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NUCLEIC ACID INSIGHTS

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Pre-clinical tools and technologies

NUCLEIC ACID INSIGHTS

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INDUSTRY UPDATE

From lab to clinic: the evolving translational landscape of nucleic acid therapeutics

Nucleic Acid Insights is delighted to present the latest updates in the nucleic acids field through this new Industry Update, which highlights recent preclinical and translational R&D advances across oligonucleotides, mRNA, DNA therapeutics, as well as associated formulation and delivery technologies.

This month we have insights from industry experts Jimmy Weterings (Vice President, Head of Oligonucleotide Therapeutics, Bonito Biosciences), Piotr Kowalski (Senior Lecturer in Advanced Therapies, University College Cork), Christian Ottensmeier (Professor of Immuno-oncology, University of Liverpool), and John Lewis (Chief Executive Officer, Entos Pharmaceuticals).

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INTRODUCTION

The nucleic acids field is rapidly expanding owing to clinical breakthroughs in mRNA, oligonucleotides, and DNA therapeutics, alongside innovations in formulation and delivery. In 2024, the global market size of the nucleic acid therapeutics sector reached $2,540.9 million and is predicted to reach $8,688.2 million by 2034 [1].

mRNA technology came into the spotlight during the COVID-19 pandemic, and is now being adapted for infectious disease, oncology, and protein replacement therapies. Oligonucleotides, including antisense (ASOs), small interfering RNA (siRNA), and aptamers, are maturing due to improved stability, potency, and tissue targeting. DNA-based therapeutics are also progressing toward greater safety and durability of expression.

Despite these advances, there are still hurdles that must be addressed in order for successful transition from laboratory to clinic. Preclinical and translational research is primarily focused on overcoming barriers to intracellular delivery, expanding extrahepatic targeting, and improving manufacturability.

This article highlights recent preclinical and translational advances in nucleic acid therapeutics, and efforts to address hurdles in targeted delivery, stability, and toxicity.

OLIGONUCLEOTIDES: CLINICAL PROGRESS AND DELIVERY INNOVATIONS

Jimmy Weterings, Vice President, Head of Oligonucleotide Therapeutics, Bonito Biosciences

"The oligonucleotide industry is as vibrant as ever. We see an increased expansion of early-stage oligonucleotide therapeutics in new tissues and indications, most particularly into CNS diseases and obesity, using a wider range of delivery vehicles. Looking forward to the day all tissues are unlocked!"

Oligonucleotide-based therapies are quickly advancing, with nearly 300 clinical trials underway across various countries [2]. ASOs, siRNAs, and other oligonucleotide therapies are benefiting from refined chemistries and novel conjugates that enhance their potency and extrahepatic delivery.

The preclinical and translational R&D field for oligonucleotide therapies mainly focuses on overcoming delivery and stability challenges. Several candidates, such as ASOs targeting neurodegenerative diseases, are already progressing toward early-phase clinical trials [3].

For example, in July 2025, Biogen and Stoke Therapeutics presented data on zorevunersen, an investigational ASO treatment for Dravet syndrome [4]. Results from Phase 1/2a and open-label extension studies showed significant and sustained reductions in major motor seizure frequency when added to standard care. The data also demonstrated improvements in cognition and behavior, supporting zorevunersen's potential as a disease-modifying therapy for Dravet syndrome.

Similarly, Aicuris has recently presented pharmacokinetic data from the first-in-human Phase 1 trial of AIC468, an ASO targeting the BK virus. The trial involving healthy volunteers showed AIC468 was well tolerated with favorable absorption and distribution [5].

With nearly 2,170 therapies in development, the oligonucleotide therapeutics field is rapidly expanding, but challenges such as tissue-specific delivery, off-target effects, and high manufacturing costs must be addressed to further advance the space [6].

mRNA THERAPEUTICS: ADVANCING BEYOND VACCINES

Piotr Kowalski, Senior Lecturer in Advanced Therapies, University College Cork

"Despite recent challenges in advancing prophylactic mRNA vaccines, therapeutic applications of mRNA continue to demonstrate strong progress, particularly in gene editing and cancer therapeutics. Novel modalities, including circular and self-amplifying mRNAs, are broadening the scope of mRNA technology and opening new therapeutic avenues."

Beyond vaccines for infectious diseases, mRNA platforms are advancing in oncology, rare diseases, central nervous system disorders, and other indications [7]. Major advances in computational design, novel RNA scaffolds, targeted delivery, and safety profiling are driving progress toward broader clinical impact in 2025 and beyond [7].

For instance, a study published in 2025 identified phosphoserine aminotransferase 1 (PSAT1) as a key factor in cardiac repair following myocardial infarction (MI) [8]. Researchers administered synthetic PSAT1-modified mRNA to mice post-MI, resulting in significant cardiomyocyte proliferation, reduced scar formation, and improved cardiac function. These findings suggest that PSAT1 could be a promising target for mRNA-based therapies in ischemic heart diseases.

Additionally, in 2025, mRNA-based therapies continued to advance through the clinical pipeline, with several candidates entering mid-stage trials. For instance, Ethris has recently started a Phase 2a clinical trial for ETH47, an mRNA-based antiviral therapy aimed at preventing asthma exacerbations caused by respiratory viruses [9]. The study will enrol 50 adult asthma patients who will receive ETH47 or a placebo following a controlled rhinovirus challenge.

However, despite the clinical progress, there are still challenges in the mRNA therapeutics field that must be addressed, such as improving delivery efficiency and stability, and managing the potential immunogenicity of mRNA to avoid adverse immune reactions [7]. Advances in lipid nanoparticle (LNP) technology and chemical modifications are helping to overcome these barriers.

EXPLORING DNA VACCINE PIPELINES

Christian Ottensmeier, Professor of Immuno-oncology, University of Liverpool

"DNA-based immunotherapeutics / DNA vaccines are beginning to deliver real clinical impact in numerous pre-malignant and malignant disease settings. In HPV-driven respiratory papillomatosis, the DNA vaccine developed by Inovio has just been granted accelerated approval by the US FDA. Additionally, a DNA-based personalised cancer vaccine has delivered meaningful clinical benefit in patients with hepatocellular carcinoma in a trial reported by Geneos; in this setting, immunotherapy had, by and large, failed to impact outcome to date prior to this trial. It is clear, therefore, that DNA as a platform for the delivery of antigens to improve immunotherapy is taking its place in the rapidly changing field of cancer immunotherapy."

Beyond oligonucleotide and mRNA-based therapeutics, DNA vaccine pipelines are also expanding and diversifying, with improved vector engineering, synthetic DNA designs, and safer integration strategies driving broader clinical translation.

In April 2025, NHS England announced that it is fast-tracking advanced melanoma patients for the iSCIB1+ cancer vaccine trial, part of the Cancer Vaccine Launch Pad (CVLP) program [10]. The needle-free DNA vaccine aims to enhance the immune system's ability to better target melanoma cells and improve immunotherapy outcomes. Patient referrals have started in May 2025, and the SCOPE Phase II trial will expand across multiple NHS sites this year. The CVLP aims to offer personalized cancer vaccines to 10,000 patients by 2030.

Therapeutic DNA vaccines are also gaining traction in indications beyond oncology, reflecting broader clinical potential. For example, PharmaJet and Immuno Cure have recently partnered to advance ICVAX, an HIV-1 therapeutic DNA vaccine, utilizing PharmaJet's Tropis® needle-free intradermal delivery system [11]. Following a successful Phase 1 trial demonstrating excellent safety and promising immune responses, this collaboration aims to improve vaccine efficacy by targeting immune cells in the skin.

In summary, DNA vaccines have become a key strategy for preventing and treating contemporary biomedical diseases, yet, similarly to oligonucleotides and mRNA, developing an effective delivery system continues to be the primary challenge in this field [12].

ADVANCES IN FORMULATION AND DELIVERY

John Lewis, Chief Executive Officer,
Entos Pharmaceuticals

"Genetic medicine is approaching a pivotal inflection point. While recent advances in molecular biology have enabled us to target the root cause of disease with unprecedented control and accuracy, a critical bottleneck remains: the safe, efficient, and tissue-specific delivery of nucleic acid-based therapeutics. Current clinically approved delivery platforms present inherent limitations, including immunogenicity, toxicity, limited tissue tropism, and challenges with redosing. To fully harness the therapeutic potential of genetic medicines, the field must advance next-generation delivery systems that are non-immunogenic, redosable, and capable of precise biodistribution across diverse cell types and tissues."

LNPs, polymeric carriers, and ligand-conjugated systems are evolving to overcome nucleic acid delivery bottlenecks, achieve tissue-specific targeting, and enable repeat dosing with reduced immunogenicity. Many companies are exploring different lipid chemistries and novel technologies such as artificial intelligence (AI) to design delivery systems that target extrahepatic tissues.

In July 2025, Etherna introduced a novel bio-reducible LNP platform designed to efficiently deliver mRNA to bone marrow stem cells and T cells. This delivery system aims to address existing bottlenecks in targeting extra-hepatic tissues, enhancing the potential of mRNA therapeutics for hematological diseases. The platform offers improved delivery efficiency with reduced toxicity. Etherna plans to further develop and apply this technology in clinical programs targeting blood and immune disorders.

Furthermore, METiS Technologies recently unveiled its AiLNP platform, an AI-driven system designed to optimize LNP formulations for nucleic acid delivery [13]. The platform utilizes a de novo lipid generation approach, enabling the development of LNPs with improved delivery efficiency and reduced toxicity profiles. AiLNP aims to advance the design of LNPs for mRNA and gene therapies, addressing challenges in targeting extra-hepatic tissues and enhancing therapeutic outcomes. METiS plans to integrate AiLNP into its development pipeline to accelerate the creation of next-generation LNP formulations.

SUMMARY

The nucleic acid therapeutics field is advancing rapidly, driven by breakthroughs in molecular design, targeted delivery, and growing clinical momentum across diverse disease areas. Oligonucleotides and mRNA therapies are expanding into new indications with improved chemistries and delivery systems, while DNA vaccines are gaining traction in oncology. Despite all this progress, challenges such as achieving consistent extrahepatic delivery, minimizing immune responses, and scaling up manufacturing without compromising stability still exist. However, ongoing innovation in lipid chemistry, formulation strategies, and data-driven design continues to push the field forward, bringing nucleic acid therapeutics closer to broader clinical impact

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EVENT PREVIEW

Biologics CDMO Europe 2025

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As part of our ongoing coverage of key gatherings in life sciences, BioInsights presents a preview of Biologics CDMO Europe 2025. Scheduled for November 19–20, 2025, in Munich, Germany, this summit will unite up to 300 senior manufacturing and external supply-chain experts from across Europe. Focusing on agile, tech-enabled biologics manufacturing, regulatory alignment, and strategic CDMO partnerships, the agenda features off-the-record case studies, executive roundtables, and deep-dive sessions.

OUTSOURCING STRATEGY AND CDMO PARTNER EVALUATION

A strategic panel including Suyamburam Sathasivam (Associate Vice President, SUN PHARMA), Ulrich Rümenapp (Head of Launch Preparation and Coordination, Bayer), and Daniel Hurni (Former Director of Manufacturing Network Strategy and Business Intelligence, Bristol Myers Squibb) will discuss outsourcing trends toward 2030. Additionally, Christopher Pawlak (External manufacturing Lead, Bayer) will outline practical tools for CDMO partner evaluation, while Andreas Schaaf (Managing Director/CSO, Eleva) will highlight innovations in biomanufacturing technologies. Key sessions will also explore risk allocation in CDMO agreements and resilient partnership models, setting a collaborative tone for navigating Europe's evolving biologics landscape.

TECH TRANSFER AND GLOBAL REGULATORY HARMONIZATION

The summit will also focus on tech transfer and regulatory compliance for advanced therapies. Christian Simon (Head of Technical Transfer External Manufacturing, Sanofi) will explore how AI-driven predictive maintenance can reduce downtime and improve equipment performance. Jenny Prange (CTO, Muvon Therapeutics) will present strategies for navigating tech transfer in regenerative therapies. Furthermore, a panel on global regulatory harmonization will follow, featuring Pavan Beleyur Narayanaswamy (Head of CMC and Regulatory Affairs, AATec Medical) and Eoin McGrath (Executive Director, ICCBBA).

COST OPTIMIZATION AND EVOLVING CONTRACT MODELS

Ulrich Rümenapp (Head of Launch Preparation and Coordination, Bayer) will address strategic approaches to outsourcing CMC development and manufacturing, including IP protection and building effective CDMO partnerships. Giulio Cavalli (Principal Lead, External Manufacturing, Johnson & Johnson Innovative Medicine) will share best practices for managing cross-border tech transfers in a globalized production landscape. The summit will also include a panel discussion on the evolution of contract models in biomanufacturing, featuring Ralf Huss (Managing Director, Biom Biotech Cluster) and Chris Baldwin (Vice President, Manufacturing and Supply, Resolution Therapeutics), who will explore shifting trends and collaborative opportunities in outsourcing agreements.

Biologics CDMO Europe 2025 will convene key stakeholders from across the biologics manufacturing landscape to address the most pressing challenges and innovations shaping the industry, from evaluating CDMO capabilities and optimizing outsourcing strategies to simplifying tech transfer and scaling single-use technologies.

