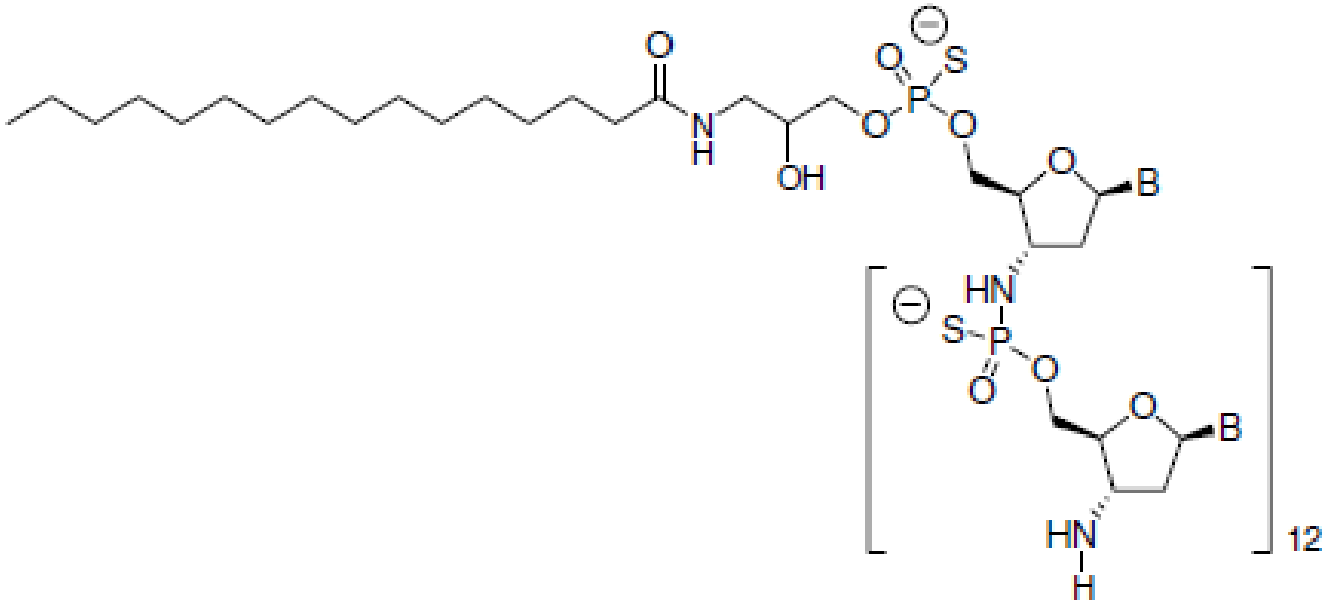

Guanine


N^2-Aminopropylguanine

Lipophilic Conjugates

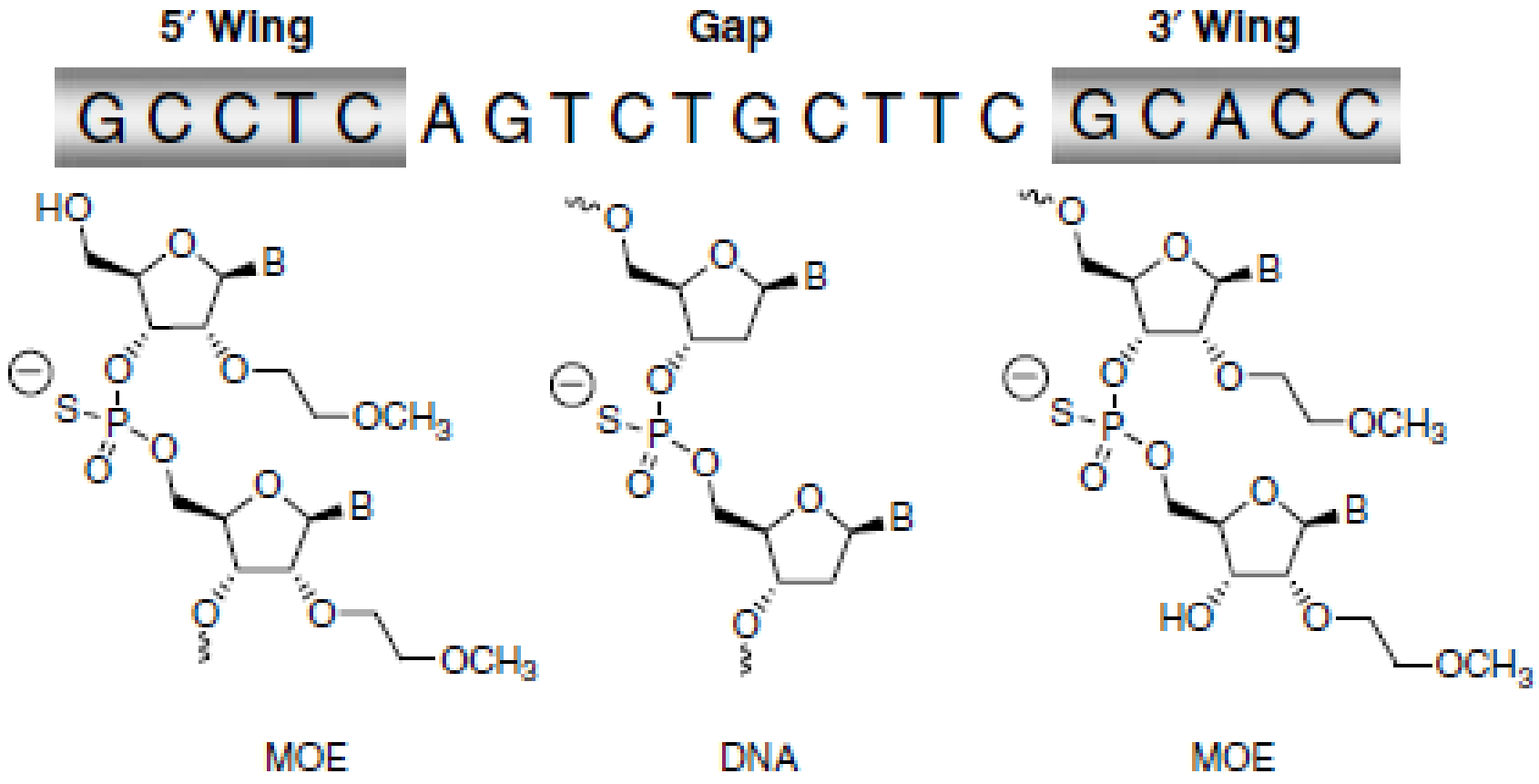


3′-Cholesterol-conjugated microRNA inhibitor



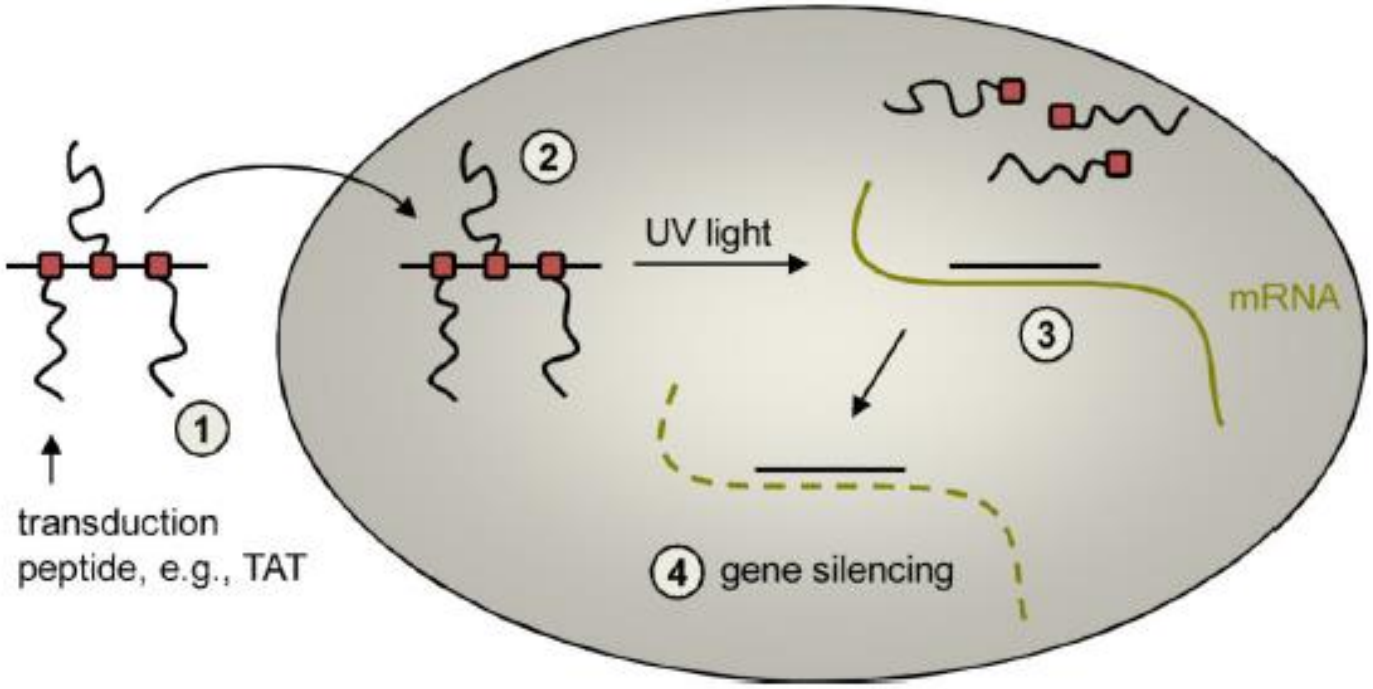
GRN163L

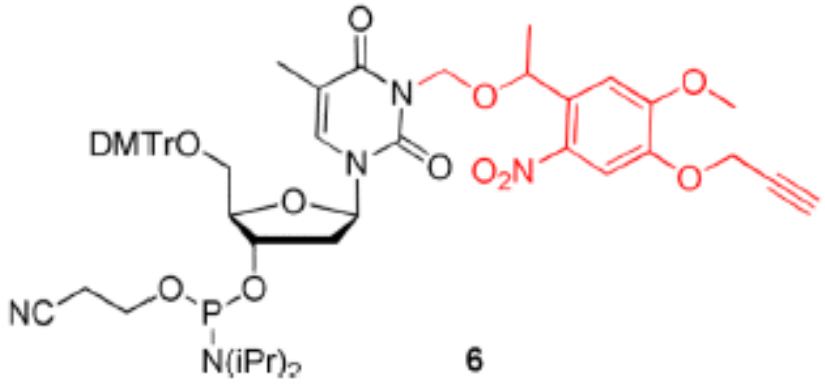
Gapmer Design



Gap design of ISIS-301012, a second-generation antisense oligonucleotide.

Light-activated ASOs





6

Govan et al. 2013

Propargyl-6-nitroveratryloxymethyl (PNVOM) **caging group** the nucleobase of a thymidine phosphoramidite.

- Delivery and **light-activation** of ASO containing caging groups **conjugated with cell-penetrating peptides.**
- PNVOM-caged thymidine nucleotides are site-specifically incorporated into antisense agents and are bioconjugated with azido-CPPs.

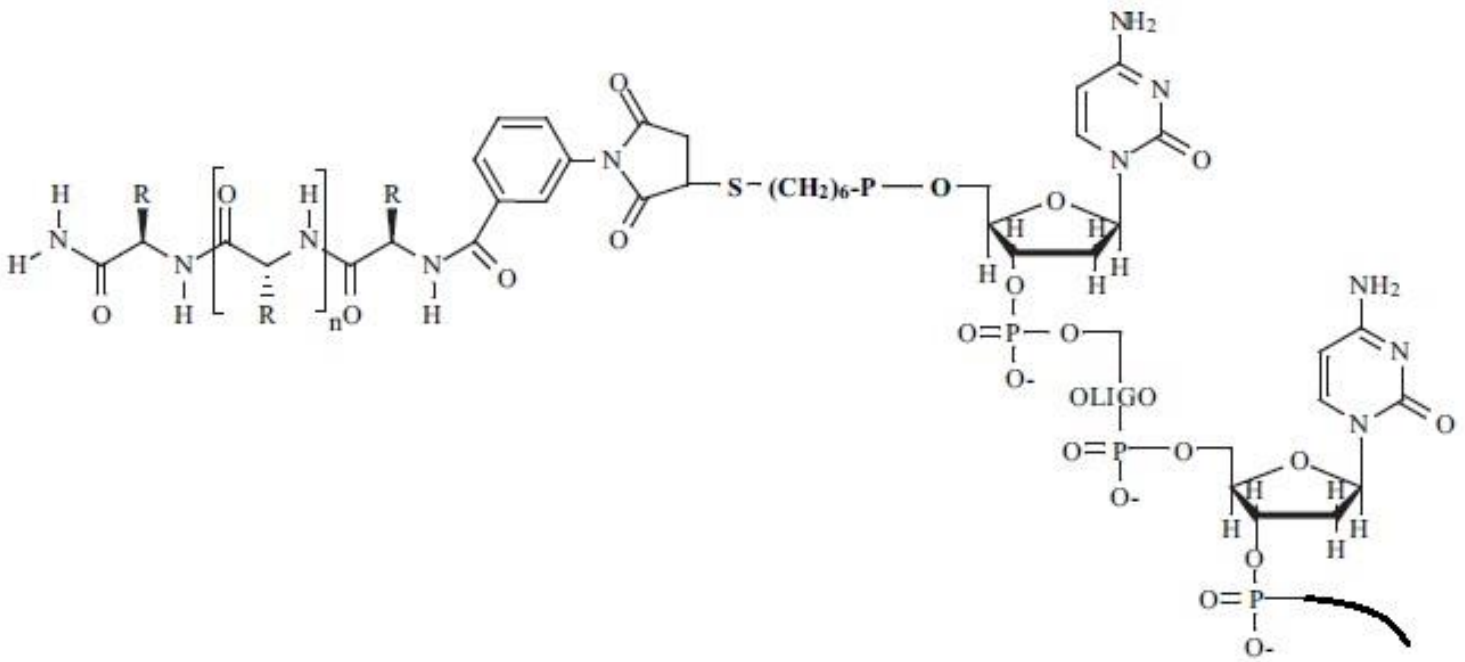
(1) CPP-caged ASO is added to the cell culture media.

(2) Taken up by mammalian cells but remain inactive due to the presence of the caging groups.

(3) UV irradiation triggers decaging, cleaving the CPPs from the antisense agent and inducing sequence-specific mRNA binding.

(4) Silencing of gene expression either by blocking the ribosome or by inducing RNase H-mediated mRNA degradation.

Peptide-Oligonucleotides as ASOs



CPP Examples for selective delivery and targeting:

Penetratin: RQIKIWFQNRRMKWKK
NLS: QAKKKKLDK

Many others!

Pierce et al. 2005

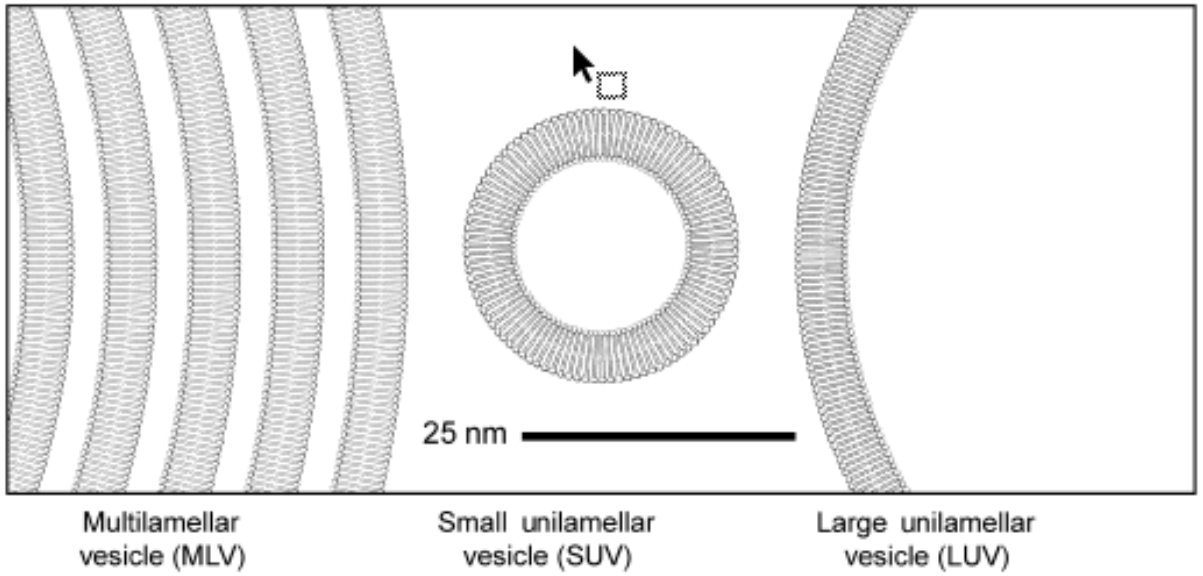
Conjugation of antisense oligonucleotides to peptide carriers

The greatest accumulation of oligonucleotides is found in the liver and kidney for mice and rats.

Modified oligonucleotides, 2’-O-(2-methoxyethyl) (2-O-MOE), also accumulate in liver and kidney.

