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Noncoding RNA therapeutics

Current challenges and potential solutions

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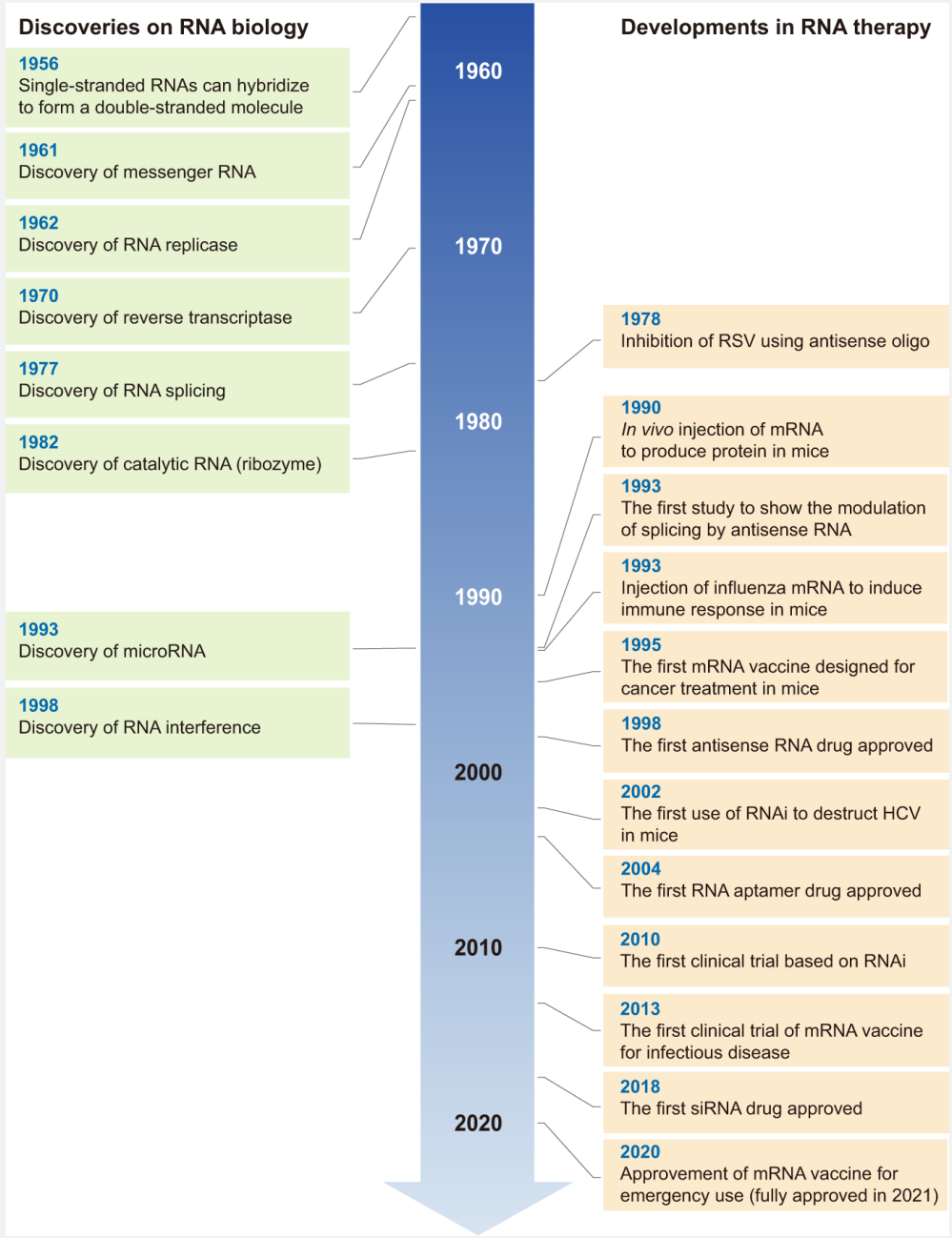
Research Center for Advanced Medicine - MedFuture

Cooperation strategy for knowledge transfer, internationalization and curricula innovation in the field of research education at the 3rd level of study - AURORA

The historical timeline of important discoveries in RNA biology and key developments in RNA therapy.



Key Points:

1.Understanding the Power of RNA: Early discoveries in mRNA research and its role in protein synthesis.

2.The Dawn of RNA Interference (RNAi):Andrew Fire and Craig Mello's Nobel Prize-winning breakthrough that opened new doors for gene regulation.

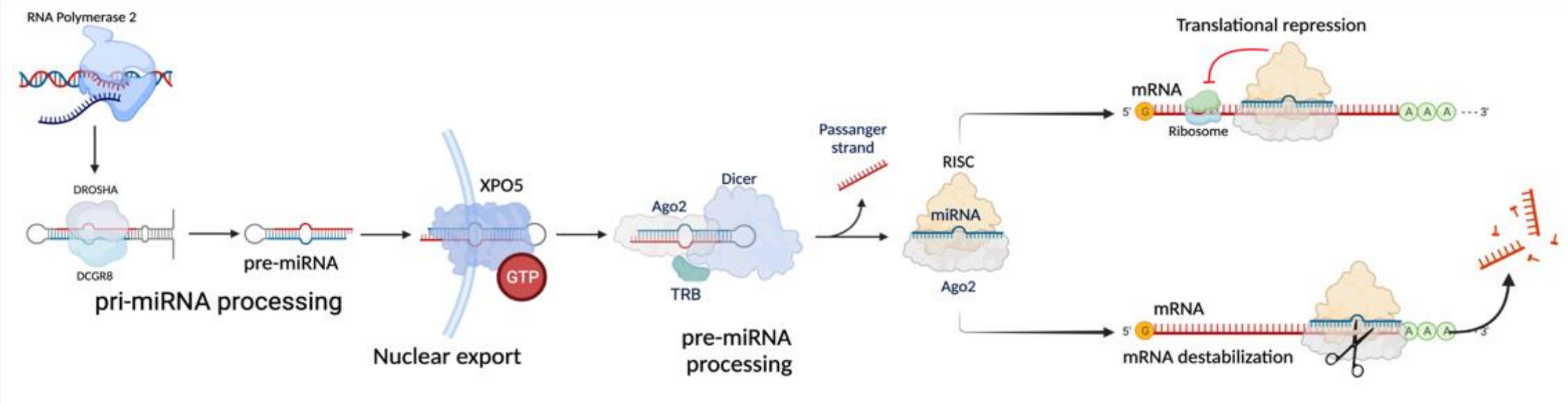
3.RNA Therapeutics Today: Exploring the latest advancements and their potential impact on treating genetic disorders and infectious diseases.