As a reader of the BioInsights journals, you're entitled to a **15% discount** on delegate tickets—just use the code **CDMO-Insights**! You can find out more about the Biologics CDMO Europe 2025 events **here**.

To learn about other events coming up in your field, you can find our online Events Calendars here:

Nucleic Acid Insights, ***Bioconjugation Insights***, ***Cell & Gene Therapy Insights***, and ***Vaccine Insights***

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EXPERT INSIGHT

From concept to clinic: the therapeutic journey of small activating RNAs

Vera Huang and Long-Cheng Li

Small activating RNAs (saRNAs) represent a novel class of oligonucleotide therapeutics capable of selectively upregulating gene expression by harnessing an evolutionarily conserved RNA-mediated transcriptional activation mechanism termed RNA activation (RNAa). Building on two decades of foundational work, initially conceptualized as a molecular tool to induce gene expression, saRNAs have successfully translated into clinical development, offering a unique therapeutic strategy for diseases where targeted gene activation could be beneficial. This article provides a comprehensive overview of the 20-year therapeutic journey of saRNAs, from early discovery and preclinical validation to their current status in clinical trials. We discuss key preclinical and clinical programs, including those targeting tumor suppressor genes as well as those targeting neuromuscular and sickle cell diseases. Finally, we outline future directions for saRNAs, emphasizing their potential to reshape the oligotherapeutic landscape and expand therapeutic options for broader indications.

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HISTORICAL OVERVIEW OF SMALL ACTIVATING RNA (saRNA)

Small activating RNAs (saRNAs) were initially discovered by Li *et al.* at the University of California, San Francisco (UCSF) in 2006 [1] as a class of 19-21-mer double-stranded RNAs (dsRNAs) capable of upregulating gene expression. Shortly after, Janowski *et al.* at UT Southwestern [2] also demonstrated a similar transcriptional activation mechanism triggered by small duplex RNAs (agRNAs) in human cells. saRNAs target promoter regions and induce gene expression in a sequence-dependent manner via a natural and evolutionarily conserved mechanism termed RNA activation (RNAa), supported by evidence in humans, mice, and other lower species [3,4]. The pioneering study led by Li *et al.* demonstrated that saRNAs targeting the promoter regions of *E-cadherin* and $p21^{CIP1}$ resulted in increased mRNA levels of these genes in human cells. While saRNAs

share similarities with small interfering RNAs (siRNAs), such as the requirement for Argonaute proteins [5], saRNAs exhibit distinct kinetics and mechanisms of action. Transcriptional activation is accompanied by changes in chromatin structure, including increased markers of active histone modifications (e.g., H3K4me3), suggesting an epigenetic mechanism underlying RNAa [1,6]. The mechanism was further elucidated for the $p21^{CIP1}$ promoter in 2016 by Portnoy et al. [7], demonstrating that promoter-targeted saRNAs bind to Ago2, a core component of the RNA-induced transcriptional activation (RITA) complex, and direct the assembly of transcriptional co-factors—including RNA helicase A (RHA) and CTR9, a component of the PAF1 complex, in the nucleus—to facilitate the recruitment of RNA polymerase II and activation of gene expression. Subsequent studies by Voutila *et al.* [8], Zhao *et al.* [9], and Musiala-Kierklo *et al.* [10] further confirmed the essential role of CTR9 in RNAa using different model genes. The primary mode of action for saRNAs is dependent on the 5′end of antisense or guide strand binding to the intended target DNA within gene promoters in a seed-region dependent manner [11]. In the case of $p21^{CIP1}$ and MBNL1, studies have ruled out the involvement of promoter overlapping antisense transcripts as the potential molecular targets of saRNAs, suggesting that RNAa is acting directly on promoter DNA and associated chromatin complexes [7,10].

Analogous to synthetic saRNAs, Place *et al.* demonstrated that a miRNA mimic (miR-373) targeting promoter regions upstream of Ecadherin and CSDC2 induced transcriptional activation in prostate cancer cell lines via miRNA-mediated gene activation (miRNAa) [12]. In 2012, Huang *et al.* further identified endogenous mouse Cyclin B1 promoter targeting miRNAs (mir-744 and mir-1186) and demonstrated that miRNAa can be a physiological mechanism exploited by tumor cells to promote cell proliferation [13]. Collectively, these studies support that endogenous miRNAs can function as bidirectional regulators, either repressing or activating, depending on context [14]. Subsequent studies expanded on these findings, identifying additional examples of miRNAa and suggesting that RNAa in general is not limited to synthetic RNA mimics but also occurs endogenously as a physiological gene regulatory mechanism [15,16].

In terms of basic science applications, saRNAs can serve as surrogate tools for overexpression studies, providing an alternative to plasmid or virus-based systems [17]. Subsequent work by Li's group and others expanded saRNA's applicability across multiple genes and cell types, establishing saRNA as a distinct class of small RNAs with therapeutic potential for a spectrum of diseases, including cancer (reviewed by [15]), metabolic diseases [18,19], cardiovascular diseases [20] and other medical conditions, such as hearing loss [21]. The first *in vivo* proof-of-concept (PoC) of RNAa was demonstrated in 2009 by Turunen *et al.* [22] where a VEGF-targeting shRNA promoted therapeutic angiogenesis in a preclinical model of hindlimb ischemia. Subsequent preclinical studies demonstrated the translational potential of saRNA-mediated gene activation targeting $p21^{CIP1}$ and C/EBPα in oncology. Activation of $p21^{CIP1}$ using a promoter-targeted saRNA led to tumor regression in xenograft prostate cancer and orthotopic bladder cancer models by inducing cell cycle arrest and senescence [23,24]. saRNAs targeting the CEBPA promoter induced C/EBPα expression in hepatocellular carcinoma (HCC) mouse models, resulting in reduced tumor burden and suppression of myeloid-derived suppressor cells (MDSCs) [25]. Following a decade of foundational research, the first saRNA therapeutics, MTL-CEBPA, developed by MiNA Therapeutics, entered clinical development in 2016, followed by RAG-01 from Ractigen Therapeutics in

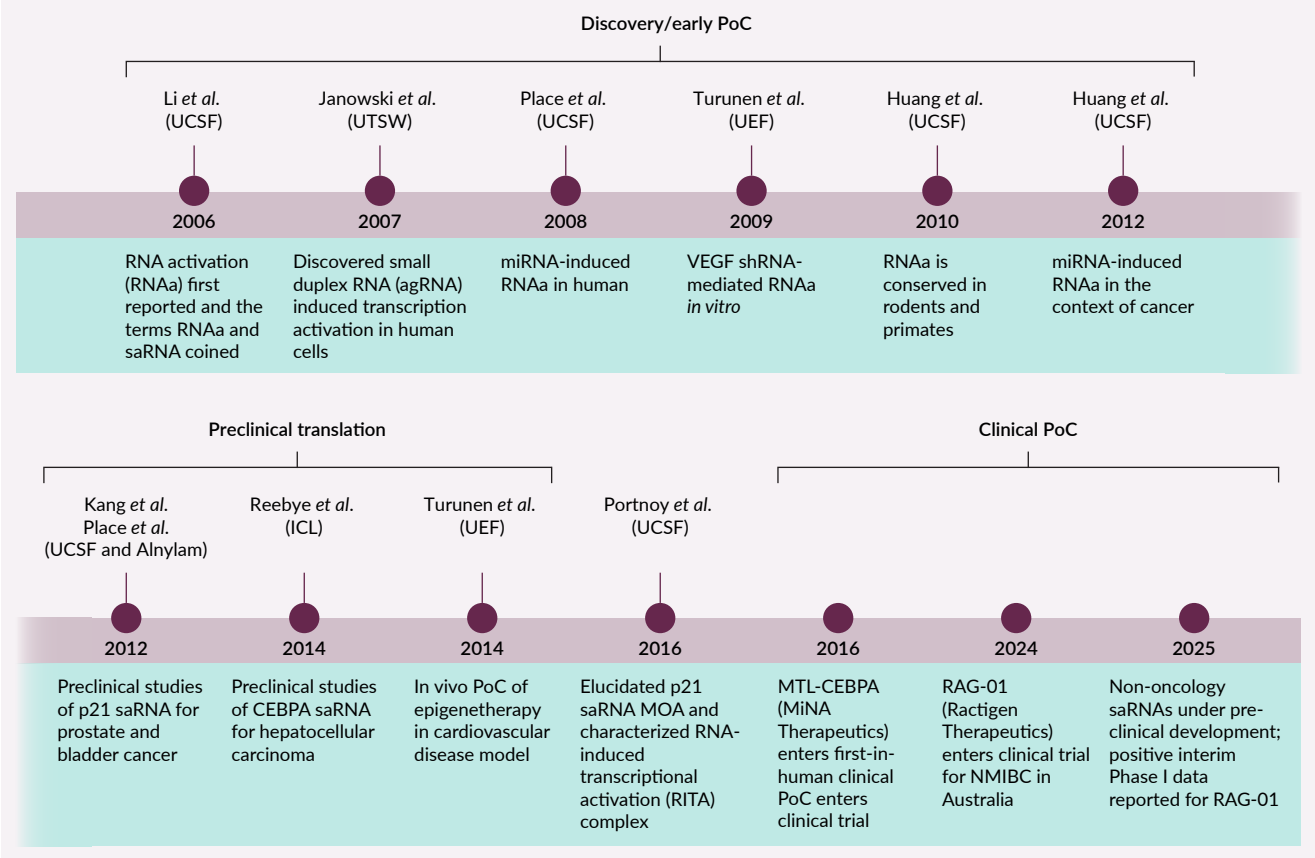
2024. These events marked pivotal milestones in the clinical translation of saRNAs **(Figure 1)**.

ADVANTAGES & CHALLENGES OF saRNA

Compared to other gene modulation approaches, saRNAs offer several unique advantages. saRNAs can be easily synthesized chemically to enable precise and transient activation of transcription from the endogenous gene locus, thereby minimizing the risk of insertional mutagenesis or long-term off-target effects from overexpression. Unlike traditional gene therapy approaches that rely on exogenous gene vector constructs, saRNAs target promoter regions of endogenous genes in a natural context to trigger transcriptional upregulation under physiological conditions and can be redosable and reversible. RNAa-mediated induction enables expression of the endogenous protein with the correct isoform(s), physiologically relevant stoichiometry, and native post-translational modification(s), which exogenous overexpression systems cannot recapitulate. Although replacement strategies using exogenous mRNA or protein are a direct solution for monogenic deficiencies, their clinical utility is constrained by significant barriers. Both modalities carry the risk of eliciting an unwanted immune response, and each faces unique limitations: efficient mRNA delivery to extrahepatic tissues remains a formidable hurdle, while protein replacement is largely confined to targets in the extracellular space. saRNAs, on the other hand, are best suited

FIGURE 1

Timeline of saRNA development from discovery to clinic (2006–2025).



for diseases where selective activation of genes that are typically underexpressed in disease states or impacted by haploinsufficiency. Therefore, the abilities of saRNAs to restore gene function without altering the genome offer a safer alternative to gene therapy in therapeutic applications, including oncology, rare diseases with haploid insufficiency, metabolic disorders, and regenerative medicine. From a practical standpoint, saRNAs offer advantages such as ease of synthesis and limited immunogenicity compared to exogenous replacement of mRNA or protein. ADAR-based RNA editing is an alternative approach that offers a reversible approach to correct single-base mutations at the transcript level; however, off-targets caused by unintended edits could be a safety concern. Wave Life Sciences' ADAR-based RNA editing therapy (WVE-006) for alpha-1-antitrypsin deficiency (AATD) has so far been well tolerated with mild to moderate adverse events (SAEs) reported in the ongoing trial. WVE-006 was able to restore the therapeutic level of SERPINA1; however, its long-term safety remains to be established. CRISPR activation (CRISPRa) is another complementary strategy for gene upregulation [26]. However, delivery of large dCas9 proteins and other components would require viral vectors, which may be limited by payload size, manufacturing complexity, and potentially cause immunogenicity to the Cas protein.

saRNAs typically exhibit EC_{50} values in the range of 1–10 nM *in vitro*, which is less potent than siRNAs, in part due to their limited nuclear entry. Efficient delivery systems are critical drivers for the clinical success of saRNA-based therapies. One of the key technical challenges is efficient nuclear entry of saRNA. This is critical because saRNAs must translocate into the nucleus to interact with the promoter regions of target genes through Ago2-containing RITA complexes. The mechanism of how the saRNA enters the nucleus remains largely unknown. Efficient nuclear delivery remains a key bottleneck for the therapeutic application of saRNAs. Conjugation strategies employing nuclear localization enhancers, as demonstrated with antisense oligonucleotides (ASOs), may be applied to saRNAs to improve their nuclear targeting [27].

saRNAs are ideal for targeting intracellular or transcriptionally silenced gene targets that are undruggable by conventional small molecules and antibodies. Compared to small molecule agonists, which require druggable proteins with ligand-binding pockets, saRNAs can directly activate the transcription of targets, thereby broadening the scope of therapeutic intervention saRNA target selection is critical in determining the success of RNAa. The efficacy of saRNAs is highly context-dependent, influenced by factors such as chromatin accessibility, promoter architecture, and the epigenetic landscape of the target gene. General guidelines for saRNA design have been described in an earlier publication by Wang *et al.* [28]. saRNAs are most effective when the target promoter sequence is within −1000 to −100, preferably within −200 to −500 relative to the transcription start site. Regions with high GC content or repetitive sequences should be avoided. Design of multiple saRNAs targeting different regions of the same promoter can further validate on-target specificity. For optimal RNAa activity, target genes should be in a transcriptionally poised state and maintain low to moderate basal expression. Promoters completely silenced by DNA methylation tend to be refractory to RNAa. Treatments with epigenetic modifying agents such as DNA demethylation agents and histone deacetylase inhibitors have been shown to enhance RNAa [1,28]. Recent work by Musiala-Kierklo *et al.* recommended prioritizing promoters with low to medium basal activity and chromatin accessibility, while avoiding multiple targets in close proximity within the same

promoter, would maximize on-target activity [10]. Despite their potential for precise gene upregulation, sequence-specific off-target effects remain a concern, particularly through partial sequence homology or miRNA-like seed region interactions or other promiscuous interactions. To address these challenges, chemical modifications to the saRNA backbone or bases can be employed to enhance specificity and mitigate off-target activity [29,30]. Comprehensive assessment of long-term safety and potential off-target effects remains to be evaluated.