The brain appears to be the greatest barrier for antisense penetration.

Liposomes for Delivery



Liposomes.

Mulilamellar vesicles (MLVs) are large (hundreds of nm in diameter) complex structures containing a series of concentric bilayers separated by narrow aqueous compartments.

Large unilamellar vesicles (LUVs) are between 50 and 500 nm in diameter, while the smallest liposomes namely small unilamellar vesicles (SUVs) are 50 nm. LUVs are the preferred systems for delivery of NA drugs. Lipids are drawn roughly to scale.

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AZD9150, a Next-Generation Antisense Oligonucleotide Inhibitor of *STAT3* with Early Evidence of Clinical Activity in Lymphoma and Lung Cancer

David Hong[1,*], **Razelle Kurzrock**[2,*,§], **Youngsoo Kim**[3,*], **Richard Woessner**[4], **Anas Younes**[5], **John Nemunaitis**[6], **Nathan Fowler**[1], **Tianyuan Zhou**[3], **Joanna Schmidt**[3], **Minji Jo**[3], **Samantha J. Lee**[3], **Mason Yamashita**[3], **Steven G. Hughes**[3], **Luis Fayad**[1], **Sarina Piha-Paul**[1], **Murali VP Nadella**[7], **Morvarid Mohseni**[4], **Deborah Lawson**[4], **Corinne Reimer**[4], **David C. Blakey**[8], **Xiaokun Xiao**[3], **Jeff Hsu**[3], **Alexey Revenko**[3], **Brett P. Monia**[3], and **A. Robert MacLeod**[3,§]

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[4]Cancer Bioscience Drug Discovery AstraZeneca Pharmaceuticals, 35 Gatehouse Drive, Waltham, MA 02451, USA

[5]Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065, USA

Signal Transducer And Activator Of Transcription 3

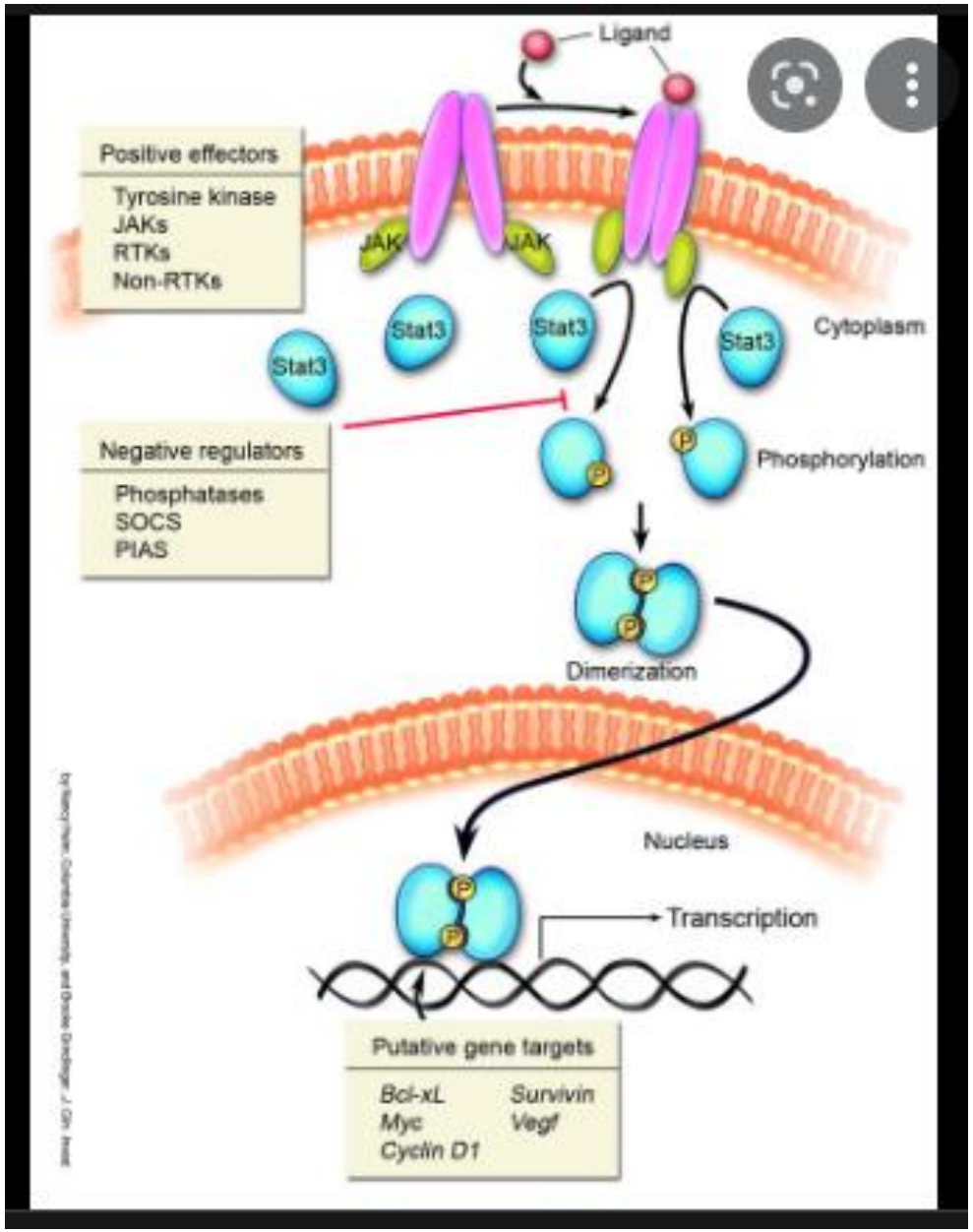
A **member of the STAT protein family**. In response to cytokines and growth factors, STAT family members are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they **act as transcription activators.**

Diseases associated with STAT3 include Hyper-Ige Recurrent Infection Syndrome 1, Autosomal Dominant and Autoimmune Disease, Multisystem, Infantile-Onset, 1. Among its related pathways are Cytokine Signaling in Immune system and IL-4 Signaling Pathways.

STAT3 (signal transducer and activator of transcription 3)

- a member of STAT family (8 members)
- Monomer stays in the cytoplasm prior to activation
- Activated by phosphorylating Tyr by various Tyr kinase receptors (EGFR, VEGFR, PDGFR) upon binding their ligand or cytokine receptor (ex. IL-6R) associating w/ Tyr kinase JAK
  → forms dimer
  → enters nucleus
  → transactivates target genes (cell proliferation, tumor invasion, survival)

-- **Persistently activated in human cancers**:
i.e. breast cancer, lung cancer, pancreatic cancer, head and neck cancer, prostate cancer, ovarian cancer, melanoma, leukemias, and lymphomas



IONIS

About | Ionis Innovation | Our Medicines | Patients & Community | Investors & media | Careers

What is Ligand-Conjugated Antisense (LICA) Technology?

LICA, or Ligand Conjugated Antisense, is a chemical technology we developed at Ionis that involves the attachment of a molecule called a ligand that binds with receptors on the surfaces of cells in a highly specific manner.

Because these receptors are often found only on certain cell types, LICA allows us to effectively deliver our antisense drugs with specificity to certain cell types that express these receptors.

LICA is leading to an emerging class of antisense medicines that can treat conditions, with both large and small patient populations, with less frequent and smaller doses.

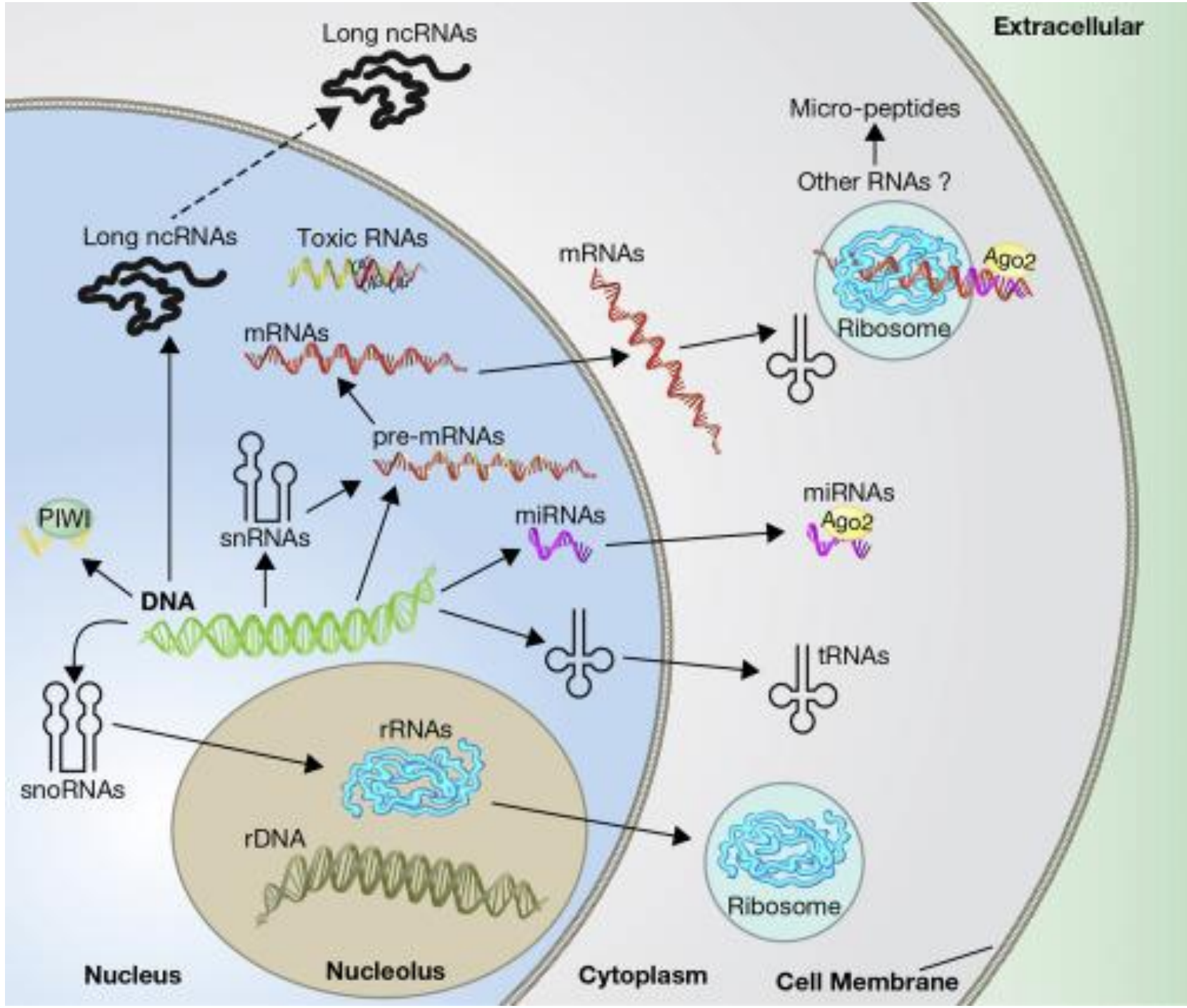
Generation 2 FDA approved hypercholesterolemia (**2′-O-methoxyethyl containing ASO**)

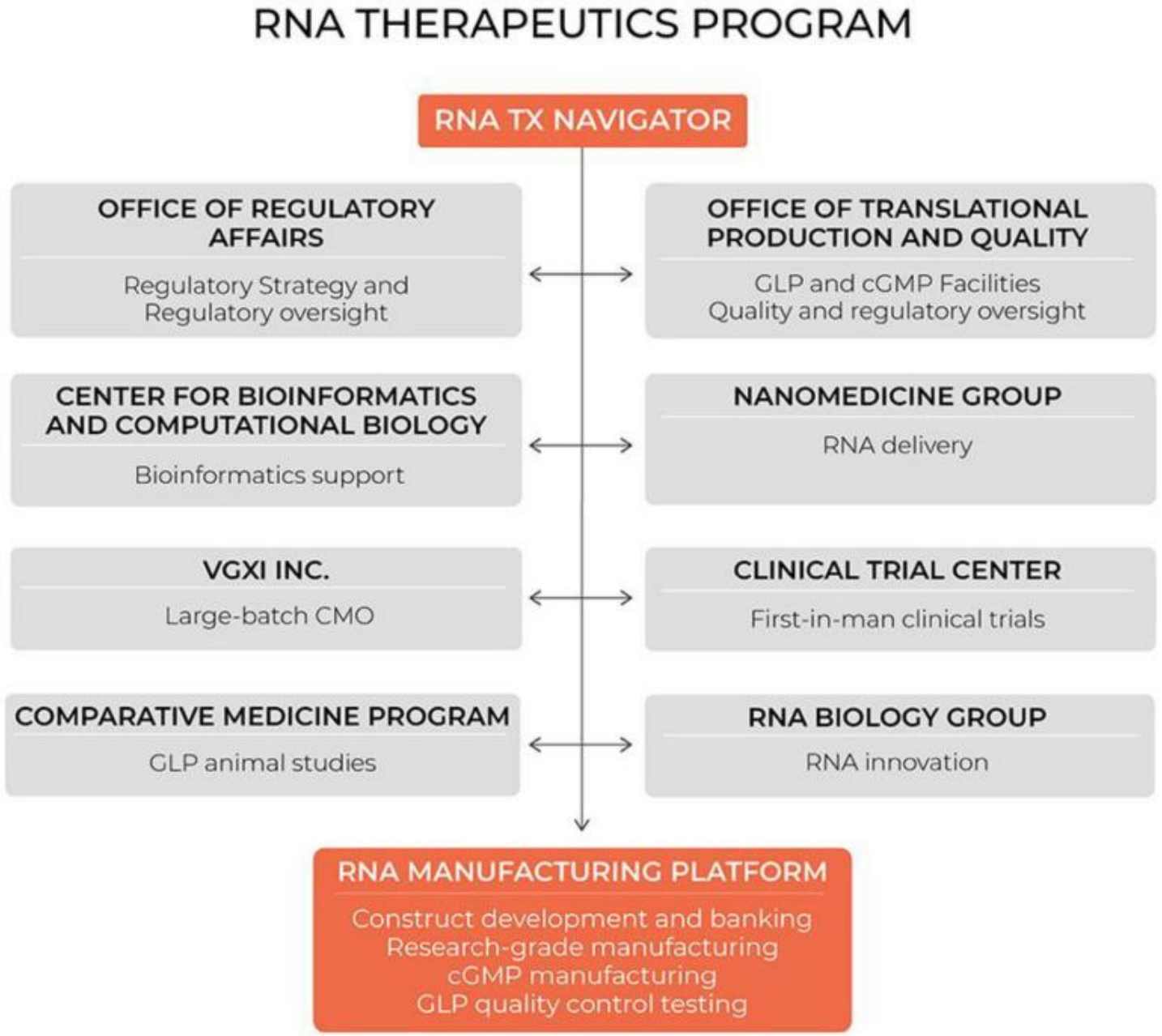
Generation 2.5 **Gapmer**; 2′-4′ constrained ethyl (cEt)-modified. AZD9150. Phase 1, II trial. Cancer (Stat3 target)

RNA-targeting Therapies Summary

- A platform for drug discovery involving chemically modified oligonucleotides.
- A wide range of RNA targets are present in the cell.
- Novel target-binding motifs governed by Watson-Crick base pairing are discovered almost daily.
- Presently, several RNA-targeted therapies are approved.

- **1978 to present!**


Extracellular
Long ncRNAs
Micro-peptides
Other RNAs ?
Long ncRNAs
Toxic RNAs
mRNAs
Ago2
Ribosome
mRNAs
pre-mRNAs
PIWI
snRNAs
miRNAs
miRNAs
Ago2
DNA
tRNAs
snoRNAs
rRNAs
rDNA
Ribosome
Nucleus
Nucleolus
Cytoplasm
Cell Membrane



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8012680/

Damase TR, Sukhovershin R, Boada C, Taraballi F, Pettigrew RI, Cooke JP. **The Limitless Future of RNA Therapeutics.** Front Bioeng Biotechnol. 2021 Mar 18;9:628137. doi: 10.3389/fbioe.2021.628137. PMID: 33816449; PMCID: PMC8012680.

[1978] A tridecamer oligodeoxynucleotide, d(A-A-T-G-G-T-A-A-A-A-T-G-G), which is complementary to reiterated 3'- and 5'-terminal nucleotides of Rous sarcoma virus 35S RNA, is an efficient inhibitor of the translation of proteins specified by the viral RNA in the wheat embryo cell-free system.

The notion of creating "antisense" oligonucleotide-based drugs was first articulated in 1978.

The mechanism that determine whether an oligonucleotide binds to its cognate RNA sequence, Watson-Crick hybridization, is well understood.

Such drugs should, in principle, be much more specific than traditional small-molecule drugs.

Stephenson ML, Zamecnik PC. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. Proc Natl Acad Sci U S A. **1978** Jan;75(1):285-8. doi: 10.1073/pnas.75.1.285. PMID: 75546; PMCID: PMC411231.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC411231/

[2008] Crooke S.T.; **Antisense Drug Technology: Principles, Strategies, and Applications.** CRC Press, 2008

The antisense concept derives from an understanding of nucleic acid structure and function and depends on Watson-Crick hybridization.

The demonstration that nucleic acid hybridization is feasible and the advances in situ hybridization and diagnostic probe technology lay the most basic elements of the foundation supporting the **antisense concept**.

