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REVIEW

Current status and trends in small nucleic acid drug development: Leading the future

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Abstract Small nucleic acid drugs, composed of nucleotides, represent a novel class of pharmaceuticals that differ significantly from conventional small molecule and antibody-based therapeutics. These agents function by selectively targeting specific genes or their corresponding messenger RNAs (mRNAs), further modulating gene expression and regulating translation-related processes. Prominent examples within this category include antisense oligonucleotides (ASO), small interfering RNAs (siRNAs), microRNAs (miRNAs), and aptamers. The emergence of small nucleic acid drugs as a focal point in contemporary biopharmaceutical research is attributed to their remarkable specificity, facile design, abbreviated development cycles, expansive target spectrum, and prolonged activity. Overcoming challenges such as poor stability, immunogenicity, and permeability issues have been addressed through the integration of chemical modifications and the development of drug delivery systems. This review provides an overview of the current status and prospective trends in small nucleic acid drug development. Commencing with a historical context, we introduce the primary classifications and mechanisms of small nucleic acid drugs. Subsequently, we delve into the advantages of the U.S. Food and Drug Administration (FDA) approved drugs and mainly discuss the challenges encountered during their development. Apart from researching chemical modification and delivery system that efficiently deliver and enrich small nucleic acid drugs to target tissues, promoting endosomal escape is a critical scientific question and important research direction in siRNA drug development. Future directions in this field will prioritize addressing these challenges to facilitate the clinical transformation of small nucleic acid drugs.

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1. Introduction to small nucleic acid drugs

Small nucleic acid drugs, or oligonucleotide drugs, are short strands of nucleic acids composed of several dozen nucleotide tandems. They share the commonality of being composed of short nucleic acid sequences designed to interact with specific target molecules within cellular pathways. This category includes various types of nucleic acids, such as antisense oligonucleotides (ASOs), and small interfering RNAs (siRNAs), which exert their therapeutic effects by interfering with the expression of target genes through their action on pre-mRNA or mRNA *via* complementary base pairing[1,2]. Aptamers, on the other hand, interact with proteins through the three-dimensional structure formation, further interfering with their function[3]. RNA therapy holds the promise of expanding the scope of drug targets to include traditional proteins as well as previously untargetable transcripts and genes[4]. Small nucleic acid drugs possess distinct characteristics when compared to conventional drugs (Table 1[5-13]). For instance, they work at the genetic or molecular level by interacting with nucleic acids (RNA or DNA) to modulate gene expression, while conventional drugs typically target proteins or other cellular components. Moreover, nucleic acid drugs can be designed to target specific genes or RNA sequences with high precision. This allows for the selective downregulation or upregulation of specific genes, potentially reducing off-target effects compared to some conventional drugs[14]. In contrast to the discovery process of traditional small molecule compounds, which often involves a high degree of serendipity in identifying lead compounds, small nucleic acid drugs can be developed as new drugs simply by designing the appropriate sequence for the specific targeted gene. This approach circumvents the blind spots encountered in the development process, resulting in faster development, reduced time and labor costs, and increased drug specificity[15]. Over the years, advancements in chemical modifications and delivery systems for small nucleic acids have not only improved their specificity and efficacy but also reduced side effects[16]. Consequently, small nucleic acid drugs have emerged as promising therapeutic tools for various diseases, owing to their unique advantages[17].

2. The development history of small nucleic acid drugs

Since the 1970s, the scientific community has made continuous advancements in understanding the underlying mechanisms of small nucleic acid drugs, which has led to significant progress in their research and development. In 1978, researchers at Harvard University, namely Zamecnik and Stephenson[18], made a groundbreaking discovery by demonstrating that a complementary nucleotide chain could effectively inhibit the replicative activity of the Rous sarcoma virus (RSV). This discovery marked a pivotal milestone in the initial development of ASOs[18]. Subsequently, in 1998, the U.S. Food and Drug Administration (FDA) approved the world's first ASO drug, Fomivirsen[19]. The drug is mainly used to treat cytomegalovirus (CMV) retinitis complicated by AIDS patients. It exerts a specific and powerful antiviral effect through antisense inhibition of human cytomegalovirus (CMV) mRNA. Although it was withdrawn for the reason of the success of antiretroviral therapy, it still demonstrated the clinical application potential and value of ASO drug[20,21].

The first miRNA was discovered in 1993, the groups of Ambros and Ruvkun identified a small RNA molecule named *lin-4* in the nematode *Caenorhabditis elegans*. This RNA was found to play a crucial role in the temporal control of development by regulating the expression of specific target genes[22,23]. Since then, miRNAs have been revealed as endogenous molecules that could post-transcriptionally regulate gene expression by binding to specific mRNAs and either inhibiting their translation or promoting their degradation. The recognition of miRNAs' functional roles sparked interest in harnessing the principles of RNAi for therapeutic purposes. In the late 1990s, a momentous achievement

Table 1 Comparison of small molecule compounds, protein replacement therapy, and small nucleic acid drugs[9-17].

Property	Small molecule compound	Antibody drug	Small nucleic acid drug
Molecular weight	Usually, <500 Da	Around >150 kDa	Usually, >7 kDa
Molecule targets	Mainly proteins	Specific proteins or their deficiencies	Mainly RNA molecules, but also proteins and DNA
Site of action	Act both intracellularly and extracellularly.	Act both intracellularly and extracellularly, depending on the target	Act intracellularly (cytoplasm or nucleus)
Administration method	Oral, intravenous, topical, etc.	Intravenous infusion	Intravenous, subcutaneous, or local delivery
Mode of action	Typically, work by binding to specific targets	Bind specifically to their target antigens, either blocking receptor−ligand interactions, inducing receptor internalization, or activating immune responses	ASOs: Modulate RNA splicing, stability, or translation, etc. siRNAs: Degrade target mRNA *via* RNA interference (RNAi), etc.
Safety/Toxicity	Off-target effects and potential toxicity	Sometimes induce immune-related adverse effects	Liver and kidney toxicities

in the field of small nucleic acid drugs occurred when Andrew Fire and Craig Mello demonstrated the gene-silencing effects of double-stranded RNA in the nematode *C. elegans*, paving the way for the discovery of RNAi and the role of siRNA in gene regulation, for which they were awarded the Nobel Prize in 2006[24]. This recognition had a profound effect on the rapid advancement of small nucleic acid drugs, attracting substantial attention from researchers, pharmaceutical companies, and investors[25]. In 2018, the FDA approved a new treatment based on this Nobel Prize-winning technology: Patisiran developed by Alnylam, is a siRNA drug that can specifically inhibit hereditary amyloid transthyretin (hATTR) protein expression. It was the first siRNA drug approved for marketing and the first gene therapy drug with a non-viral drug delivery system[26].

In 2014, the development of *N*-acetylgalactosamine conjugation (GalNAc), a small nucleic acid coupled delivery system, contributed to a rapid recovery in the development of small nucleic acid drugs[27]. With advancements in chemical modification and delivery system technologies, small nucleic acid drugs have entered a new era of steady and rapid development. The chemical modification of nucleotides serves to enhance the stability and mitigate the immunogenicity of nucleic acid molecules. Concurrently, the development of delivery systems is instrumental in preventing nucleic acid drug degradation by endonucleases within the body and improving the efficiency of cellular uptake[28]. The breakthroughs achieved in chemical modification technology and the advent of the GalNAc delivery system have collectively addressed the challenges of stability and the absence of effective delivery systems for small nucleic acid drugs[29]. Since 2016, a succession of groundbreaking small nucleic acid drugs has been introduced, marking significant achievements in the treatment of genetic rare diseases and chronic conditions. These developments have further underscored the therapeutic potential of small nucleic acid drugs (Fig. 1)[30].

