RNA Therapeutics: How Far Have We Gone?

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Abstract

In recent years, the RNA molecule became one of the most promising targets for therapeutic intervention. Currently, a large number of RNA-based therapeutics are being investigated both at the basic research level and in late-stage clinical trials. Some of them are even already approved for treatment. RNA-based approaches can act at pre-mRNA level (by splicing modulation/correction using antisense oligonucleotides or U1snRNA vectors), at mRNA level (inhibiting gene expression by siRNAs and antisense oligonucleotides) or at DNA level (by editing mutated sequences through the use of CRISPR/Cas). Other RNA approaches include the delivery of *in vitro* transcribed (IVT) mRNA or the use of oligonucleotides aptamers. Here we review these approaches and their translation into clinics trying to give a brief overview also on the difficulties to its application as well as the research that is being done to overcome them.

Keywords

Antisense oligonucleotides · Aptamers · CRISPR-Cas gene editing · Modified mRNA replacement therapy · siRNA-based drugs · U1snRNA vectors

Abbreviations

2′-F	2′-fluoro
2′-MOE	2′-O-methoxyethyl
2′-O-CH2Py(4)	2′-O-methyl-4-pyrimidine
2′-OMe	2′-O-methyl
AADC	Aromatic L-amino Acid Decarboxylase
AAVs	Adeno-associated Viruses;
AGO	Argonaute
AMD	Age-related Macular Degeneration
AONs	Antisense Oligonucleotides
ASGPR	Asialoglycoprotein receptor
ASGR	Asialoglycoprotein Receptor
BBB	Blood Brain Barrier
BMD	Becker Muscular Dystrophy
CNS	Central Nervous System
CPPs	Cell-penetrating Peptides
CRISPR	Clustered Regulatory Interspaced Short Palindromic Repeats

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L. Romão (ed.), *The mRNA Metabolism in Human Disease*, Advances in Experimental Medicine and Biology 1157, https://doi.org/10.1007/978-3-030-19966-1_7

CRISPR-Cas 9	CRISPR-associated protein 9 (Cas9)
CSF	Cerebrospinal Fluid
DLin-MC3-DMA	Anionic lipid dilinol eylmethyl-4-dimethyl-aminoburyate
DMD	Duchenne Muscular Dystrophy
dsRNAs	double-stranded RNAs
ExSpeU1s	Exon-Specific U1 snRNAs
FH	Familial Hypercholesterolemia
GalNAc	N-acetylgalactosamine
GPCRs	G Protein-coupled Receptors
HCV	Hepatitis C Virus
hFVII	Human Factor VII
hTTRA	Hereditary Transthyretin Amyloidosis
I2S	Iduronate 2-sulfatase
IVT	*in vitro* transcribed
LDL-C	Low-density Lipoprotein Cholesterol
LNAs	Locked Nucleic Acids
LNPs	Lipid-based Nanoparticles
miRNAs	microRNAs
mNIS+7	Modified Neurologic Impairment Score +7
mRNA	messenger RNA
NDA	New Drug Application
PCK9	Proprotein Convertase Subtilisin/Kexin Type 9
PD	Pharmacodynamics
PILs	Pegylated immunoliposomes
PIWI	P-element induced wimpy testis
PK	Pharmacokinetics
PMOs	Phosphoroamidate Morpholino Oligomers
PNAs	Peptide Nucleic Acids
PS	Phosphorothioate
RIS	RNA-induced silencing complex
RNAi	RNA interference
RNP	Ribonucleoprotein
RTK	Human Receptor Tyrosine Kinase
SELEX	Systematic Evolution of Ligands by Exponential enrichment
sgRNA	single guide RNA
shRNA	short hairpin RNA
siRNAs	small interference RNAs
SLNs	Solid Lipid Nanoparticles
Sm	Smith antigen
SMA	Spinal Muscular Atrophy
SNALPs	Nucleic-acid-lipid-particles
SSOs	Splice Switching Oligonucleotides
TLRs	Toll-like Receptors
TTR	Transthyretin
U.S. FDA	U.S. Food and Drug Administration

7.1 Introduction

The RNA molecule has traditionally been viewed as an intermediate between DNA and protein. Recently though, this reductive view has been abandoned as more classes and functions of RNA have been discovered as well as therapeutic applications involving this molecule are being developed. RNA therapeutics can either mimic or antagonize the endogenous RNA functions and have several advantages. They can act even on targets that were previously "undraggable" and, most importantly, they are easy to design, cost effective, stable and easy to combine with other drugs presenting also low immunogenicity. Despite these advantages, the use of RNAs as drugs requires the overcoming of two major obstacles: the poor pharmacological properties of RNA, which is rapidly degraded by RNases and the difficulties in its delivery to the target organs and tissues. In this chapter we present the major RNA-based therapeutics currently under research, discussing the challenges to their translation into the clinic and the recent advances in delivery strategies. RNA tools such as ribozymes, riboswitches and SINE-UP strategy are no less important but will not be discussed in this chapter.

7.2 Antisense Oligonucleotides

7.2.1 Brief Overview

Antisense oligonucleotides (AONs) are short synthetic oligonucleotides that bind to RNA through standard Watson-Crick base pairing and can modulate the function of their target RNA [1, 2]. AONs can function in various ways (Fig. 7.1). For example, AONs can mediate targeted gene knockdown through the recruitment of endogenous RNAse H to degrade mRNA at sites of DNA:RNA hybridization caused by AON binding (Fig. 7.1a). They can also be designed to bind to translation initiation sites on mRNAs in cytosol to block translation (Fig. 7.1b). Another approach uses single-stranded AONs to modulate miRNAs expression; these AONs directly bind target miRNAs to inhibit their function (anti-miRs), and thus depress their target gene (Fig. 7.1c). Moreover, AONs can also be used to modulate pre-mRNA splicing in the target gene bypassing the disease-causing mutation (Fig. 7.1d) [3, 4]. These AONs are designated Splice Switching Oligonucleotides (SSOs) and are single stranded 15–25 nucleotides long, which direct pre-mRNA splicing to a new pathway by binding sequence elements and sterically blocking access to the transcript by the spliceosome and other splicing factors [1, 5–7].