Similar to other RNA therapeutics modalities, extrahepatic delivery remains a major hurdle. While lipid nanoparticles (LNPs) and conjugates (e.g., GalNAc for liver targeting) have revolutionized hepatic delivery, equivalent platforms for efficient, safe, and targeted delivery to extrahepatic tissues via tissue-specific conjugate, lipid, LNP, dendrimers, and aptamers are still under active development [31]. In addition, unlike protein-coding sequences, promoter regions are often poorly conserved across species; thus, establishing *in vivo* PoC for saRNA therapeutics typically requires humanized mouse models carrying the relevant human promoter sequence, or higher species with closer promoter homology to humans (e.g., non-human primates). Ongoing research and development are essential to address current challenges and technical constraints to fully realize the therapeutic potential of saRNAs.

CLINICAL DEVELOPMENT OF saRNA

MiNA Therapeutics: MTL-CEBPA

MTL-CEBPA represents the most clinically advanced example of saRNA-based therapeutics to date. Developed by UK-based MiNA Therapeutics, MTL-CEBPA is designed to upregulate expression of CCAAT/enhancer-binding protein alpha (CEBPA), a transcription factor with critical roles in regulating liver function, inflammation, and cellular differentiation, and master regulator of the myeloid cell lineage [8]. Loss of CEBPA is frequently found in cancer, leading to an accumulation of immunosuppressive MDSCs. CEBPA upregulation by saRNA inhibits tumor growth in multiple tumor models [9,25]. MTL-CEBPA is a CEBPA 21-mer 2′O-Me saRNA oligonucleotide duplex packaged in myeloid-directed Liposomal SMARTICLES® nanoparticles (MTL-CEBPA), which modulates the tumor microenvironment (TME) by relieving myeloid-driven immunosuppression as the main mode of action in HCC [8,32].

In the first-in-human Phase 1/2 trial (OUTREACH study NCT02716012), MTL-CEBPA was administered to 38 patients with advanced HCC via monotherapy or in combination with sorafenib, a standard-of-care multi-kinase inhibitor. The study assessed the safety of dose escalation, tolerability, pharmacokinetics, and preliminary efficacy [33]. The dose-escalation phase of the study followed a standard 3+3 design with the goal of determining the maximum tolerated dose (MTD). Six cohorts (cohorts 1–6) of eligible participants in each cohort received sequential doses of 28, 47, 70, 98, 130, and 160 mg/m^2 weekly (QW). Patients off-treatment were followed up for survival every 3 months. Tumor response and progression were evaluated using the revised RECIST1.1. MTL-CEBPA was well tolerated with no MTD reached. The toxicity profile was comparable with that of the other tyrosine kinase inhibitors (TKIs) used in HCC. Antitumor activity was evident; a mean progression-free survival of 4.6 months was observed in pretreated patients with a modest overall response rate of 4% as monotherapy.

Preclinical models suggest that CEBPA upregulation may reduce MDSCs and enhance antitumor immunity, providing a rationale for such combination strategies [25]. In a subsequent study in OUTREACH-2

Phase 2 (NCT04710641), MTL-CEBPA plus sorafenib versus sorafenib alone was compared in 150 advanced HCC patients. Subsequent clinical efforts have explored MTL-CEBPA in combination with immune checkpoint inhibitors.

TIMEPOINT (NCT04105335) is a Phase 1a+b clinical trial including a total of 100 patients with a wide range of advanced pre-treated cancers resistant to immune checkpoint inhibitors. The study included dose-escalation and expansion primarily to determine the safety, tolerability, and efficacy of MTL-CEBPA in combination with the anti-PD-1 antibody, pembrolizumab. Consistent with previous clinical data of the OUTREACH trial, MTL-CEBPA has an overall favorable safety profile when used as a combination agent with pembrolizumab. Among all tumor types, clinical activity and disease stabilization were best demonstrated in intrahepatic cholangiocarcinoma (disease control rate by RECIST: 80%). Mechanistically, MTL-CEBPA and pembrolizumab promoted increased T cell infiltration, activity, and a reduction in myeloid cells with an immunosuppressive phenotype in the TME and periphery. Additional data with a larger number of patients per group to associate the on-target activity of MTL-CEBPA with clinical outcome. Changes to immunosuppressive myeloid cells reduction and increases in pro-inflammatory infiltrates are in line with the previous observations in the OUTREACH trial, suggesting they may be specifically linked to MTL-CEBPA mechanism of action. However, without the inclusion of a monotherapy control group with anti-PD-1 only, it was difficult to determine if the effects were solely attributable to MTL-CEBPA [34].

Ractigen Therapeutics: RAG-01

RAG-01 is an saRNA therapeutic developed by China-based Ractigen Therapeutics, engineered to activate the $p21^{CIP1}$ tumor suppressor gene for the treatment of non-muscle invasive bladder cancer (NMIBC). $p21^{cip1}$, encoded by the CDKN1A gene, is a key regulator of cell cycle arrest and DNA repair, traditionally considered an undruggable target. RAG-01 is locally administered into the bladder by intravesical instillation leveraging Ractigen's proprietary LiCO™ (lipid-conjugated oligonucleotide) platform, which efficiently penetrates the urothelial lining to locally deliver the saRNA with minimal systemic exposure.

The ongoing Phase 1 study (NCT06351904) is an open-label, multi-center, Phase 1 study designed to evaluate the safety, tolerability, pharmacokinetics (PK), pharmacodynamics (PD), and preliminary efficacy of intravesically delivered RAG-01 in patients with NMIBC who have failed Bacillus Calmette Guérin (BCG) therapy. As of December 2024, 9 patients were enrolled across 3 dose-escalation cohorts (30–300 mg) for 6 weeks followed by maintenance instillations up to week 72. No dose-limiting toxicities (DLTs) at any of the dose levels evaluated were reported. RAG-01 showed minimal systemic exposure with a dose-dependent maximum urine concentration. A dose-dependent, on-target, increase in $p21^{CIP1}$-positive urothelial cells was observed. Preliminary efficacy analysis revealed a 66.7% (2/3) complete response (CR) rate for carcinoma *in situ* (CIS) at any time and a 66.7% (2/3) disease-free survival (DFS) rate for papillary tumors at 3 months [35].

Intravesical RAG-01 demonstrated a favorable safety profile. Dose-dependent $p21^{CIP1}$ protein induction in urothelial cells confirmed target engagement. Preliminary anti-tumor efficacy supports further clinical investigation of saRNA as a novel therapeutic approach for NMIBC. In recognition of its promise, RAG-01 received FDA Fast Track Designation in December 2024.

These encouraging clinical findings of MTL-CEBPA and RAG-01 demonstrated

robust therapeutic activity of saRNAs in patients, supporting the feasibility of this approach. Many more programs are anticipated to advance over the coming decade (Table 1).

EMERGING PRECLINICAL saRNA PROGRAMS

Ractigen Therapeutics- RAG-18

Several promising preclinical saRNA programs have emerged in recent years. Ractigen is advancing another saRNA program (RAG-18) targeting Duchenne and Becker muscular dystrophies (DMD and BMD) through the upregulation of utrophin, a functional analog of dystrophin, to compensate for dystrophin loss in DMD/BMD patients. Current therapeutic approaches for DMD include ASO-mediated exon skipping, gene therapy, and gene editing, with exon skipping being the most common strategy but with limited efficacy. Muscle-targeted oligonucleotide delivery has achieved only limited success, with four phosphorodiamidate morpholino oligomers (PMOs) approved for DMD. RAG-18 is intended to function through targeted activation of the *UTRN* gene in muscle cells, leading to increased production of utrophin, the protein encoded by UTRN. Utrophin is structurally and functionally analogous to dystrophin, which is deficient in patients with DMD and BMD. Unlike mutation-specific exon-skipping therapies, upregulation of utrophin offers a mutation-agnostic therapeutic strategy to restore muscle function. Several human-specific UTRN saRNAs have been identified to induce UTRN expression at both the mRNA and protein levels and demonstrated durable activity in cell lines derived from skeletal muscle. Treatment of mdx mice bearing human UTRN promoter (hUTRNp KI/KI × *mdx*) with a lead UTRN saRNA ameliorated muscle damage. RAG-18 has a favorable safety profile in rodents [36].

RAG-18 is administered via intravenous injection and leverages LiCO™ technology to target skeletal and cardiac muscles to activate the endogenous *UTRN* gene. RAG-18 has been granted Orphan Drug Designation (ODD) and Rare Pediatric Disease Designation (RPDD) by FDA [37,38] and is currently at the IND-enabling stage.

TABLE 1

saRNAs in clinical development.

Company	Program ID	Target	Indications	Delivery vehicle	ROA	Clinical status	Clinical trial number
MiNA Therapeutics	MTL-CEBPA*	CEPBA	HCC	LNP	IV	Phase 1	OUTREACH NCT02716012
MiNA Therapeutics	MTL-CEBPA†	CEPBA	HCC	LNP	IV	Phase 2	OUTREACH-2 NCT04710641
MiNA Therapeutics	MTL-CEBPA‡	CEPBA	Advanced solid tumors	LNP	IV	Phase 1b	TIMEPOINT NCT04105335
MiNA Therapeutics	MTL-HBG	HBG	SCD	LNP	IV	IND-enabling	
Ractigen Therapeutics	RAG-01	CDKN1A (p21^{CIP1})	NMIBC	LiCO	Intra-vesical	Phase 1	NCT06351904
Ractigen Therapeutics	RAG-18	UTRN	DMD/BMD	LiCO	IV	IND-enabling	

*Monotherapy. †Ongoing randomized trial comparing MTL-CEBPA plus sorafenib versus sorafenib alone. ‡Combination with pembrolizumab. DMD/BMD: Duchenne and Becker muscular dystrophies. HCC: hepatocellular carcinoma. IV: intravenous. LiCO: lipid conjugated oligonucleotide. LNP: SMARTICLES® lipid nanoparticles. NMIBC: non-muscle invasive bladder cancer. ROA: route of administration. SCD: sickle cell disease.

Mina Therapeutics: MTL-HBG

MiNA Therapeutics is developing MTL-HBG as a potential treatment for sickle cell disease (SCD), which is caused by a mutation in the HBG gene, producing hemoglobin S (HbS). Clinical studies have shown that the expression of fetal gamma globin (HbF) can compensate for the dysfunctional HbS, supporting a validated therapeutic approach for managing beta-hemoglobinopathies in SCD patients. The investigational therapy aims to increase the production of the fetal hemoglobin (HbF) gene (HBG), which can help alleviate SCD symptoms. MTL-HBG works directly targeting the HBG gene to induce HbF production by saRNA and has shown promising preclinical results in SCD. As the current approved HbF inducers (e.g., hydroxyurea) have limitations and safety concerns, MTL-HBG offers safer and superior treatment for the majority of SCD patients.

In highly translatable preclinical models of SCD, MTL-HBG induced HbF to a therapeutically relevant level and demonstrated potential for best-in-class *in vivo* induction of fetal hemoglobin. Elevated HbF levels could potentially protect patients from disease symptoms, including sickling and vaso-occlusive crises, without the need for stem cell transplantation, gene editing, or intensive preconditioning. MTL-HBG comprises an saRNA that directly targets the HBG gene encapsulated in NOV340 liposomes. MTL-HBG induced persistent, pancellular levels of % HBF significantly exceeding the protective threshold of 20%, superior to that of hydroxyurea. MTL-HGB has a clean off-target profile and does not induce cytotoxicity. Based on quantitative systems pharmacology modeling, once monthly IV infusions of MTL-HBG were predicted to be sufficient to maintain HbF levels above the protective threshold of 20% for the majority of SCD patients. This approach offers superiority in safety, tolerability, and accessibility over CRISPR and small-molecule therapies currently in development. MTL-HBG is currently in IND-enabling preclinical development [39].