Main characteristics of microRNAs and long noncoding RNAs

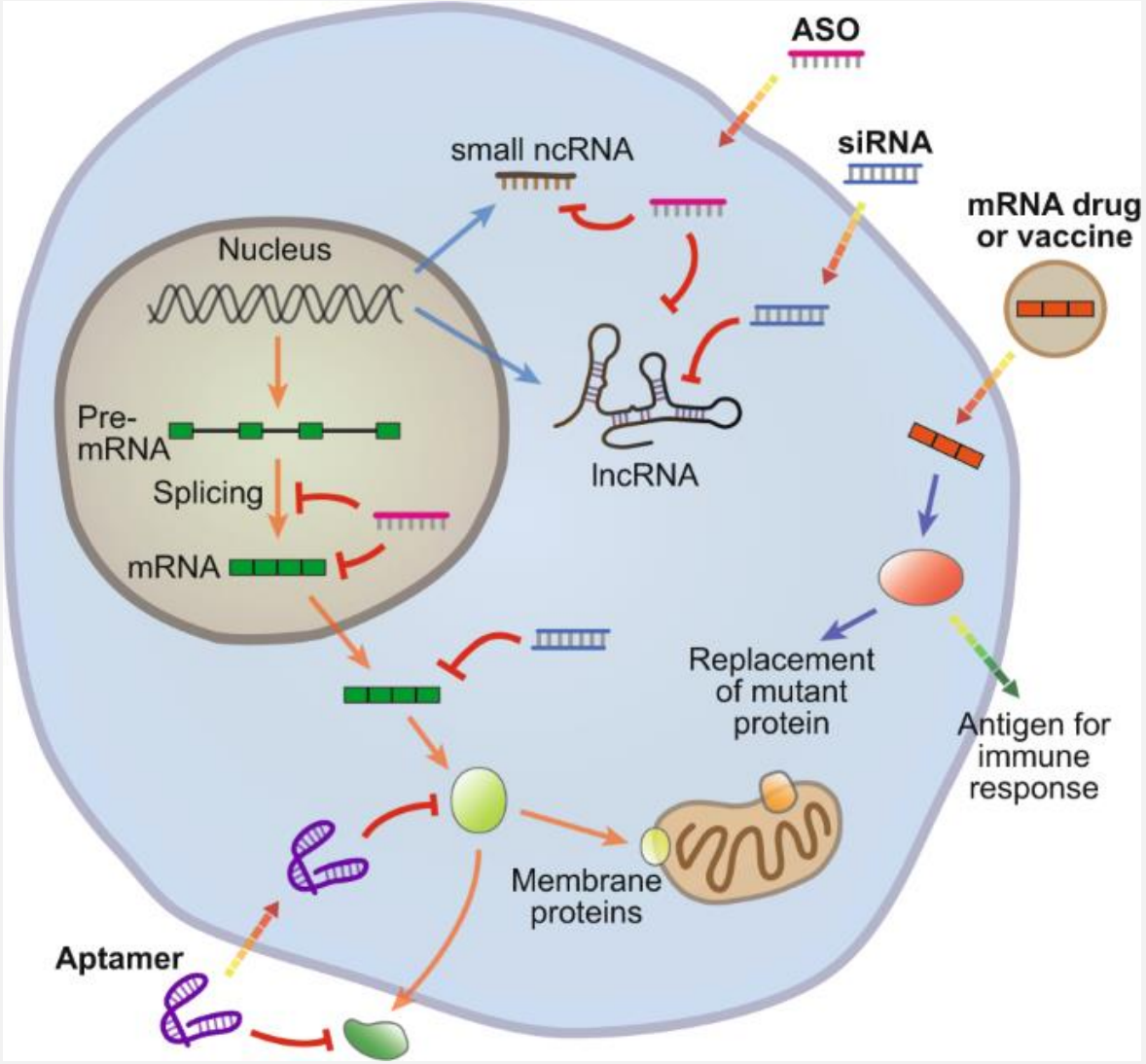
Noncoding RNAs (ncRNAs) are generated from the larger part of the genome that does not encode proteins but produces noncoding transcripts that regulate gene expression and protein function.

The two major classes of ncRNA are the well-studied short microRNAs (miRNAs) and the more recently identified long ncRNAs (lncRNAs).

- **MicroRNAs (miRNAs)** are highly conserved, small, 17- to 25-nucleotide (nt), single-stranded ncRNAs that act as gene regulators. Seed sequence binding with perfect complementarity results in the degradation of the targeted mRNA, whereas binding with imperfect complementarity, which is more common, results in translational inhibition, both of which are facilitated by RISC.
- **Long noncoding RNAs (lncRNAs)** are larger transcripts (>200nt in size) that are synthesized similarly to mRNAs, but not translated into protein. LncRNAs contain two types of functional element, the **interactor** elements involved in direct physical interaction with other nucleic acids, with proteins or lipids, and the **structural** elements, leading to the occurrence of secondary and/or tertiary 3D RNA structures, which direct their functional interactions.



Why are they useful therapeutically ?

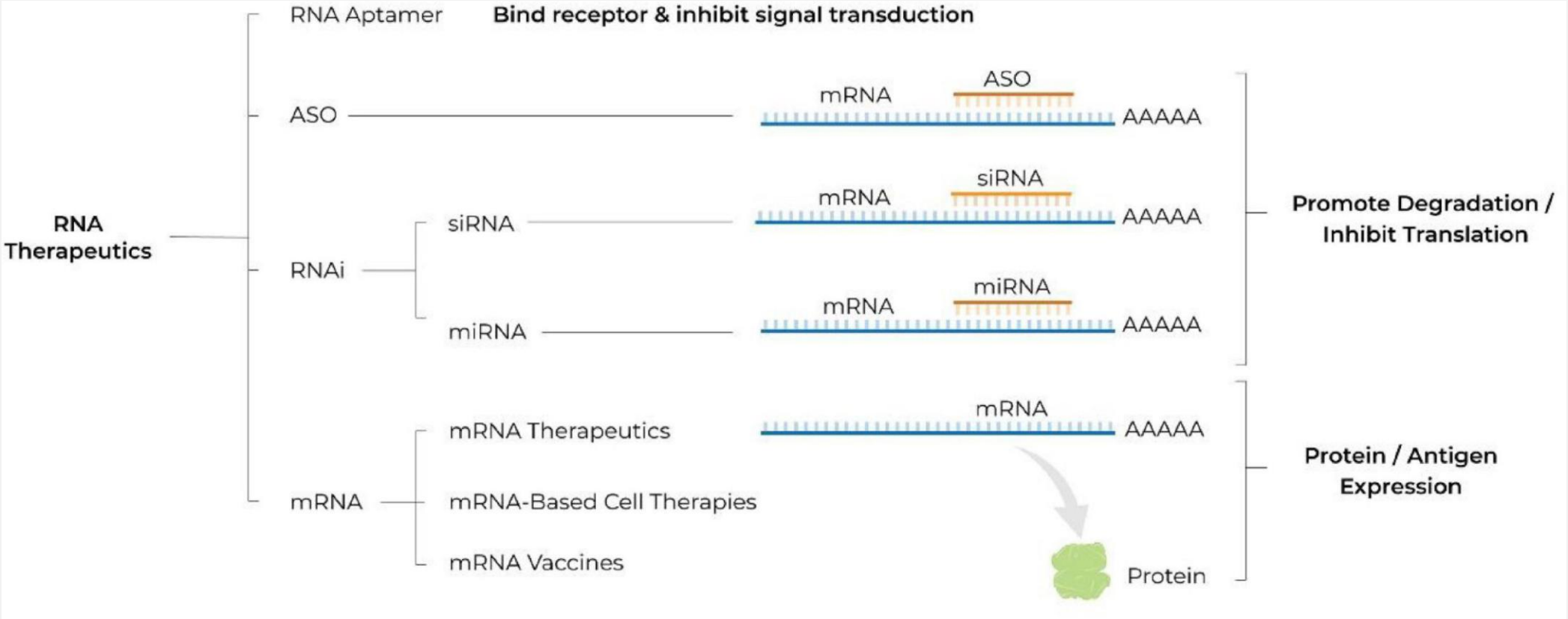


The use of miRNA-based therapeutics has dual advantages.

- **First**, miRNAs are naturally occurring molecules in human cells, unlike man-made chemotherapy compounds or ASOs, and therefore have all the mechanisms in place for their processing and downstream target selection.
- **Second**, miRNAs act by targeting multiple genes within one pathway, thus causing a broader yet specific response.

- LncRNA targeting may include transcriptional inhibition, post-transcriptional inhibition, steric hindrance of secondary structure formation or protein interactions, introduction of synthetic (for example, circular) lncRNAs,
- An interesting development is the exploration of natural antisense transcripts (NATs): lncRNAs that are transcribed in the antisense direction to coding genes, and negatively regulate them in cis.

Types of RNA-targeting therapeutics



ASOs are single-stranded DNA molecules with full complementarity to one select target mRNA and may act by blocking protein translation (via steric hindrance), causing mRNA degradation (via RNase H cleavage).

Small interfering RNAs (siRNAs) may be single or double stranded and exploit the endogenous miRNA pathway and mediate silencing of one, fully complementary mRNA via their loading into the RNA-induced silencing complex (RISC)

MiRNA mimics exploit the main advantage of endogenous miRNAs being able to target multiple mRNAs at once. miRNA mimics have the same sequence as an endogenous miRNA while the passenger strand carries a few mismatches to prevent RISC loading and potential action as an anti-microRNA (antimiR)

AntimiRs are essentially ASOs designed to be fully or partially complementary to an endogenous miRNA to prevent the interaction with its target genes. AntimiRs may also be referred to as 'antagomiRs' if they are conjugated to cholesterol to improve intracellular delivery

Short hairpin RNAs (shRNAs) exploit the miRNA maturation pathway, being cleaved by Dicer into a double-stranded mature product before RISC loading. ShRNAs are traditionally introduced into cells using viral vector systems such as adenovirus-associated viruses, retroviruses or lentiviruses.