3. Classification and mechanism of small nucleic acid drugs

Small nucleic acid drugs represent a burgeoning class of therapeutic agents that harness the inherent regulatory capacities of nucleic acids for precise and targeted interventions. This classification encompasses a spectrum of molecules, each distinguished by its structural characteristics and underlying mechanisms of action[31] (Figs. 2–4, Table 2[19,22,24,26,32-39]).

3.1. ASO

ASOs are short, single-stranded nucleic acid sequences designed to selectively bind to target RNA either in the nucleus or cytoplasm *via* Watson-Crick hybridization[13]. The functionality of ASOs is contingent upon diverse mechanisms, influenced by both the targeted region within the RNA sequence and the design-chemical properties of the ASO[40]. The selection of mRNA sequences for ASO targeting is guided by considerations of binding accessibility. Optimal regions include terminal sequences, internal loops, hairpins, joint sequences, and bulges comprising 10 or more bases[32]. ASOs exert their effects primarily through two major mechanisms: Ribonuclease H (RNase H) enzyme-mediated activity and steric hindrance. Based on these two modes of action, ASOs can give rise to a range of different application strategies, including RNase H enzyme-mediated degradation, exon skipping, exon inclusion, inhibition of mRNA translation, etc (Fig. 2A). In the process of RNase H enzyme-mediated activity, oligonucleotides composed of DNA bind to homologous mRNA transcripts, forming DNA–RNA duplexes. The endogenous nucleic acid enzyme RNase H1 recognizes these DNA–RNA duplexes as substrates and catalyzes the degradation of RNA. Cleavage at the binding site with the ASO disrupts the target RNA, leading to silencing of the target gene expression[41,42]. The RNase H enzyme-mediated activity has been widely employed to downregulate

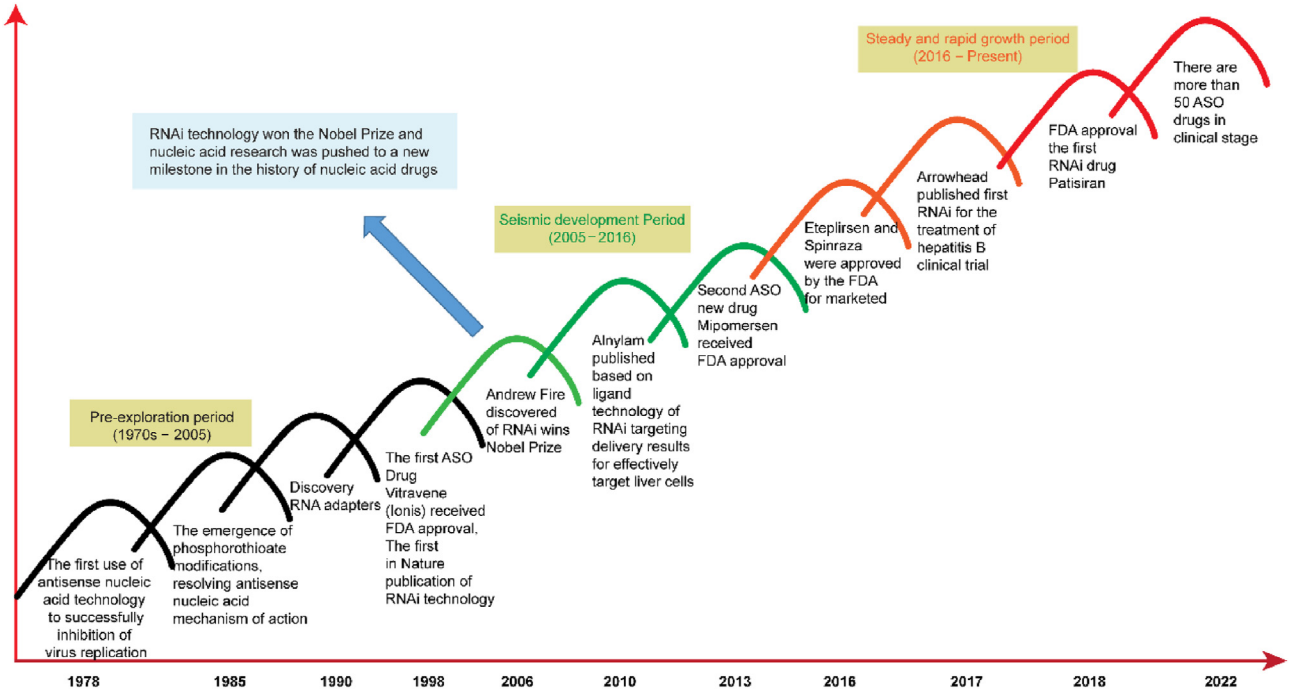

Figure 1 The key milestones in the small nucleic acid drug industry.

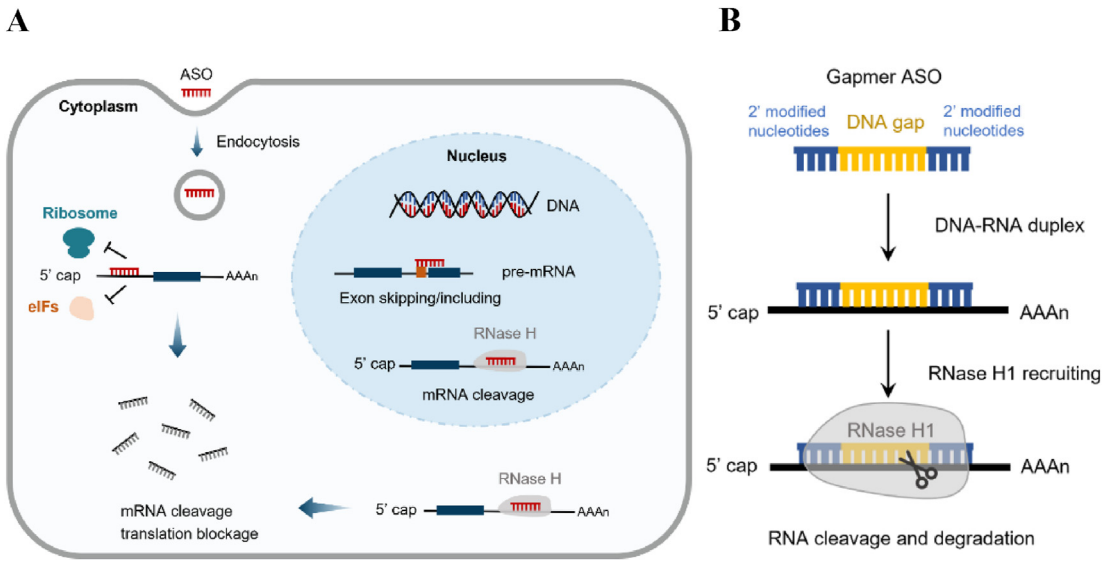

Figure 2 Schematic illustrations of ASOs. (A) ASOs can modulate the mRNA cleavage *via* recruiting RNase H. The translation process can be regulated by ASOs binding with the 5′UTR region to prevent the ribosome or eIFs from entering and triggering the translation. Moreover, ASOs can also interact with pre-mRNA to achieve the splicing altering by exon skipping or including. (B) A gapmer ASO consists of three main components: Central DNA "Gap" segment, specifically hybridize to the target mRNA sequence. Flanking RNA Wings, serve to enhance binding affinity to the target mRNA and facilitate the recruitment of RNase H for mRNA degradation. Chemical modifications, enhance the stability, binding affinity, and specificity of the gapmer ASO.