The modification of gene expression, using a synthetic single stranded DNA, resulting in inhibition of mRNA translation was demonstrated for the first time by Paterson and colleagues in 1977 in a cell-free system [8]. Almost a year later, Zamecnik and Stephenson showed that in chicken fibroblast tissue culture containing Rous Sarcoma virus, the addition of a synthetic 13-mer oligonucleotide complementary to the 3′ end of the virus, could inhibit its replication and the subsequent transformation of fibroblasts into sarcoma cells [9]. Since then, remarkable progress

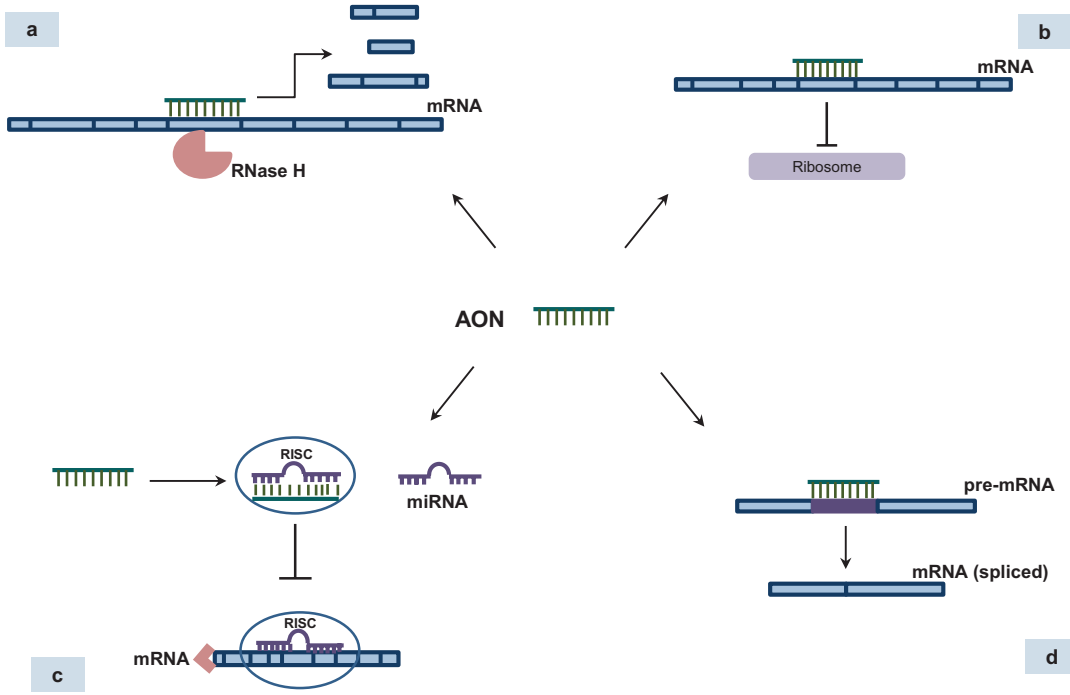

Fig. 7.1 Antisense mechanisms of RNA-based drugs. Antisense oligonucleotides (AONs) impact in gene expression through four different mechanisms: (**a**) RNase H-mediated mRNA degradation; (**b**) steric block of ribosome binding; (**c**) complementary binding to target microRNAs (miRNAs) in order to inhibit their function (antagomirs); and (**d**) splicing modulation

has been made in oligonucleotide drug development and currently, antisense technology is a powerful tool that can be used for target validation and to correct or alter RNA expression for therapeutic benefit [1, 10–12].

Initially, AONs were just synthetic unmodified DNA or RNA molecules, which despite delivering some promising results, would prove to be quite ineffective in biological systems due to their susceptibility to degradation by nucleases, poor affinity for their target mRNA, multiple off-target effects, inability to cross the cell membrane given their negative charge, and weak binding to plasma proteins, leading to rapid clearance by the kidney [1, 3, 13]. Therefore, a wide variety of chemically modified analogues of nucleotides have been developed since then. These chemical modifications were made in the oligonucleotides, generating three categories of AONs, commonly known as generations, with different chemical and pharmacological properties.

7.2.2 Antisense Oligonucleotides Chemistry

Several characteristics need to be fulfilled for the clinical application of antisense oligonucleotides. First, the sequence of the antisense oligonucleotide must be specific enough to avoid off-targets. Other important aspects to be taken into account are the chemistry of the AON and its resistance to degradation by nucleases in order not only to maintain the integrity of the molecule but also to ensure that it is present in an amount, which is sufficient for a true efficacy. In addition, ideal AON should have good pharmacokinetic (PK) and pharmacodynamic (PD) properties and, above all, should not be toxic. Finally, and a very fundamental thing to check, is whether the designed AONs are possible to deliver to target tissues or organs. To try to cope with these desired AON properties, several chemical modifications have been made to the backbone, ribose sugar moiety or nucleobase components, which have a profound effect on the enhanced stability, binding strength and specificity to the target RNA sequence [13] (Fig. 7.2).