RNatives, a Finland-based company, is developing novel promoters targeting miRNAs to treat ocular, central nervous system (CNS), cancer, cardiovascular, and metabolic diseases. The technology is built on the work pioneered by researchers from the University of Eastern Finland, including the development of VEGF-activating saRNAs and nuclear miRNAs (e.g., miR-466c) that promote angiogenesis and tissue regeneration [40]. Upregulation of endogenous VEGF-A has been demonstrated to reduce myocardial infarction in a mouse disease model, suggesting its translational potential [22,41].

TRANSLATIONAL OUTLOOK

Over the past 20 years, saRNA has been rapidly developing as a cutting-edge technology in RNA therapeutics. The clinical advancement of MTL-CEBPA and RAG-01 marks significant progress for the saRNA field, demonstrating clinical PoC for gene activation-based therapeutics. These first-in-human studies provide validation that RNA activation is clinically feasible and safe. Ongoing and future trials will be critical in determining its therapeutic potential across broader indications. While initial efforts have focused on oncology, saRNA technology holds tremendous therapeutic potential across a wide range of indications. For example, saRNAs can be harnessed to upregulate key transcription factors involved in metabolic regulation, tissue remodeling, and inflammation. By reactivating haploid insufficient, epigenetically silenced genes, or normally expressed genes, saRNAs offer a promising therapeutic approach for rare genetic disorders such as myotonic dystrophy, where a modest increase in gene expression is sufficient to mitigate disease phenotypes [10]. Enhancing neuroprotective

genes via saRNAs can also be applied to broader indications such as neurodegenerative diseases. Ongoing efforts are needed to optimize delivery technologies, target specificity, and further understanding of the gene activation mechanism, which will be key to unlocking their full clinical potential. Next-generation saRNAs are expected to incorporate advanced chemical modifications to enhance their RNAa activity, improve their stability, and specificity. Advancements in saRNA design through the integration of artificial intelligence will enable more precise targeting of gene promoters, allowing for the activation of target genes with high specificity and minimal off-targets. From a manufacturing perspective, lessons learned from siRNA production can be directly applied to saRNAs, including implementing cost-effective synthesis strategies and robust quality control measures to ensure the consistent production of high-quality saRNAs at a commercial scale.

Similar to siRNAs, innovation in saRNA delivery systems will include the development of platforms capable of delivering saRNAs to specific tissues or cell types. Efficient extrahepatic delivery to the CNS, skeletal, and cardiac muscles will open new avenues for therapeutic intervention in diseases with high unmet needs. This will be achieved through the conjugation of delivery vehicles with targeting ligands, such as peptides, lipids, antibodies, and aptamers that can recognize and bind to specific cell surface markers. In this regard, anti-tumor effects of C/EBPα-saRNA by using transferrin receptor (TfR) aptamers for targeted delivery have been demonstrated in an advanced pancreatic cancer mouse model [42].

With favorable safety profiles, continuous advancement in delivery technologies, and increasing clinical validation, we envision that saRNAs are poised to become a key modality in the evolving field of oligonucleotide-based therapies.

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NUCLEIC ACID INSIGHTS

DOWNSTREAM/PURIFICATION: ISSUES AND RAMIFICATIONS

SPOTLIGHT

Safety first: advancing siRNA therapeutics with AI-driven design and off-target minimization

INTERVIEW

"Being able to bring molecules to patients with diseases that start outside the liver is incredibly inspirational."

Jokūbas Leikauskas, Editor, BioInsights, speaks to **Marie Wikström Lindholm**, Chief Scientific Officer at Silence Therapeutics, about advances in short-interfering RNA (siRNA) design, the role of artificial intelligence (AI)/machine learning (ML) in oligonucleotide development, strategies for minimizing off-target risks, and future goals for safer, broader therapeutic applications beyond the liver.

Nucleic Acid Insights 2025; 2(8), 167–172 · DOI: 10.18609/nuc.2025.025

Q What are you working on right now, and what are the key focus areas at Silence Therapeutics?

MWL At Silence, we currently work on our own therapeutic pipeline, where our most advanced drug, Zerlasiran, is intended for broad cardiovascular indications. This drug targets lipoprotein(a) (Lp[a]), which is a genetically determined independent risk factor for cardiovascular disease. I started as an academic scientist working on cardiovascular diseases myself and was an Associate Professor

in experimental cardiovascular medicine before I entered the industry. Therefore, this program is something close to my heart, and it has been exciting to see the Phase 1 and Phase 2 data demonstrating the intended effect on Lp(a).

We also have another interesting siRNA molecule, Divesiran, which targets TMPRSS6, aiming to increase hepcidin production. Hepcidin is a key regulator of iron metabolism, and Divesiran can therefore be considered as a therapeutic for a whole series of indications where the goal is, for instance, to reduce the risk of tissue iron overload in patients. This drug is currently in a Phase 2 trial for an indication called polycythemia vera, a type of rare blood cancer. We anticipate that recruitment will be finalized before the end of this year.

At Silence, I am delighted to be working alongside a team of biologists, chemists, bioinformaticians, and data scientists who are all making sure that we create the best possible molecules for different indications. Our intention is to develop molecules with the best possible safety, potency, and duration of action, regardless of what cell type we are targeting. Notably, we have been working on trying to get them into cell types other than hepatocytes.

Q With more than 15 years of experience in oligonucleotide therapeutics, what major shifts or breakthroughs have you witnessed in the field that have most changed the way we design and develop siRNAs?

MWL Before I worked with siRNAs, which I have been doing for almost 8 years now, I spent 10 years working with single-stranded antisense oligonucleotides (ASOs), including GalNAc-conjugated, as well as with anti-miRNA and splice-switching oligonucleotides.

When I first got into the industry, we thought, at least for single-stranded ASO gapmers, that we only needed to consider the exons and the untranslated regions (UTRs) when choosing what part of the transcript to hit. Now we know that when we design molecules that are supposed to be completely unique for the intended targets, we must consider the whole transcriptome. That insight, together with the realization that some of the most potent molecules actually hit in the UTRs, and alongside the enormous increase in available information, changed the landscape. When I started, we often used only the RefSeq database, whereas nowadays we are handling much larger amounts of data as we try to find unique sequences. We can also predict potency and figure out which sequences should be the most effective using ML tools. The goal is to find unique sequences—the only full-match transcript in the entire transcriptome—starting with the human one.

Additionally, there has been a realization that you can also have some activity with single mismatches to sequences in the transcriptome, and Silence has been quite active in this area. For example, it is now known that it is possible to have activity with two mismatches, or in some cases three mismatches, depending on where along the transcript they are. The search for unique sequences, combined with a risk score for potential off-targets, has become increasingly complex. On top of that, we are dealing with a huge amount of available data and a much larger catalogue of single-nucleotide polymorphisms (SNPs) that we are now aware of. All of this means the computing power necessary to really understand how to pick the most potent and unique sequences has increased significantly.

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"...AI/ML tools will help us move toward new chemistries that truly explore the interface between the physical-chemical properties of molecules and the biology they interact with."

It has been a very interesting journey. I think our increase in understanding of 'how to pick the best sequences to hit' is more incremental now, as our understanding of the types of interactions that can happen continues to grow. Back then, it was a little bit more like the 'wild west'.

Q Where do you see the greatest potential for AI and ML to transform oligonucleotide design over the next 5–10 years?

MWL We are already there—I am assuming that everyone in the field is already using AI/ML to pick the best sequences. The next big breakthrough, though, might be in areas where we do not necessarily think of ML and big data as useful today, such as clinical trial design, as well as process development for the CROs or CMOs that are making the oligonucleotides.

Generally, it is becoming less about trial and error and much more about learning from previous experience—for instance, getting faster in consistent batch-to-batch composition. Even though I am personally early in the value chain, I always think through: if we try something novel with the chemical modifications and design of the molecules, will it also be possible to synthesize them at scale? The development we are seeing—applying AI/ML tools on the manufacturing side—may help bring more innovative chemistries to market.

Another area where we will hopefully see significant progress is molecular modelling. Across the industry, hundreds of ribose and other modifications for therapeutic oligonucleotides have been tested for their effect on activity and other key parameters. But up until recently, it was just trial and error of single modifications, not necessarily combined with insights about how those chemical changes might interfere with, for example, the initial interactions with AGO2.

I believe AI/ML tools will help us move toward new chemistries that truly explore the interface between the physical-chemical properties of molecules and the biology they interact with. Currently, it is still more of a blunt-force approach than a sophisticated understanding behind the design of new modification patterns.

Q Off-target effects are often mentioned as one of the key challenges in siRNA therapeutics. From your perspective, how important is rigorous off-target risk minimization for advancing siRNA therapeutics? How can this challenge be addressed?

MWL The key reason why I pay extra attention to off-target risks is because one of the first ASO gapmers I worked on, many years ago, resulted in a serious adverse event in a clinical trial. At that time, we thought the main

"...siRNAs can act like microRNAs through the seed region and seed-mediated interactions, posing additional risks."

patient safety risk would be dose-limiting liver toxicity. However, what we actually experienced with that ASO was kidney toxicity. I looked back at it many times, and there was simply no way we could have predicted it with the tools available at the time.

That experience really made me very aware that, on one hand, oligonucleotide therapeutics give us the possibility to treat diseases where there are no other options. But on the other hand, safety, particularly now when I am working with siRNAs, is crucial. The reason for me stating that is that siRNAs have a very long duration of action, which is fantastic with the right target and the right chemistry. Therefore, we can design molecules where a single injection produces an effect that lasts for many months, which is excellent for on-target activity. However, it can also be very risky if you also get off-target effects. From a patient safety perspective, this is obviously critical.

As an industry and with the knowledge we have today, we have three main areas to consider regarding toxicity. Firstly, there is the chemistry itself, which can lead to acute immune and toxicity reactions. However, this can be designed and screened away before reaching patients, and therefore, there are few examples of long-term negative effects that are chemistry-dependent but sequence-independent. Those risks can typically be designed out through careful selection of chemical modifications.

Secondly, we have the direct Watson-Crick base pairing-dependent toxicities. Of course, you would not pick a sequence with more than one full match target, but as mentioned already, you also need to watch out for mismatches depending on where they are. They can still lead to downregulation via RNA-induced silencing complex (RISC), and that requires a risk assessment. To address this risk, most predictions can be done *in silico*. As mentioned earlier, we are applying AI/ML to refine those cycles of predictions continuously.

Thirdly, siRNAs can act like microRNAs through the seed region and seed-mediated interactions, posing additional risks. If we removed every possible 6/7-mer site in the transcriptome that could interact with the siRNA guide strand through this mechanism, there would likely be nothing left. Therefore, the best strategy is to either minimize seed-mediated effects through chemical modification or rely on a cascade where you first design to avoid as much off-target as possible, then narrow down to a smaller set of candidates, and finally add experimental data. We use RNA-Seq for an unbiased assessment of 'differentially expressed genes' (DEGs; i.e., off-target effects) after having boiled it down to a small set of candidates in the discovery cascade. Most likely, there will be some DEGs induced by miRNA-like activity, and some that can be identified as downstream of the intended biological effect, but that is a separate issue. The point is to add experimental data and carry out a transcriptomic analysis of treated versus untreated samples in a model system that is relevant for humans. Of course, this gets further complicated because we are used to relying on rodents and primates as toxicity models. But when it comes to microRNAs and the transcripts they interact with, or where an siRNA might do the same, it is very species dependent. These interactions usually occur in the UTRs, where there is little correlation between species.

Lastly, I should also mention that there have been oligonucleotide safety working groups producing industry recommendations for many years, and there is a recently published paper on industry recommendations on applying *in silico* designs and transcriptomic

data further down the line to help ensure safer molecule selection [1]. My team contributed to that paper, and Silence is fully behind its conclusions.

Q For oligonucleotides, toxicology models are proving particularly challenging – what progress is being made here?

MWL There is much relevant work going on, not just for therapeutic oligonucleotides but also to improve translation for other modalities with respect to toxicology model systems. Silence partners with a consortium funded by the Medical Research Council in the UK called TransNAT to develop and validate patient-derived model systems for testing oligonucleotide safety. I think the development of patient-derived inducible pluripotent stem cell organoids, for tissues such as muscle, heart, and central nervous system, is particularly important. The idea is not only that they can be designed with better predictive value for toxicity, but especially that they can help us assess off-target reactions. Having systems developed from patient-derived material provides supplementary value to the blunter instruments we currently rely on.

Before joining this consortium, I did not realize it could take up to half a year to create a 'micro-brain' that can then be used for experimentation. But seeing that development and knowing how close we are to being able to use these systems for drug discovery is very encouraging.

Q Finally, what are your goals and priorities over the next 1–2 years, both for yourself and for Silence as a whole?

MWL To begin with, I hope that we can take all the knowledge we have accumulated for liver targeting and apply oligonucleotide therapeutics to various cell types—'extrahepatic delivery' is more than just a buzzword. Everyone working in this field has a platform that could, in theory, be applied to any disease caused by the upregulation of a disease-causing protein. To get there, we need safe and specific delivery to other cells. I can already say that we now have evidence in mouse models that the designs we have been applying for liver targeting can also work in other cell types.