RNA targeting multiplied as new classes of RNAs and new roles for RNAs were discovered.

Cech T.R., Steitz J.A.; The noncoding RNA revolution-trashing old rules to forge new ones. *Cell.* **2014**; **157**: 77-94.

Noncoding RNAs (ncRNAs) accomplish a remarkable variety of biological functions.

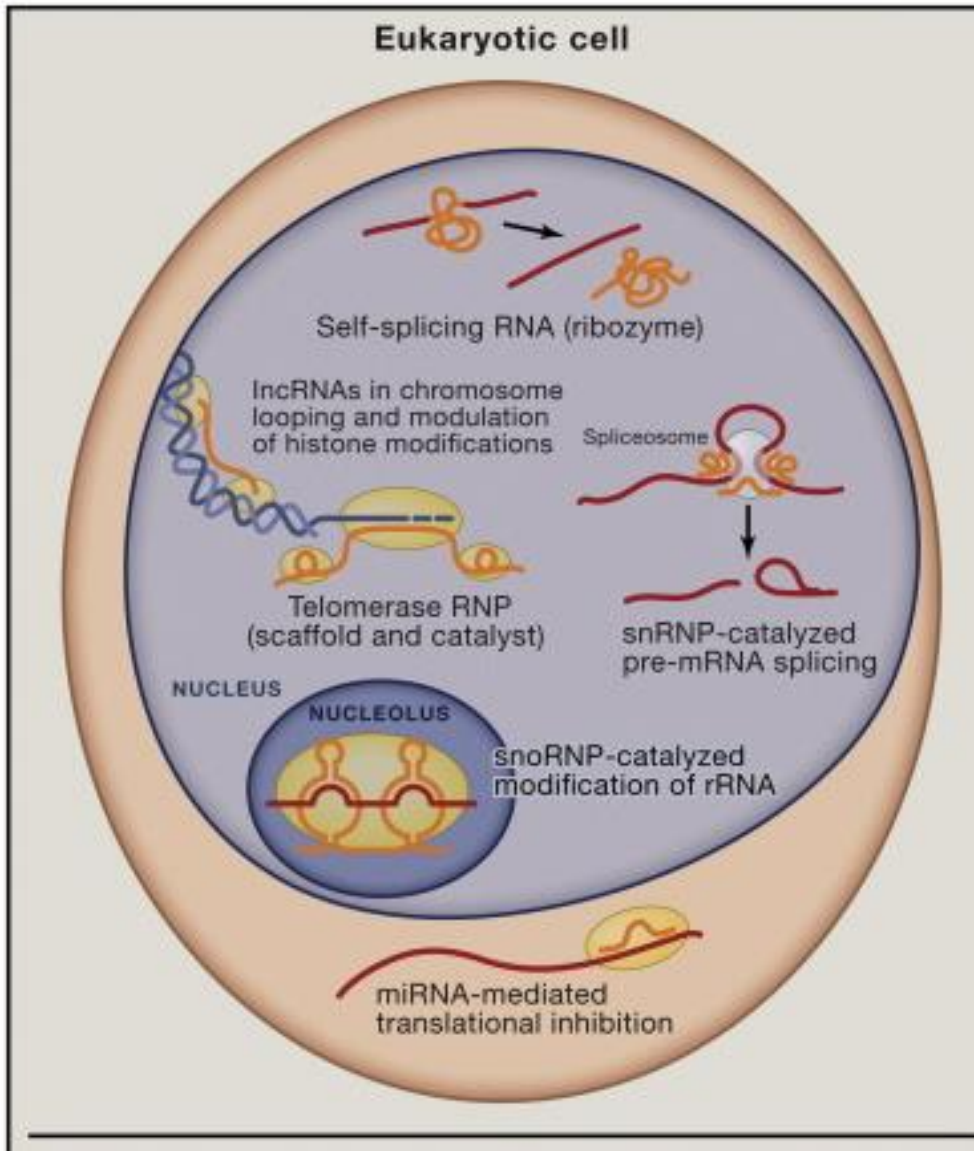
They regulate gene expression at the levels of transcription, RNA processing, and translation.

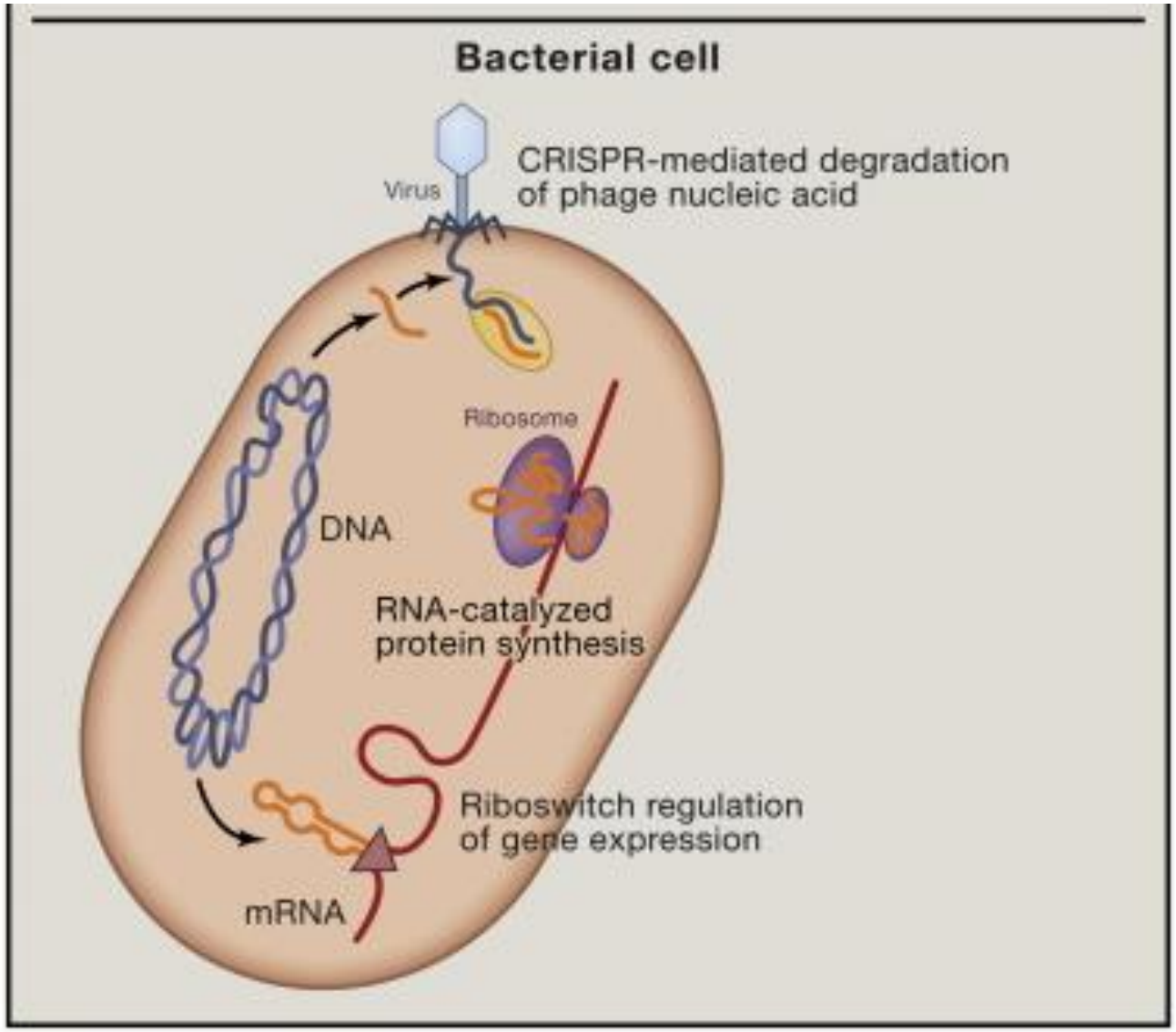
They protect genomes from foreign nucleic acids. They can guide DNA synthesis or genome rearrangement.

For ribozymes and riboswitches, the RNA structure itself provides the biological function, but most ncRNAs operate as RNA-protein complexes, including ribosomes, snRNPs, snoRNPs, telomerase, microRNAs, and long ncRNAs.

Many, though not all, ncRNAs exploit the power of base pairing to selectively bind and act on other nucleic acids.

Noncoding RNAs Function in Diverse Contexts





ncRNA functions

The **human proteome contains hundreds of different RNA-binding proteins**, each binding a few RNA nucleotides, so even a 200 nt ncRNA is likely to engage multiple RNA-binding proteins.

In some cases, the proteins will bind cooperatively, in other cases they will compete for overlapping binding sites.

Biochemistry is good at examining one-protein-one-RNA interactions and can even be stretched to examine several proteins at a time.

Biochemistry cannot deal with 1,000 purified proteins. Transcriptome-wide approaches such as CLIP-seq, on the other hand, always interrogate interactions within the full complexity of the cell.

The challenge here is that these technologies reveal what is happening, but not how and why.

New experimental and computational approaches are needed to understand RNPs as dynamic systems. And who knows what rules will be overturned then?

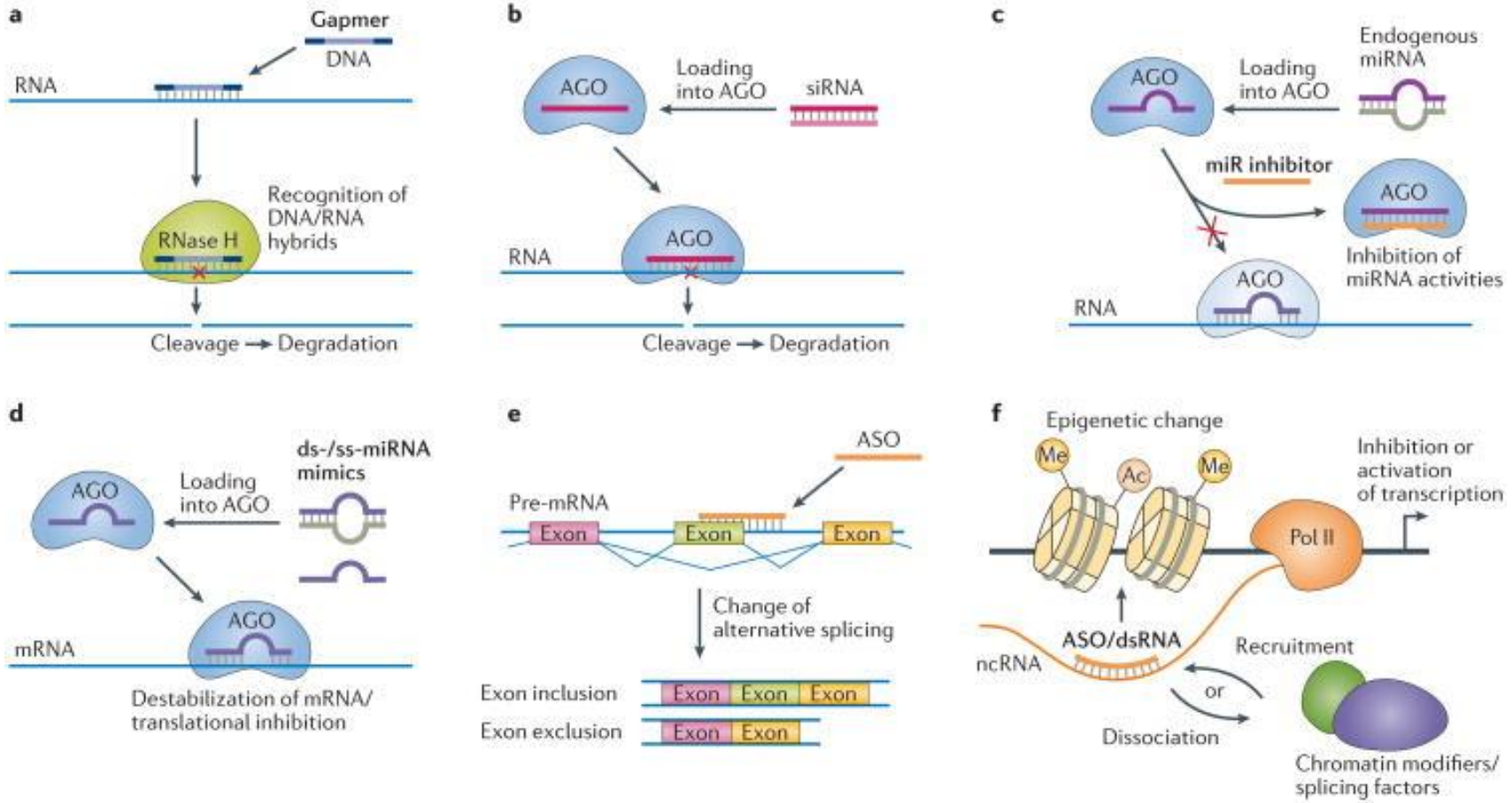
RNA targeting multiplied as new classes of RNAs and new roles for RNAs were discovered.

Matsui M., Corey D.R.; Non-coding RNAs as drug targets.*Nat. Rev. Drug Discov.* **2017**; **16**: 167-179.

Most of the human genome encodes RNAs that do not code for proteins.

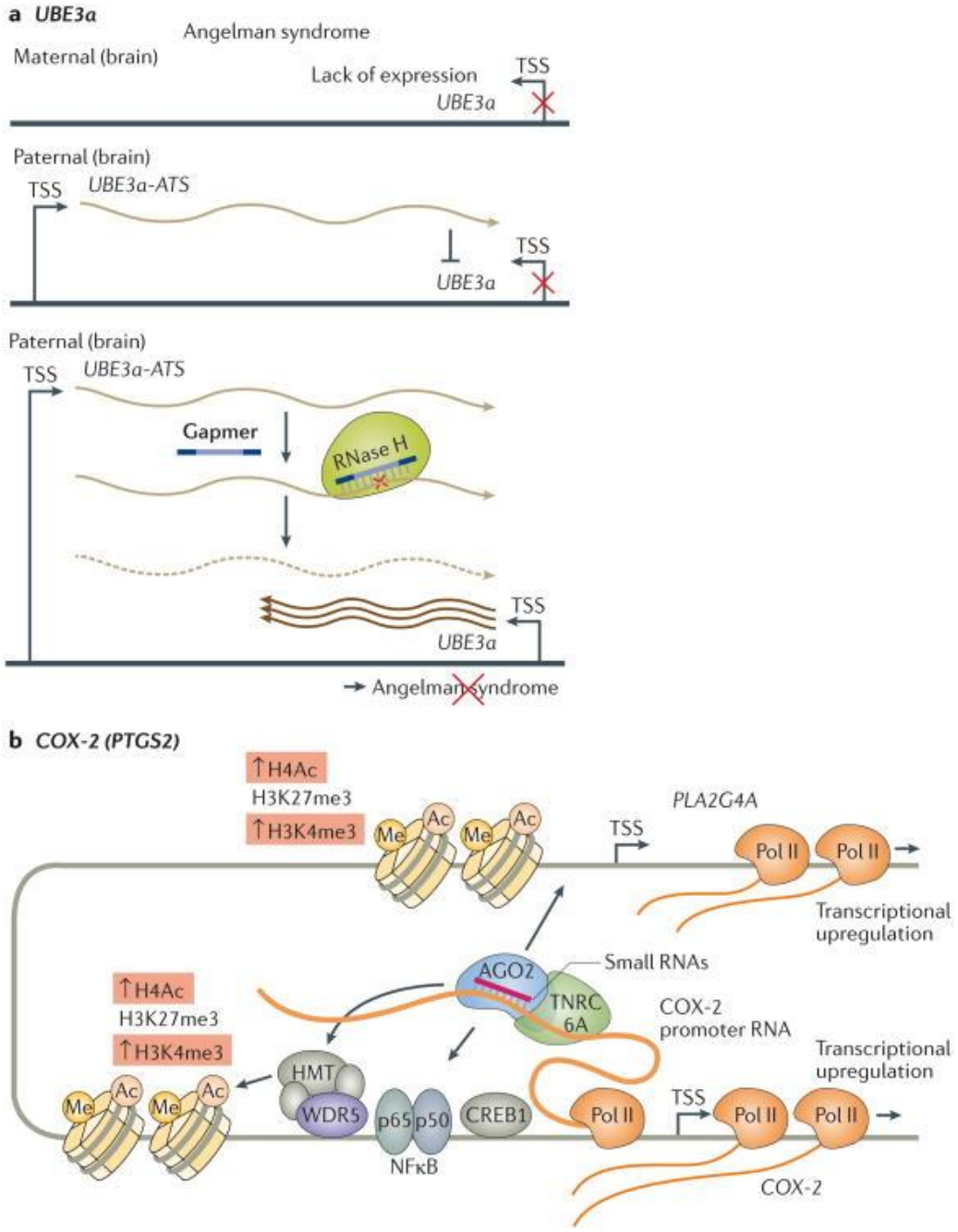
These non-coding RNAs (ncRNAs) may affect normal gene expression and disease progression, making them a new class of targets for drug discovery. Because their mechanisms of action are often novel, developing drugs to target ncRNAs will involve equally novel challenges.

However, many potential problems may already have been solved during the development of technologies to target mRNA. Here, we discuss the growing field of ncRNA - including microRNA, intronic RNA, repetitive RNA and long non-coding RNA - and assess the potential and challenges in their therapeutic exploitation.