The first generation of AONs is characterized by alterations in the backbone, the most common being the phosphorothioate (PS) backbone, accomplished by the replacement of one of the non-bridging oxygen atoms by a sulphur atom. AONs bearing PS linkages are compatible with recruitment of RNase H, which cleaves the target of AONs. This modification allows for an improved nuclease resistance, as well as strong binding to plasma proteins, reducing renal clearance, but still presents poor binding affinity, low specificity and poor cellular uptake [1, 7, 10, 13–15]. Despite these disadvantages, PS oligonucleotides are still the most commonly used AONs and were the first antisense-based drug approved for clinical use in 1998 with fomivirsen (Vitravene®) used for repression of cytomegalovirus mRNA translation [16]. It gained U.S. FDA (U.S. Food and Drug Administration) approval for intraocular treatment of cytomegalovirus retinitis in immunosuppressed patients in 1998 [16] and was discontinued later due to commercial considerations.

In order to surpass the downsides of the first generation oligonucleotides, a second generation was developed through modifications at the 2′ position of the ribose. The most widely studied second generation AONs are 2′-O-methyl (2′-OMe) and 2′-O-Methoxyethyl (2′-MOE), which present higher nuclease resistance and higher affinity for the target RNA, while also reducing non-specific protein binding and toxicity [7, 10, 13, 15]. These second generation AONs, however, do not support RNase H-mediated cleavage of the target mRNA, which impairs their usage for purposes of gene downregulation [1, 14, 17]. This limitation has been minimized with the development of "gapmer" structures where 2′ sugar-modified residues are present on either side of a central "gap" region comprising 8–10 PS-modified nucleotides. The external sugar modified residues thus increase affinity and nuclease resistance, while the internal "gap" region allows RNase H-mediated cleavage of the target RNA [1, 6, 18, 19].

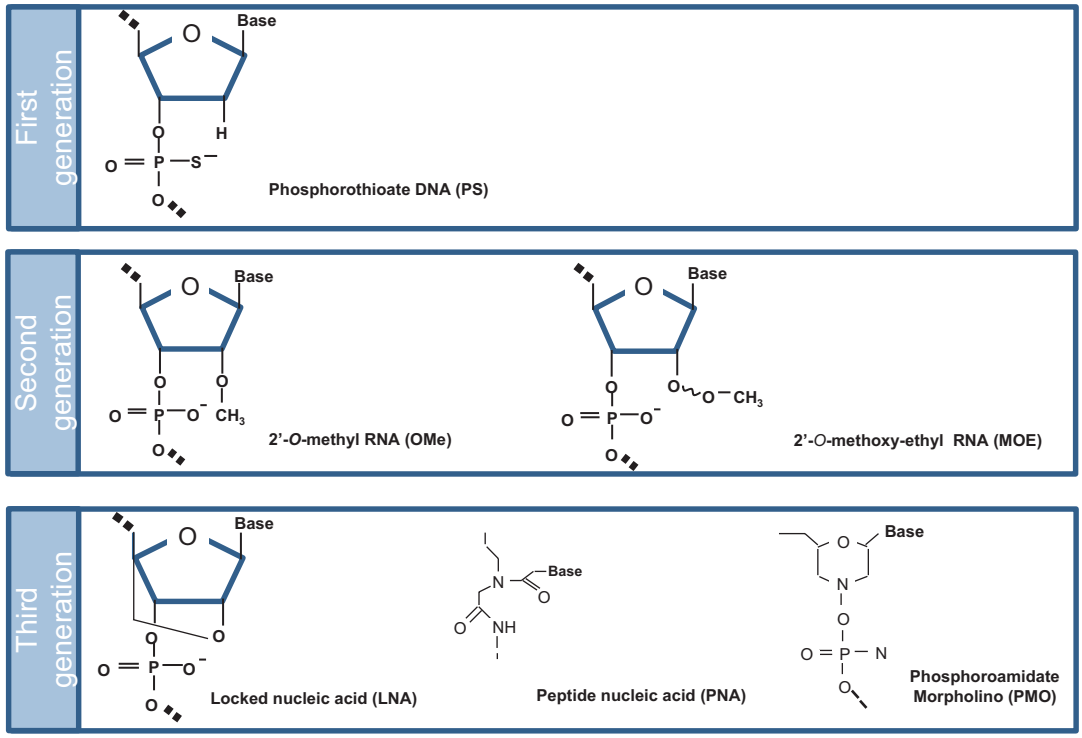

Fig. 7.2 Chemical modifications of antisense oligonucleotides. First generation antisense oligonucleotides (AONs) are characterized by phosphorothioate (PS) backbone; second generation AONs contain a methyl or methoxyethyl group at the 2′ position of the ribose; finally, third generation AONs are characterized by modifications of the furanose ring structure

Finally, the third generation of oligonucleotides is characterized by modifications of the furanose ring of the nucleotide, with the most common being peptide nucleic acids (PNAs), locked nucleic acids (LNAs) and phosphoroamidate morpholino oligomers (PMOs) [12, 14]. These modifications further increase nuclease and protease resistance, target affinity, specificity and *in vivo* stability of antisense drugs, while reduce non-specific interactions with proteins [1, 13, 14, 20, 21]. Nevertheless, PNAs and PMOs present poor cellular uptake, low water solubility and are rapidly cleared from the blood due to their uncharged nature [1, 10, 20, 22] whereas LNAs appear to generate higher toxicity than other chemical modifications, questioning their safety for therapeutic applications [1, 20].