Making medicines for genetically driven diseases in cells other than hepatocytes and applying everything we know about biology, bioinformatics, chemistry, synthesis, and scaling up so the molecules can become medicines is extremely exciting.

At Silence, one of our core values is seeing the patient in every action we take—no matter if you are a chemist, a bioinformatician, or a data scientist. We are always keeping that in mind, whether it is improving an assay with predictive value for the clinic or developing a new chemistry.

Being able to bring molecules to patients with diseases that start outside the liver is incredibly inspirational. For me personally, it is about making sure we maintain that long-term focus, while also ensuring that people can use their knowledge, their expertise, and, importantly, have fun while doing it.

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BIOGRAPHY

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AUTHORSHIP & CONFLICT OF INTEREST

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NUCLEIC ACID INSIGHTS

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EVENT PREVIEW

SPOTLIGHT

TIDES Europe 2025

Nucleic Acid Insights 2025; 2(8), 163–164 · DOI: 10.18609/nuc.2025.023

As part of our ongoing coverage of major gatherings in the advanced therapeutics space, *Nucleic Acid Insights* presents a preview of TIDES Europe 2025. Scheduled for November 11–13, 2025, in Basel, Switzerland, this conference will spotlight key strategies, trends, and technologies in oligonucleotide and peptide discovery, development, and manufacturing. In this preview, we highlight key sessions, speakers, and themes that will shape the conversation at one of Europe's most anticipated nucleic acid events.

TIDES EUROPE | Oligonucleotide & Peptide Therapeutics

KEYNOTE SESSIONS: DRIVING THE FUTURE OF NUCLEIC ACID-BASED DRUG DEVELOPMENT

The keynote sessions will spotlight cutting-edge innovations in the nucleic acids field, including oligonucleotide therapeutics for cardiovascular diseases and AI-driven drug discovery. Shalini Andersson (VP of Oligonucleotide Discovery, AstraZeneca) will showcase how antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) can precisely modulate gene expression and protein synthesis to treat heart disease with greater efficacy and fewer side effects. Her talk will emphasize the potential of targeted delivery to expand the druggable space in cardiovascular medicine. Additionally, Brendan Frey (Founder & Chief Innovation Officer, Deep Genomics) will explore how AI foundation models are transforming pharmaceutical R&D by integrating diverse tasks, scaling with data, and improving target accuracy. He will highlight applications in ASOs, siRNAs, mRNAs, and RNA/DNA editing, drawing on Deep Genomics' internal programs and partnerships to illustrate how these models are accelerating drug development and unlocking new therapeutic possibilities.

EXPANDING THERAPEUTIC IMPACT THROUGH NOVEL TECHNOLOGIES

The event also includes sessions that will showcase transformative strategies for advancing next-generation oligonucleotide therapeutics through platform innovation, regulatory insight, and molecular engineering. Chris Hart (VP, Data Science and AI/ML, Eli Lilly and Company) will highlight

how oligonucleotide medicines are evolving into best-in-class treatments for both rare and prevalent diseases, emphasizing collaborative efforts in delivery technologies and molecular engineering to expand therapeutic impact. Jennifer Franklin (Executive Director, CMC Regulatory Affairs, Ionis Pharmaceuticals) will share practical insights into applying platform data for oligonucleotide development, including regulatory engagement and future directions for health authority interactions. Lastly, Firoz Antia (VP, Oligonucleotide & Small Molecule CMC, Denali Therapeutics) will present key CMC considerations for Denali's Oligonucleotide Transport Vehicle (OTV) platform and antibody-ASO conjugates, offering a deep dive into how these innovations are shaping delivery and manufacturing strategies. Together, these sessions will provide a comprehensive view of how oligonucleotide platforms are driving therapeutic breakthroughs and regulatory evolution.

TIDES Europe 2025 is one of Europe's leading events for nucleic acid therapeutics, bringing together over 750 attendees and over 100 speakers. Held in Basel, the event will feature cutting-edge presentations on therapeutic innovation, scalable manufacturing, and advanced nucleic acid delivery technologies. With a focus on expanding therapeutic impact and overcoming development bottlenecks, TIDES Europe offers a comprehensive view into the future of nucleic acid-based medicines and next-generation biologics.

As a reader of *Nucleic Acid Insights*, you're entitled to a **10% discount** on delegate tickets—just use **this link**! You can find out more about the TIDES Europe 2025 events **here**.

Want to keep up to date with all of the latest nucleic acid events you might want to attend or exhibit at? Explore our free online Events Calendar **here**.

Nucleic Acid Insights 2025; 2(8), 163–164 · DOI: 10.18609/nuc.2025.023

NUCLEIC ACID INSIGHTS

mRNA

SPOTLIGHT

COMMENTARY

Engineering nanotechnology for *in vivo* CAR cell therapy

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Chimeric antigen receptor (CAR)-T cell therapy has revolutionized the treatment of B cell malignancies over the past decades. Despite its success, CAR-T cell therapy remains limited by high costs, complex *ex vivo* manufacturing, and the risk of severe adverse effects. *In vivo* generation of CAR-T cells is emerging as a promising alternative, offering a less invasive, off-the-shelf approach that streamlines production while reducing logistical and financial burdens. To enable the delivery of CAR-encoding nucleic acids to immune cells, a range of delivery platforms is being explored, including non-viral lipid and polymeric nanoparticles as well as viral vectors based on lentiviruses and adeno-associated viruses (AAVs). Among these, lipid nanoparticles (LNPs) encapsulating mRNA present distinct advantages: they enable scalable, standardized manufacturing–exemplified by the rapid development of COVID-19 mRNA vaccines—and offer improved cost–effectiveness and accessibility. This commentary highlights the current landscape of *in vivo* CAR-T cell therapy and discusses key bioengineering strategies for the successful implementation of LNP-mRNA platforms.

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INTRODUCTION

Over the past decades, chimeric antigen receptor (CAR)-T cell therapy has profoundly transformed the treatment of various B cell malignancies. The engineered CAR combines a cell targeting domain, typically a single-chain variable-fragment (scFv), with intracellular CD3ζ and co-stimulatory (or activation) domains such as CD28 or CD4-1BB, thereby redirecting T cells toward specific tumor-expressed antigens [1,2]. While most clinical successes have been achieved in the treatment of hematological cancers–including leukemia, lymphoma, and myeloma [3]—developing effective CAR-T cell therapies for the treatment of solid tumors has proven more challenging [4]. In addition, the high costs associated with CAR-T therapy greatly limit its accessibility: the acquisition price for approved drug products ranges from €350,000 to €500,000 per patient, excluding further expenses such as pretesting, hospitalization, lymphodepletion, and imaging studies. The total cost of implementing CAR-T cell therapy has

been estimated to exceed €1,000,000 per patient [5,6], and the manufacturing process is time-consuming, typically requiring 3–6 weeks [7]. Furthermore, specific toxicities have emerged since CAR-T cell therapy became a standard of care. Cytokine release syndrome (CRS) and immune effector cell–associated neurotoxicity syndrome (ICANS) are among the most prominent adverse events, usually developing within the first months after treatment [8]. Long-term complications, such as persistent cytopenias, B cell aplasia, and hypogammaglobulinemia, have also been reported, increasing the risk of severe infections [8,9]. To overcome these therapeutic, logistical, and financial challenges, novel strategies in CAR cell engineering are being explored. Direct *in vivo* generation of CAR cells is emerging as a less invasive, off-the-shelf approach that simplifies manufacturing and logistics while reducing costs [10]. In this Commentary, we discuss the current state of *in vivo* CAR cell therapy and highlight key optimization strategies for engineering nanotechnology-based platforms, including payload optimization, delivery vehicle design, and targeting strategies.

IN VIVO CAR-T CELL THERAPY: STATE OF THE ART

To generate CAR immune cells *in vivo*, delivery vehicles are required to insert genetic material encoding for the synthetic receptor into specific cells following systemic administration [11]. Several classes of nucleic acid delivery vehicles are currently being explored, including lipid and polymeric nanoparticles, as well as lentiviral and adeno-associated vectors (AAV) [10–12]. The latter are produced by cloning the CAR genes into the viral vector, which can then be further engineered to display T cell-targeting ligands. Such moieties (i.e., CD3, CD4, or CD8) may be incorporated into the viral envelope through genetic fusion or chemical conjugation [11,13].

Umoja Biotech has developed a proprietary lentiviral vector platform, VivoVec™, which incorporates a CD19-CAR transgene and is surface-engineered with a multi-domain fusion protein composed of T cell-activating and costimulatory ligands. In non-human primates, a single dose of VivoVec induced functional CAR-T cells, resulting in persistent B cell aplasia for up to 76 days [14]. Similarly, EsoBiotec is advancing a third-generation nanobody-targeted lentiviral vector (ESO-T01) for *in vivo* generation of BCMA-specific CAR-T cells. In an ongoing Phase 1 trial, initial case series reported complete responses in two patients with multiple myeloma; however, CRS was observed in all treated patients to date, underscoring both the promise and the safety challenges of this approach [15]. Beyond T cells, Interius BioTherapeutics is developing an *in vivo* CAR natural killer (NK) cell platform using a CD7-targeted lentiviral vector to deliver a CD20-specific CAR transgene [16].

Although lentiviral vectors enable stable integration of CAR genes, their permanent nature raises concern about germline transmission and disruption of host gene expression upon insertion [13]. By contrast, AAVs persist as episomal DNA without integrating into the host genome, supporting long-term transgene expression without the risk of insertional mutagenesis [10]. For instance, an AAV carrying CD4-CAR plasmid DNA successfully generated cytotoxic CAR-T cells *in vivo*, resulting in tumor remission in a humanized mouse model of leukemia following a single infusion. However, prolonged presence and memory formation of CD4-CAR-T cells were also observed, which may cause sustained $CD4^+$ T cell aplasia in clinical settings. This highlights the importance of incorporating effective safety switches into such therapies [17].

These limitations have motivated the exploration of non-integrating carriers such as nanoparticles for CAR gene delivery [18,19]. Lipid nanoparticles (LNPs) are

composed of ionizable cationic lipids, cholesterol, helper lipids, and polyethylene glycol (PEG)-lipids, which encapsulate nucleic acid cargo. Alternatively, cationic poly (β-amino ester) (PBAE) polymers can electrostatically complex with negatively charged DNA or mRNA [10,13,18,19]. Nanoparticles offer scalability, compatibility with freeze-drying for long-term storage, and cell-free manufacturing when mRNA is used as cargo [11,13,18]. These features make mRNA-loaded nanoparticles cost-effective and well-suited for off-the-shelf therapeutic applications, enabling rapid deployment in patients with aggressive disease [11,13]. Recent technological advances have further improved mRNA stability, translation efficiency, and immunogenicity control [19]. After nanoparticle uptake by cells, mRNA can escape the endosome and undergo translation in the cytoplasm. Its confinement to the cytoplasm and inherent instability support a favorable safety profile. While transient expression may necessitate repeated dosing, it allows for tighter dose control and proactive management of potential adverse effects such as CRS and ICANS [13,19,20]. Moreover, transient CAR expression may help limit T cell exhaustion [13].

Compared with PBAE-based nanoparticles, LNPs demonstrate superior encapsulation efficiency, greater protection against nucleic acid degradation, and a well-established safety record, exemplified by their success in mRNA vaccines [10,13,21]. FDA approval further validates their safety and highlights their potential for broader therapeutic applications, including *in vivo* CAR-T cell engineering [21]. While this Commentary emphasizes LNP-mRNA approaches, PBAE nanoparticles carrying CAR mRNA or DNA have also been successfully engineered and shown efficacy in preclinical models [20,22].

Table 1 summarizes selected preclinical and clinical efforts employing LNP-RNA for *in vivo* CAR cell therapy [9,23–36]. One of the most advanced clinical programs to date is CPTX2309 by Capstan Therapeutics—an LNP-mRNA formulation targeting $CD8^+$ T cells and encoding a CD19-CAR, being developed for autoimmune disease [9]. In cynomolgus monkeys, up to 85% of $CD8^+$ T cells expressed the CAR construct after the third dose, which was associated with deep B cell depletion. Upon recovery, repopulating B cells were predominantly naïve, suggesting an immune reset [9]. A first-in-human Phase 1 clinical trial is currently underway to evaluate the safety and tolerability of CPTX2309 in healthy adults (NCT06917742).

Orna Therapeutics is pursuing a different approach, employing a circular RNA (circRNA) payload encoding a CD19 panCAR™ within a non-targeted LNP, with the goal of simultaneously reaching T cells, NK cells, and macrophages [23,24]. The company expects to advance this candidate to the clinic in 2026 [24]. Similarly, Orbital Therapeutics' lead asset, OTX-201, delivers CD19 CAR circRNA in a targeted LNP and is being developed for B cell-driven autoimmune diseases. In non-human primates, OTX-201 achieved complete B cell depletion across blood, spleen, and lymph nodes [25].