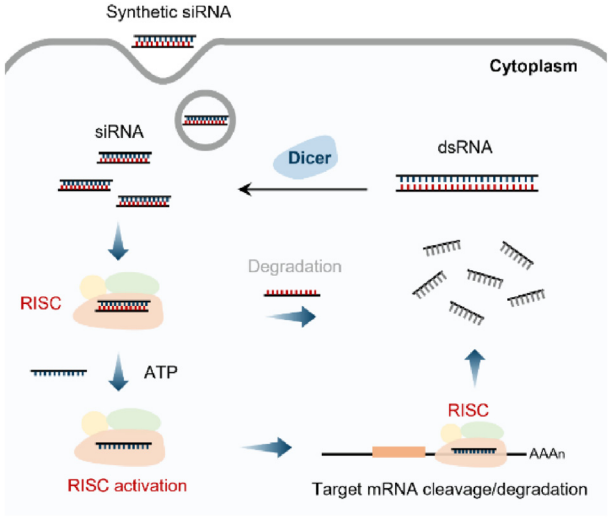

Figure 3 RNAi mechanism of siRNA. dsRNA (either transcribed or artificially introduced) is processed by Dicer into siRNA which is loaded into the RISC. The guide strand then guides the active RISC to the target mRNA. The full complementary binding between the guide strand of siRNA and the target mRNA leads to the cleavage of mRNA.

pathogenic genes and is exemplified by marketed antisense oligonucleotide drugs such as fomivirsen, mipomersen, and inotersen[43]. Due to the requirement of forming DNA−RNA duplexes as enzyme substrates, ASO drugs employing the RNase H1 enzyme-mediated mechanism adopt a "gapmer" structure, consisting of two parts: a central "gap" segment composed of DNA and a flanking region composed of RNA on either side, formed an RNA−DNA−RNA structure (Fig. 2B). The role of the RNA fragments in the wings is to facilitate binding to the target transcript. To enhance affinity, RNA modifications are typically introduced. The DNA portion of the gapmer is designed to be complementary to the target mRNA sequence, which is recognized by the RNase H1 enzyme as substrates, leading to the degradation of the target RNA. It is worth noting that the RNase H1 enzyme exhibits activity both in the cytoplasm and in the nucleus of cells. It can target not only cytoplasmic RNA but also nuclear RNA[8]. This mechanism of action allows ASO drugs to specifically target and degrade disease-causing mRNA, making them a promising approach for treating various genetic disorders[9,42-44].

Additionally, ASOs can prevent the formation of the 5′ cap in mRNA by strategically targeting sequences in the 5′ UTR, inhibiting the binding of essential proteins such as translation initiation factor eIF-4α and subsequently prohibiting 5′ cap-dependent translation[32,45]. Alternatively, designing ASOs that bind near the initiation codon forms a steric hindrance, which prevents the mRNA from entering the ribosome for protein translation, causing the down-regulated expression of the target mRNA[46,47]. Moreover, there is also an intranuclear way. In the process of forming mRNA, ASO binds to a certain exon region of pre-mRNA, causing the exon skipping or altering the splicing position, which can correct errors and restore the correct RNA sequence, with promising applications in genetic diseases such as Duchenne muscular dystrophy[48]. These multifaceted approaches reveal the versatility of ASOs in molecular intervention, offering a promising strategy for targeted therapeutic applications. However, their susceptibility to circulatory degradation, rapid renal clearance, and adverse immune-stimulating effects greatly limits their clinical application. Various strategies of chemical modification

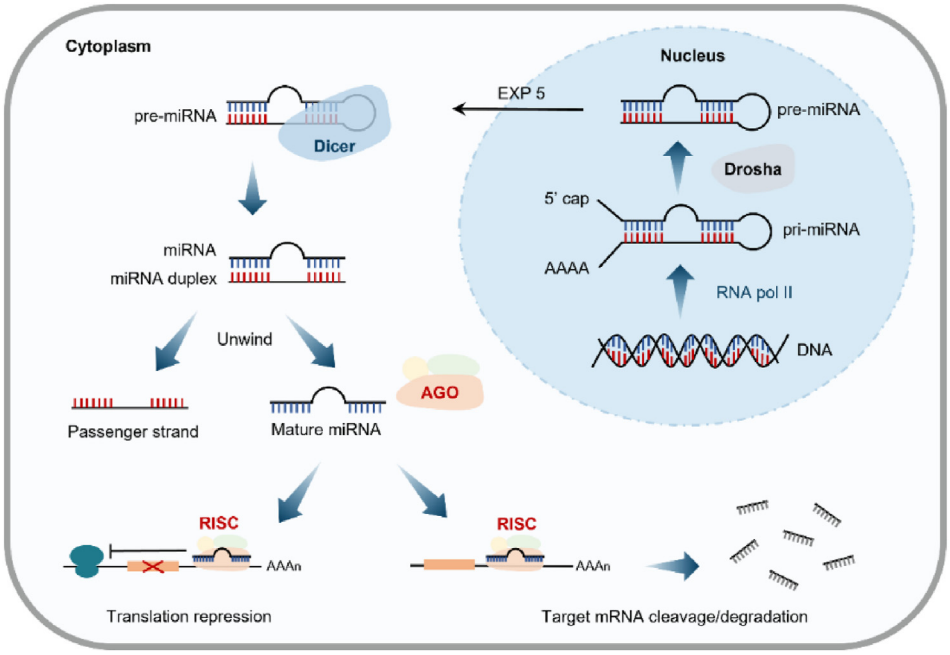

Figure 4 RNAi mechanism of miRNA. Transcription of miRNA gene is carried out by RNA polymerase II in the nucleus to give pri-miRNA, which is then cleaved by Drosha to form pre-miRNA. The pre-miRNA is transported by Exportin 5 to the cytoplasm where it is processed by Dicer into miRNA. The miRNA is loaded into the RISC where the passenger strand is discarded, and the miRISC is guided by the remaining guide strand to the target mRNA through partially complementary binding. The target mRNA is inhibited *via* translational repression, degradation or cleavage.

Table 2 Structural characteristics and mechanisms of action of different types of small nucleic acid drugs.

Category	Structure	First discovery	The first approved drug	Mechanism	Ref.
ASO	Antisenseoligonucleotides (13−30 nt)	1978	1998	RNase H1 recruitment induced mRNA cleavage, steric hindrance in the process of translation, etc.	19,32,33
siRNA	dsRNA (21−25 nt)	1998	2018	RNA interference	24,26,34
miRNA	dsRNA (∼21 nt)	1993	−	RNA interference	22
Aptamer	ssDNA or ssRNA	1990	2004	Inhibit the biological activity of target proteins.	35
saRNA	dsRNA (∼21 nt)	2006	−	RNA activation	36
sgRNA	ssRNA (Around 20 nt)	2011	−	CRISPR/Cas9 gene editing	37
tRNA	ssRNA	1965	−	Gene silencing, RNA processing, etc.	38,39

−Not available.

and delivery system can significantly extend the half-life of ASO and minimize toxicity. After certain specific chemical modifications, ASO drugs can enter cells and specifically regulate the expression of the target gene[42,49,50].