7.2.3 Recent Successful Applications of Antisense Oligonucleotides

The therapeutic application of AONs is very promising. A huge amount of preclinical data has been produced in recent years and many studies have even undergone clinical trials (Table 7.1). Of those, four drugs with different AON chemistries and treatment targets reached, or almost reached clinical practice [12] (Fig. 7.3). One of them is Mipomersen (Kynamro®; Genzyme) that was approved by the U.S. FDA in 2013 for the treatment of familial hypercholesterolemia (FH). Mipomersen is a gapmer of 20 nucleotides and has a sequence complementary to a segment of the Apo b-100 mRNA. Its binding creates a

Table 7.1 Summary table of clinical trials for Antisense Oligonucleotides (AONs)

Chemistry	Drug	Target/organ	Dose/route	Indication	Key observations	References
Commercialized						
2′-H (PS backbone)	Fomivirsen	HCMV *UL122*, Eye	330 μg/eye once every 4 weeks, ITV	CMV retinitis	(1) Effective for CMV retinitis in AIDS patients	[23–25]
2′-MOE (PS backbone)	Mipomersen	*APOB*, Liver	200 mg once weekly, SC	HoFH	(1) Reduction of apoB100 containing lipoprotein particles; (2) Target-related ALT elevations; (3) Clinically meaning full tolerability issues	[26–29]
2′-MOE, fully modified (PS backbone)	Nusinersen	*SMN2* (Intron 7), CNS	12 mg once every 4 months, IT	SMA	(1) Correction of SMN2 splicing defect; (2) Two phase 3 studies terminated early for significant benefit; (3) No drug related adverse events	[30–33]
PMO	Eteplirsen	*DMD* (Exon 51), Muscle	30 mg/kg once weekly, IV	DMD	(1) Increased production of dystrophin; (2) Interpretation of results controversial	[34, 35]
Phase 3						
2′-H	Alicaforsen	*ICAM1*, Colon	240 mg once daily, Enema	Chronic pouchitis	Phase 2 findings included reduction in Pouchitis Disease Activity Index and endoscopy subscore	NCT02525523 [36]
2′-H	Mongersen	*SMAD7*, Intestine	160 mg once daily, Oral	Crohn's disease	Phase 3 failed to demonstrate benefit in patients with Crohn's Disease	NCT02596893 [37] NCT02601300
2′-MOE	Inotersen	*TTR*, Liver	300 mg once weekly, SC	Hereditary ATTR	Phase 3 findings included (1) significant benefit in inotersen-treated patients in both neuropathic disease progression and quality of life measures compared to placebo-treated patients, (2) reduction of mutant and normal TTR, (3) thrombocytopenia (1 fatal bleeding event) and (4) renal events	NCT01737398 [38] (Ionis Pharmaceuticals, press release, Nov 2, 2017)
2′-MOE	Volanesorsen	*APOC3*, Liver	300 mg once weekly, SC	FCS	Phase 3 findings included (1) consistent reduction of triglycerides, (2) mean reduction in triglycerides of 1712 mg/dLin FCS patients, (3) reduced abdominal pain and pancreatitis, and (4) FCS-related thrombocytopenia	NCT02211209 [39–42]

PMO	SRP-4045	*DMD* (Exon 45), Muscle	30 mg/kg once weekly, IV	DMD	Not published	NCT02500381
PMO	Golodirsen (SRP-4053)	*DMD* (Exon 53), Muscle	30 mg/kg once weekly, IV	DMD	On clinical hold due to serious adverse event	NCT02500381
Phase 2						
2′-MOE	IONIS-FXI$_{Rx}$/BAY 2306001	*F11*, Liver	100–300 mg once weekly, SC	Clotting disorders	Phase 2 findings included (1) reduction of Factor XI protein, and (2) reduction of thrombotic events without increase in bleeding	NCT02553889 NCT01713361 [43]
2′-MOE	IONIS-PTP1B$_{Rx}$	*PTPN1*, Liver	200 mg once weekly, SC	T2D	Phase 2 findings included (1) reduction of HbA1c, (2) improved leptin and diponectin levels, and (3) decreased body weight	NCT00455598
2′-MOE	IONIS-GCCR$_{Rx}$	*NR3C1*, Liver	60–420 mg once weekly, SC	T2D	Phase 1 findings included (1) improvement in lipid profile, and (2) attenuation of dexamethasone-induced hepatic insulin resistance	NCT01968265 [44]
2’-MOE	IONIS-GCGR$_{Rx}$	*GCGR*, Liver	50–200 mg once weekly, SC	T2D	Phase 2 findings included (1) attenuation of glucagon-induced increase in blood glucose levels, and (2) reduction of HbA1c	NCT02583919 [45]
2′-MOE	IONIS-FGF$_{R4}$	*FGFR4*, Liver	100–200 mg once weekly, SC	Obesity	Not published	NCT02463240
2′-MOE	IONIS-HTT$_{Rx}$	*HTT*, CNS	10–120 mg once every 4 weeks, IT	Huntington’s disease	In progress	NCT02519036
2′-MOE	IONIS-DGAT2$_{Rx}$	*DGAT2*, Liver	once weekly, SC	NASH	In progress	NCT03334214
2′-MOE	Apatorsen	*HSPB1*, Tumor Cells	200–1000 mg once weekly, IV	Cancer	Phase 1 findings included decrease in tumor markers and decline in CTCs	NCT01454089 [46]
2′-MOE	ATL1102	*ITGA4*, Immune Cells	200 mg twice weekly, SC	MS	Phase 2 findings included reduction in new active lesions	[47]
2′-MOE	Atesidorsen/ ATL1103	*GHR*, Liver	6 mg/kg twice weekly, SC	Acromegaly	Not published	ACTRN12615000289516

(continued)

Table 7.1 (continued)