Beyond CAR-T-cell-based approaches, Myeloid Therapeutics is developing *in vivo* CAR macrophages and monocyte (CAR-M) therapies. To enable selective myeloid cell targeting, their platform fuses a tumor-recognition scFv to the α-chain of the human Fc receptor. Functional activity requires the endogenously expressed Fc receptor γ-chain, which is predominantly restricted to the myeloid lineage. Their lead candidate, an LNP-mRNA CAR against tumor-associated calcium signal transducer 2 (TROP2), is currently being evaluated in a Phase 1 dose-escalation study in adults with advanced epithelial cancers (NCT0596904) [37]. Initial biomarker analyses showed CAR-positive myeloid cells co-localized with TROP-2 expressing tumor cells, along

TABLE 1

Clinical and pre-clinical candidates for *in vivo* LNP-RNA CAR cell approaches.

Company	Asset	CAR target antigen	Payload	Targeting moiety	Stage	Indication
CAR-T						
Capstan Therapeutics	LNP CPTX2309 [9]	CD19 CAR	Sequence-optimized CAR mRNA	Human CD8 antibody	Phase 1; NCT06917742	Autoimmune disease
	LNP [29]	FAP CAR	mRNA	Mouse CD5 antibody	Pre-clinical (mice)	Cardiac injury
Orna Therapeutics	panCAR™ [23,24,30]; ORN-145; ORN-328; ORN-252	CD19 CAR BCMA CAR	Circular RNA	N/A	Pre-clinical (NHP); Phase 1 expected 2026	Autoimmune disease; B-cell driven malignancies
Orbital Therapeutics	LNP [25,31]; OTX-201	CD19 CAR	Circular RNA	Undisclosed	Pre-clinical (NHP) Phase 1 expected 2026	Autoimmune disease
	LNP [32]	CD19 CAR	mRNA	Mouse CD3 Ab fragment; mouse CD7 Ab fragment	Pre-clinical (mice)	B-cell malignancies
	SORT-LNP [33]	CD19 CAR	mRNA	N/A	Pre-clinical (mice)	B-cell lymphoma
	LNP [34]	TRP CAR	CAR mRNA and IL-7 mRNA	Biotinylated CD3 antibody	Pre-clinical (mice)	Melanoma
	CAMP LNP [35]	uPAR CAR	Circular RNA	N/A	Pre-clinical (mice)	Inflammaging
CAR-M						
Myeloid Therapeutics	LNP; MT-302 [26]; MT-303 [26]	TROP2 CAR; GPC3 CAR	mRNA	N/A	Phase 1; NCT05969041; NCT06478693	Epithelial cancer; hepatocellular carcinoma
Carisma and Moderna	LNP [36]	GPC3 CAR	mRNA	N/A		Hepatocellular carcinoma
	Oxidized LNP [27]	CD19 CAR	mRNA	N/A	Pre-clinical (mice)	
	LNP [28]	SasA CAR	mRNA and CASP11 siRNA	CRV peptide	Pre-clinical (mice)	Sepsis/MRSA

BCMA: B-cell maturation antigen. CAMP: cardiolipin-mimic phosphoramide. FAP: fibroblast activation protein. GPC3: glypican 3. MRSA: methicillin-resistant *Staphylococcus aureus*. SasA: S. aureus surface protein A. TRP1: tyrosinase-related protein 1. TROP2: tumor-associated calcium signal transducer 2. uPAR: urokinase-type plasminogen activator receptor.

with upregulation of antigen presentation, pro-inflammatory signaling, and activation of adaptive immune responses [37]. In addition, Myeloid's second LNP-mRNA candidate, MT-303, encodes an anti-glypican 3 (GPC3) CAR and is under investigation in a first-in-human Phase 1 trial [38]. In collaboration with Moderna Therapeutics, Carisma is also developing an *in vivo* GPC3 CAR-M therapy using LNP-mRNA for hepatocellular carcinoma, which has demonstrated significant suppression of disseminated tumor growth in both humanized and syngeneic tumor mouse models [36].

ENGINEERING NANOTECHNOLOGY FOR *IN VIVO* CAR CELL THERAPY

The development of innovative nanotechnology to enable *in vivo* CAR cell therapy is essential for overcoming the limitations of

traditional *ex vivo* approaches. Delivering CAR mRNA via LNPs offers several advantages, including streamlined manufacturing, reduced costs, and improved patient accessibility [13,39]. By contrast, *ex vivo* CAR-T cell therapy is a time-consuming, multi-step procedure that requires T cell extraction and expansion, lymphodepletion and subsequent CAR-T cell reinfusion. In comparison, LNP production is a more straightforward process, enabling the generation of a universal, off-the-shelf product that is immediately available [13].

Despite these benefits, a major challenge in LNP manufacturing lies in the complexity of the formulations, particularly the precise mixing of components at defined ratios. Scaling up production while maintaining the same level of precision and reproducibility remains difficult. Nevertheless, the high modularity of LNPs provides a significant advantage. This modularity can be divided into three critical components:

- LNP design and composition
- Surface modification with cell-targeting moieties, and
- The CAR-encoding RNA or DNA payload **(Figure 1)**

To achieve efficient nucleic acid delivery to immune cells and ensure robust expression of the construct, each of these elements must be carefully optimized.

LNP DESIGN & COMPOSITION

Efficient screening of LNP libraries is essential for understanding how lipid composition influences physicochemical properties and biological behavior. LNPs possess several favorable characteristics—including a high surface-to-volume ratio, biodegradability, and tunable design—that make them well-suited for CAR cell therapy [40]. By varying the ratios and types of phospholipids, ionizable cationic materials, sterols, and PEG-lipids, LNPs can be optimized to incorporate large CAR mRNA constructs (~1500–3000 nucleotides). These components largely define the LNPs' physicochemical properties—such as size, morphology, and surface charge—which in turn shape tissue selectivity based on the distinct physiological and anatomical features of different organs [41].

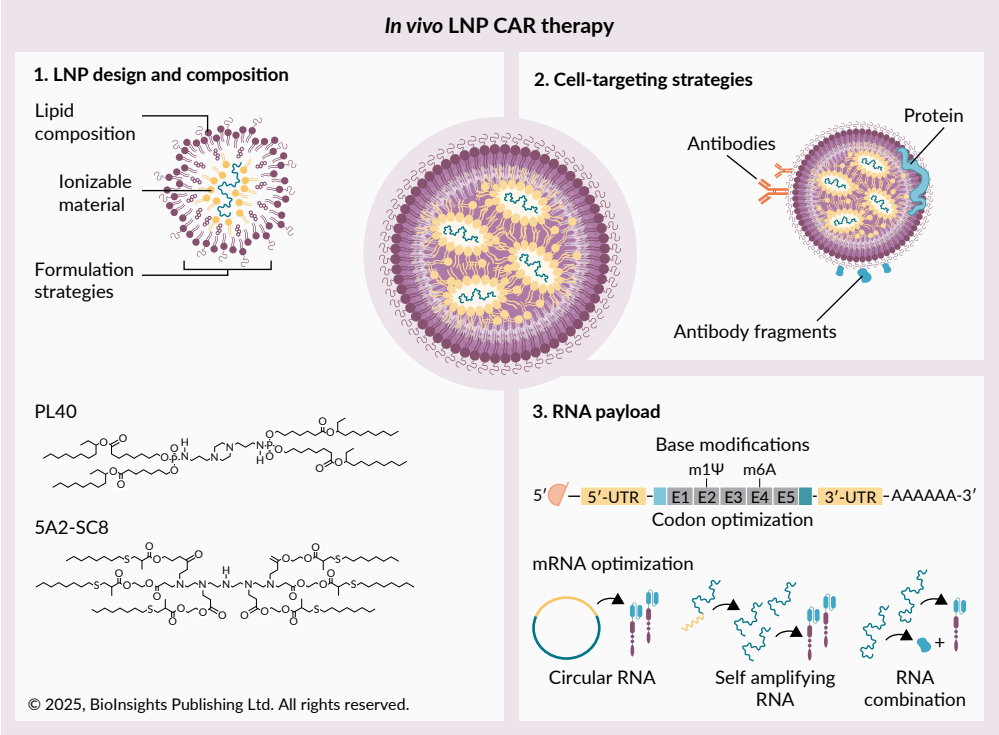
Following intravenous injection, LNPs typically accumulate in the liver due to its fenestrated endothelium and the adsorption of apolipoprotein E, which promotes interaction with the low-density lipoprotein receptor on hepatocytes [41]. However, extrahepatic delivery strategies have demonstrated that compositional optimization or incorporation of targeting lipids and ligands can redirect LNP tropism. For example, Billingsley *et al.* screened a library of 24 distinct LNPs and observed marked variability in delivery efficiency to T cells, underscoring how lipid composition directly impacts functional delivery and therapeutic effect [42]. Similarly, other groups have reported that the inclusion of charged lipids or cardiolipin-mimic phosphoramide (CAMP) lipids can effectively reroute LNPs toward T and B cell populations [35,43]. Selective organ targeting (SORT) lipids have also shown promise in directing LNPs to $CD3^+$, $CD4^+$, and $CD8^+$ cells, as well as delivering CAR mRNA to the spleen [36]. These strategies harness naturally occurring serum protein adsorption—i.e., biomolecular corona formation—to redirect LNPs without requiring direct ligand conjugation [41,44].

LNP SURFACE MODIFICATION WITH CELL-TARGETING MOIETIES

Aside from compositional modifications, LNP tropism can be redirected toward cell populations relevant for *in vivo* CAR cell therapy, such as splenic T cells, by incorporating targeting moieties. This can be

FIGURE 1

Modular design of LNP technology for *in vivo* CAR cell therapy.



Modular design of LNP technology for *in vivo* CAR cell therapy, comprising three key elements: (1) LNP design and composition, (2) LNP surface modification with cell-targeting moieties, and (3) CAR-encoding RNA payload. LNPs can be optimized by altering the lipid composition, ionizable cationic material, or using different formulation strategies. For example, incorporation of PL40 or 5A2-SC8 skewed LNP-mRNA's tropism toward splenic T cells. Immune cell-specificity can be enhanced by surface functionalization with targeting moieties such as antibodies, antibody fragments, or ligands. Finally, the RNA payload can be engineered through base modifications, codon optimization, or utilizing advanced RNA entities.

achieved, for example, by conjugating antibodies via PEG-maleimide coupling or by using biotinylated antibodies [34,45]. Several antibody-conjugated LNPs have been evaluated in preclinical studies for *in vivo* CAR-T cell generation, including those functionalized with anti-CD3, anti-CD4, and anti-CD5 [29,34,40,46,47].

To minimize nanoparticle size, reduce conjugation complexity, and avoid Fc region exposure–which can accelerate phagocytic clearance–smaller targeting moieties such as scFvs or nanobodies are being explored [45]. For instance, conjugating anti-CD3, anti-CD5, and anti-CD7 antibody fragments to LNPs enabled efficient *in vivo* CAR mRNA delivery to T cells, achieving up to 90% B cell depletion [32]. In another study, antigen presenting cell (APC)-mimicking LNPs were engineered by conjugating CD3 and CD28 antibody fragments to promote T cell activation; however, this strategy has only been tested *ex vivo* and requires validation *in vivo* [48]. Alternatively, a dual-targeting approach combining a CD7-specific antibody and a CD3-targeting scFv on LNPs demonstrated effective T cell-specific targeting *in vivo* [49].

For clinical translation, challenges such as variability in conjugation efficiency, degree of antibody modification, and suboptimal target selection must be carefully addressed. To overcome these limitations, antibody-free targeting strategies have emerged [50–52]. For example, Hofstraat *et al.* developed an apolipoprotein

nanoparticle (aNP) platform designed for RNA delivery to immune cells and their progenitors in the bone marrow [52]. Incorporating fusion proteins into the aNP platform further expands its potential for *in vivo* CAR cell applications [51,53].

CAR-ENCODING RNA PAYLOAD

Using mRNA to induce CAR expression offers several advantages, including straightforward modification of the CAR construct and a scalable, cell-free manufacturing process via *in vitro* transcription (IVT). Standard mRNA modifications—such as 5′ cap, 3′ poly(A) tail, and base and backbone modifications—reduce immunogenicity, increase resistance to exonuclease degradation, and enhance mRNA stability and protein production [19,54–56]. Further optimization can be achieved by incorporating untranslated regions (UTRs) derived from α- and β-globin mRNAs and by applying codon design strategies that minimize secondary structure formation and boost expression levels [54,56].

Advanced RNA platforms, including self-amplifying RNA (saRNA) and circRNA, are emerging in novel CAR cell therapy studies. saRNA, derived from RNA virus genomes, contains a replication element that enables intracellular RNA amplification, resulting in prolonged protein expression [54]. This extended expression supports the persistence of CAR-expressing cells, which is essential for maintaining long-term therapeutic efficacy. CircRNAs are produced by chemical, enzymatic, or ribozyme ligation to covalently circularize the RNA [23,54]. Similar to saRNA, circRNAs provide prolonged protein expression but also offer greater efficiency, enhanced stability, and increased resistance to RNases, while exhibiting lower cytotoxicity and fewer side effects compared to linear mRNA [57,58].