3.2. *RNAi*

RNA interference refers to the phenomenon of introducing double-stranded RNA (dsRNA) composed of sense RNA and antisense RNA corresponding to mRNA into cells, which can cause specific degradation of mRNA, leading to the silencing of its corresponding genes. RNAi technology can be divided into two mechanisms: siRNA and miRNA[51].

3.2.1. *siRNA*

Small interfering RNA (siRNA), also known as short interfering RNA or silencing RNA, is a class of double-stranded RNA molecules, 20−25 base pairs in length[52]. The modulation of gene expression through siRNA-mediated RNAi represents a significant mechanism for gene silencing (Fig. 3). This intricate process involves the enzymatic cleavage of exogenous double-stranded RNA by the Dicer enzyme, generating siRNA or directly introducing synthetic siRNA. Subsequently, the siRNA engages with the cytoplasmic endonuclease Argonaute (AGO) protein, forming a silencing complex known as the RNA-induced silencing complex (RISC). Within the RISC, the siRNA undergoes unwinding, leading to the degradation of its sense strand. Concurrently, the RISC complex, bound to the antisense strand, becomes activated[34,53,54]. The activated RISC complex with its antisense strand selectively associates with the target mRNA, inducing a precise cleavage event. This targeted cleavage results in the specific degradation of the mRNA, thereby impeding the translation process of specific genes, leading to a consequential inhibition of gene expression, achieving the effect of treating diseases, and underscoring the potency and precision of siRNA-mediated RNAi as a tool for gene regulation in biomedical applications[55].

However, siRNA is a negatively charged bioactive macromolecule that has an extremely poor ability to penetrate cell membranes and is greatly unstable in physiological environments[56,57]. In the absence of a protective delivery vehicle, siRNA must be chemically modified to ensure stability in the circulation after parenteral administration. Therefore, the siRNA delivery system is the most critical factor restricting the development of siRNA drugs.

3.2.2. *miRNA*

MicroRNAs (miRNAs) have emerged as noteworthy candidates in the realm of nucleic acid-based therapeutics due to their pivotal roles in post-transcriptional gene regulation. These short, single-stranded RNA molecules, typically comprising 21 to 23 nucleotides, exert their influence on gene expression not only by binding to specific mRNA molecules, thereby modulating protein synthesis, but also by regulating gene transcription in the nucleus[58-60]. This functional intricate regulation is similar to siRNAs, together composed of the two primary parts of RNA interfering technology (Fig. 4). However, the specificity of siRNA and miRNA targeting is due to their different modes of interaction with mRNA. siRNAs are highly specific because they require near-perfect complementarity to their target mRNA sequences for efficient cleavage and degradation. Therefore, siRNAs usually have a single intended target. miRNAs typically recognize target mRNAs through partial complementarity. The imperfect base pairing between miRNA and its targets allows for a broader range of interactions and potential regulatory functions[53].

The transcription of DNA by RNA polymerase II produces primary miRNA (pri-miRNA) with a hairpin structure, subsequently cleaved by Drosha, which is a dsRNA-specific ribonuclease, to generate precursor miRNA (pre-miRNA). The pre-miRNA is longer containing a hairpin loop structure and double-strand region. After transportation to the cytoplasm, pre-miRNAs undergo further processing by Drosha and Dicer, resulting in mature miRNAs incorporated into the RISC complex. It is worth noting that although siRNA and miRNA are both non-coding RNAs with similar roles in gene silencing and regulation, siRNA is fully complementary to a single gene at a specific location, while a miRNA usually has multiple targets and can regulate hundreds or thousands of genes. Moreover, a gene can be regulated by several different miRNAs. Based on the multiple biological functions of miRNAs described above, make them promising therapeutic agents in a wide range of pathological processes. Currently, miRNA drugs consist of two main forms: miRNA analogs (miRNA mimics) and targeted miRNA drugs (antimiRs). The functions of miRNA mimics are similar to those of miRNA in the human body, introducing them to enhance the negative regulatory effects of endogenous miRNAs, mediate mRNA degradation, and reduce intracellular protein expression. AntimiRs are designed to be complementary to the target miRNA sequence, primarily inhibiting miRNA function by competitively binding to the target miRNA, preventing it from exerting its regulatory effects on target mRNAs. This inhibition can lead to the stabilization and increased expression of the targeted mRNAs[61-63].

3.3. *Aptamer*

Nucleic acid aptamers are synthetic short single-stranded DNA or RNA sequences that can bind with high affinity and specificity to a variety of target molecules such as small organic molecules, DNA, RNA, polypeptides or proteins[64,65]. The nucleic acid aptamer technology was born in 1990, together with ASO and RNAi technology, as major discoveries in the field of small nucleic acids at the end of the 20th century[66]. Unlike other nucleic acid drugs, aptamers do not work through base pairing, but are similar to antibodies, relying on their three-dimensional structure to bind to the ligand. Nucleic acid aptamers play a role in three ways: as inhibitors to block the relevant effect of disease-related targets; as agonists to activate target receptors; and as targeted molecular carriers to deliver other drugs to target cells or tissues[67]. Compared with antibodies, aptamers are more difficult and less expensive to obtain, less immunogenic, have better tissue penetration, and have a wider range of targets[3].

Aptamers can be enriched through *in vitro* screening from a nucleic acid molecule library and enrichment using an *in vitro* screening technique: Systematic Evolution of Ligands by Exponential Enrichment (SELEX), which consists of several key steps: binding, isolation, recapture, and amplification[68]. Nucleic acid aptamers are widely used as biosensors because of their binding ability with high specificity and selectivity to a wide range of target substances. The conformation of the nucleic acid aptamer will change once binding specifically to a target substance. Researchers have applied nucleic acid aptamers as probes and developed many electrochemical sensors based on conformational changes, also known as electrochemical aptamer-based (E-AB) sensors[69,70]. The combination with electrochemical detection methods makes them portable, easy to operate, and economical, which remarkably increased the application of nucleic acid aptamers in the sensor field[71-73].

3.4. *Others*

3.4.1. *saRNA*

RNA activation (RNAa) is a small molecule RNA-mediated gene expression up-regulation mechanism. It was in 2006 that saRNA was formally discovered and named by Li et al.[36] Small activating RNA (saRNA) refers to a double-stranded RNA that activates gene expression and is structurally similar to siRNA however exerts a different role[74]. RNAa also requires the involvement of AGO proteins, especially Ago2, to process and form endogenous transcription complexes to target the gene promoter/enhancer regions and upregulate gene expression at the transcriptional level[75].

3.4.2. *sgRNA*

Single guide RNA (sgRNA) is a single-stranded RNA, approximately 20 nucleotides in length[76], which is a part of the CRISPR gene editing tool. The CRISPR/Cas9 gene editing system includes sgRNA and Cas9 nuclease. sgRNA guides Cas9 (or Cpf1) nuclease to perform double-stranded cleavage at a specific gene site to achieve site-directed editing of genes by introducing point mutations, gene insertion or deletion, thereby correcting pathogenic genes or introducing beneficial genes to achieve the purpose of treating diseases[37].