Therapeutic	Type	Modification and delivery	Route of administration	Target organ	Disease	Target gene and/or pathway	Reason for termination	Ref.
Aganirsen (GS-101)	ASO	1st gen; PT	Topical	Eye	Ischaemic central retinal vein occlusion, neovascular glaucoma	Insulin receptor substrate 1 (*IRS1*) mRNA	Formulation stability issues	
Cobomarsen (MRG-106)	AntimiR	3rd gen; LNA	Subcutaneous or intravenous	Blood or lymphoid organs	Various lymphomas	miR-155	Non-safety or efficacy-related company decision	
PRO-040201 (TKM-ApoB, ApoB SNALP)	siRNA	Liposomal (stable nucleic acid lipid particle)	Intravenous	Liver	Hypercholesterol-aemia	Apolipoprotein B (*APOB*) mRNA	Potential for immune stimulation (flu-like symptoms)	267
AGN 211745 (AGN-745, siRNA-027)	siRNA	Chemical composition unclear; carrier-free	Intravitreal	Eye	Age-related macular degeneration, choroidal neovascularization	Vascular endothelial growth factor receptor 1 (*VEGFR1*) mRNA	Lack of clinical efficacy, TLR3 stimulation (sequence-independent TLR3-mediated therapeutic effect)	3
RG-101	AntimiR	GalNAc conjugated	Subcutaneous	Liver	Hepatitis C infection	miR-122	High levels of bilirubin in the blood	
MRX34	miRNA mimic	Liposomal	Intravenous or intratumour	Tumour	Primary liver cancer, advanced or metastatic cancer with or without liver involvement, haematological malignancies	miR-34a targetome	Immune-related adverse events	2
Oblimersen sodium (G3139, Genasense)	ASO	1st gen; PT	Subcutaneous	Tumour	Various malignancies	*BCL2* mRNA	Lack of clinical efficacy, insufficient delivery, primary end points not met	2
Suvodirsen (WVE-210201)	ASO	1st gen; PT, stereopure	Intravenous	Muscle	Duchenne muscular dystrophy	Dystrophin (*DMD*) pre-mRNA splicing (exon 51 skipping)	Lack of clinical efficacy, failure to increase dystrophin levels	
DCR-MYC (DCR-M1711)	siRNA	Liposomal	Intravenous	Tumour	Advanced solid tumours, multiple myeloma, lymphoma	*MYC* mRNA	Lack of clinical efficacy despite MYC reduction	
DCR-PH1	siRNA	Liposomal	Intravenous	Liver	Primary hyperoxaluria type 1 (PH1)	Lactate dehydrogenase A (*LDHA*) mRNA	Development shifted to GalNAc-conjugated variant (DCR-PHXC)	
Custirsen (ISIS 112989, OGX-011, TV-1011)	ASO	2nd gen; 2′-MOE gapmer	Intravenous	Tumour	Prostate cancer, breast cancer	Clusterin (*CLU*) mRNA	Lack of clinical efficacy, primary end points in phase III trials not met	268
Bevasiranib (Cand5)	siRNA	1st gen; PT	Intravitreal	Eye	Age-related macular degeneration, diabetic macular oedema	Vascular endothelial growth factor (*VEGF*) mRNA	Lack of clinical efficacy, TLR3 stimulation (sequence-independent TLR3-mediated therapeutic effect)	3
AEG35156 (AEG 161, GEM 640)	ASO	Mixed backbone oligonucleotides	Intravenous	Tumour	Various malignancies	X-linked inhibitor of apoptosis (*XIAP*) mRNA	Lack of clinical efficacy, increased incidence of chemotherapy-induced peripheral neuropathy	15
ISIS 329993 (ISIS-CRPRx)	ASO	2nd gen; 2′-MOE	Subcutaneous or intraperitoneal	Heart or joints	Paroxysmal atrial fibrillation, rheumatoid arthritis	C-reactive protein (*CRP*) mRNA	Lack of clinical efficacy despite CRP mRNA reduction	26

RNA therapeutics for which clinical development was halted

Main issues:

- Low efficiency
- Formulation and stability issues
- Tolerability and adverse reactions

The translation of RNA-based therapeutics into the clinic

The translation of all RNA-based therapeutics into the clinic has been hampered by issues associated with specificity, delivery and tolerability

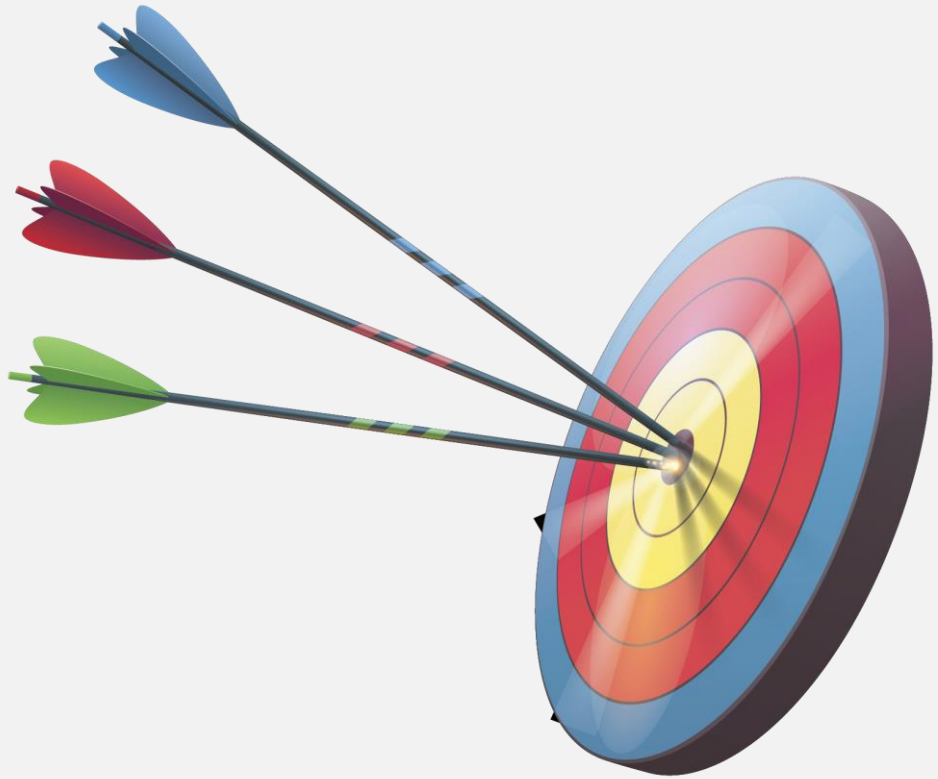
Specificity issues include undesired on-target effects due to uptake in cells other than the cells of interest, or off-target effects caused by either sequence similarities or overdosing to levels much higher than expected endogenously

Delivery issues are related to three major points: the instability of 'naked', chemically unmodified RNA structures; their inefficient intracellular delivery, which requires the exploitation of endosomal escape mechanisms; and the lack of delivery vehicles suitable for targeting the organ and cell type of interest.

Tolerability issues are caused by the recognition of RNA structures by pathogen-associated molecular pattern (PAMP) receptors, such as Toll-like receptors (TLRs), leading to adverse immune effects.

The hurdle of specificity

The quality of an RNA therapeutic is determined by the strength of its on-target specificity as well as the absence of off-target and undesired on-target effects.