Chemistry	Drug	Target/organ	Dose/route	Indication	Key observations	References
2′-MOE	IONIS-PKK$_{Rx}$	*KLKB1*, Liver	200 mg once weekly, SC	HAE, chronic migraine	In progress	NCT03108469
2′-MOE	IONIS-HBV$_{Rx}$	*HBV S*, Liver	SC	HBV, chronic atypical	In progress	NCT02981602
cET	AZD9150/IONISSTAT3-2.5$_{Rx}$	*STAT3*, Cancer and Stromal Cells	2–4 mg/kg once weekly, IV	Cancer	Phase 1 findings included (1) reduction of STAT3, (2) reduction in serum IL6, and (3) reduction in tumor burden	NCT02549651 NCT01563302 [48]
cET	AZD5312/ IONISAR-2.5$_{Rx}$	*AR*, Cancer Cells	150–1150 mg once weekly, IV	Prostate cancer	Phase 1 findings included declines in PSA and circulating tumor cells in some patients	NCT02144051 [49]
LNA	Miravirsen	*MIR122*, Liver	3–7 mg/kg once weekly, SC	HCV	Phase 2 findings included inhibition of miR-122 function.	NCT01200420 [50, 51]
2′-MOE, GalNAc	IONIS-APO(a)-L$_{Rx}$	*LPA*, Liver	10–40 mg once weekly, SC	CVD	Phase 1 findings included reduction of Lp(a)	NCT03070782 [52]
2′-MOE, GalNAc	IONIS-ANGPTL3-L$_{Rx}$	*ANGPTL3*, Liver	10–60 mg once weekly, SC	Dyslipidemias	Phase 1 findings included reduction of ANGPTL3, LDL cholesterol and triglycerides	NCT02709850 [53]
2′-MOE, GalNAc	IONIS-APOCIII-L$_{Rx}$	*APOC3*, Liver	once weekly, SC	CVD	In progress	NCT02900027
2′-MOE, GalNAc	GSK3389404/ IONIS-HBV-L$_{Rx}$	*HBV S*, Liver	30–120 mg single dose/once weekly, SC	Chronic HBV	In progress	NCT03020745
Undisclosed, GalNac	AZD4076/RG-125	*MIR107*, Liver	SC	Diabetic NASH	In progress	NCT02826525
Undisclosed	RG-012	*MIR21*, Kidney	110–220 mg once weekly, SC	Alport syndrome	In progress	NCT02855268
Phase 1						
2′-MOE	Eluforsen	Lung/ respiratory epithelium	Intranasal (three times weekly for 4 weeks)	Cystic fibrosis	Phase findings included (1) eluforsen was well tolerated (2) improved CFTR function	NCT02564354; [54]
2′-MOE	BIIB067/ IONISSOD1$_{Rx}$	*SOD1*, CNS	IT	Familial ALS	In progress	NCT02623699

LNA	MRG-106	*MIR155*, Cancer Cells	75–900 mg once weekly, ITM/SC/IV	Hematological malignancies	Phase findings included (1) improvements in cutaneous lesions, and (2) transcriptional changes consistent with target activity	NCT02580552 [55]
LNA	ISTH0036	*TGFB2*, Eye	6.75–225 μg single dose, IVT	Glaucoma	Phase 1 findings included (1) dose-response trend observed in post-operative intraocular pressure, and (2) no adverse events	NCT02406833 [56]
PO, 2′-OMe, ENA	DS-5141b	*DMD* (Exon 45), Muscle	0.1–6.0 mg/kg once weekly, SC	DMD	In progress	NCT02667483
2′-MOE, GalNAc	IONIS-FB-L_{Rx}	*CFB*, Liver	10–40 mg once every 2 weeks, SC	Ocular disease	Phase 1 findings included (1) dose-dependent reduction in factor B levels accompanied by similar reduction in factor B function and complement split factor Bb, and (2) no drug related adverse events	ACTRN12616000335493 [57]
2′-MOE, GalNAc	IONIS-AGT-L_{Rx}	*AGT*, Liver	once weekly, SC	Treatment resistant Hypertension	In progress	NCT03101878
2′-MOE, GalNAc	IONIS-PKK-L_{Rx}	*KLKB1*, Liver	SC	HAE, chronic migraine	In progress	NCT03263507
Discontinued						
2′-OMe	Drisapersen	*DMD* (Exon 51), Muscle	6 mg/kg once weekly, SC	DMD	Rejected by FDA	[58, 59] (Biomarin Pharmaceutical, press release, May 31, 2016)
2′-MOE	Custirsen	*CLU*, Tumor Cells	640 mg once weekly, IV	Prostate cancer and NSCLC	Failure to meet primary endpoints in phase 3 trials	NCT01578655 NCT01630733 [60] (OncoGeneX, press release, Nov 10, 2016)
2′-MOE	IONIS-APO(a)$_{Rx}$	*LPA*, Liver	300 mg once weekly, SC	CVD	Replaced with GalNac conjugate	[61]
2′-MOE	ISIS 388626	*SLC5A2*, Kidney	50–200 mg once weekly, SC	T2D	Availability of small-molecule inhibitors of SGLT2	[62]

(continued)

Table 7.1 (continued)

Chemistry	Drug	Target/organ	Dose/route	Indication	Key observations	References
2′-MOE	ISIS 333611	*SOD1*, CNS	0.15–3.0 mg single dose, IT	Familial ALS	Replaced by more potent compound	[63]
2′-MOE	ISIS 104838	*TNF*, Immune Cells	0.1–6 mg/kg IV, 200 mg once weekly, SC	Inflammatory disease	Inadequate activity	[64]
2′-MOE	ISIS 113715	*PTPN1*, Liver	100–600 mg once weekly, SC	T2D	Replaced by more potent compound	
cET, 2′-MOE	IONIS-DMPK$_{2.5Rx}$	*DMPK*, Muscle	100–600 mg once weekly, SC	DM1	Inadequate activity	NCT02312011 [65]
LNA	EZN-4176	*AR*, Cancer Cells	0.5–10 mg/kg once weekly, IV	Prostate cancer	ALT elevations	NCT01337518 [66]
Undisclosed, GalNac	RG-101	*MIR122*, Liver	SC	HCV	Cases of hyperbilirubinemia	EudraCT 2016-002069-77 (Regulus, press release, Jun 12, 2017)