3.4.3. *tRNA*

In addition to the above, there is also transfer RNA (tRNA) which is a single-stranded RNA with a typical ‘cloverleaf’ structure, typically 73−93 nucleotides in length, involved in the translation of proteins[77]. Recent studies have shown that under some stress conditions, tRNAs can produce large amounts of small non-coding RNAs, which are produced by specific cleavage of pre- and mature tRNAs. These tRNAs, named tRNA-derived fragments, or tRNA fragments, have a variety of molecular functions. Some of

them were found to be involved in cell proliferation, progression, and invasive metastasis in several malignant human tumors. Several newly identified tRNA-derived fragments have been considered as the new biomarkers and therapeutic targets for the treatment of cancer and virus infection[78,79].

4. The industry development status of small nucleic acid drugs

To date, a total of 19 small nucleic acid drugs have been approved by the FDA for marketing worldwide, containing 11 ASO drugs, 6 siRNA drugs, and two nucleic acid aptamers (including products that have been withdrawn from the market)[80] (Table 3[19,26,81-103]). Genetic diseases are currently the most approved indication category, with thirteen of the marketed nucleic acid drugs targeting genetic diseases, three for ophthalmic diseases, one targeting cardiovascular disease, one for neurodegenerative disease and one for metabolic disease[104]. These 11 ASO drugs all use chemical modifications of the backbone, bases and sugars. The latest marketed Eplongtersen is a liver-targeted GalNAc-conjugated ASO drug based on the Ligand-Conjuagated Antisense (LICA) technology. Among the six siRNA drugs that utilize delivery systems, five employ GalNAc-conjugated delivery systems, while one drug utilizes a lipid nanoparticle (LNP) delivery system[105].

According to statistics, a total of 108 small nucleic acid drugs have entered the clinical pipeline in the world[106]. The global indications include neoplastic diseases, genetic diseases, sensory organ diseases, cardiovascular diseases, and digestive and metabolic diseases. Among these, oncology and genetic disorders are the most represented indications in the clinical pipeline[107-109].

Regarding the indications, treating spinal muscular atrophy (SMA) is the most commercially successful one[42]. SMA is a rare

Table 3 FDA-approved small nucleic acid drugs.

Type	Generic name	Trade name	Target	Indication	Modification	Delivery system	Company	Year of approve[a]	Ref.
ASO	Fomivirsen	Vitravene	*CMV IE2*	CMV	PS	/	Ionis	1998[b]	19
	Mipomersen	Kynamro	*APOB*	HoFH	PS, 2′-*O*-MOE,5 mC gapmer	/	Ionis	2013[b]	81
	Nusinersen	Spinraza	Exon 7 of *SMN2*	SMA	PS, 2′-*O*-MOE,5 mC	/	Ionis&Biogen	2016	82
	Eteplirsen	Exondys 51	Exon 51 of *DMD*	DMD	PMO	/	Sarepta	2016	83
	Inotersen	Tegsedi	*TTR*	hATTR-PN	PS, 2′-*O*-MOE gapmer	/	Ionis	2018	84
	Volanesorsen	Waylivra	*APOC3*	FCS	PS, 2′-*O*-MOE gapmer	/	Ionis	2019	85
	Golodirsen	Vyondys 53	Exon 53 of *DMD*	DMD	PMO	/	Sarepta	2019	86
	Vitolarsen	Viltepso	Exon 53 of *DMD*	DMD	PMO	/	Nippon Shinyaku	2020	87
	Casimersen	Amondys 45	Exon 45 of *DMD*	DMD	PMO	/	Sarepta	2021	88
	Tofersen	QALSODY	*SOD1*	ALS	PS, 2′-*O*-MOE gapmer	/	Ionis	2023	89,90
	Eplontersen	Wainua	*TTR*	hATTR-PN	PS, 2′-*O*-MOE	GalNAc	Ionis & AstraZeneca	2023	91−94
siRNA	Patisiran	Onpattro	*TTR*	hATTR-PN	2′-*O*-Me, 2′F, PS	LNP	Alnylam	2018	26,95,96
	Givosiran	Givlaari	*ALAS1*	AHP	2′-*O*-Me, 2′F, PS	GalNAc	Alnylam	2019	95−97
	Lumasiran	Oxlumo	*HAO1*	PH1	2′-*O*-Me, 2′F, PS	GalNAc	Alnylam	2020	98
	Inclisiran	Leqvio	*PCSK9*	FH	2′-*O*-Me, 2′F, PS	GalNAc	Alnylam & Novartis	2020	99
	Vutrisiran	Amvuttra	*TTR*	hATTR-PN	2′-*O*-Me, 2′F, PS	GalNAc	Alnylam	2022	100
	Nedosiran	Rivfloza	*LDHA*	PH	2′-*O*-Me, 2′F, PS	GalNAc	Dicerna Pharmaceuticals	2023	101
Aptamer	Pegaptanib	Macugen	VEGF-165	wAMD	/	/	Eyetech & Pfizer	2004[b]	102
	Avacincaptad pegol	Izervay	C5	GA	/	/	Iveric Bio & Archemix	2023	103

/Not available.

CMV, cytomegalovirus retinitis; HoFH, homozygous familial hypercholesterolemia; SMA, spinal muscular atrophy; DMD, Duchenne muscular dystrophy; hATTR-PN, hereditary transthyretin-mediated amyloidosis with polyneuropathy; FCS, familial coeliac disease; ALS, amyotrophic lateral sclerosis; AHP, acute hepatic porphyria; PH1, primary hyperoxaluria yype I; FH, familial hypercholesterolemia; wAMD, wet age-related macular degeneration; GA, geographic atrophy. PS, phosphorothioate; 2′-F,2′-fluoro; 2′-MOE, 2′-*O*-methoxyethyl; 2′-*O*-Me, 2′-*O*-methyl; PMO, phosphorodiamidate morpholino oligonucleotide; 5 mC, 5-methylcytosine.

[a]US FDA-approved time.

[b]The drug has been delisted.

disease that mostly affects children. It is caused by mutations in motor neuron survival genes and can cause permanent damage to neurons[110]. Nusinersen (Trade name: Spinraza) is the first SMA treatment ASO drug approved by the FDA, which can alter the splicing of *SMN2* pre-mRNA, thereby increasing the production of full-length SMN protein. Nusinersen applies to a wide range of populations, from 3-day-old babies to 80-year-old people[82].

Inclisiran, co-developed by Novartis and Alnylam, has received approval as an adjunctive drug when utilized in combination with statins or other lipid-lowering therapies. This approval is specifically indicated for patients who are unable to attain target levels of low-density lipoprotein cholesterol despite being administered the maximum tolerable dosage of statins. The introduction of Inclisiran marks a noteworthy milestone in the application of small nucleic acid drugs for the management of common chronic diseases[99].

Vutrisiran (Amvuttra) is the first drug to treat patients with hereditary transthyretin amyloidosis with polyneuropathy (hATTR-PN). Approved by the FDA in 2022, this drug is the first and only FDA-approved long-acting RNAi treatment drug that can reverse nerve damage. Compared with Patisiran (Onpattro), Vutrisiran has low manufacturing cost, does not require frequent administration, and has the convenience of subcutaneous injection[26,100].