The hurdle of specificity

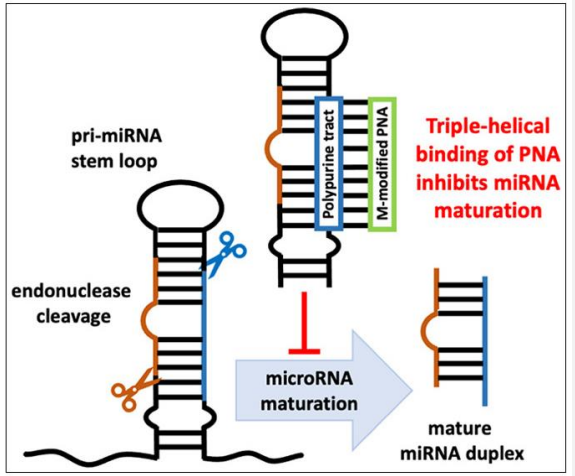
Cell-specific miRNA modulation

Using a suitable vector under the control of a specific promoter, which is overexpressed in the cells of interest/

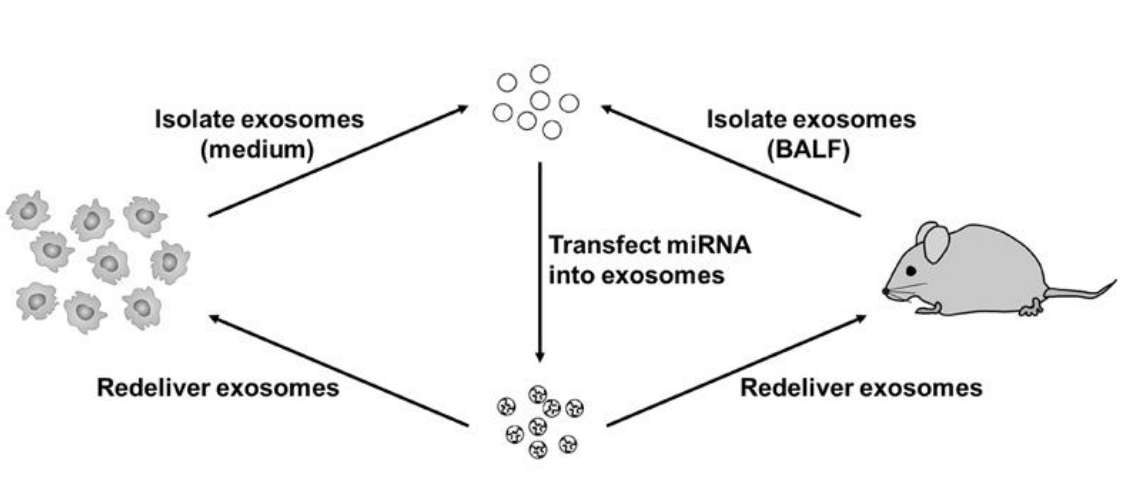
ex. *For instance, MYCN is a well-known driving oncogene in neuroblastoma. Therefore, more selective expression of a therapeutic miRNA in neuroblastoma cells could be achieved by cloning the miRNA into a vector under the promoter for MYCN.*

Targeting precursor miRNAs.

Peptide nucleic acid (PNA) oligomers that target pre-miRNA are exploited to inhibit miRNA maturation. PNAs bind to their RNA targets with high specificity as even a single mismatch strongly influences the association constant of PNA–RNA interactions.



Ex vivo manipulations.



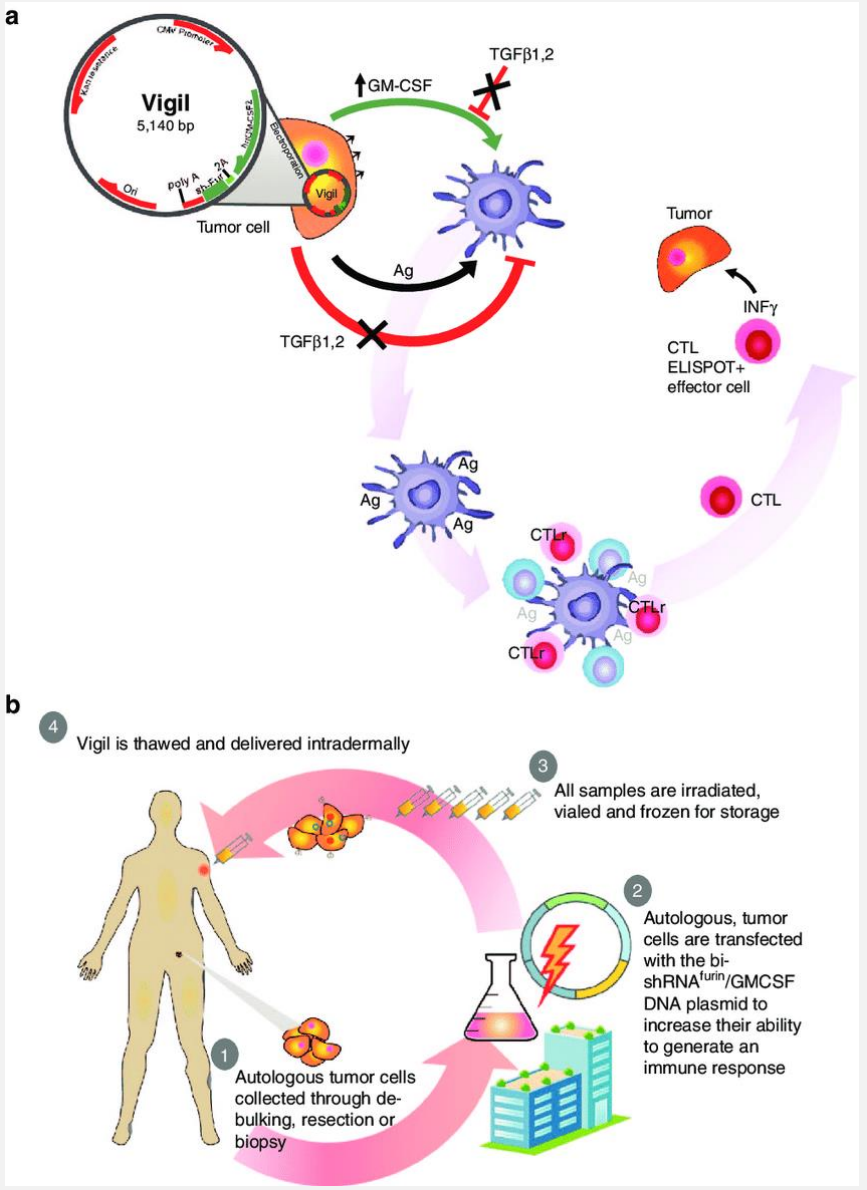
The hurdle of specificity

Ex vivo manipulations.
Ex vivo manipulations may be more appreciated after successes such as the use of chimeric antigen receptor (CAR) T cells and with new possibilities offered by the CRISPR–Cas9 system

The autologous tumour cell immunotherapeutic Vigil has shown promising results in advanced malignancies in phase II and III clinical trials.

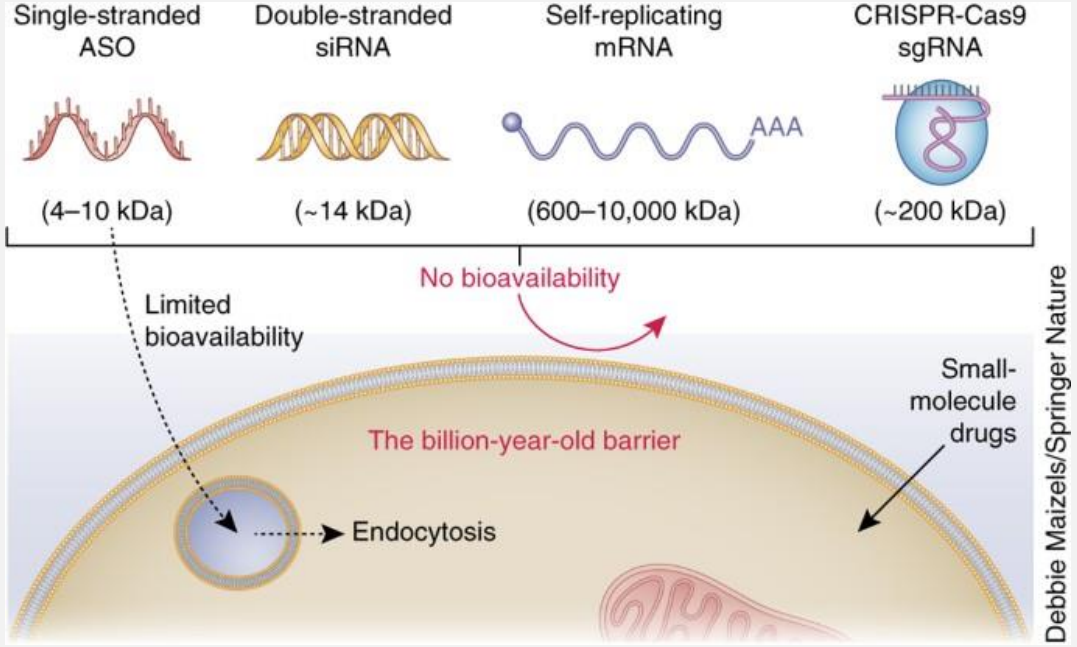
Vigil is generated from the patient's tumour cells modified to express recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF) and a bifunctional shRNA that targets furin.