Regulating RNA levels or splicing with ASOs and duplex RNAs

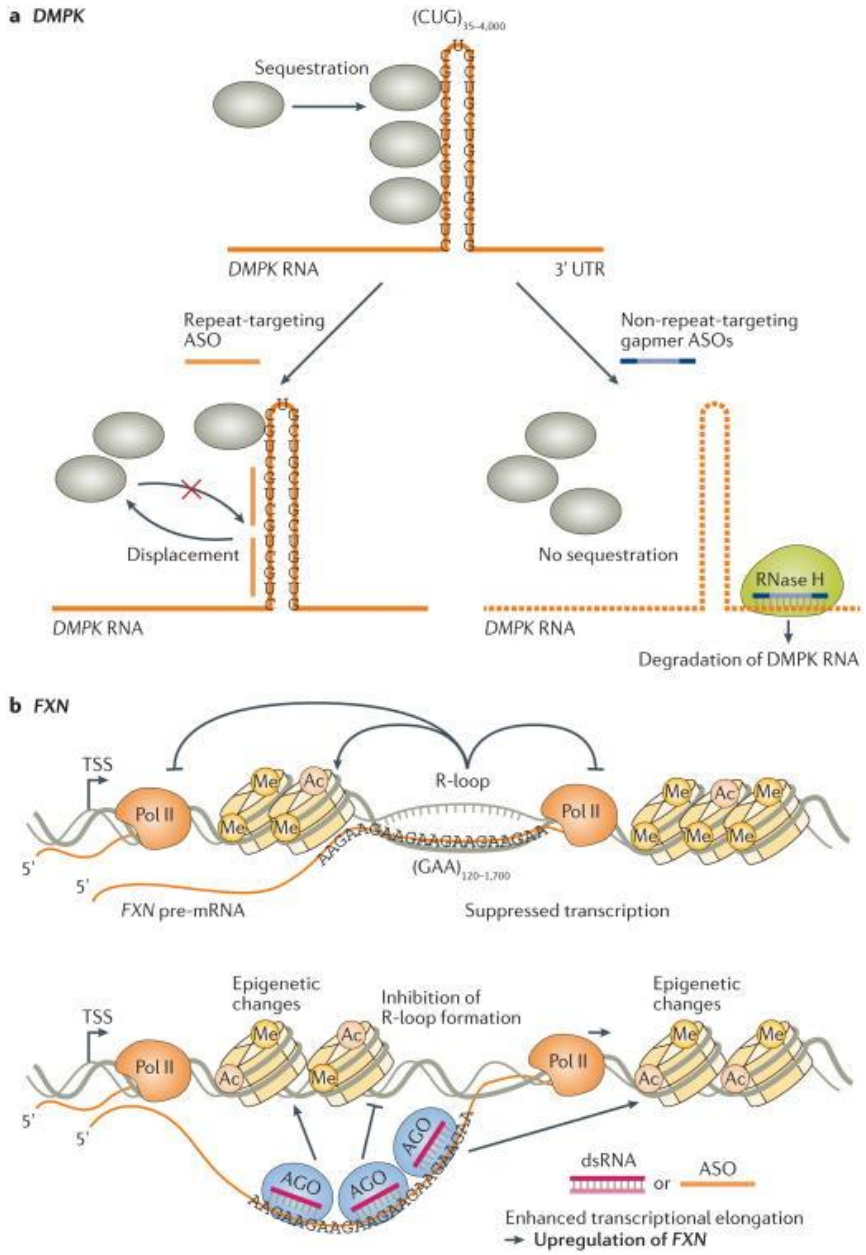
a) Reduction of cellular RNAs by **ASO gapmers**. RNase H recognizes the DNA-RNA duplex and cleaves the target. **b) Reduction** of cellular RNAs by **small silencing RNAs (siRNAs).** One strand (guide strand) is loaded into argonaute 2 (AGO2) protein and an active RISC complex is formed. The complex binds to the complementary sequences on a target RNA and cleaves it. **c) miRNA inhibitors** complementary to specific endogenous miRNAs bind to specific miRNAs and inactivate them. **d) Gene expression regulation** by miRNA mimics. Double-stranded or chemically modified single-stranded miRNA mimics reduce target gene expression by destabilizing target RNAs and/or inhibiting translation. **e) Regulation of alternative splicing by ASOs** targeting regions close to intron-exon junctions. **f) Modulation of transcription and epigenetic status** by ASOs or dsRNAs targeting promoter-associated RNAs.



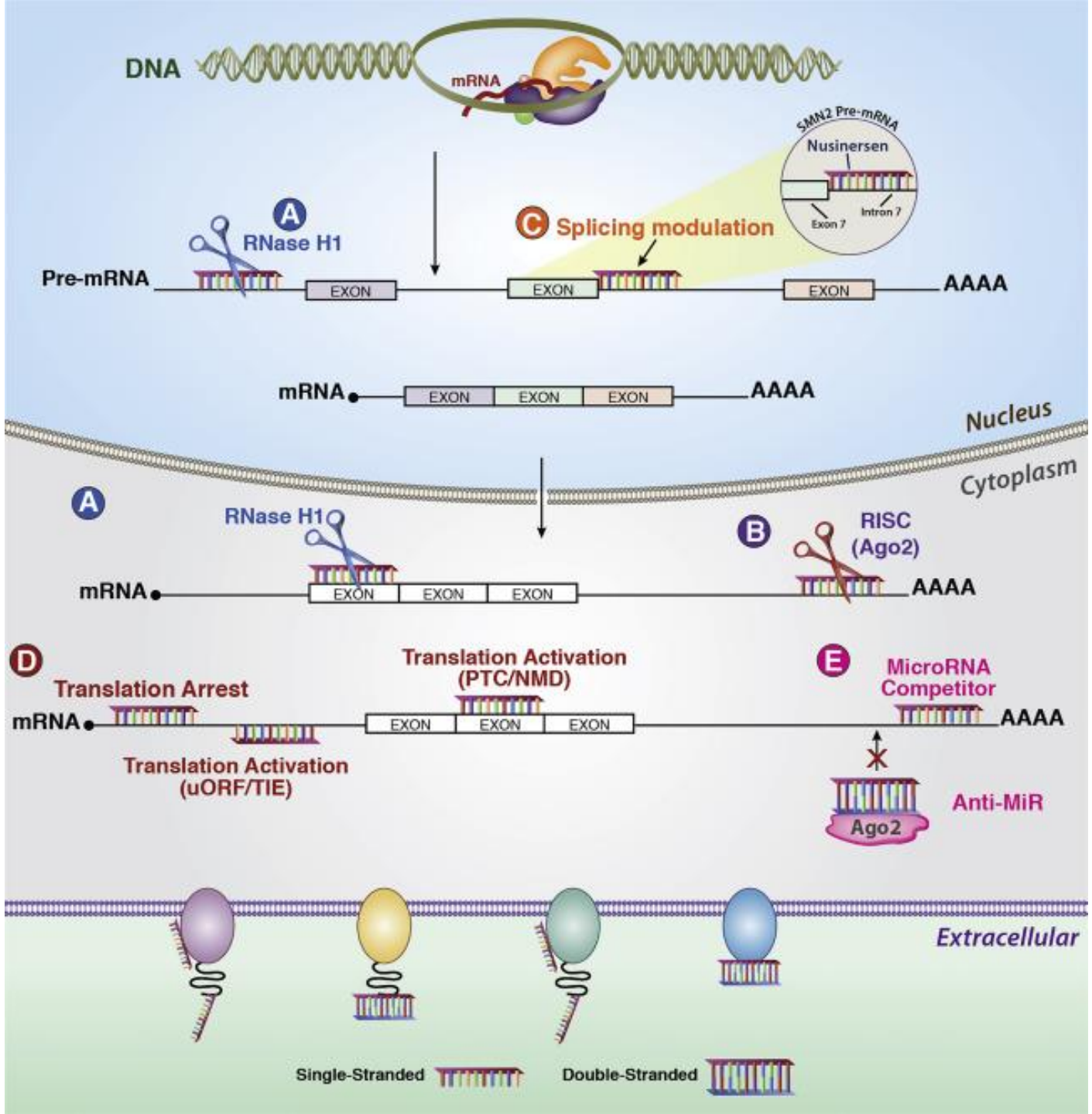
Modulating gene expression by targeting cis-acting ncRNAs or promoter RNAs

a) Targeting *UBE3a-ATS* ncRNAs using ASOs. *UBE3a* encoding an E3 ubiquitin ligase is the imprinted gene. In the brain, paternal *UBE3a* is silenced by antisense transcripts *UBE3a-ATS* and maternal deficiency of *UBE3a* causes Angelman syndrome. Depletion of nuclear *UBE3a-ATS* using specific gapmers activates paternal *UBE3a* expression[126].

b) *COX-2* expression regulation mediated by promoter RNAs produced from the upstream of the *COX-2* promoter. The transcript could function as a scaffold for interaction of complementary small RNAs in complex with AGO and TNRC6A. Binding of the protein-small RNA complex to the promoter RNAs could further trigger recruitment of some histone modifiers (e.g. WDR5) and transcription factors (e.g. NFκB, CREB1) to the COX-2 promoter, causing transcriptional upregulation of *COX-2*. These molecular recognitions also affect expression of the adjacent *PLA2G4A* gene[108]. Image 3b was modified, with permission, from REF. 108.



ASOs and duplex RNAs targeting repetitive RNAsa) Antisense oligonucleotides used for therapy of myotonic dystrophy type 1 (DM1). DM1 is caused by gain-of-function toxicity from CUG-repeat containing DMPK transcripts. ASOs targeting the CUG repeats can be used to prevent binding of muscleblind like splicing regulator 1 (*MBNL1*) to the repeats. Gapmer ASOs targeting outside the repeat are also used for depleting toxic nuclear DMPK transcripts. **b)** Activating *FXN* expression using GAA repeat-targeting oligonucleotides. *FXN* encoding frataxin is a causative gene for FA and its expression is suppressed at least partly because of the R-loop formation between the expanded GAA repeats within the intron1 of *FXN* pre-mRNA and the genomic DNA in patients (upper). Double-stranded RNAs or single-stranded locked nucleic acid (LNA) ASOs targeting the GAA repeats can inhibit the R-loop formation in the repeat region and change histone modifications surrounding the repeats, leading to activation of *FXN* gene expression (lower)[78]. Image 2b was modified, with permission, from REF. 77.



Antisense Mechanisms Commonly Used to Modulate Gene Expression

Chemistry Glossary:

2′-H, 2′-deoxy;
2′-OMe, 2′-methoxy;
2′-MOE, 2′-O-methoxy ethyl;
cET, (*S*)-constrained ethyl;
ENA, 2′-*O*,4′-*C*-ethylene-bridged nucleic acid;
LNA, locked nucleic acid; BNA, bridged nucleic acid;
PMO, phosphorodiamidate 2.5 ASO drugs contain cEt modification;
L, ligand conjugate.
Target/Organ:
AGT, angmorpholino oligomer;
PO, phosphodiester linkage;
PS, phosphorothioate linkage.

[2021] The Limitless Future of RNA Therapeutics

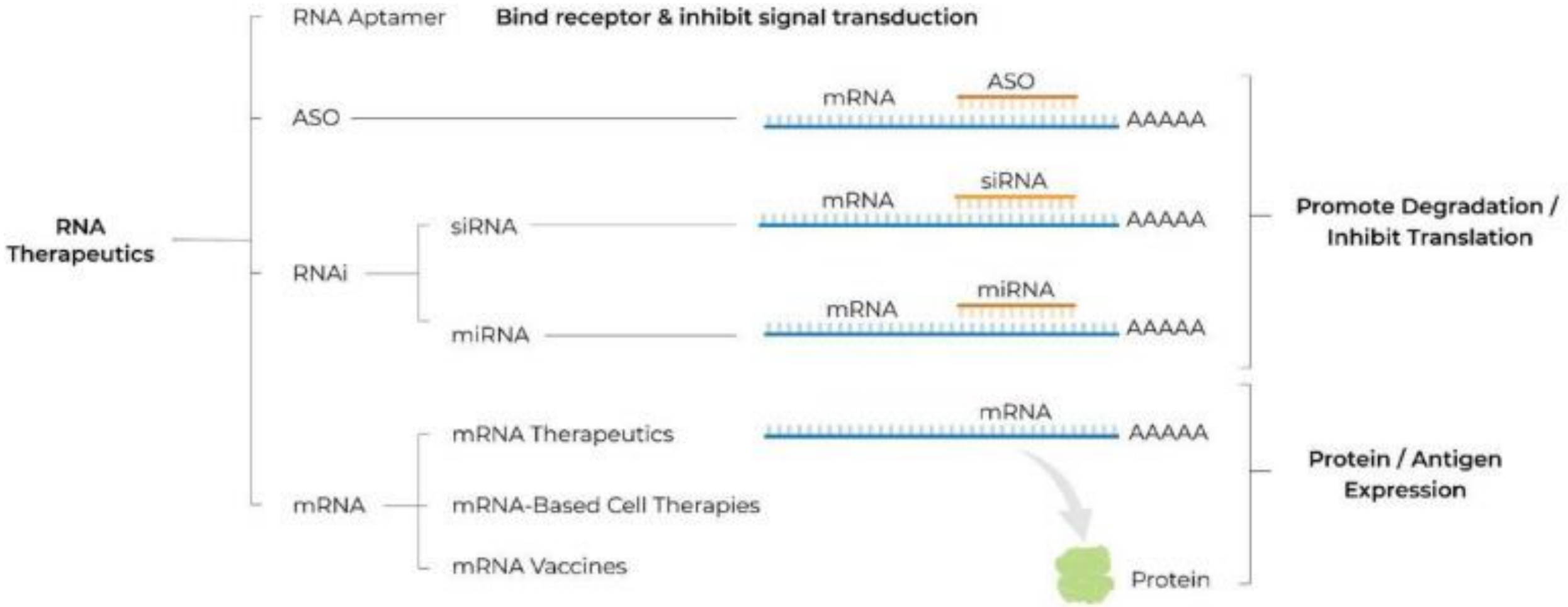
Recent advances in the generation, purification and cellular delivery of RNA have enabled development of RNA-based therapeutics for a broad array of applications. RNA therapeutics comprise a rapidly expanding category of drugs that will change the standard of care for many diseases and actualize personalized medicine.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8012680/

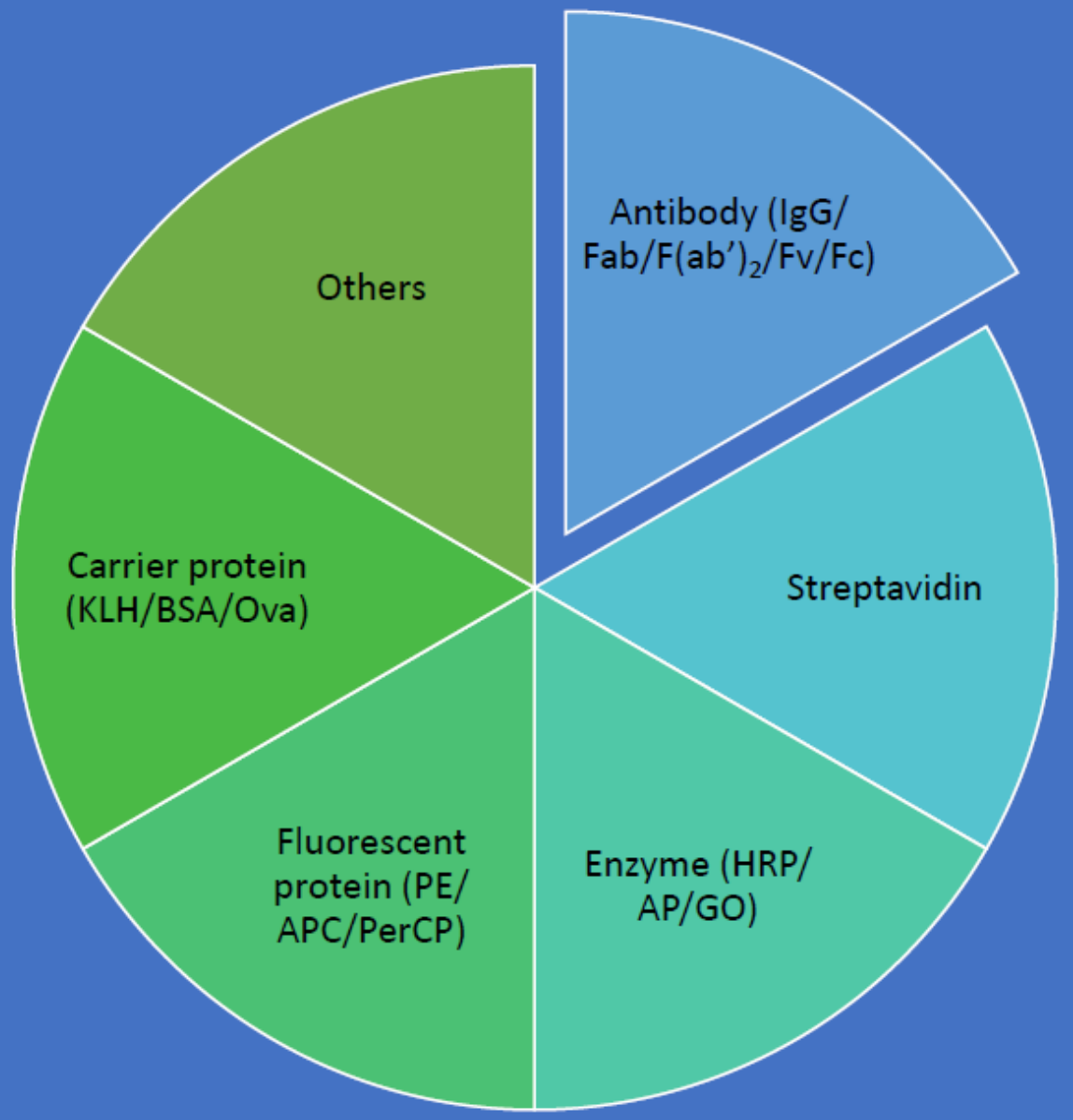
RNA therapeutics, delivery of RNA therapeutics, hospital-based RNA therapeutics, messenger RNAs (mRNAs), self-amplifying mRNA.