In general, the products on the market are all focused on rare disease indications, the successful commercialization also fulfills the original intention of addressing clinical requirements and pain points. However, it also reveals the current lack of indications with large patient populations in the small nucleic acid drug market.

5. The advantages of small nucleic acid drugs

Compared to the current mainstream small molecule drugs and antibody drugs, small nucleic acid drug technology operates through a distinct mechanism. Over several decades of continuous and in-depth research along with technological breakthroughs, small nucleic acid drug technology has gradually demonstrated unique advantages and significant potential for development[111-113].

5.1. *Higher efficiency in drug development*

The mechanism of action of small nucleic acid drugs primarily relies on forming Watson-Crick base-pairing interactions with the target RNA sequence, making the drug development process simpler. Traditional small molecule compound development requires fitting into the complex structure of proteins, which are not static and may undergo conformational changes *in vivo*, further increasing the difficulty of drug development[114]. Therefore, the early stages of drug development and screening are complex, with the discovery of lead compounds often relying on chance, and molecular optimization requiring extensive screening efforts[115]. Small nucleic acid drugs only require information about the target gene sequence to rationally design highly specific oligonucleotide lead compounds targeting that gene sequence, avoiding the blind spots in the development process[116]. Thus, the speed of early-stage development is faster than traditional drug technologies.

5.2. *Enhanced targeting and specificity*

Small nucleic acid drugs regulate gene expression at the post-transcriptional level and can specifically target the mRNA of the pathogenic gene with high precision, regulate the expression from upstream, and achieve sequence specificity at the single-base level[117]. This specificity and targeting ability are central to their therapeutic efficacy and safety, allowing them to modulate gene expression or protein function in a highly targeted manner, selectively inhibit the expression of a particular gene or modulate specific biological pathways without affecting unrelated genes or pathways, minimizing off-target effects on non-disease-related genes or cellular processes. Overall, the enhanced targeting and specificity of small nucleic acid drugs make them promising candidates for the development of highly selective and efficacious therapies with potentially fewer side effects compared to conventional drugs.

5.3. *A longer-lasting effect*

Small nucleic acid drugs have longer efficacy than traditional small molecule drugs and antibody drugs. Taking siRNA drugs as an example, after completing a round of mRNA degradation, the RISC loaded with the siRNA guide strand can circulate in the body and bind to the next target mRNA for degradation[118]. Therefore, following a single administration, the efficacy of oligonucleotide drugs typically lasts for several months, achieving a long-term effect within the cell. For instance, Inclisiran targets PCSK9, and its indications cover chronic diseases such as mixed hyperlipidemia, hypercholesterolemia, and atherosclerosis. It can be administered subcutaneously twice a year, while monoclonal antibodies with the same target, such as evolocumab, and alircumab need to be administered every 2 weeks. This long-acting treatment is subverting the traditional treatment model for chronic cardiovascular diseases and opens up more possibilities for the clinical application of small nucleic acid drugs[83,86-88].

5.4. *A wide range of target options*

Both small molecule and antibody drugs exert their therapeutic effects by binding to target proteins, thus their development difficulty is largely influenced by the druggability of the target protein. In the human genome, only about 1.5% encode proteins, corresponding to approximately 20,000 types; among these, currently accounting for only about 0.05% of the encoded human genome have successfully developed corresponding therapeutic drugs[119-121]. The mechanism of small nucleic acid drugs is based on the principle of complementary base pairing, not limited by the binding of target proteins, and therefore has a richer pool of candidate targets. For example, small nucleic acid drugs can target pathogenic gene sequences that are specific to patients, including sequences that cause rare diseases, or specific alleles of gene sequences with nucleotide variations. Theoretically, any disease caused by specific gene overexpression can be treated by small nucleic acid drugs, which provides a wealth of candidate targets for the development of small nucleic acid drugs, including many targets that conventional drugs cannot make. For example, as mentioned in Section 4, Nusinersen is the world's first drug to

treat the fatal disease SMA. It achieves this by targeting the backup gene *SMN2* to promote and compensate for the loss of the full-length SMN protein normally produced from the mutated gene *SMN1*. This wide range of target options provides strategies for the treatment of rare diseases[82,122].

5.5. Potential for personalized medicine

Milasen is a groundbreaking example of personalized medicine in the field of ASO drugs. It is an ASO drug designed to target a specific genetic mutation of *MFSD8* in a six-year-old girl, Mila, with a rare form of Batten disease. The patient's whole-genome sequencing revealed a retrotransposon insertion within the *MFSD8* gene, resulting in a transcript with an in-frame stop codon. Thus, a steric block type ASO was designed to bind to a predicted splice enhancer upstream of the SVA retrotransposon in intron 6, restoring the normal splicing (exon 6−7)[123]. It turned out that Milasen reduced the frequency of the seizures, stabilized worsening symptoms, and improved the life quality of the patient. This approach represents a significant advancement in the customization of medical treatments to an individual's unique genetic makeup and corrects the genetic defect responsible for her condition. It highlights the potential of personalized medicine approaches, particularly using small nucleic acid drugs, for rare genetic disorders. This approach opens the door to a new era of precision medicine, where treatments can be tailored to the specific genetic characteristics of each patient, offering hope for those with previously untreatable genetic conditions[9,124].

6. Challenges in the research and development of small nucleic acid drugs

In the pursuit of cutting-edge medical solutions, small nucleic acid drugs are at the forefront, pushing the limits of conventional approaches. Their development involves advanced technologies in RNA design, chemical modifications, delivery systems, synthesis, and formulation (Table 4). Despite these advancements, a central impediment confronted by small nucleic acid drugs lies in their intricate journey to reach target cells. Unmodified small nucleic acid drugs, especially ASOs, are not only easily degraded by nucleases in the body, but also easily induce immune responses. In addition, without the help of a targeted delivery system, it is difficult for negatively charged siRNA drugs to enter cells and exert their effects[125,126]. For both ASO drugs and siRNA-based drugs, the main developing difficulties lie in making structural modifications to increase their stability and in choosing an optimized drug delivery system. Furthermore, after entering the target cells, small nucleic acid drugs are typically encapsulated within endosomes for transport to lysosomes, where they are usually degraded and unable to exert their effects in the active form within the cytoplasm[127]. Therefore, there is a need to enhance the ability of small nucleic acid drugs to escape from endosomes and lysosomes.

6.1. Stability issues: multi-site modification combination

Unmodified small nucleic acid drugs have low cellular uptake efficiency and are prone to degradation by endonucleases within cells. To overcome these shortcomings and improve druggability, modifications are needed on the phosphate backbone, ribose, or bases of oligonucleotides. Currently, after several generations of technological advancement, chemical modification techniques have effectively addressed issues such as nucleic acid drug instability, susceptibility to clearance and degradation, and short half-life, making them more long-lasting and greatly improving the compliance of patients[135]. The commonly used modification sites are as follows (Fig. 5).