Furin inhibition results in downregulation of immunosuppressive cytokines TGFβ1 and TGFβ2, thereby supporting T cell responses in the tumour.



The hurdle of delivery

Efficient delivery of RNA therapeutics not only to the organ and cell type of interest but also across the cell membrane to perform their intracellular functions is one of the greatest challenges in the field.



Feature	Challenge for delivery
Oligonucleotide size and charge	Too large or too charged to passively diffuse across the lipid bilayer
RNase susceptibility	Rapid degradation by blood and tissue RNases.
Reticuloendothelial system	Rapid clearance from the blood by the kidneys and liver scavenger receptors
Immunogenicity	Oligonucleotides activate extracellular and intracellular innate immune responses
Endocytosis	Oligonucleotides are taken up, but trapped inside endosomes

First steps, know what to modify

As RNA therapeutics are naturally unstable and unable to cross cell membranes owing to their negative charge, various chemical modifications are applied to improve their pharmacokinetics and pharmacodynamics

First-generation modifications

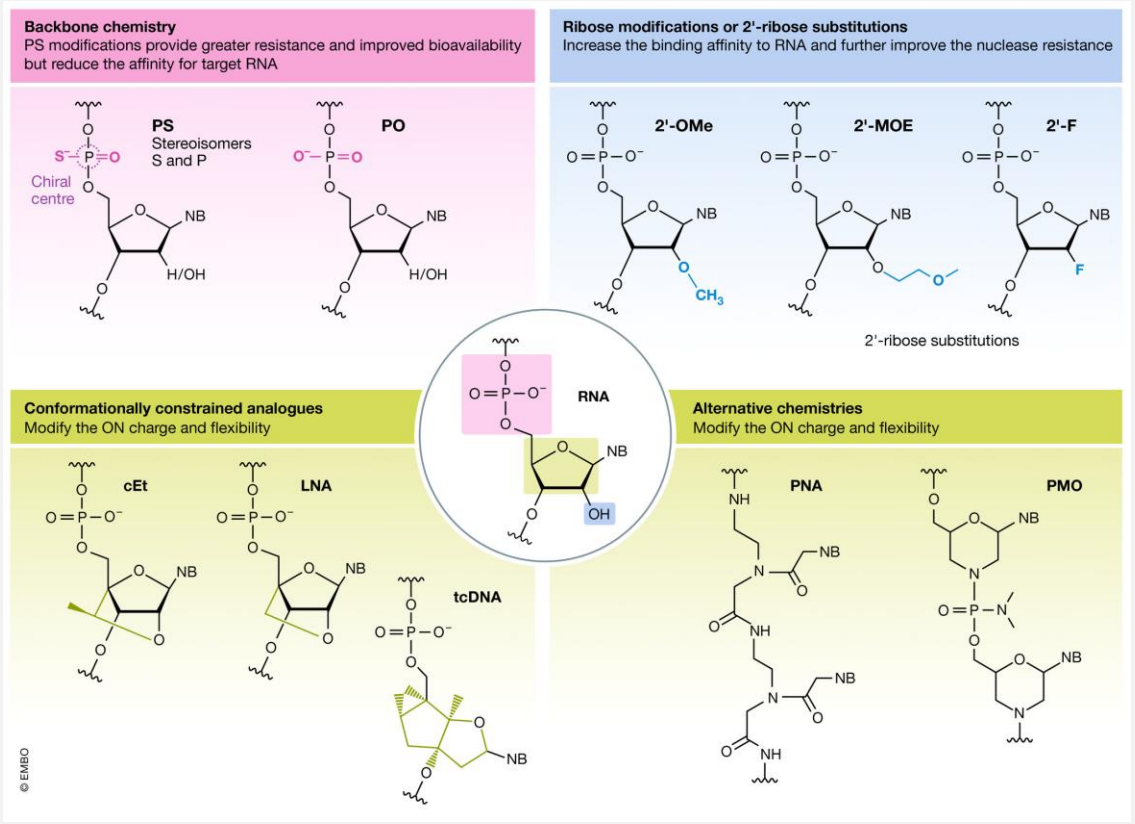
- Replace phosphodiester with phosphothiorate (PT) backbone linkages to improve stability.

Second generation modifications

- Replace the 2′-O-alkyl group of the sugar moieties with, for example, 2′-O-Me, 2′-MOE or 2′-F to improve bioavailability, enhance efficacy and reduce toxicity and immunostimulation.

Third-generation modifications

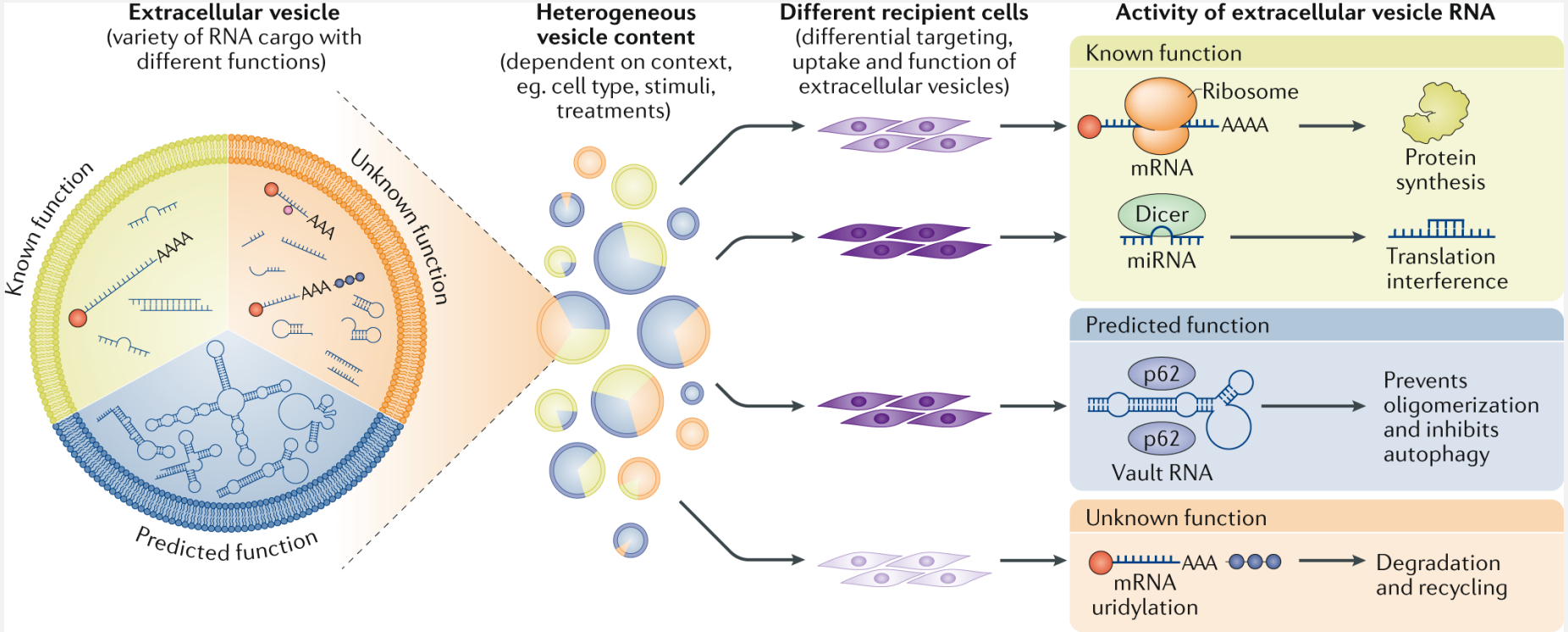
- Apply changes to the furanose ring to create, for example, locked nucleic acids (LNAs), peptide nucleic acids (PNAs) and phosphoramidate morpholino oligomers (PMOs).



The hurdle of delivery

Exosome-mediated miRNA delivery.

Exosomes are considered natural carriers of miRNAs and may present an ideal delivery system owing to their negligible antigenicity, minimal cytotoxicity and their ability to bypass the endocytic pathway and circumvent phagocytosis.