Different classes of RNA therapeutics.

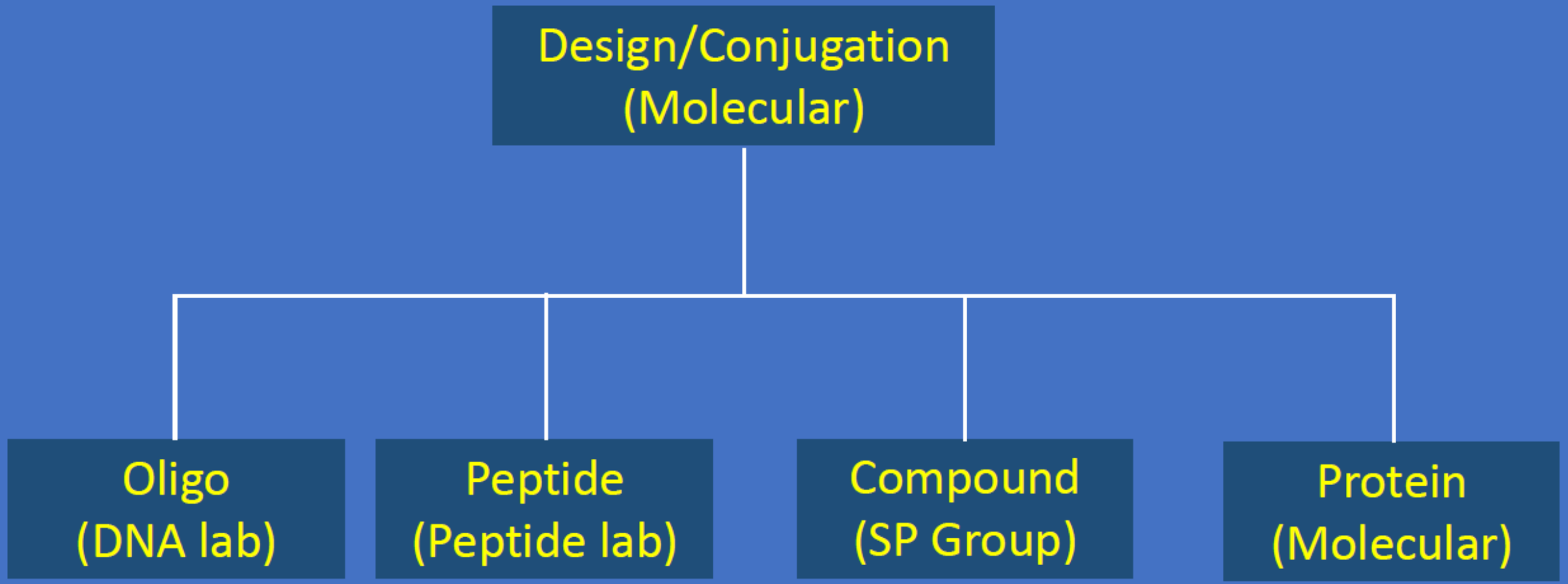
ASO, antisense oligonucleotide; **RNA**, ribonucleic acid; **RNAi**, RNA interference; siRNA, small interfering RNA; **miRNA**, microRNA; **mRNA**, messenger RNA; **A**, adenosine molecule; **AAAAA**, poly A tail.

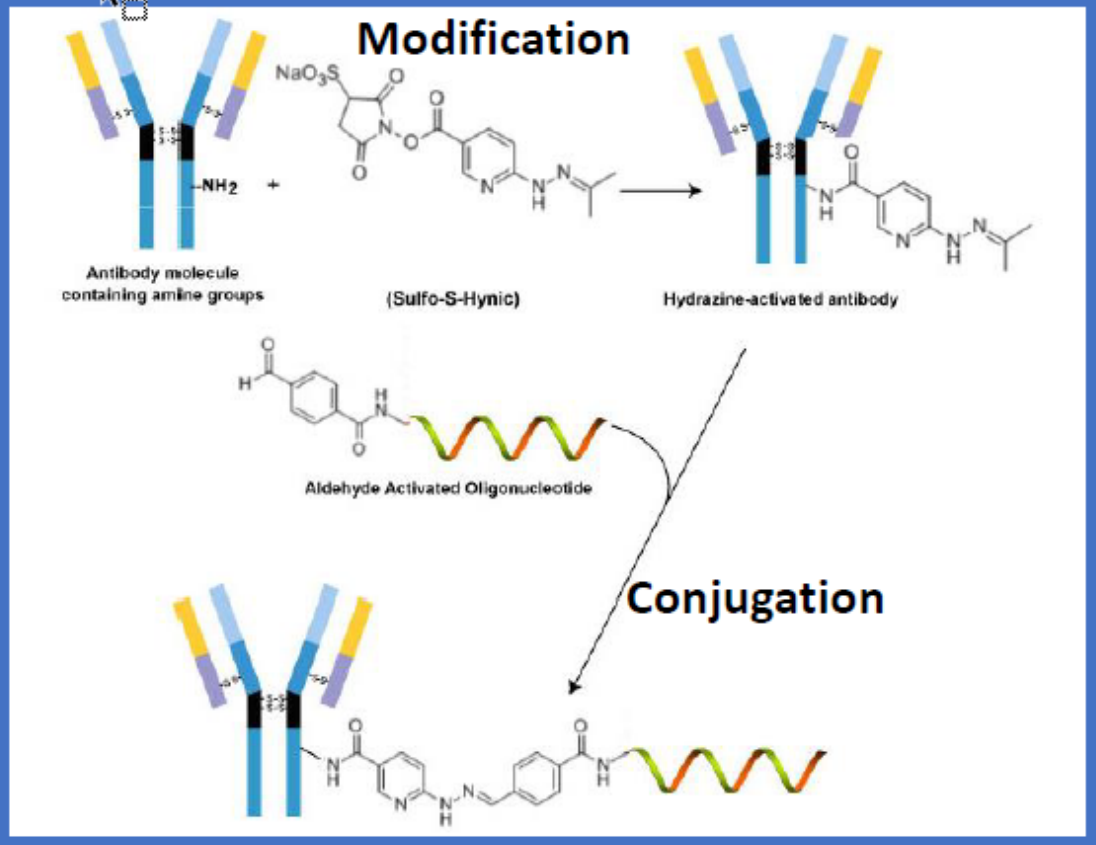


Custom Protein-Oligonucleotide Conjugation



Protein-Oligo Bioconjugation Service at BSI





Purification

(Dialysis, Diafiltration, Affinity, SEC, IE, RP)

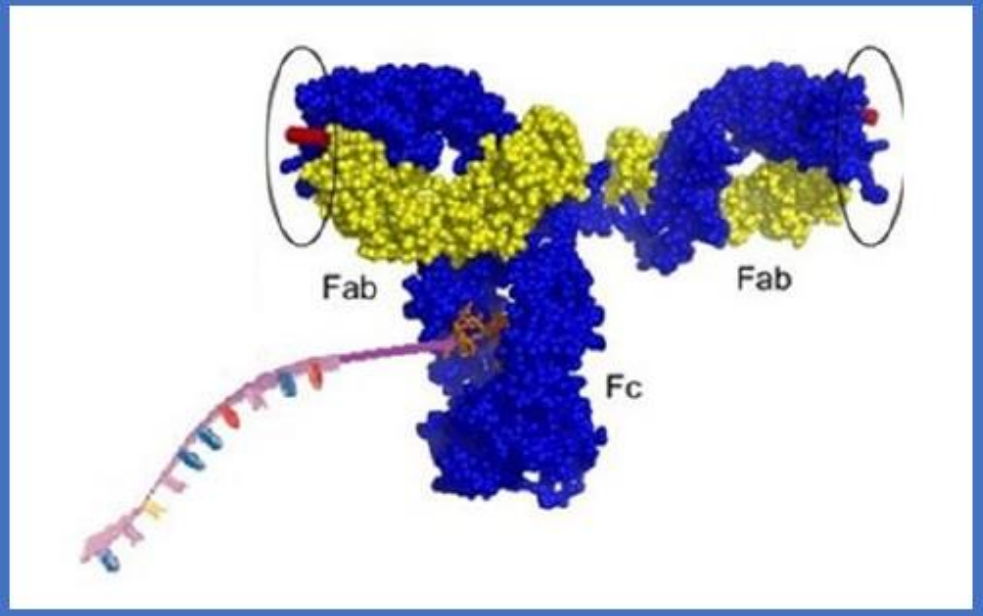
Quality assurance

(Gel, HPLC, Mass, PCR, Function)

- Protein (NH_2, Cys, COOH)
  - > Cleavable: S-S, hydrazide, CHO
  - > Non-cleavable: maleimide, DBCO, azide
- Oligonucleotide

  NH_2, COOH, SH, maleimide, hydrazide, CHO, DBCO, azide
- Bifunctional Crosslinker (X----Y)
  - > NH2, COOH, SH
  - > Hydrazide, CHO, DBCO, azide, maleimide

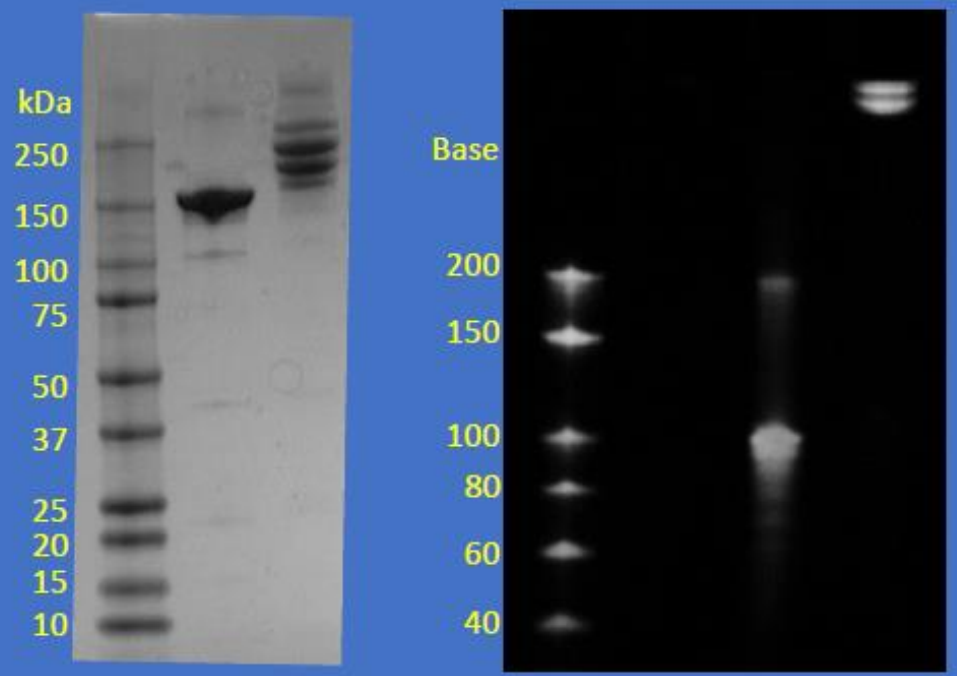
Antibody-Oligonucleotide Conjugation



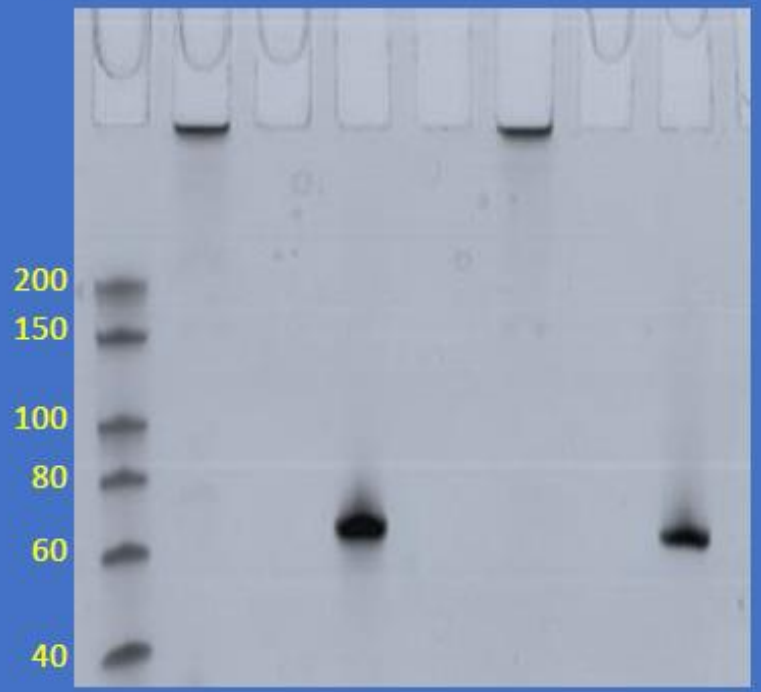
- High specificity to antigen
- High affinity to antigen
- Sensitive detection
- Diagnostic agents
- Drug delivery
- *In vivo* targeted therapy

Conjugation of ssDNA to Antibody

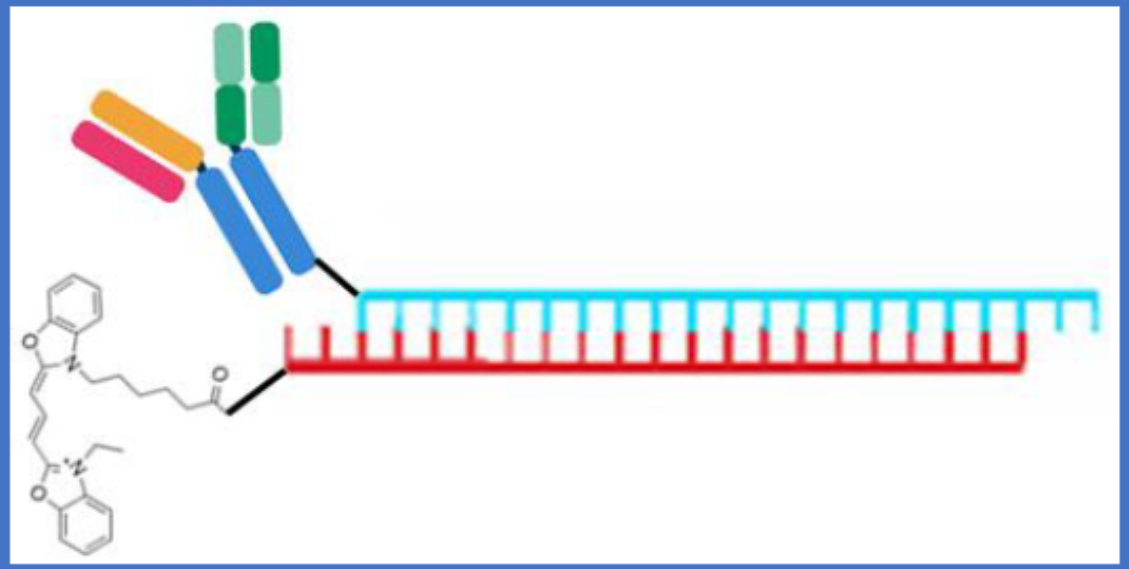
5’-[Antibody]-[oligo]$_{100}$-3’



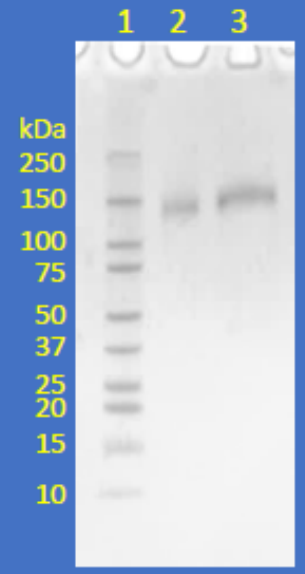
5’-[Acrydite]-[Oligo]$_{68}$-Antibody-3’