6.1.1. Phosphate backbone modifications

Phosphorothioate (PS) modification of the backbone can increase the resistance of phosphodiester bonds to nucleases, thereby enhancing nucleic acid stability. Additionally, the PS backbone can increase the protein binding ability of nucleic acids, such as binding to albumin in plasma, thus delaying renal clearance rates[42,136]. Phosphorodiamidate morpholino oligomers (PMOs) feature a neutral backbone without charge, applying a more complex modification method, where phosphodiester bonds are substituted with phosphorodiamidate linkages, and ribose is replaced by morpholino[137]. Significantly, PMOs exhibit resistance to numerous enzymes present in biological fluids, rendering them exceptionally well-suited for *in vivo* applications[138]. As of now, PMO-based treatment for Duchenne muscular dystrophy (DMD) has demonstrated significant success. Specifically, this therapeutic approach restores the synthesis of functional dystrophin by modulating RNA splicing to exclude the mutated exon 51 of the dystrophin gene. This exclusion enables the translation of downstream full-length dystrophin protein from the modified mRNA, thereby ameliorating the disease phenotype[139].

6.1.2. 2′ sugar modifications

The 2-hydroxyl modification of RNA/DNA plays a crucial role in enhancing the pharmacological properties of ASOs. The most commonly used substituents are the fluorine group (2′-F), methyl group (2′-*O*-Me), and methoxyethyl group (2′-*O*-MOE). Generally, modification at the 2′ position increases nucleic acid resistance to nucleases and enhances thermal stability during complementary hybridization, thereby improving affinity[42].

6.1.3. Base modification

The most commonly used base modification involves replacing the hydrogen atom at the C5 position of cytosine with a methyl group (5-methylcytosine, 5 mC). 5 mC can enhance thermal stability, increase affinity for targets, and reduce the immunogenicity of PS ASOs[140].

6.2. Tissue-specific targeting challenges: extrahepatic delivery

Small nucleic acid drugs need to enter cells and escape endosomes to exert their effects. siRNA drugs, due to their large molecular weight and strong hydrophilicity, cannot directly enter cells. While ASO drugs can be delivered directly after certain chemical modifications, the dosage also limits their application methods and safety. Therefore, efficient *in vivo* delivery systems are required.

Patisiran, the first siRNA therapeutic drug approved by the FDA, based on a lipid nanoparticle (LNP) delivery system, and is the first gene therapy drug with a non-viral vector delivery system for the treatment of ATTR. Encapsulating small nucleic acid drugs in lipid nanoparticles protects the encapsulated drugs from degradation and clearance, and promotes their transport across the cell membrane to the target site[141]. Since then, the development of GalNAc conjugated delivery technology has greatly improved the liver-targeted delivery effect of nucleic acid drugs, which is currently the most mature siRNA drug delivery method. GalNAc is a ligand for the asialoglycoprotein receptor (ASGPR) which

Table 4 Technical challenges in the development of small nucleic acid drugs[93,94,126,128-134].

Technical item	Definition	Core technical challenges	Purpose
Design technology	Design and screening of corresponding RNA drug molecule sequences based on target disease genes.	• Accurate access to functional information on pathogenic genes for proactive, unambiguous, and targeted small nucleic acid drug sequence design	• Improving effectiveness • Improved specificity
Modification technology	Techniques for modifying and optimizing the *in vivo* properties of small nucleic acid drugs by chemically modifying their structure.	• Phosphoric acid skeleton modification • Ribose modifications • Ribose pentacyclic modification • Base modifications • End-to-end transformation	• Improves serum stability and intracellular stability • Reduction of specific or non-specific drug toxicity
Delivery technology	Technology to enhance the biological activity of nucleic acid drugs using vectors, improve distribution *in vivo* and increase drug concentration and bioavailability in target tissues.	• Lipid-based nano-delivery systems, polymer delivery systems, nucleic acid coupled delivery systems, exosome delivery systems, etc. • Optimized synthesis design to address the delivery system's toxicity and tendency to aggregate and leak	• Enhance cell or tissue targeting • Improving delivery efficiency • Reducing delivery system-mediated drug toxicity
Synthesis technology	Oligonucleotides are obtained by adding nucleotides one by one to the synthesized oligonucleotide chain, removing the protecting groups from the bases and phosphate groups, purifying and quantifying.	• Research on liquid phase synthesis technology for future commercial production of large varieties • New small nucleic acid monomer synthesis technology • Small nucleic acid fermentation technology	• Ensure the supply of nucleoside monomers of the required quality on time • Towards the development and commercialization of small nucleic acid drug products
Formulation technology	Technology for optimizing small nucleic acid drugs at the formulation level and developing individual or compounded drugs.	• Development of multi-targeted small nucleic acid complexes for silencing multiple signaling pathways	• Achieving multi-targeted action regulation • Enhancing the effects of small nucleic acid drugs

is an endocytic receptor that is highly specific and expressed on the membrane surface of hepatocytes and is barely expressed in other cells[142,143], making it an ideal receptor for hepatic siRNA delivery[1,144]. In 2022, Alnylam launched the second-generation ATTR upgraded product vutrisiran, which uses an enhanced stabilization chemistry (ESC)—GalNAc delivery system to improve drug efficacy and metabolic stability[100]. From the perspective of convenience of administration, vutrisiran has great advantages over patisiran. The former only needs to be injected subcutaneously once every 3 months, while the latter requires intravenous infusion every 3 weeks, each time taking 80 min[26].

Selective and efficient ASGPR targeting ligands, optimized siRNA design and favorable administration routes are key factors for the clinical translation of GalNAc−siRNA[145]. Except for patisiran, the rest five siRNA drugs use the GalNAc delivery system to achieve liver precise target delivery, which made significant progress in specific targeting, but they cannot solve the problem of extrahepatic delivery. Currently, there are no cases of extrahepatic targeting with clinical small nucleic acid drugs. Therefore, further research and development are still needed to develop nucleic acid drugs with extrahepatic targeting. Some of the strategies that have been explored include peptide-based nanoparticles, which are self-assembled complexes of peptides and oligonucleotides that can target specific receptors on the cell surface and facilitate endosomal escape[146]. Antibody−oligonucleotide conjugates are covalent linkages of monoclonal antibodies and nucleotides that can exploit the natural binding affinity and internalization of the antibodies to deliver the nucleotides to the desired cells[147]. Ligand-mediated delivery, which involves attaching ligands, such as aptamers, peptides, or small molecules, to the nucleotides to enhance their stability, specificity, and uptake by the target cells[148].

These and other novel approaches have demonstrated the potential of extrahepatic delivery of nucleotide therapeutics for the treatment of various diseases, such as cancer, muscular dystrophy, and metabolic disorders. However, there are still many challenges and limitations that need to be overcome, such as optimizing