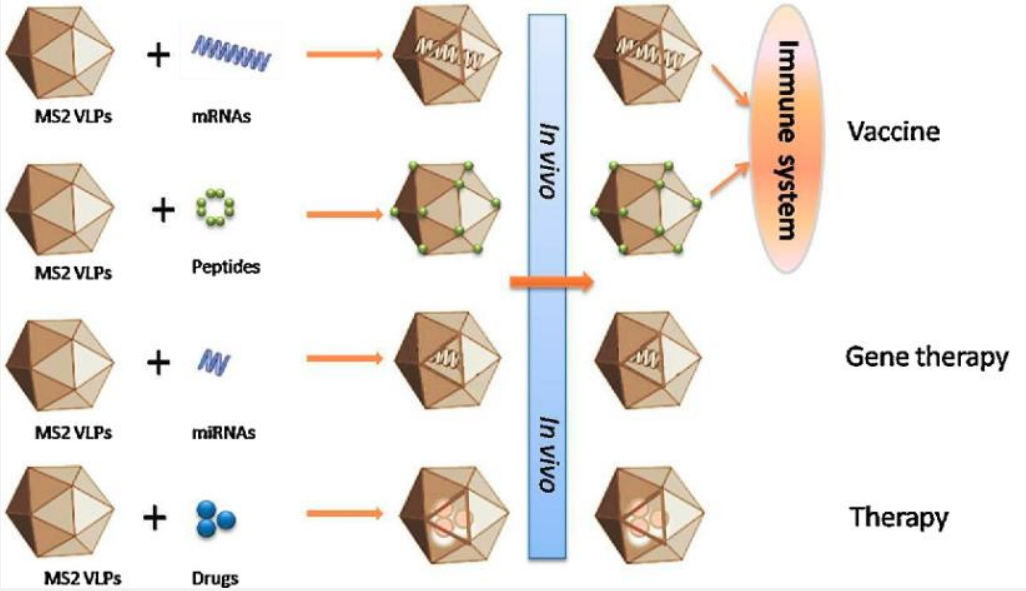


From: [RNA delivery by extracellular vesicles in mammalian cells and its applications]

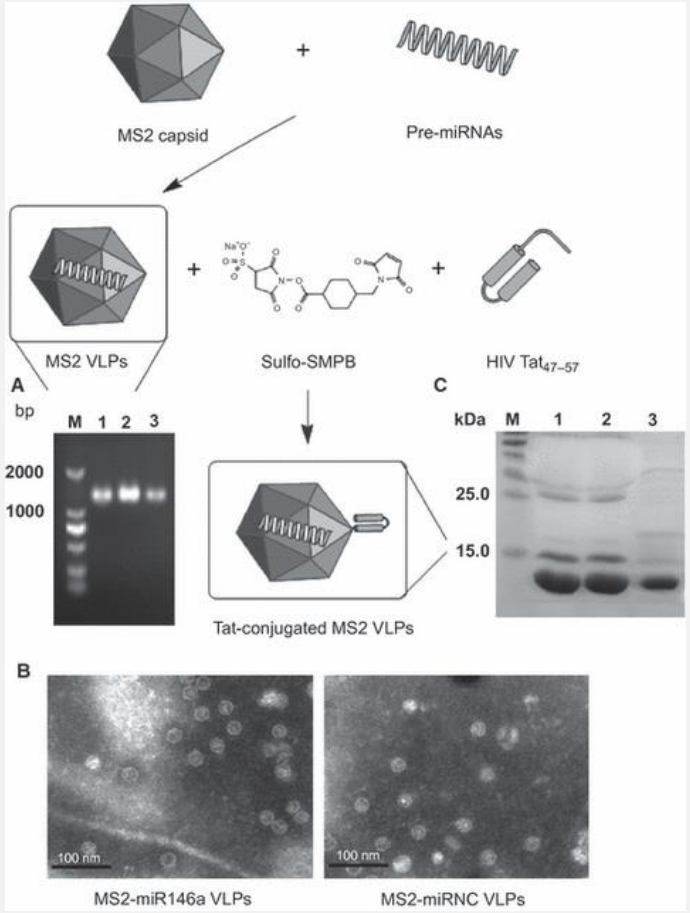
The hurdle of delivery

Bacteriophage and bacterial minicell delivery vehicles

Several studies have reported bacteriophages as a safe and effective delivery vehicle that can be loaded with RNAs or other drugs. For example, modification of MS2 bacteriophages to be used as virus-like particles (VLPs) could protect miRNAs against degradation.



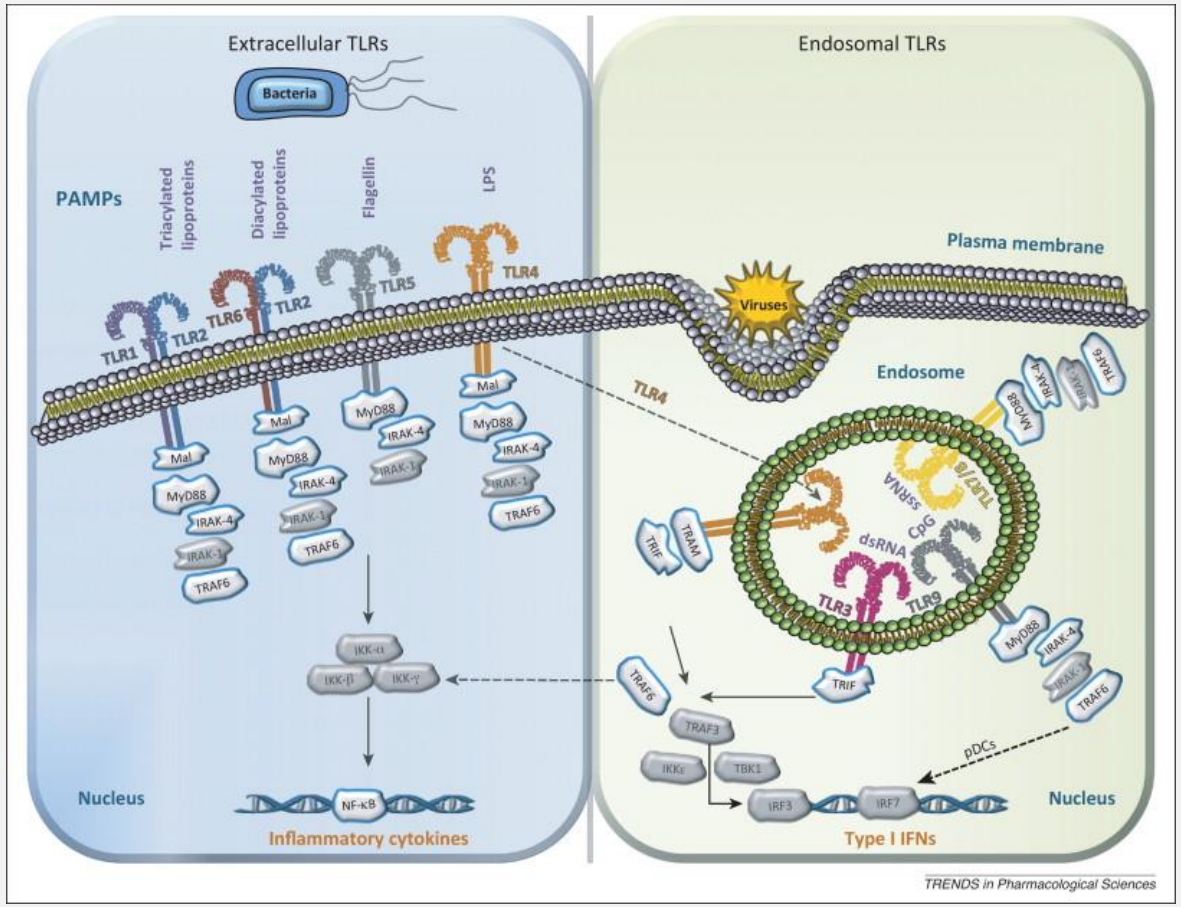
VLPs are nanoparticles devoid of viral genetic material and can self-assemble from the coat protein into an icosahedral capsid.



Self-assembly of MS2 by interaction between a specific MS2 cistron, a 19 nt sequence known as a pac site, and the bacteriophage coat protein allows the bacteriophage particles to be loaded with RNAs or other molecules.