Abbreviations: **Chemistry:** *2′ – H* 2′- deoxy, *2′ – MOE* 2′ –O- methoxy ethyl, *PMO* phosphorodiamidate morpholino oligomer, *cET* (S)- constrained ethyl, *LNA* locked nucleic acid, *GalNAc* N-Acetilgalactosamine, ENA, 2′ –O, 4′ –C- ethylene-bridged nucleic acid. **Target/organ:** *HCMV UL122* Human cytomegalovirus with IE-2, *APOB* apolipoprotein B, *SMN2* survival of motor neuron 2, *CNS* central nervous system, *DMD* dystrophin, *ICAM1* intercellular adhesion molecule, *SMAD7* SMAD family member, *TTR* transthyretin, *APOC3* apolipoprotein 3, *PTPN1* protein tyrosine phosphatase, non-receptor type1, *NR3C1* nuclear receptor subfamily 3, group C member 1 (glucocorticoid receptor), *GCGR* glucagon receptor, *FGFR4* fibroblast growth factor receptor 4, *HTT* huntingtin, *DGAT* diacylglycerol O-acyltransferase 2, *HSPB1* heat shock protein family B (small) member 1 (heat shock protein27), *ITGA4* integrin subunit alpha 4, *GHR* growth hormone receptor, *KLKB1* kallikrein B1, *HBV S* hepatitis surface antigen (HBsAg), *STAT3* signal transducer and activator of transcription 3, *AR* androgen receptor, *MIR* microRNA, *LPA* lipoprotein (a), *ANGPTL3* angiopoietin like 3, *SOD1* superoxide dismutase 1, *TGFB2* transforming growth factor beta 2, *CFB* complement factor B, *AGT* angiotensinogen, *CLU* clusterin, Solute Carrier Family 5 Member 2, *TNF* tumor necrosis factor alpha, *DMPK* DM1 protein kinase. **Dose/route:** *IVT* intravitreal, *SC* subcutaneous, *IT* intrathecal, *IV* intravenous. **Indication:** *CMV* cytomegalovirus, *HoFH* homozygous familial hypercholesterolemia, *SMA* spinal muscular atrophy, *ATTR* transthyretin amyloidosis, *FCS* familial chylomicronemia, *T2D* type 2 diabetes, *NASH* non-alcoholic steatohepatitis, *MS* multiple sclerosis, *HAE* hereditary angiodema, *HCV* hepatitis C virus, *CVD* cardiovascular disease, *ALS* amyotrophic lateral sclerosis, *NSCLC* non-small cell lung cancer, *DM1* myotonic dystrophy type 1. **Key observations:** *ALT* alanine aminotransferase, *HbA1c* hemoglobin A1c, *CTC* circulating tumor cells, *PSA* prostate specific antigen, *FDA* food and drug administration, *SGLT2* sodium-glucose co-transporter 2, *ALT* alanine aminotransferase