Several companies are advancing these technologies. For instance, Orna and Orbital Therapeutics are developing *in vivo* CAR-T cell therapies for B cell malignancies and autoimmune disease (Table 1). Orna incorporates CAR-encoding circRNA into immunotropic LNPs, enabling targeted *in vivo* delivery to immune cells without lymphodepletion, thereby offering a potentially safer and more cost-effective treatment [23]. In another example, Zhang *et al*. reported that incorporating circRNA into CAMP-LNPs not only prolonged protein expression but also enhanced splenic and T cell targeting, thereby increasing the spleen-to-liver ratio of protein expression [35]. CAR therapies may be further advanced by combining multiple RNA modalities within LNP formulations. For example, co-delivery of CAR mRNA and siRNA targeting programmed cell death protein (PD-1) achieved robust CAR expression and PD-1 knockdown in primary human T cells *ex vivo*, mitigating PD-1-mediated inhibition of T cell activation [59]. While demonstrated *ex vivo*, this approach holds promise for *in vivo* applications. In a different strategy, Li *et al*. designed a dual mRNA construct encoding both tyrosine-related protein 1 (TRP1) CAR and interleukin-7 (IL-7). This combination enabled *in vivo* CAR-T cell generation while sustaining T cell proliferation and activity via IL-7 secretion, thereby enhancing therapeutic efficacy [34]. Recently, a T-cell-targeted LNP platform was reported that delivered both CAR minicircle DNA and transposase-encoding mRNA, enabling stable genomic integration of the CAR construct and persistent CAR-T cell production [49].

Together, these strategies represent a growing toolbox for optimizing *in vivo* LNP targeting, offering increasingly flexible, scalable, and clinically translatable approaches.

OUTLOOK

In vivo CAR cell therapy using non-viral vectors is gaining momentum due to cost-effective production, suitability for

large-scale manufacturing, and minimally invasive administration. Its off-the-shelf nature enables faster treatment regimens and reduces inter-patient variability. Furthermore, the modularity of CARs and LNP technology supports the development of innovative therapeutic strategies that improve specificity while reducing CAR-related toxicities such as CRS and ICANS.

Several strategies to regulate CAR cell activity are being explored [60]. One approach introduces 'killing switches' that trigger programmed cell death upon induction by a small molecule [61]. For instance, inducible caspase 9 (iC9) switches consist of fusion proteins combining modified human caspase 9 with FK506 protein. Binding of small-molecule inducers such as AP1903 or AP20187 promotes iC9 dimerization, leading to rapid T cell apoptosis [62]. Another strategy incorporates a cell-surface antigen into the CAR construct, allowing antibody-dependent cell-mediated cytotoxicity upon administration of a specific antibody [63–65].

Logic-gated CARs represent an additional regulatory strategy, combining synthetic receptors with distinct functions to achieve precise tumor targeting and minimize on-target off-tumor toxicity (OTOT). AND-gated CARs, for example, require multiple signals to activate CAR-T cell function [65]. Synthetic notch (SynNotch) receptors provide one approach, where binding to a primary antigen induces expression of a CD3ζ-based CAR targeting a secondary antigen [66]. Alternatively, instantaneous and reversible AND-gated CARs can be engineered by pairing endogenous signaling adaptors such as SLP-76 and LAT [67]. Inhibitory or NOT-gated CARs add another safeguard by incorporating recognition domains for healthy cell antigens, coupled to inhibitory signaling domains like CTLA-4 [68].

CAR expression regulation can also be achieved at the mRNA level. For example, incorporating microRNA (miRNA) binding sites into UTRs allows tissue-specific suppression; hepatocyte-expressed miR-122 degrades mRNA to reduce liver off-target expression [69]. At the genomic level, CRISPR/Cas9 technology has emerged as a powerful tool for precise CAR-T cell engineering [70,71]. Beyond *ex vivo* applications, Hamilton *et al.* demonstrated *in vivo* gene editing using Cas9-enveloped delivery vehicles (Cas9-EDVs) that simultaneously expressed a CD19 CAR and disrupted the T cell receptor α constant (*TRAC*) gene, enabling persistent CAR expression while limiting off-target effects [72]. CRISPR-mediated base editing further enhances safety by altering single nucleotides within epitopes such as CD45 [73] or CD123 [74], protecting healthy hematopoietic cells without impairing protein function.

Computational modeling is increasingly used to accelerate the design of nanotechnology-based delivery systems [75,76]. In parallel, machine learning tools are being applied to optimize both CAR architecture and RNA or DNA payloads. For example, artificial intelligence (AI) can predict tumor recognition efficacy of multi-antigen logic-gated CARs and facilitate *de novo* antigen binder design [77,78]. Xia *et al.* used protein algorithms to generate novel antigen binders solely from target structures, bypassing the need for scFv [79]. This strategy can be generalized to diverse tumor-associated antigens, potentially enhancing CAR cell efficacy against solid tumors [80]. AI also aids mRNA optimization, including codon usage, UTR structure, and stability [81]. Hunter *et al.* showed that optimized UTRs increased CAR expression and improved $CD19^+$ tumor cell lysis *in vitro*, while LNPs carrying anti-CD19 CAR mRNA enhanced CAR expression *in vivo* in humanized mice [9]. Deep learning approaches further enable rational design of 5'UTRs to maximize translation efficiency [82,83].

Despite these advances, major challenges remain. Non-viral vectors such as LNPs suffer from limited endosomal escape,

leading to suboptimal transfection efficiency. As a result, higher doses are often required, raising the risk of toxicity and immune responses to the carrier [84–86]. Repeated dosing is typically necessary due to transient CAR expression, although immune activation from carrier administration may paradoxically promote antitumor immunity by stimulating APCs, cytokine release, and T cell priming [87].

Most current *in vivo* CAR-T cell systems rely on antibody-conjugated LNPs, but targeted formulations present significant manufacturing hurdles, complicating good manufacturing practice (GMP)-compliant scale-up and increasing costs [88]. No targeted nanoparticle systems are commercially available to date. Alternatively, LNP tropism towards T cells can be achieved by tuning lipid composition or designing novel ionizable cationic lipids [33,42]. Coupling high-throughput screening with machine learning may allow reverse-engineering of LNP-mRNA systems to achieve optimal T cell targeting [76,89,90].

In summary, *in vivo* CAR cell engineering holds great promise for advancing immunotherapy. Realizing its potential will require integration of nanotechnology, immunology, and bioinformatics to overcome current barriers and translate preclinical innovation into effective therapies.

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AUTHORSHIP & CONFLICT OF INTEREST

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Willem JM Mulder and Roy van der Meel are listed as inventors on patent applications related to apolipoprotein nanoparticle and fusion protein technology discussed in this manuscript.

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NUCLEIC ACID INSIGHTS

mRNA
EVENT PREVIEW

RNA Therapeutics & Manufacturing Asia 2025

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As part of our ongoing coverage of major gatherings in the advanced therapeutics space, *Nucleic Acid Insights* presents a preview of RNA Therapeutics & Manufacturing Asia 2025. Scheduled for November 5-6, 2025 at the Amari Bangkok, Thailand, this conference will spotlight advanced RNA drug development, scalable manufacturing, and innovative delivery technologies. In this preview, we highlight key sessions, speakers, and themes that will shape the conversation at one of Asia-Pacific's most anticipated nucleic acid events.

RNA THERAPEUTICS & MANUFACTURING Asia 2025

ADVANCING RNA INNOVATION THROUGH COLLABORATION AND NOVEL CLINICAL STRATEGIES

A variety of leading experts will explore the evolving landscape of RNA-based therapies, diagnostics, and public health strategies. Felicia Pradera (Director of Regional Research Centre for Respiratory Medicines and Tropical Diseases, Moderna) will explore how regional partnerships and translational research are accelerating RNA innovation across the Asia-Pacific. Additionally, Subhash Thuluva (Senior VP & Head-Clinical Development, Biological E. Limited) will present a comprehensive roadmap for RNA vaccine development, focusing on how adaptive trial designs and accelerated pathways will shape future clinical strategies. He will explain how developers can select endpoints, assess immunogenicity, and implement safety monitoring. The session will also highlight how teams can prepare for regulatory review and mitigate risks as RNA vaccine candidates move toward approval.

BREAKTHROUGHS IN RNA DELIVERY TECHNOLOGIES

The event will also showcase next-generation RNA delivery technologies poised to transform therapeutic development. Ying Bo (CEO, Abogen Biosciences, China) will introduce Abogen's advanced RNA platform and next-gen lipid nanoparticles (LNPs), which will enable safe and effective mRNA delivery in preclinical models. He will also share clinical updates on how these innovations will support vaccines and treatments for cancer and autoimmune diseases. Poon Hung Fai (CEO, Sirnaomics) will present Sirnaomics' proprietary siRNA delivery systems—PNP and GalNAc—that

will help to enable precise targeting for conditions like cancer and fibrosis. He will highlight promising clinical data from trials STP705 and STP122G and reveal how novel approaches such as AODC will expand RNAi therapies to the CNS, lung, and gut. Together, these leaders will outline how RNA delivery breakthroughs will drive the next wave of precision medicine.

ADVANCED RNA MODALITIES FOR NEXT-GEN CANCER IMMUNOTHERAPY

The conference will also highlight advancements, challenges, and recent breakthroughs in novel RNA modalities. Wonil Kim (Chief Scientific Officer, Aston Sci.) will introduce Th-Vac®, an AI-powered platform for designing mRNA cancer vaccines that target antigens like TROP2, PD-L1, IDO, and KRAS to activate CD4+ T cells. The approach aims to overcome limitations in current immunotherapies.

The RNA Therapeutics & Manufacturing Asia 2025 conference promises to be a landmark gathering spotlighting the latest innovations in RNA-based drug development, scalable production, and delivery technologies across the Southeast Asia region. Uniting leading scientists, biotech executives, and regulators, the event will delve into strategies for overcoming manufacturing bottlenecks, advancing next-gen RNA platforms, and accelerating clinical translation. Key sessions will feature breakthroughs in mRNA and siRNA therapeutics, precision delivery systems, and CRISPR-RNA integration, offering a comprehensive view into the future of RNA medicines and personalized healthcare in the region.

As a reader of *Nucleic Acid Insights*, you're entitled to a **15% discount** on delegate tickets—just use the code **RNAxBioinsights15**! You can find out more about the RNA Therapeutics & Manufacturing Asia 2025 event **here**.

Want to keep up to date with all of the latest nucleic acid events you might want to attend or exhibit at? Explore our free online Events Calendar **here**.

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NUCLEIC ACID INSIGHTS

REVIEW

The toxicological landscape of short nucleic acid therapeutics: a short review of toxicity mechanisms, clinical translation, and regulatory frameworks

Alice Ghidini

RNA-targeting therapies, particularly antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs), represent a crucial advancement in molecular medicine. This review examines the therapeutic potential and inherent challenges of these modalities, highlighting their distinct advantages, design flexibility, a broad range of addressable targets, and high specificity. The critical role of chemical modifications in ASO and siRNA design is discussed, emphasizing the necessity of advanced computational tools for toxicity prediction. While numerous therapies have received regulatory approval, their clinical translation has revealed a landscape of modality-specific adverse events. Obstacles encountered in clinical trials, such as delivery difficulties and off-target effects, are investigated, with a focus on differentiating the safety profiles of distinct therapeutic classes like ASO gapmers, splice-switching oligonucleotides (SSOs), and siRNAs. Furthermore, it explores the translational relevance of preclinical models by examining species-specific toxicological differences and discusses the evolving regulatory frameworks designed to guide their development. The integration of computational methods, enhanced chemical modifications, and innovative delivery strategies is clearly paving the way for more efficacious and safer therapeutics. As our understanding of RNA biology deepens, a corresponding evolution in safety science and regulatory oversight will be paramount for realizing the full clinical potential of RNA based therapeutic modalities.

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ADVANTAGES & CHALLENGES OF OLIGONUCLEOTIDE THERAPEUTICS

In comparison to traditional small-molecule drugs, oligonucleotide therapeutics present several benefits, including facile design, a wide array of addressable targets, and considerable specificity [1–3].

While immunogenicity was an initial concern, advancements in medicinal chemistry have generated sophisticated modifications to mitigate many of these issues. Marketed oligonucleotide drugs typically incorporate a specific set of chemical modifications, including backbone alterations like phosphorothioates (PS) and 2′ ribose modifications like 2′-O-methoxyethyl (MOE), or sugar moiety changes such as locked nucleic acids (LNA) [4]. These chemistries are instrumental in defining the mechanism of action, which can range from simple steric hindrance (SSOs) to the recruitment of cellular machinery for targeted transcript degradation, such as RNase H1 for ASO gapmers or the RNA-induced silencing complex (RISC) for siRNAs.

These modifications are crucial for optimizing pharmacological properties [1]. Nevertheless, safety concerns have arisen, categorized as either hybridization-dependent (sequence-specific binding to unintended RNAs) or hybridization-independent (non-specific interactions with biological entities other than RNA, such as proteins). These phenomena represent clinical safety liabilities requiring resolution [2,5]. Beyond toxicity, delivery to tissues other than the liver remains a formidable challenge [6,7].

MECHANISMS OF TOXICITY

The safety considerations for oligonucleotide drugs fall into three main categories:

- Hybridization- and sequence-dependent effects
- Hybridization-independent but sequence-dependent effects, and
- Hybridization- and sequence-independent phenomena [1,5,8]

Hybridization- and sequence-dependent effects: on- and off-target safety

These effects are mediated by the oligonucleotide sequence hybridizing to either its intended target (on-target) or unintended RNAs (off-target).