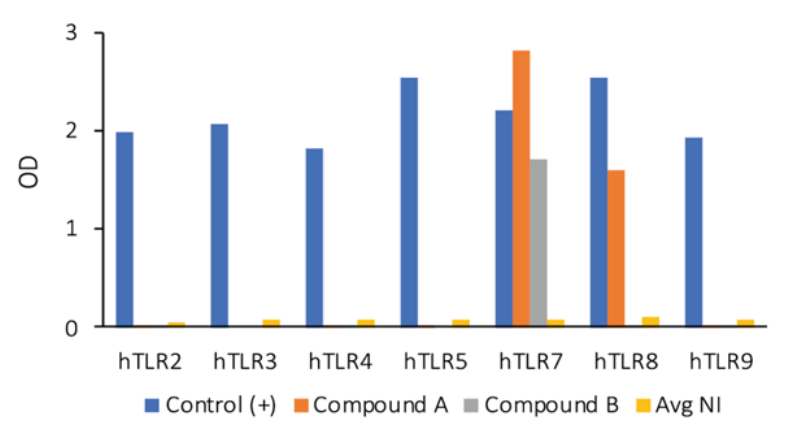
The hurdle of tolerability/immunogenicity

As a viral defence mechanism, our immune system recognizes both single-stranded (ss) and double-stranded (ds) RNA via diverse extra- and intracellular PAMP receptors.

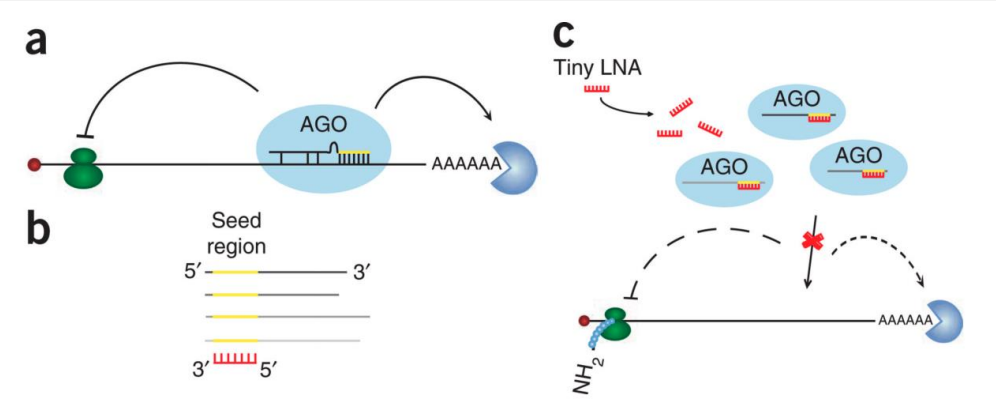


The predominant pathway recognizing RNA therapeutics is TLR signalling, which is mediated through myeloid differentiation factor 88 (MyD88) and activates various pathways, resulting in nuclear factor-κB (NF-κB) activation and the production of pro-inflammatory cytokines and type I interferon response.

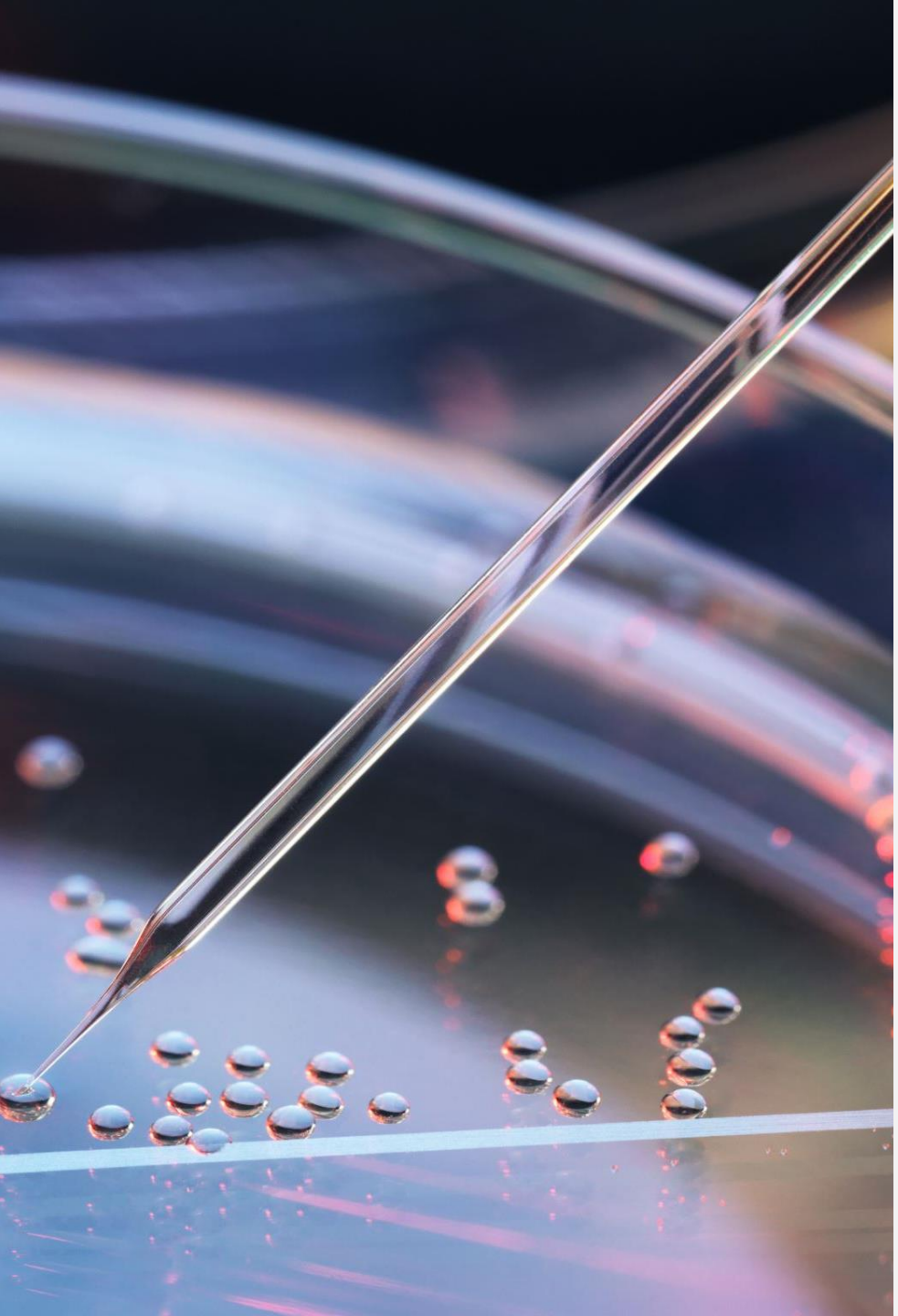
POTENTIAL SOLUTIONS



- **Expanding immune-related adverse reaction screening with TLR interaction methods.**
  - Not all miRNAs and miRNA therapeutics similarly induce immunogenicity, and the apparent difficulty of predicting such responses prompts the use of broader screening methods in preclinical studies with appropriate models.



- **Using ‘tiny’ antisense RNAs**
  - As efficient activation of TLRs requires a length of at least 21 nucleotides for ssRNA, the design of smaller RNA therapeutics could circumvent the issue. Locked nucleic acid (LNA)-modified antimiRs with a short sequence of 7–8 nucleotides, termed ‘tiny’ LNAs, target the 5′-seed region of miRNAs and can enable antagonism and inhibition of an entire miRNA family sharing that seed sequence.



Modifications at clinical level

- **Applying metronomic miRNA therapy.**
  - By analogy with metronomic chemotherapy, we define metronomic RNA therapy as regular and frequent administration of limited drug doses over a prolonged period, to achieve a low, but active, dose range without inducing excessive toxicity or immunogenicity.

- **Combinatorial RNA therapeutics.**
  - Combination therapies can also be used to reduce the required dose of RNA therapeutics and thus their immunogenicity, for example, if used in synergy with chemotherapy, radiotherapy, small-molecule-based or immunotherapies.

OK, BUT ARE THERE ANY IMPLEMENTED RNA THERAPEUTICS IN THE CLINIC NOW ?