Conjugation of siRNA duplex to Antibody

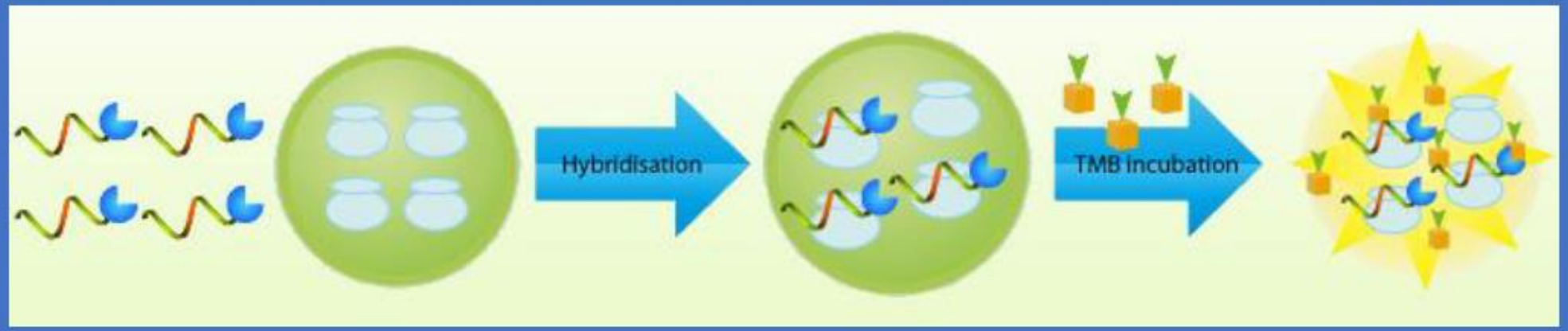


Lane 1, ssRNA sense strand
Lane 2, ssRNA antisense strand (Cy2)
Lane 3, Annealed siRNA duplex



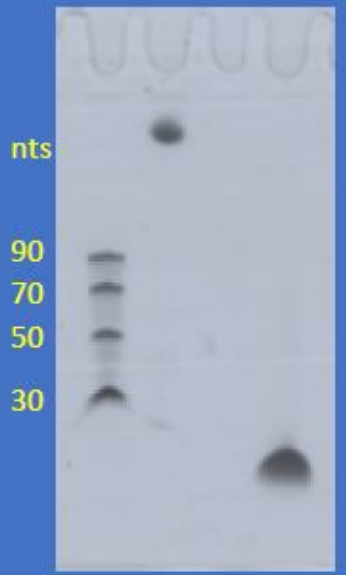
Lane 1, Protein Standards
Lane 2, Antibody control
Lane 3, Antibody-siRNA conjugate

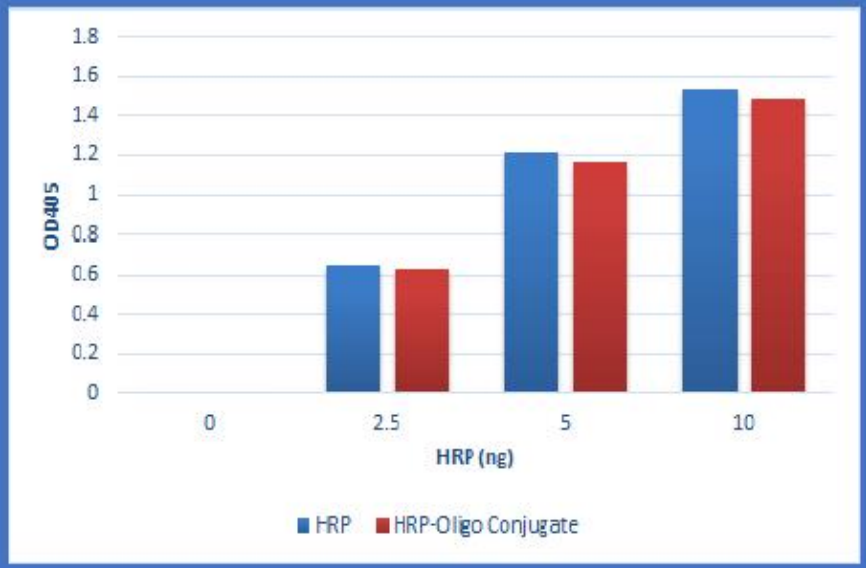
Enzyme (HRP) -Oligonucleotide Conjugation



- Signal amplification
- Sensitive detection
- Diagnostic agents

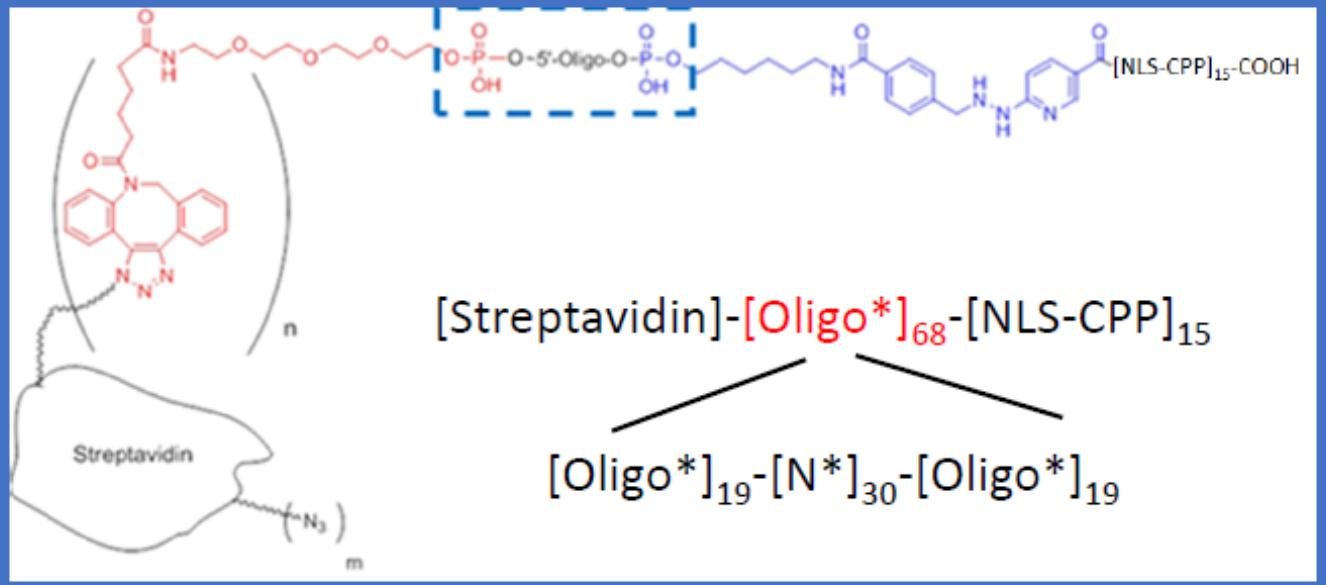
5’-[Oligo]$_{16}$-[HRP]-3’ Conjugate

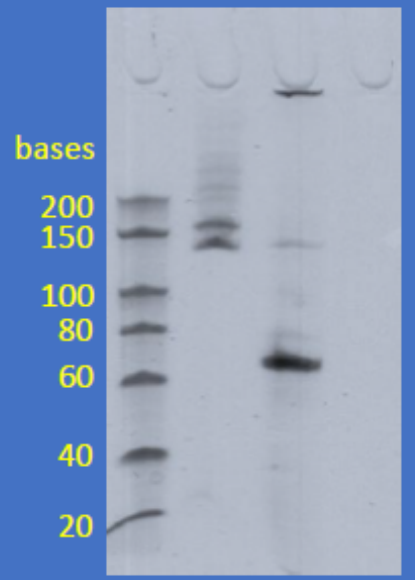


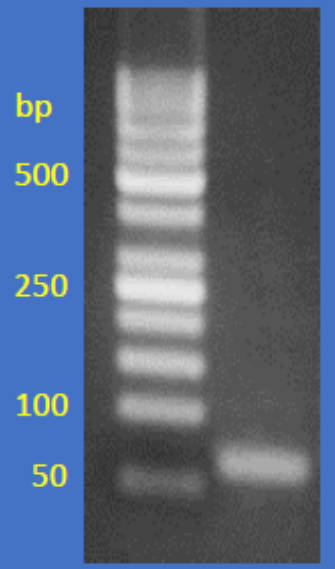


Streptavidin-Oligonucleotide Conjugation

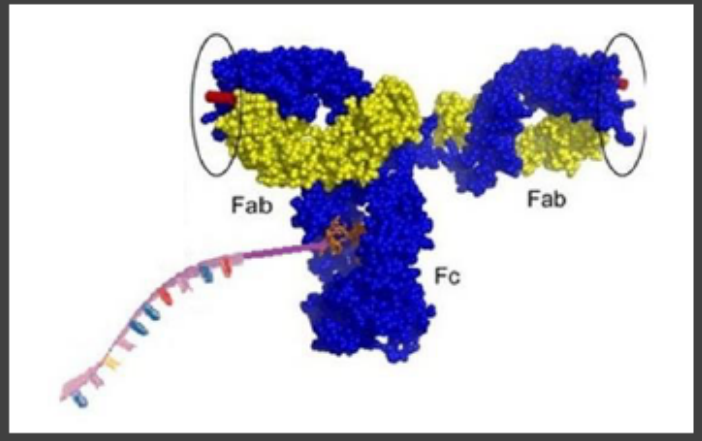
- Tetrameric, non-covalent protein (52kDa)
- Very tight binding to biotin
- Sensitive detection
- Diagnostic agents





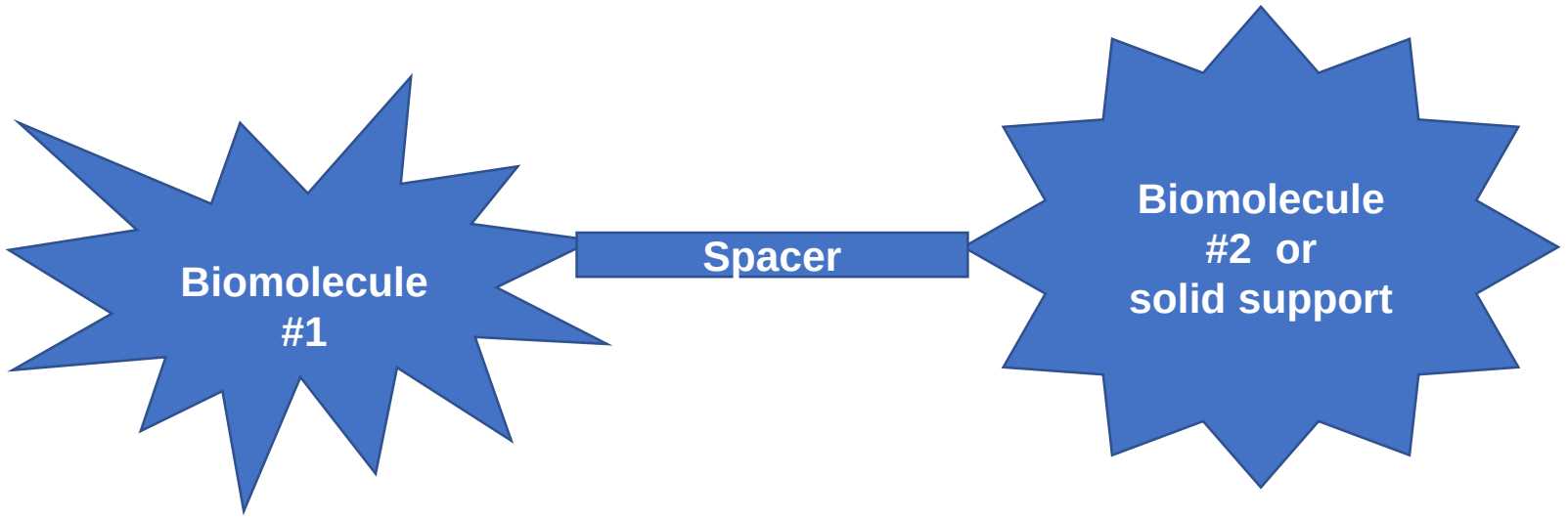


Summary



- Whatever protein
- DNA, RNA, hybrid, BNA
- ss- and ds-stranded
- 5’-, 3’- and internal
- Complicated modifications
- Whatever oligo length (>100-mer)
- High quality by purification
- QC by functional assay

Bioconjugation Services Available from Biosynthesis, Inc.



Strategy Considerations

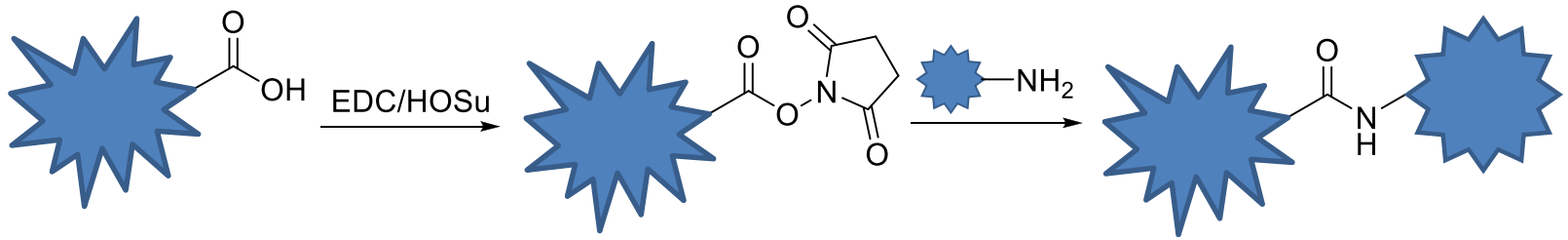
- Customer’s requirements
- **Structure considerations**: Available functional groups for modification and conjugation
- **Reagent considerations**: Available low-cost chemicals and cross-linkers for modification and conjugation
- **Chemistry considerations**: As straightforward as possible for low risk of failure and low cost to the customer
- **Consideration of orthogonal methods** if conjugation involves multi-components: amine/thiol

Chemistry

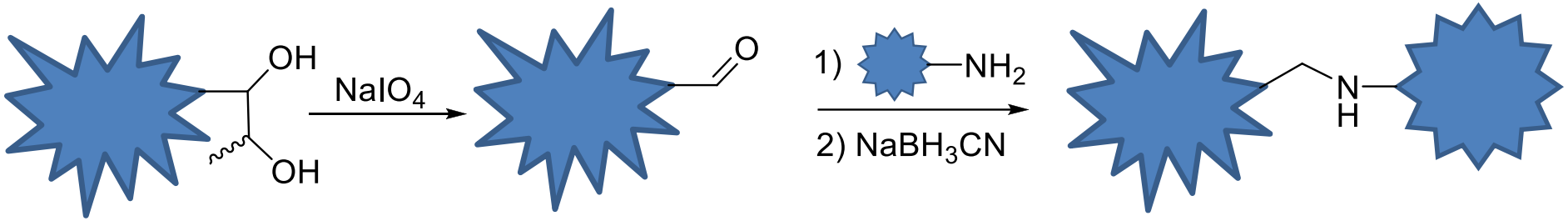
(Easy handling, mild condition, and high yield)

Amine-Involving Reactions

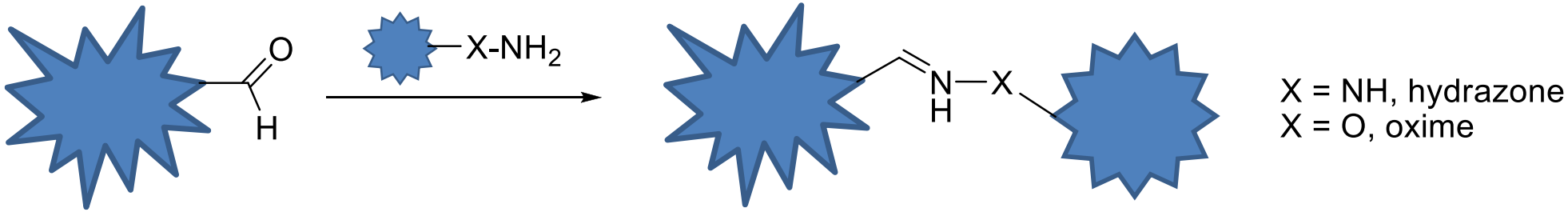
1) Amide bond formation: EDC activation and active esters



2) Reductive amination



3) Hydrazone and oxime formation

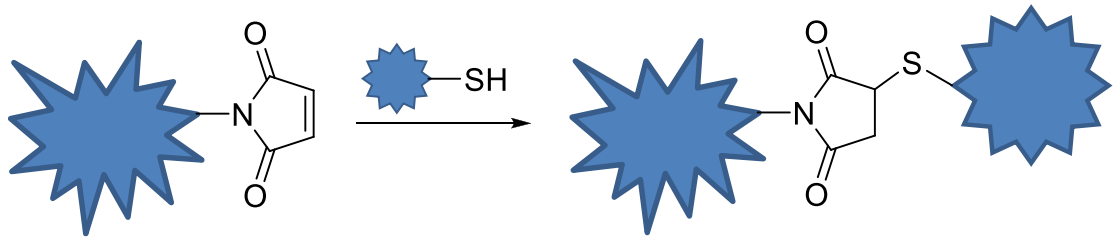


Chemistry

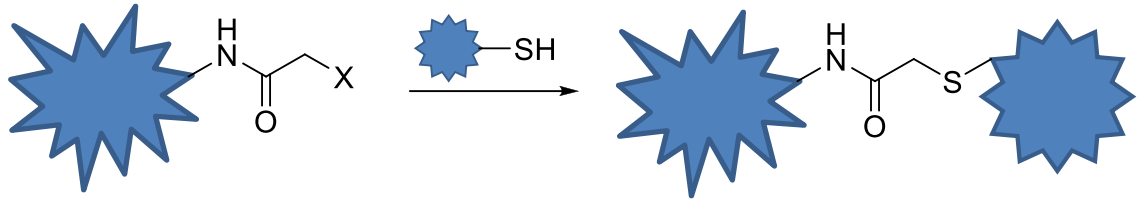
(Easy handling, mild condition, and high yield)