- On-target toxicity: this may manifest as an exaggerated pharmacological response or an adverse effect in a non-target tissue. The prolonged duration of action of oligonucleotides, potentially weeks or months, makes this a notable concern [5,9]
- Off-target toxicity: this remains a primary challenge across all oligonucleotide classes
  - ASO gapmers and SSOs: these single-stranded oligonucleotides can bind to partially complementary sequences in unintended mRNAs. For gapmers, this can lead to RNase H1-mediated degradation of unintended transcripts, while for SSOs, it can cause unintended splicing alterations, sometimes resulting in severe outcomes like acute hepatotoxicity [10–12]. Assessing these effects now involves computational models that incorporate RNA secondary structure, moving beyond simple homology searches [13,14]
  - siRNAs: as double-stranded molecules, siRNAs primarily mediate off-target effects when the guide strand tolerates mismatches in its ‘seed’ region (nucleotides 2–8),

leading to the silencing of hundreds of unintended genes [15]. Chemical modifications in this region are a key strategy for mitigating these effects [16]

Hybridization-independent, sequence-dependent effects

This category includes toxicities influenced by specific nucleotide motifs interacting with cellular proteins, rather than by Watson-Crick base pairing.

- Immunostimulation: the innate immune system can be activated via pattern recognition receptors (PRRs)
  - ASOs: the activation of TLR9 by ASOs containing unmethylated CpG motifs is a well-characterized phenomenon that is significantly potentiated by the presence of a phosphorothioate (PS) backbone. This can be mitigated through sequence design [17,18]
  - siRNAs: being double-stranded RNA, siRNAs can activate TLR3, TLR7/8, and cytosolic sensors, triggering a potent interferon response. Chemical modifications like 2′-O-methyl are widely used to dampen this recognition [19]
  - SSOs: SSOs using a neutral backbone, such as phosphorodiamidate morpholino oligomers (PMOs), generally have low immunogenic potential as they do not effectively engage PRRs [20]
- Hepatotoxicity: the liver, a primary site of accumulation, is a major organ for toxicity
  - ASO gapmers: a safety concern, particularly for gapmers with high-affinity modifications like LNA, is sequence-specific hepatotoxicity, likely caused by promiscuous RNase H1 activity on off-target transcripts [11,12]
  - siRNAs: hepatotoxicity with siRNAs is generally less related to the chemistry and more to off-target silencing or the delivery vehicle, such as lipid nanoparticles (LNPs) [21]
  - SSOs: Due to their steric-blocking mechanism, SSOs exhibit a more favorable hepatotoxicity profile compared to gapmers [22]
- Nephrotoxicity: the kidneys, particularly the proximal tubule cells, are another major site of accumulation
  - ASOs: PS-ASOs can accumulate in the kidney and lead to renal toxicity. High-affinity gapmers can induce a more acute nephrotoxicity, possibly through off-target mechanisms similar to those in hepatotoxicity [5,23,24]
  - siRNAs: for siRNAs conjugated with N-acetyl galactosamine (GalNAc), delivery is highly targeted to hepatocytes, reducing renal exposure and mitigating nephrotoxicity-related considerations [25,26]
- Thrombocytopenia: a decrease in platelet count is a clinically adverse event
  - ASOs: this toxicity is primarily associated with PS-ASOs. The mechanism appears to be sequence-dependent, involving direct interaction with platelet glycoprotein VI and potential immune-mediated pathways [27,28]
  - siRNAs and SSOs: clinically, thrombocytopenia has not been a

common finding for siRNAs or neutral-backbone SSOs [20,25]

Hybridization- and sequence-independent effects

These are class effects of certain chemical modifications, most notably the PS backbone.

- Inhibition of coagulation: PS-ASOs can bind to proteins in the coagulation cascade, prolonging clotting times. This is a transient, concentration-dependent effect with generally low clinical risk [29]
- Complement activation: the PS backbone can activate the alternative complement pathway. This effect is more pronounced in non-human primates than in humans and is also transient and concentration-dependent [30]

Toxicity of impurities

An often-overlooked aspect of oligonucleotide safety is the toxicity from impurities generated during synthesis, such as shorter (n−1) or longer (n+1) sequences. These can have their own toxicological profiles, sometimes proving more toxic than the parent drug. A thorough characterization and toxicological assessment of the impurity profile is a critical component of safety evaluation and a key regulatory expectation [31–33].

THE DEVELOPMENT PATHWAY: FROM PRECLINICAL ASSESSMENT TO REGULATORY GUIDANCE

Successfully navigating the development of an oligonucleotide therapeutic requires an integrated approach that combines robust preclinical safety assessment with strategies to mitigate identified risks, all within a structured regulatory framework.

A robust preclinical safety strategy is essential and typically involves a tiered approach, starting with computational analysis and progressing through *in vitro* and *in vivo* models. Computational tools are used to predict off-target hybridization and flag immunogenic motifs. *In vitro* assays then screen for cytotoxicity (caspase activation), hepatotoxicity (LDH, ATP levels in primary hepatocytes), and nephrotoxicity, with an increasing use of advanced models like kidney-on-a-chip platforms. Immunostimulatory potential is assessed by measuring cytokine release from peripheral blood mononuclear cells (PBMCs) [18,34]. Following this, *in vivo* studies in rodents and non-human primates (NHPs) remain a cornerstone for identifying target organs of toxicity and establishing a safe starting dose for human trials, involving assessments of clinical pathology, urinalysis, and histopathology [5,9].

Significant progress has been made in developing strategies to mitigate the toxicities identified in these preclinical studies. A primary approach is the rational application of chemical modifications. For example, incorporating 5′-methyl DNA into an ASO gap has been shown to reduce hepatotoxicity [35], and the development of stereopure oligonucleotides, where the stereochemistry at each PS linkage is controlled, has also shown promise in improving the therapeutic index [36–38]. Careful *in silico* screening to optimize the oligonucleotide sequence is another critical and straightforward strategy [5,12,17]. Furthermore, enhancing delivery to the target tissue can substantially improve the safety profile. The use of GalNAc conjugates to target hepatocytes has increased potency and markedly reduced the kidney toxicity associated with systemically administered ASOs, becoming the standard for liver-targeted siRNAs [25,26].

This entire process has been increasingly guided by regulatory agencies worldwide. The US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have developed specific guidance for

the nonclinical safety evaluation of oligonucleotide therapeutics [39]. These guidelines emphasize a risk-based approach tailored to the specific modality and chemistry. The forthcoming International Council for Harmonisation (ICH) S13 guideline, whose drafting initiation was announced in November 2024, is expected to provide unified international standards, further clarifying regulatory expectations for preclinical studies, including assessments of genotoxicity, carcinogenicity, and reproductive toxicology [40,41].

TRANSLATIONAL CHALLENGES & CLINICAL REALITIES: TOXICOLOGY IN PRACTICE

Translating preclinical safety findings to humans is complicated by species-specific differences in toxicological responses, with clinical trials serving as the ultimate test of a drug's safety. For instance, non-human primates (NHPs) exhibit greater sensitivity to complement activation by PS-ASOs than humans [30], while rodents can show exaggerated inflammatory responses that do not translate [9]. The clinical experience with both approved and failed oligonucleotide drugs provides an invaluable, practical lens through which to view the toxicities discussed previously.

The PS-ASO platform: a case study in managing inherent toxicities

Although the phosphorothioate (PS) backbone is the most common modification in therapeutic oligonucleotides, its associated toxicities are well-documented. The clinical development of inotersen (Tegsedi®) and volanesorsen (Waylivra®) serve as prime examples of these safety challenges.

- Inotersen, an ASO gapmer for hereditary transthyretin amyloidosis (hATTR), demonstrated efficacy but was also associated with safety concerns. Its clinical trials revealed cases of severe, sometimes fatal, thrombocytopenia and glomerulonephritis. This led to the inclusion of a black box warning on its label, necessitating a strict and burdensome patient monitoring program [42]. This clinical outcome is a direct reflection of the sequence-dependent platelet interactions and potential immune-mediated renal damage associated with PS-ASOs

- Volanesorsen, an ASO targeting APOC3 for familial chylomicronemia syndrome (FCS), faced a similar challenge. While effective at lowering triglycerides, its development was marred by a high incidence of severe thrombocytopenia (grade 4 in some patients). This toxicity profile ultimately led the FDA to reject its marketing application in the USA, although it gained approval in Europe under strict conditions. The divergent regulatory outcomes for volanesorsen underscore how the very same toxicity can be weighed differently in a risk-benefit analysis, depending on the disease's severity and the availability of alternative treatments [43]

Impact of chemistry and modality: contrasting profiles in DMD and hATTR

Direct comparisons of drugs with different chemistries for the same indication offer powerful insights.

- Duchenne muscular dystrophy (DMD): the development of splice-switching oligonucleotides for DMD provides a stark contrast in safety profiles based on backbone chemistry. Drisapersen, which utilized a 2′-O-methyl PS chemistry, was ultimately discontinued not only for lack of definitive efficacy but also for a challenging safety profile

that included persistent injection site reactions and signals of renal toxicity (thrombocytopenia and proteinuria) [44]. In contrast, eteplirsen (Exondys 51®), which employs a neutral phosphorodiamidate morpholino oligomer (PMO) backbone, received accelerated approval and has a markedly more benign safety profile, largely devoid of the class-related toxicities associated with the PS backbone [45]. This comparison serves as a clinical validation of how moving away from the PS backbone can mitigate inherent safety risks

- Hereditary transthyretin amyloidosis (hATTR): the treatment landscape for hATTR offers a compelling comparison between ASO and siRNA modalities. While the ASO inotersen is effective, its use is constrained by the aforementioned renal and platelet toxicities. The siRNA therapeutic patisiran (Onpattro®), delivered via a lipid nanoparticle (LNP), demonstrated a more favorable safety profile in its pivotal trials. Its primary adverse events were manageable infusion-related reactions, with a conspicuously lower risk of severe thrombocytopenia or glomerulonephritis [46]

The critical role of delivery: the GalNAc-siRNA platform vs LNP and CNS challenges

Targeted delivery has proven to be a transformative strategy for improving both efficacy and safety.

- The GalNAc-siRNA platform: the conjugation of N-acetyl galactosamine to siRNAs has enabled highly efficient, hepatocyte-specific delivery. This has led to a wave of successful therapies, including givosiran (Givlaari®) for acute hepatic porphyria [47], lumasiran (Oxlumo®) for primary hyperoxaluria type 1 [48], and inclisiran (Leqvio®) for hypercholesterolemia [49], that share a remarkable safety profile. Their toxicity is generally limited to mild-to-moderate injection site reactions, as the targeted delivery minimizes exposure and potential toxicities in other organs like the kidneys. This platform exemplifies how solving the delivery challenge can concurrently solve major safety challenges

- Delivery vehicle toxicity: the delivery system itself can introduce toxicity. Revusiran, an early siRNA therapeutic for hATTR-CM that used a GalNAc delivery moiety, was discontinued after an imbalance in mortality was observed in the treatment arm. This was suspected to be related to off-target effects of the siRNA or toxicity from the delivery platform, underscoring that the safety of the entire drug-delivery complex must be considered [2]

- CNS-directed therapies: delivering oligonucleotides to the central nervous system presents unique challenges. Tofersen (Qalsody®), an intrathecally delivered ASO for SOD1-ALS, has a safety profile marked by serious neurologic events, including myelitis, radiculitis, and papilledema [50]. The high-profile discontinuation of tominersen for Huntington's disease due to an unfavorable risk–benefit profile further highlights the complexities and potential for unexpected adverse outcomes when targeting the CNS [51]. Even the successful SSO nusinersen (Spinraza®) for SMA is associated with procedural risks and post-marketing reports of hydrocephalus, reminding the field that CNS safety requires vigilant, long-term monitoring [52]

FUTURE PERSPECTIVES & CONCLUSION

The field of RNA therapeutics is rapidly expanding beyond ASOs and siRNAs to include emerging modalities such as RNA editing oligonucleotides, RNA-based PROTACs (RiboTACs), and circular RNAs [53]. Each of these new platforms will present a unique set of safety challenges that will require the development of novel assessment strategies. For example, RNA editing oligonucleotides carry the risk of off-target edits, while the immunogenicity of large circular RNA constructs remains to be fully characterized. Addressing these future challenges will require continued innovation in safety science.

Oligonucleotide therapeutics have emerged as a powerful new class of medicines, but their development is accompanied by a unique set of safety challenges linked to their chemistry, sequence, and mechanism of action. A deep understanding of the diverse mechanisms of toxicity is essential for the rational design of safer and more effective drugs. The clear distinctions in the safety profiles of ASO gapmers, SSOs, and siRNAs underscore the importance of a tailored preclinical assessment strategy for each modality. Future progress will be driven by the continued integration of advanced computational models, the development of more predictive safety assays, and the discovery of novel chemical modifications and delivery technologies. By proactively addressing safety liabilities through rational design and a comprehensive preclinical and clinical evaluation, the full therapeutic potential of oligonucleotide-based medicines can be realized.

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