RNA therapeutics approved by the FDA and/or the European Medicines Agency

Therapeutic	Type	Modification and delivery	Route of administration	Target organ	Disease	Target gene and pathway	FDA and/or EMA approval year
Fomivirsen (Vitravene)	21-mer ASO	1st gen; PT	Intravitreal	Eye	Cytomegalovirus (CMV) retinitis in immunocompromised patients	CMV IE-2 mRNA	1998 (FDA), 1999 (EMA)[a]
Mipomersen (Kynamro)	20-mer ASO	2nd gen; 2′-MOE gapmer	Subcutaneous	Liver	Homozygous familial hypercholesterolaemia	Apolipoprotein B mRNA	2012 (EMA), 2013 (FDA)
Nusinersen (Spinraza, ASO-10-27)	18-mer ASO	2nd gen; 2′-MOE	Intrathecal	Central nervous system	Spinal muscular atrophy	Survival of motor neuron 2 (*SMN2*) pre-mRNA splicing (exon 7 inclusion)	2017 (EMA), 2016 (FDA)
Eteplirsen (Exondys 51)	30-mer ASO	3rd gen; 2′-MOE PMO	Intravenous	Muscle	Duchenne muscular dystrophy	Dystrophin (*DMD*) pre-mRNA splicing (exon 51 skipping)	2016 (FDA)
Inotersen (Tegsedi, AKCEA-TTR-LRx)	20-mer ASO	2nd gen; 2′-MOE; GalNAc-conjugated	Subcutaneous	Liver	Hereditary transthyretin amyloidosis	Transthyretin (*TTR*) mRNA	2018 (EMA), 2018 (FDA)
Patisiran (Onpattro)	21 nt ds-siRNA	2nd gen; 2′-F/2′-O-Me; liposomal	Intravenous	Liver	Hereditary transthyretin amyloidosis	Transthyretin (*TTR*) mRNA	2018 (EMA), 2019 (FDA)
Golodirsen (Vyondys 53, SRP-4053)	25-mer ASO	3rd gen; 2′-MOE PMO	Intravenous	Muscle	Duchenne muscular dystrophy	*DMD* pre-mRNA splicing (exon 53 skipping)	2019 (FDA)
Givosiran (Givlaari)	21 nt ds-siRNA	2nd gen; 2′-F/2′-O-Me; GalNAc-conjugated	Subcutaneous	Liver	Acute hepatic porphyria	Delta aminolevulinic acid synthase 1 (*ALAS1*) mRNA	2020 (EMA), 2019 (FDA)
Viltolarsen (Viltepso, NS-065, NCNP-01)	21-mer ASO	3rd gen; 2′-MOE PMO	Intravenous	Muscle	Duchenne muscular dystrophy	*DMD* pre-mRNA splicing (exon 53 skipping)	2020 (FDA)
Volanesorsen (Waylivra)	20-mer ASO	2nd gen; 2′-MOE gapmer	Subcutaneous	Liver	Familial chylomicronaemia syndrome	Apolipoprotein CIII (*APOC3*) mRNA	2019 (EMA)
Inclisiran (Leqvio, ALN-PCSsc)	22 nt ds-siRNA	2nd gen; 2′-F/2′-O-Me; GalNAc-conjugated	Subcutaneous	Liver	Atherosclerotic cardiovascular disease, elevated cholesterol, homozygous/ heterozygous familial hypercholesterolaemia	Proprotein convertase subtilisin/kexin type 9 (*PCSK9*) mRNA	2020 (EMA)
Lumasiran (Oxlumo, ALN-GO1)	21 nt ds-siRNA	2nd gen; 2′-F/2′-O-Me; GalNAc-conjugated	Subcutaneous	Liver	Primary hyperoxaluria type 1	Hydroxyacid oxidase 1 (*HAO1*) mRNA	2020 (EMA), 2020 (FDA)

Currently, 12 RNA-based therapeutics are approved by the FDA and/or the European Medicines Agency (EMA) aiming at gene modifications in liver, muscle or the central nervous system.

All these therapeutics are either siRNAs or ASOs that cause specific gene downregulation, or ASOs that target pre-mRNA splicing (that is, inducing exon skipping or inclusion).

RNA therapeutics in phase II or III clinical development

A plethora of RNA therapeutics are in phase II or III clinical development, including newer entities such as miRNA mimics and antimiRs, but no lncRNA-based therapeutics have entered the clinic.

Therapeutic	Type	Modification and delivery	Route of administration	Target organ	Disease	Target gene and pathway	Phase	Identifier
rAAV5-miHTT (AMT-130)	Pri-miR-451 backbone	Adeno-associated viral vector (AAV5)	Intrastriatal	Brain	Huntington disease	Huntingtin (*HTT*) mRNA	I/II	NCT04120493
WVE-120102	ASO (allele-selective)	Stereopure ASO	Intrathecal	Brain	Huntington disease	U-variant of SNP rs362331 (SNP2) in *HTT* mRNA	I/II	NCT03225846, NCT04617860
RG-125 (AZD4076)	Anti-miR-103/107	GalNAc-conjugated antagomiR	Subcutaneous	Liver	Type II diabetes, nonalcoholic fatty liver disease	miR-103/107	I/II	NCT02612662, NCT02826525
Remlarsen (MRG-201)	miR-29 mimic	Cholesterol conjugated	Intradermal	Skin	Keloid (pathological fibrosis)	miR-29 targetome	II	NCT02603224, NCT03601052
siG12D-LODER	siRNA	Biodegradable polymeric matrix (PLGA)	Intratumoral	Tumour	Advanced pancreatic cancer	G12D-mutated *KRAS* mRNA	II	NCT01188785; NCT01676259
Prexigebersen (BP1001-A)	ASO	Liposomal	Intravenous	Blood and/or immune cells	Acute myeloid leukaemia, chronic myeloid leukaemia	*GRB2* mRNA	II	NCT01159028; NCT04196257; NCT02781883
Olpasiran (AMG 890, ARO-LPA)	siRNA	GalNAc conjugated	Subcutaneous	Liver	Cardiovascular disease	Apolipoprotein A (*LPA*) mRNA	II	NCT03626662, NCT04270760
Vupanorsen (AKCEA-ANGPTL3-LRx)	ASO	GalNAc conjugated	Subcutaneous	Liver	Dyslipidaemias, hyperlipidaemias, hyperlipoproteinaemias	Angiopoietin-like 3 (*ANGPTL3*) mRNA	II	NCT04459767, NCT03371355, NCT04516291
Miravirsen (SPC3649)	Anti-miR-122	PS-β-D-oxy-LNA gapmer ODN	Subcutaneous	Liver	Hepatitis C virus infection	miR-122	II	NCT01646489, NCT01727934, NCT01872936, NCT01200420
Donidalorsen (IONIS-PKK-LRx, ISIS 721744)	ASO	GalNAc-conjugated PS-2′-MOE ODN	Subcutaneous	Liver	Hereditary angio-oedema, COVID-19	Prekallikrein (*PKK*) mRNA	II	NCT03263507, NCT04030598, NCT04307381, NCT04549922
BMT 101 (cp-asiRNA)	Cell-penetrating asymmetrical siRNA	Carrier-free	Intradermal	Skin	Hypertrophic scar	Connective tissue growth factor (*CTGF*) mRNA	II	NCT03133130, NCT04012099
Danvatirsen (IONIS-STAT3-2.5Rx, AZD9150)	ASO	GalNAc conjugated	Intravenous	Tumour	Metastatic NSCLC, resectable early-stage NSCLC, pancreatic cancer, mismatch repair-deficient colorectal cancer	*STAT3* mRNA	II	NCT03819465, NCT03794544, NCT02983578
Bamosiran (SYL040012)	siRNA	Carrier-free	Topical	Eye	Ocular hypertension, glaucoma	β-Adrenergic receptor 2 (*ADRB2*) mRNA	II	NCT00990743, NCT01227291, NCT01739244, NCT02250612
Cemdisiran (ALN-CC5)	siRNA	GalNAc conjugated	Subcutaneous	Blood	Paroxysmal nocturnal haemoglobinuria, IgA nephropathy, Berger disease, glomerulonephritis	Complement 5 mRNA	II	NCT04601844, NCT02352493, NCT03841448, NCT03999840
Apatorsen (OGX-427)	ASO	2′-O-MOE-PTO gapmer	Intravenous	Tumour	Squamous cell lung cancer, non-squamous NSCLC, urological neoplasms, metastatic bladder cancer, urinary tract neoplasms, castration-resistant prostate cancer	*HSP27* mRNA	II	NCT01120470, NCT01454089, NCT01829113, NCT02423590

FUTURE PERSPECTIVES AND OUTLOOK

- Although their delivery to target organs and their efficient introduction into cells remain challenging, the many advantages of RNA drugs make the development of these technologies a worthwhile investment.
- Inspired by the development of various novel RNA drugs, including the novel COVID-19 vaccines in 2020, many researchers are making unprecedented efforts to develop new RNA-based drugs.

THANK YOU FOR YOUR ATTENTION !