Thiol-Involving Reactions

1) Michael addition reaction: Maleimide and acrydite

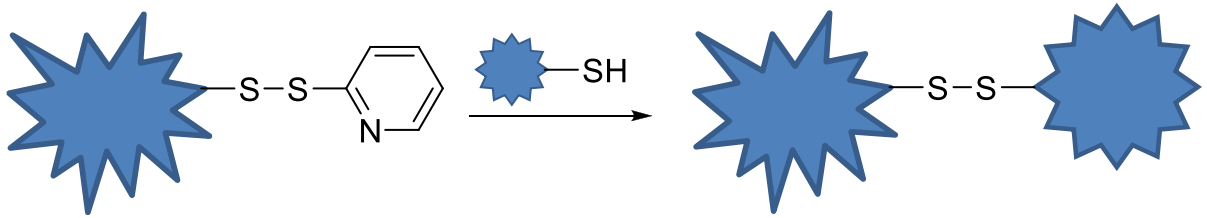


2) α-Substitution of haloacetamide



X = Br or I

3) Thiol exchange

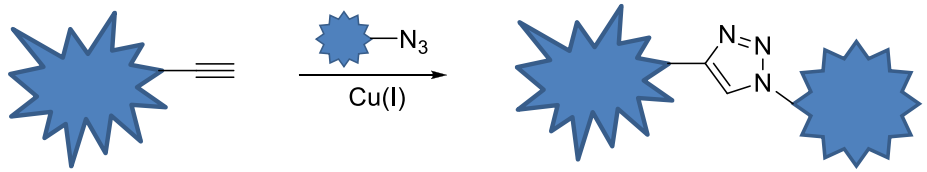


Chemistry

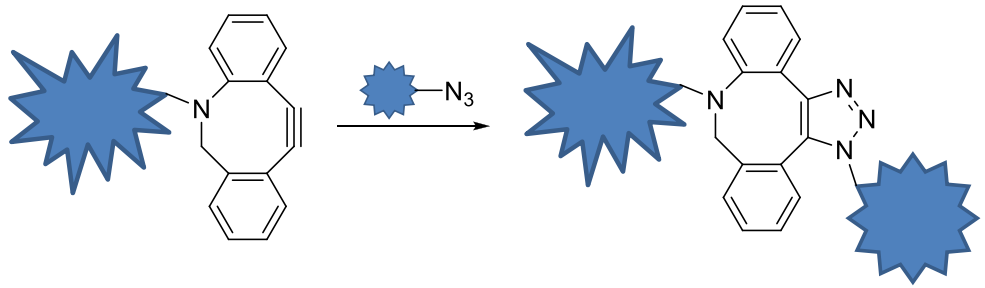
(Easy handling, mild condition, and high yield)

Click Chemistry

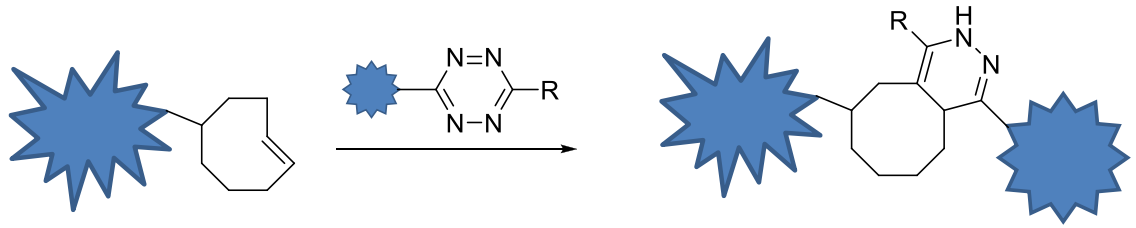
1) Copper-assisted click chemistry



2) Copper-free click chemistry: DBCO and BCN



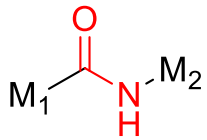
3) Inverse Diels-Alder reaction: TCO/Tetrazine reaction



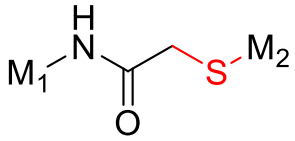
Linker (Spacer)

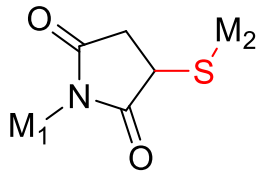
Non-cleavable spacers

- **Amide bond**

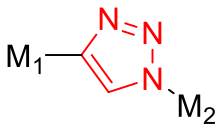


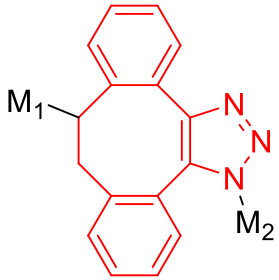
- **Thioether bond**

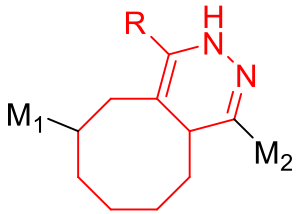




- **Click chemistry linker**



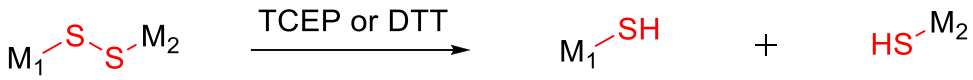




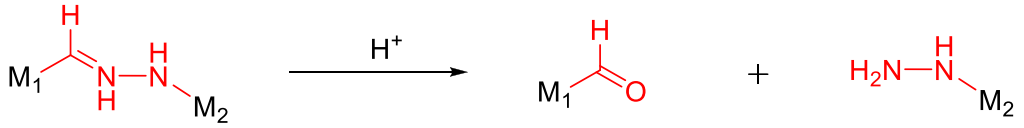
Linker (Spacer)

Cleavable spacers

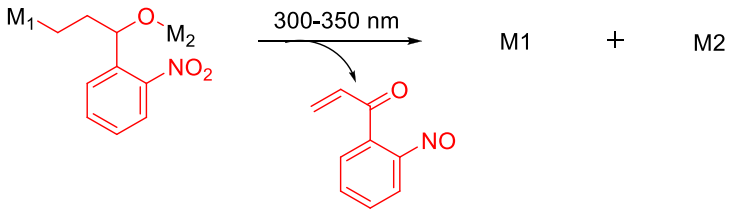
- **Reductive cleavable linker**



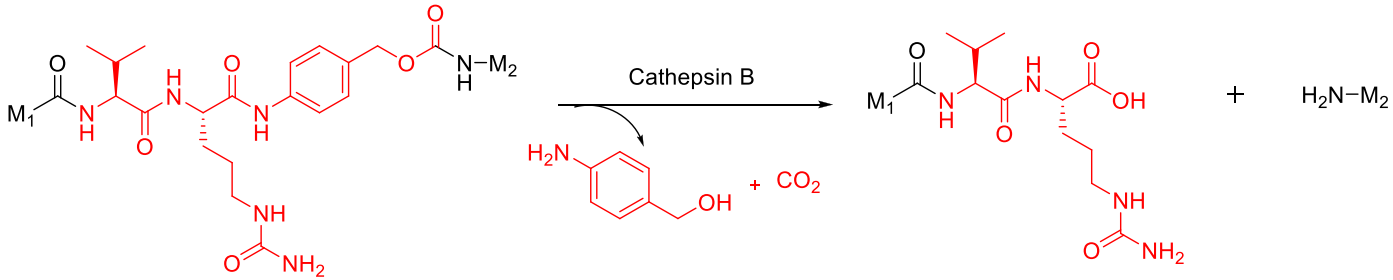
- **pH sensitive linker**



- **Photocleavable linker**



- **Enzymatic cleavable linker**

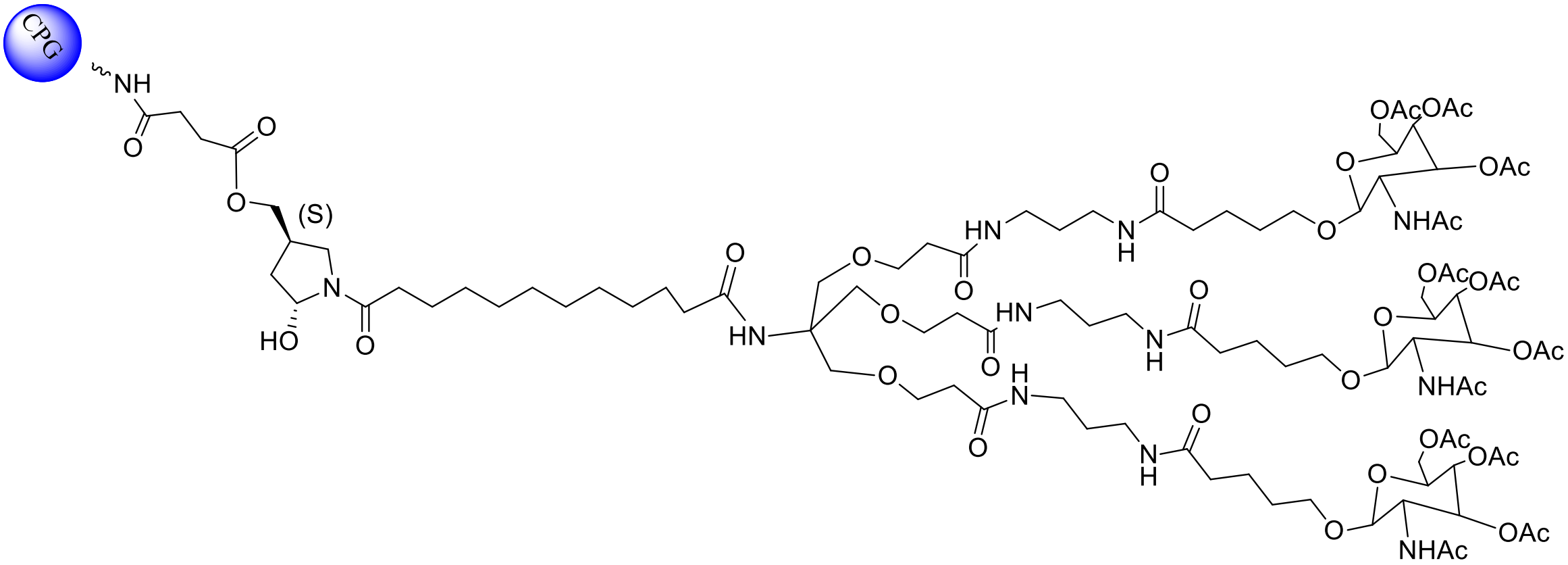


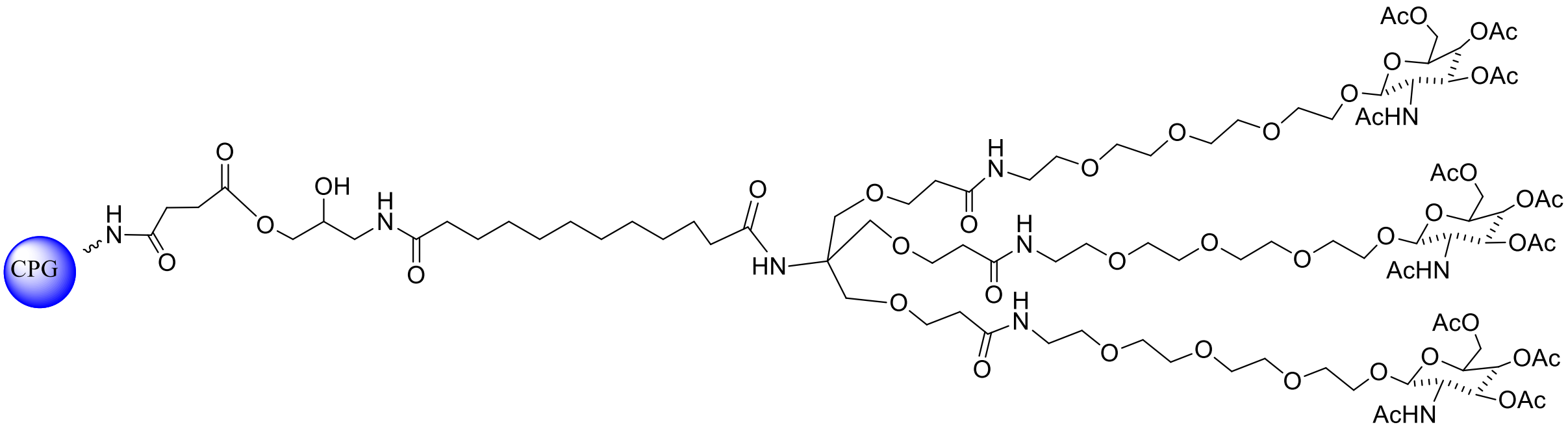
Substrates

- Small molecules (drugs, fluorescent dyes, *etc.*)
- Oligonucleotides
- Peptides
- Lipids
- Carbohydrates
- Polymers (PEGs, PGAs, dextrans, dendrimers, *etc.*)
- Nanoparticles (gold nanoparticles, Q-dots)
- Solid supports (magnetic beads, agarose, silica gel, microspheres, *etc*.)
- proteins (carrier proteins, antibodies, enzymes,

I. GalNAc modification of oligos

Triantennary GalNAc



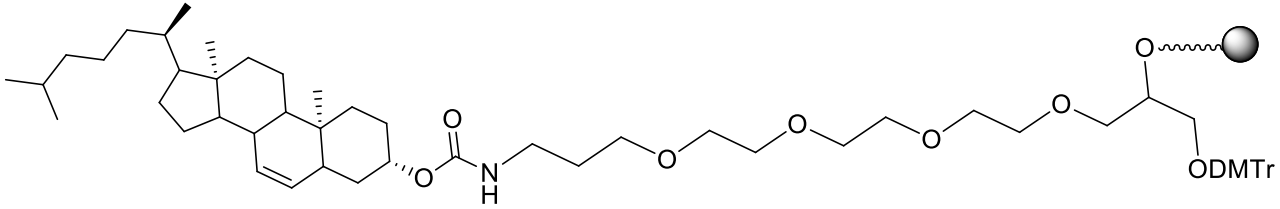


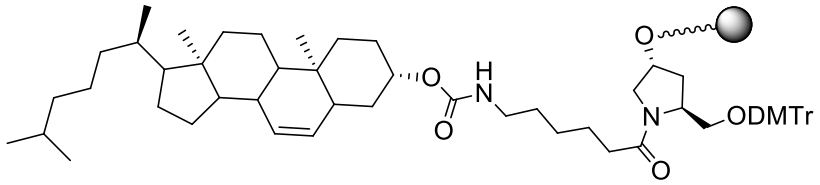
Tri-GalNAc CPG from Belarus, open market