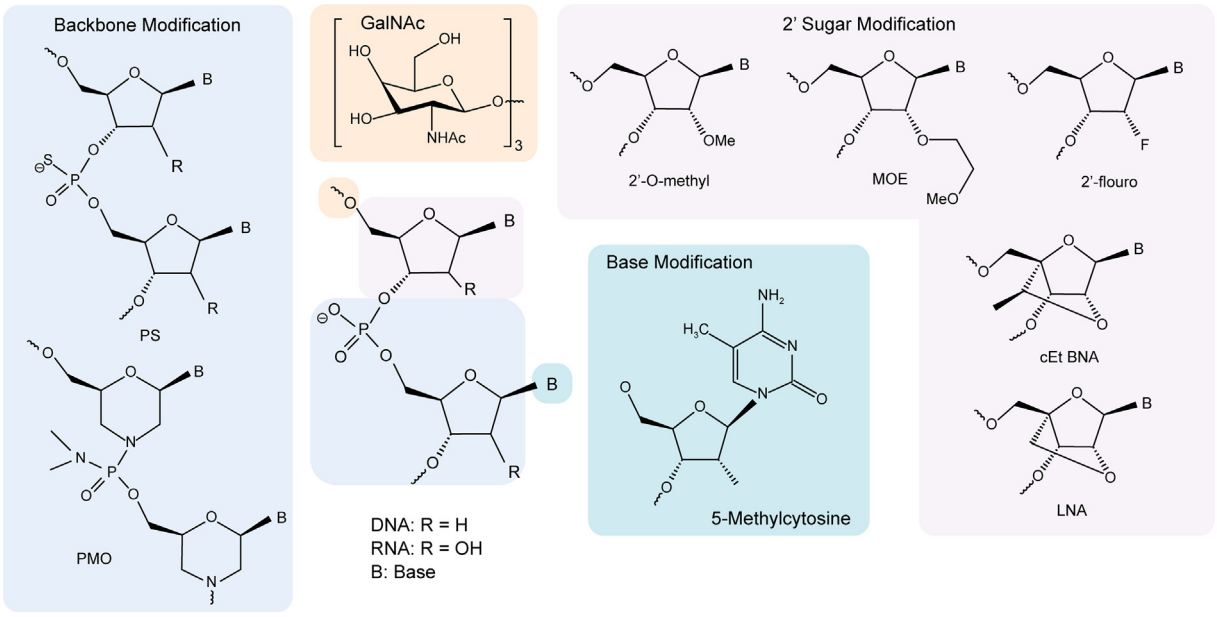

Figure 5 Common methods of chemical modification of small nucleic acid drugs.

the pharmacokinetics, biodistribution, safety, and efficacy of the delivery systems[149].

6.3. *Endosomal escape*

ASO and siRNA drugs must be successfully delivered to target sites within cells, such as the cytoplasm or nucleus before they can exert their gene-silencing effects. Small molecule drugs can passively diffuse across the cell membrane based on extracellular concentration gradients, RNA molecules, due to their large size, hydrophilicity, and/or charge, cannot penetrate the cell membrane through passive diffusion. Thus, cellular uptake of ASO and siRNA drugs primarily occurs through endocytosis, followed by entry into the endolysosomal system. Escape from the endosome is necessary to avoid degradation in the lysosomal environment. This process involves the release from early endosomes, late endosomes, and lysosomes, as these organelles are involved in the degradation and recycling of internalized molecules. The lipid bilayer of endosomes can capture and retain approximately 99% of RNA molecules. Once ASOs and siRNA drugs are trapped in these organelles or degraded, they will not be able to reach the target RNA and will reduce therapeutic efficacy and increase toxicity[150,151]. Research indicates that only 0.3%−1% of small RNA conjugates are capable of escaping the endosome and entering the target cells. Several factors influence the subcellular trafficking and release of ASO and siRNA drugs, such as their chemical modifications, delivery vehicles, receptor-mediated endocytosis, endosomal escape mechanisms, and intracellular transport proteins[152]. For example, ASO drugs with cEt modifications have shown enhanced endosomal escape and cytoplasmic delivery compared to unmodified ASO drugs[153]. Similarly, siRNA drugs can be delivered by various nanoparticles, liposomes, or polymers that can facilitate their endocytosis and endosomal escape[151]. Therefore, for small nucleic acid drug therapeutics to be applicable for the treatment of prevalent human diseases, resolving the bottleneck issue of endosomal escape in a non-toxic manner is imperative[154].

6.4. *Overcoming adverse reactions (ADRs)*

Although small nucleic acid drugs have the potential to treat some diseases that are difficult to target by conventional drugs, they also have some ADRs and toxicity that limit their clinical use[155,156]. For example, mipomersen, the third ASO drug approved by the FDA in 2013 for the treatment of familial hypercholesterolemia, was withdrawn from the market in 2019 because of the unacceptable risk of liver damage[157]. Eteplirsen is the first ASO drug to treat Duchenne muscular dystrophy. The most common ADRs of eteplirsen were balance disorder, vomiting, and contact dermatitis. In the clinical trials, some patients treated with eteplirsen presented with hypersensitivity reactions[155]. Pharmacodynamically, low concentrations of small nucleic acid drugs at the target site caused by poor tissue-targeted delivery, low delivery efficiency, and insufficient targeting forced higher dosages to be administered[151]. The binding of small nucleic acid drugs to non-target RNAs or enrichment in non-target organs or tissues can both result in the toxicity[128]. In addition, potential toxic effects can be triggered by the nano-delivery component or the degraded component[94]. Therefore, overcoming the adverse reaction is crucial for unlocking the transformative potential of small nucleic acid drugs in medicine.

6.5. *Other technical challenges*

The raw materials and equipment used in the production of small nucleic acid drugs require large-scale productive capacity. Therefore, it is essential to consider the quality, speed, and cost of production in the process of scaling up production[129]. The small nucleic acid drug industry chain covers upstream nucleic acid monomer and reagent production, midstream new drug development, and drug production to downstream product commercialization for patients. Small nucleic acid monomers are one of the key raw materials upstream of small nucleic acid drug development. They often need to be chemically modified after synthesis for subsequent use, which will directly affect the stability and

other performance indicators of small nucleic acid drugs[158]. The synthesis of small nucleic acid monomers involves multiple technical means, and the production technology has complex process requirements in terms of purity, throughput, automation, and speed, requiring a long period of technical accumulation. Solid-phase synthesis is often not sufficient for commercial mass production, while liquid-phase synthesis technology can greatly enhance production capacity[159]. Different customers have diverse research objectives and have highly individual production requirements on sequence, length, purity, and modification methods, requiring well-developed production equipment and methods. There are strict GMP production requirements for small nucleic acid monomers used in clinical research[160]. The performance of small nucleic acid monomers is susceptible to change and the requirements for supply chain transport technology are stringent. An increasing number of ASO-based therapies are being tested in clinical trials. Improvements in ASO drug delivery may change the treatment landscape for many diseases in the near future.

7. Future perspective

Small nucleic acid drugs mainly act on mRNA in cells through the principle of base complementary pairing and achieve the purpose of treating diseases by regulating the expression of proteins. Compared with the traditional small molecule drugs and antibody drugs targeting the proteins, small nucleic acid drugs offer a diverse pool of candidate targets for new drug development. Additionally, they boast a shorter development cycle, better targeting and specificity, longer-lasting effects, wider applicability in therapeutic fields and more potential for personalized medicine. Thus far, there is no doubt that small nucleic acid drugs have demonstrated impressive market performance in treating rare diseases with a limited population. However, the industry's key concern is whether these drugs can transcend the confines of rare diseases and find applications in broader fields. Future research and development efforts will focus on further optimizing the chemical modification and delivery system for improving safety and potency, reducing the frequency of toxic and side effects, promoting endosomal escape and targeting extrahepatic tissues. Currently, individual modifications or delivery vehicles alone are insufficient to overcome numerous obstacles. Combining nucleic acid chemical structure modifications with drug delivery systems holds promise for achieving better therapeutic outcomes, but this approach also increases the technical difficulty and clinical translation costs[161–165].

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Author contributions

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Conflicts of interest

The authors declare no conflicts of interest.

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