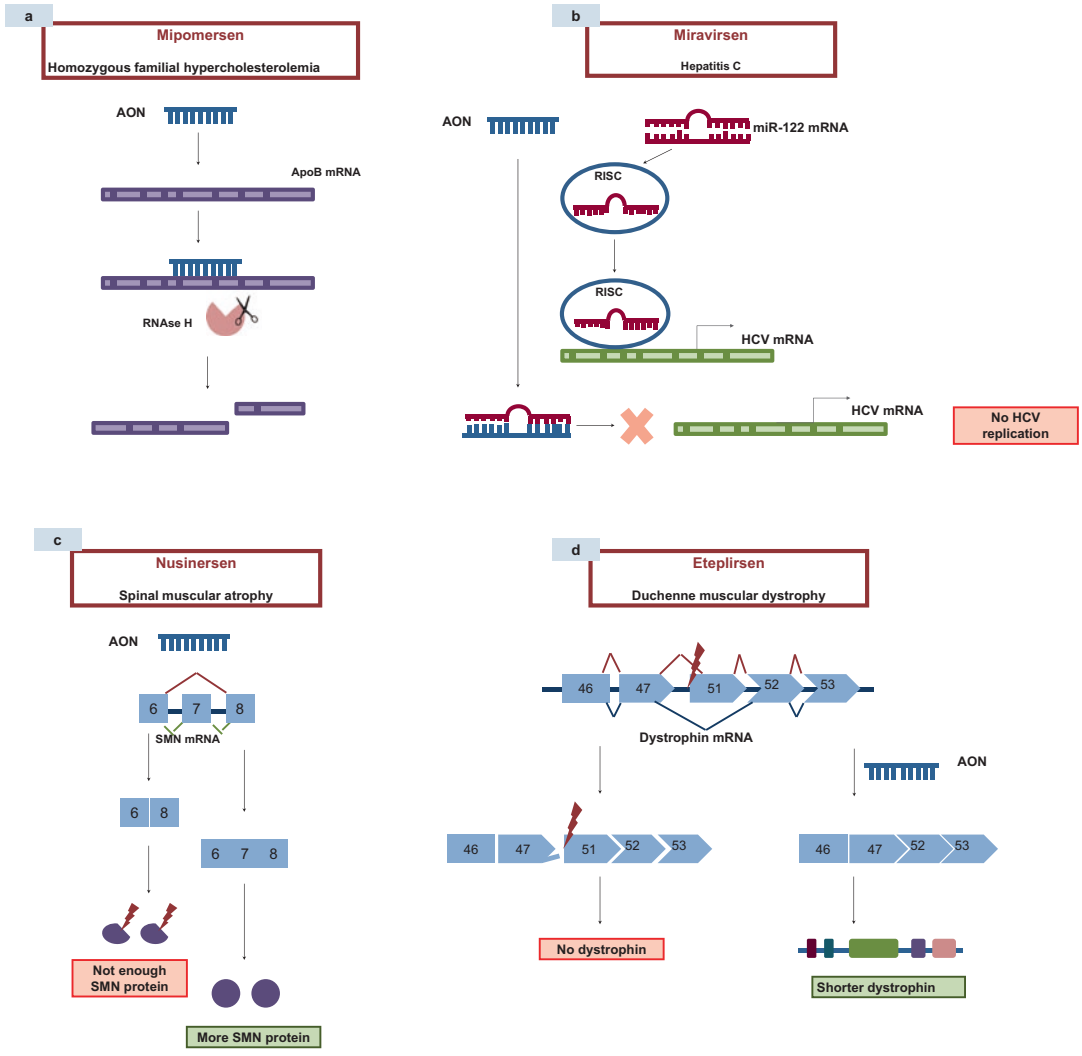

Fig. 7.3 Antisense drugs for clinical practice. Currently, four drugs with different AON chemistries and mechanisms of action have either obtained U.S. FDA approval (Mipomersen, Nusinersen and Eteplirsen) and reached clinical practice, or are seeking accelerated approval soon, with significant pre-clinical data supporting their rapid translation into clinic (Miraversen). (**a**) Mipomersen (Kynamro®; Genzyme), a 2′-MOE-modified AON approved by the U.S. FDA in 2013 for the treatment of familial hypercholesterolemia (FH); (**b**) Miravirsen (SPC3649, Santaris Pharma), an antagomir to miR-122, seeking approval for hepatitis C treatment; (**c**) Nusinersen (Spinraza®, Biogen), a fully modified 2′-MOE AON, approved by U.S. FDA in 2016 for the treatment of spinal muscular atrophy (SMA) and, (**d**) Eterplirsen® (EXONDYS 51TM, Sarepta), a PMO approved by the U.S. FDA in 2016 for the use in Duchenne muscular dystrophy (lightning symbol means the existence of a pathogenic alteration)

DNA:RNA hybrid that is substrate for the enzyme RNase H thus inducing the cleavage of the human Apo b-100 mRNA. The drug has a PS backbone, with 2′-MOE-modified ends, which when compared with earlier antisense technologies, provides greater biological stability and higher affinity to the target mRNA [12, 67]. When administered subcutaneously at a dose of 200 mg per week, it was shown to reduce ApoB-100 production and low-density lipoprotein cholesterol (LDL-C) in a dose-dependent fashion [68]. In general, the results achieved with Mipomersen

point to its efficacy, safety, and tolerability, demonstrating its suitability for use in the target patient population, and providing a tangible tool for use in the management of FH and severe hypercholesterolemia [11, 12, 68]. However, mild-to-moderate injection site reactions, flu-like symptoms and hepatic effects (despite transient and generally reversible) limited its utilization and therefore its commercial success [11].

AONs complementary to mature miRNAs (antagomirs) are also being developed to counteract miRNAs implicated in disease pathogenesis. An example is Miravirsen (SPC3649, Santaris Pharma), an antagomir to miR-122, a liver-specific microRNA that the hepatitis C virus (HCV) requires for replication. Miravirsen is designed to recognize and sequester miR-122, making it unavailable to HCV. As a result, viral replication is inhibited, and the level of HCV infection is reduced [12]. Positive results were observed in a phase II study. The use of Miravirsen in patients with chronic HCV genotype 1 infection showed prolonged dose-dependent reductions in HCV RNA levels without evidence of viral resistance [50]. The updated results revealed no long-term safety issues among 27 Miravirsen-treated patients [51, 69]. Moreover, there was a prolonged decrease in miR-122 plasma levels in patients dosed with Miravirsen but the plasma levels of other miRNAs were not significantly affected by antagonizing miR-122 [69, 70].

The above examples use AONs to alter gene expression, either directly or indirectly, to change disease progression. Another precise method to alter gene expression is to manipulate pre-mRNA splicing using SSOs. This is the case of Nusinersen (Spinraza®, Biogen), a fully modified 2′-MOE AON, approved by U.S. FDA in 2016 for the treatment of spinal muscular atrophy (SMA). SMA is an autosomal recessive neuromuscular disease caused by progressive loss of alfa-motor neurons in the anterior horn of the spinal cord [12, 71]. The most severe form, infant onset or type 1, is the most common one, representing 50% of all SMA cases. Type 2 is less severe, but also very debilitating. These infants never walk, and as they grow the disease progresses and patients begin to lose the capacity of lift even their arms. In humans, a parolog gene of SMA exists, the *SMN2* gene that differs of the *SMN1* by 5–11 nucleotides. However, in the majority of the *SMN2* transcripts the exon 7 is lacking, resulting in a truncated protein, which is rapidly degraded [72]. Nusinersen induces the inclusion of exon 7 in the *SMN2* mRNA by targeting and blocking an intron 7 internal splice site. This action increases SMN protein production, thus improving its function [73]. Intrathecal injection of Nusinersen (every 4 months) allows therapeutic delivery directly into the cerebrospinal fluid (CSF) bathing the spinal cord, the site of motor neuron degeneration and, substantially prolonging survival of type 1 infants, while also resulting in improvements in all measures evaluated [32]. Similar benefit was demonstrated in patients with later onset type 2 SMA [33]. More remarkable, treatment of type 1 pre-symptomatic infants with Nusinersen has been demonstrated to result, in many cases, in achievement of motor milestones at the age expected for healthy infants. Moreover, 92% of the infants treated prior to the development of symptoms were able to sit without support, a milestone never achieved by a type 1 SMA infant before Nusinersen treatment was introduced and 50% were able to walk without support [74].