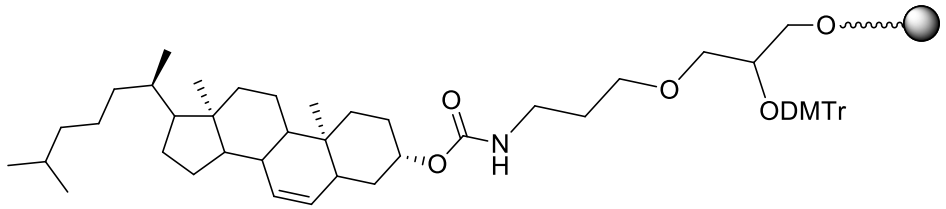
III. Lipid modification

Cholesterol

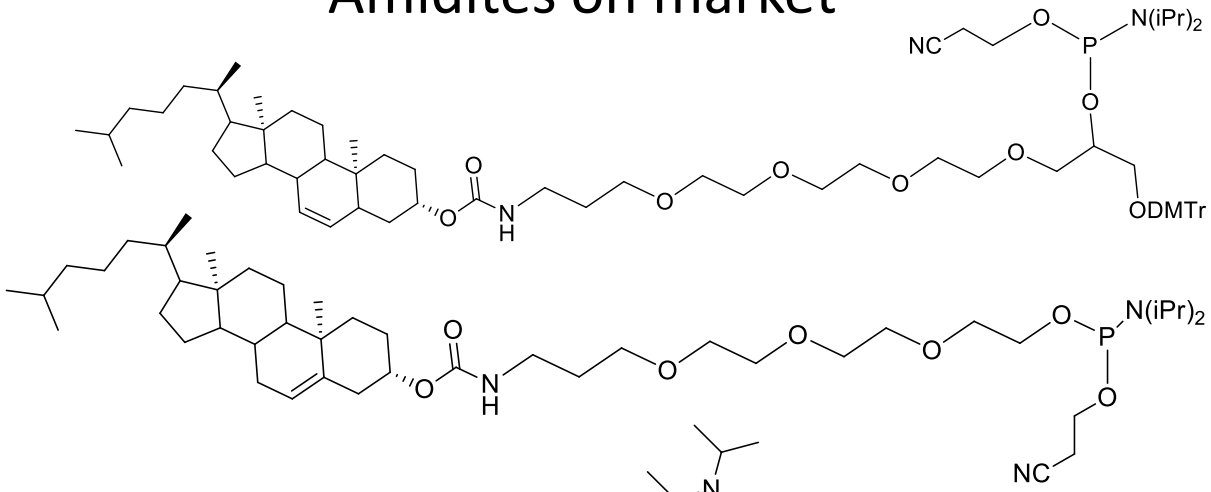
CPGs on market

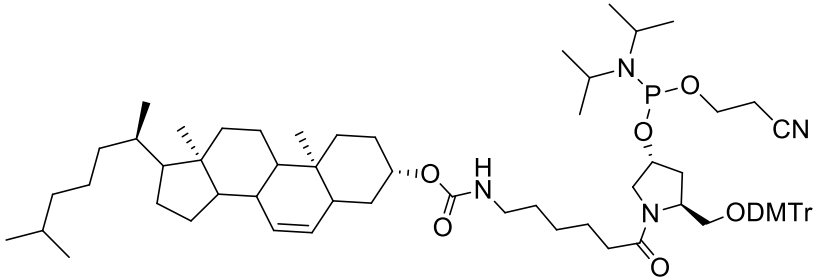


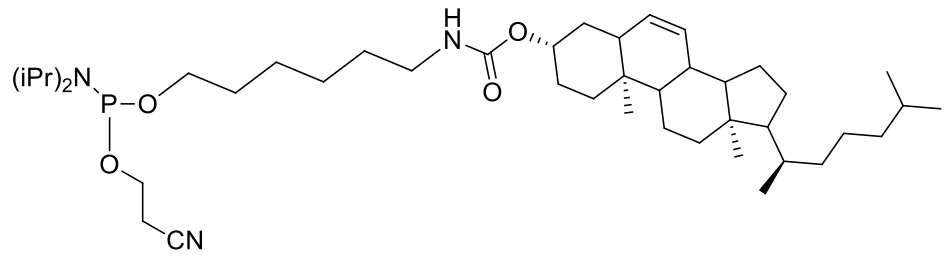




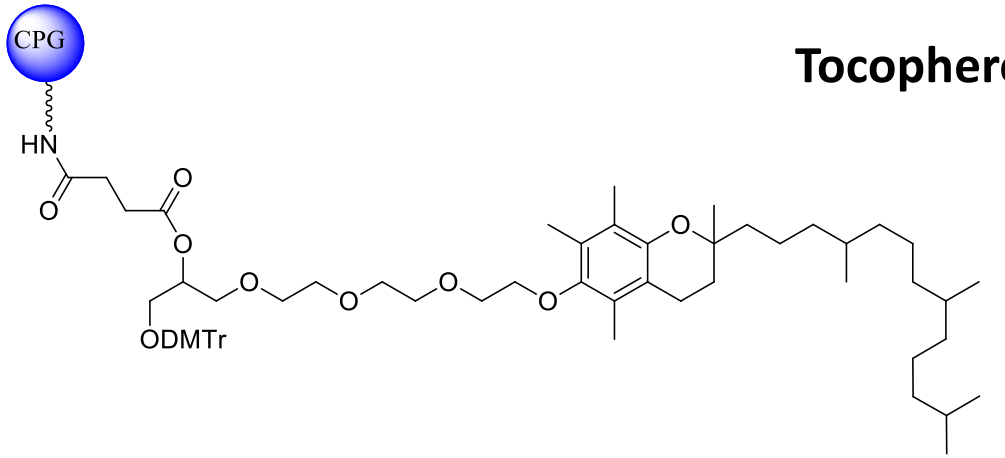
Amidites on market

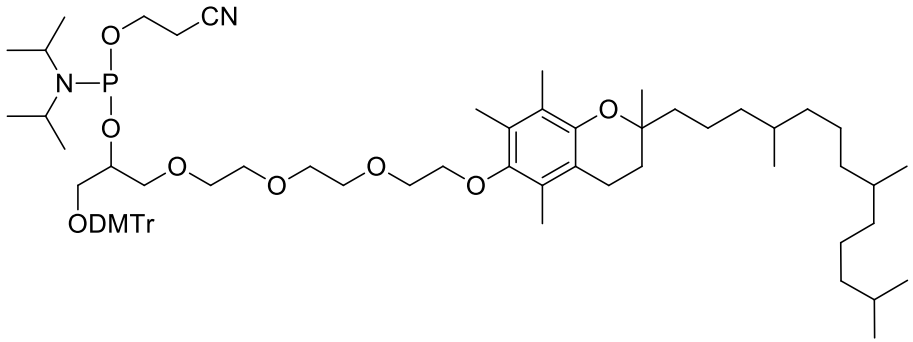






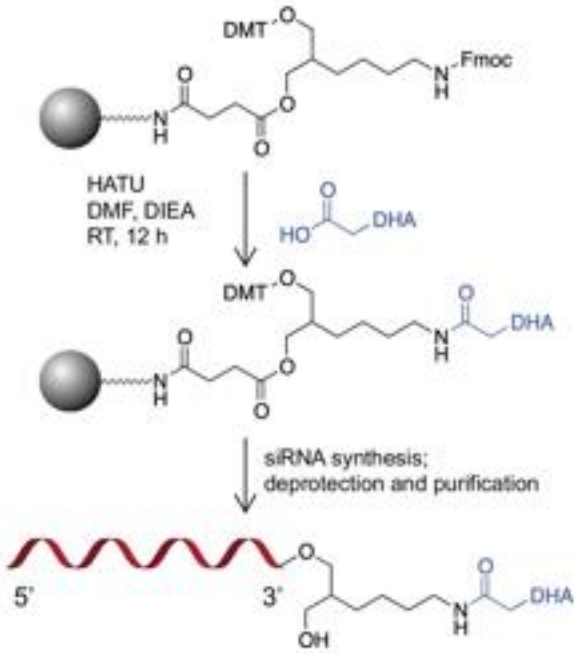
Tocopherol



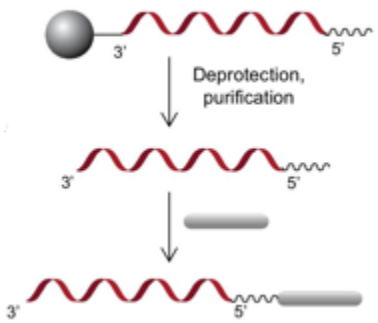


Other lipids

3’ modification



5’ or internal mod

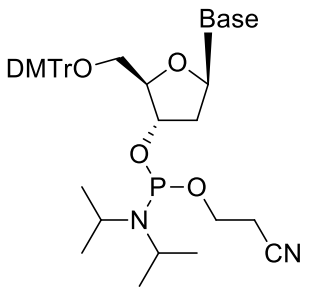


NHS, Maleimide or click-chem

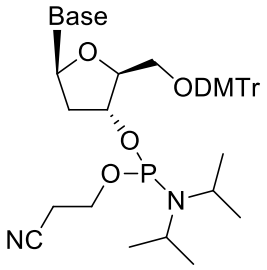
Other methods

Lipid amidite;
Staudinger reaction;
etc.

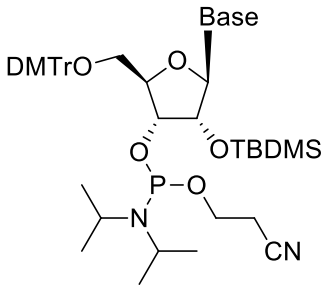
IV. Variety of bases



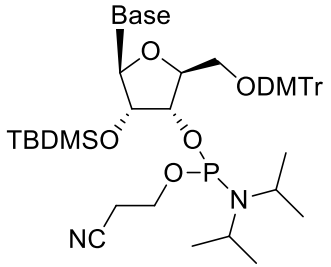
DNA



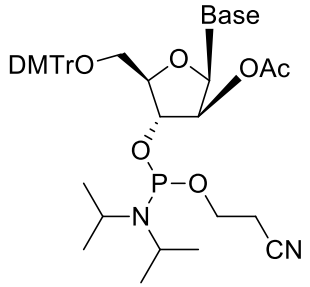
L-DNA



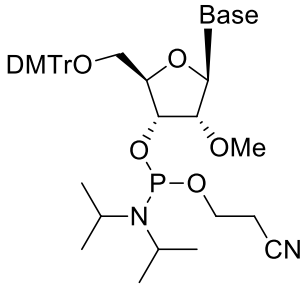
RNA



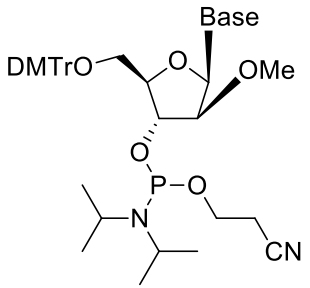
L-RNA



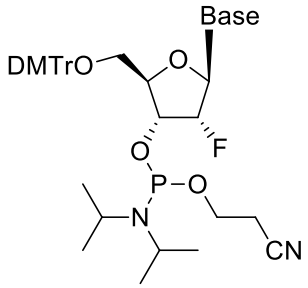
Ara-RNA



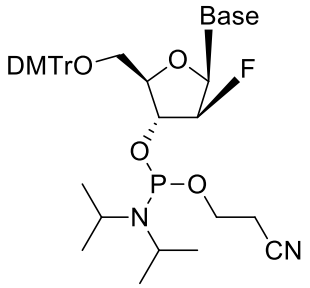
2'-OMe RNA



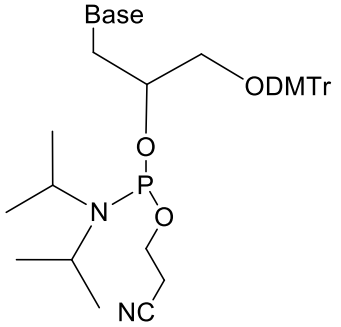
Ara-2'-OMe RNA



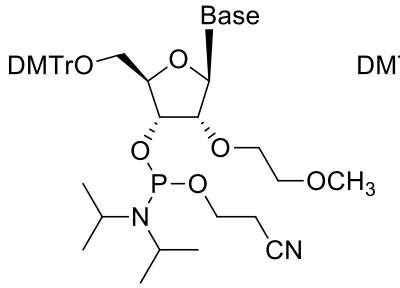
2'-F RNA



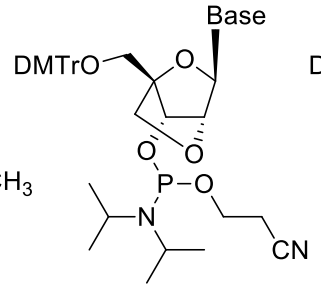
FANA



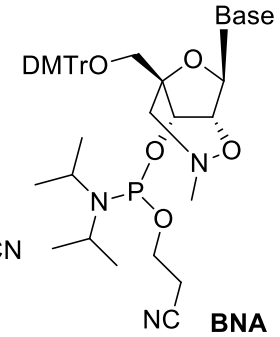
GNA



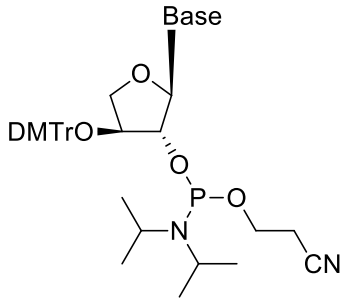
MOE RNA



LNA



BNA



TNA

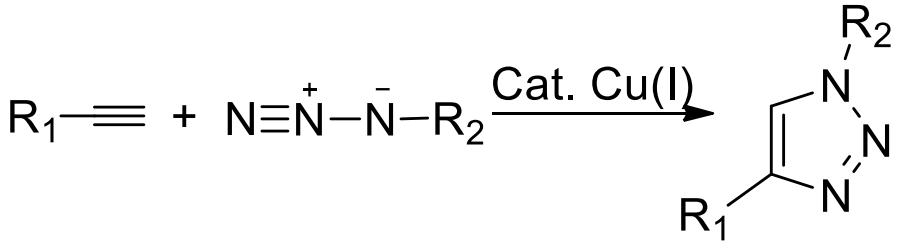
Special bases

5-Me-2’-fC
5-Me-2’-fU (fT)
MOE-C
MOE-U
LNA-C
LNA-U
2’-Ome I
2’,2’-diF C
GNA
TNA

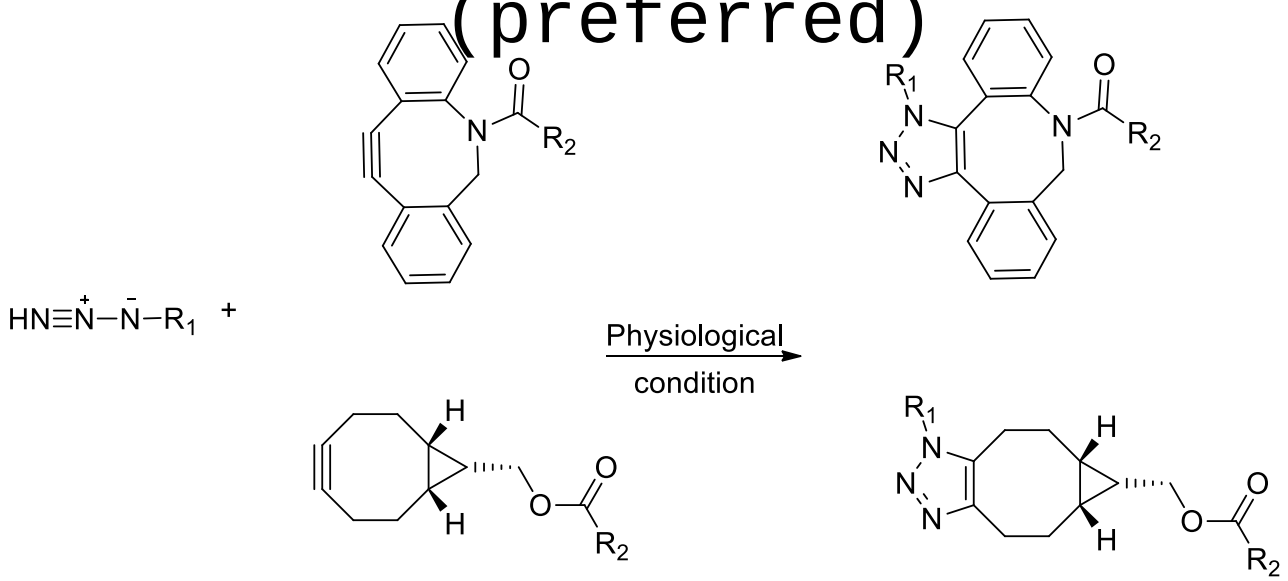
V. Click-chemistry

Alkyne-Azide

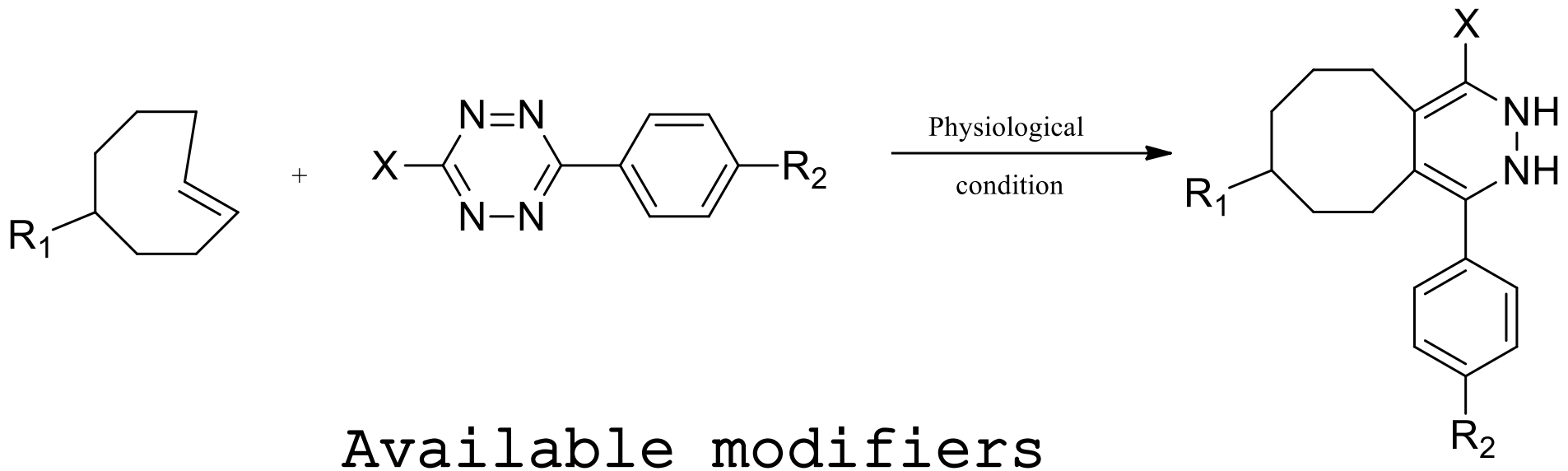
Strain-Promoted Alkyne-Azide Cycloaddition (SPAAC, patented)



Copper-free click-chemistry (preferred)



Tetrazine-TCO



Available modifiers

Tetrazine

Tetrazine;
Tetrazine PEG5;
MethylTetrazine;
MethylTetrazine PEG4;
MethylTetrazine PEG5;
etc.

TCO (trans-cyclooctene)

TCO;
TCO PEG4;
TCO PEG12;
Etc.

Thank you!