Another SSO, already in the market is Eterplirsen® (EXONDYS 51™, Sarepta) that was approved by the U.S. FDA in 2016 for use in Duchenne muscular dystrophy (DMD) patients, a severe, childhood-onset disease that results mostly from deletions within the dystrophin gene. DMD is a progressive, neuromuscular disease, occurring mainly in males (1 in 3500–5000 males born worldwide) [75, 76]. It is caused by an absence of the protein dystrophy, a membrane-associated protein that forms a network with sarcolemmal glycoproteins by linking the cytoskeleton actin in muscle fibers within the first few extracellular matrix [77], which results in altered myocyte integrity, muscle wasting and relentlessly progressive weakness. Becker muscular dystrophy (BMD) is a milder disease

caused by dystrophin truncations (due to "in frame" deletions) rather than its absence. A viable strategy for generating truncated, but functional, dystrophin protein involves the skipping of exons to correct DMD-linked mutations (which includes 83% of mutations in DMD) [78]. This can reduce the severity of the disease and produce a milder phenotype, similar to that of BMD. Eteplirsen, the first PMO drug ever approved, binds to the exon/intron splice site at the beginning of exon 51, resulting in its skipping, giving origin to an in frame transcript, which prevents the unwanted degradation of the mutant transcripts by the nonsense-mediated mRNA decay (NMD) pathway, and allowing the production of an internally deleted but functional dystrophin protein [79]. Eterplirsen is applicable for approximately 14% of patients with DMD mutations. It is administered via intravenous infusion and was found to be well tolerated, with no adverse effects, in several clinical trials [79]. In addition, over 3 years of follow-up, Eterplirsen-treated patients showed a slower rate of decline in ambulation assessed by the 6-min walk test compared to untreated matched historical controls from two DMD natural history cohorts: the Leuven Neuromuscular Reference Center (LNMRC) and the Italian Telethon registry [80]. Previously, the ability of Eteplirsen to induce expression of dystrophin had been demonstrated by an observed increase of dystrophin-positive fibers in skeletal muscle of DMD patients [35]. Recently, Kinane and coworkers compared the pulmonary function data from DMD patients, who received Eteplirsen in studies 201/202 (included 12 patients treated with Eteplirsen over 5 years) with the natural history data published. This study verified that the deterioration of respiratory muscle function with Eteplirsen treatment as measured by forced vital capacity was half of that seen in natural history. Maximum expiratory pressure and maximum inspiratory pressure also declined more slowly in Eteplirsen treated patients compared to natural history, thus demonstrating its potential to preserve respiratory function in patients with DMD [81].

7.2.4 Antisense Oligonucleotides Delivery

One of the major issues for the use of AONs for therapeutic purposes is the efficient delivery to their target site. AONs need to reach the target tissue and, once there, they must reach the appropriate intracellular compartment [2, 7]. Parenteral injection, such as intravenous infusion or subcutaneous injection is the main method at the moment of delivery of PS modified single-stranded AONs formulated in a simple saline solution [15, 21]. However, even though AON activity has been observed in many tissues such as lung, stomach, bladder, and heart, AONs predominantly accumulate in liver, kidney, bone marrow, adipocytes, and lymph nodes [21]. Therefore, delivery problems must be considered in terms of sets of barriers to movement of AON within the body. Tissue barriers to delivery include the vascular endothelial barrier, first-pass renal excretion (which strongly affects PK and bio-distribution of AONs), and the blood brain barrier (BBB) that AONs cannot cross due to their size and charge, limiting their access to the central nervous system (CNS), in the case of CNS diseases. The one exception to this is intrathecal injection of single-stranded AONs with specific chemical modifications into the CSF, which allows AONs into the CNS [21].

Two main strategies are being developed to improve AON delivery: viral and non-viral delivery. Despite viral vectors are efficient systems for the delivery of genetic material and for the capability to infect a large number of cell types, they also showed some constraints, such as immunogenicity, tumorogenicity risks, limited loading capacity and scaling-up problems [7]. However, adeno-associated viruses (AAVs), which are non-integrative vectors and therefore present a low risk of genomic insertions, have been used in *in vitro* cells and in animal models to efficiently deliver AONs sequences embedded into modified snRNA systems (modified U1 snRNAs and modified U7 snRNAs). Indeed, promising therapeutic results were obtained with this strategy to induce exon-skipping in diseases like Leber Congenital

Amaurosis [82] and DMD [83–87] and exon-inclusion in SMA [88, 89] (for a more extended review on this subject see [90]). Non-viral delivery also represents a good alternative and the conjugation of free AONs with non-viral delivery carriers can be achieved by different strategies. One option is to conjugate AONs (or their carrier) to a ligand that interacts selectively with a cell surface receptor. Ideally, one such receptor should be expressed only in the tissue to be targeted. Additionally, it should also be abundantly expressed, rapidly and extensively internalized, and have a high affinity to its ligands, so that they become readily available. Some receptors used to target AONs include integrins, G protein-coupled receptors (GPCRs), human receptor tyrosine kinase (RTK), scavenger receptors, asialoglycoprotein receptor (ASGR), Toll-like receptors (TLRs) and folate receptor [91–97], as reviewed in [98]. However, receptors fulfilling all the above-referred criteria are not available for the majority of tissues and, once the AONs reach the cell surface of the target cell, they must be internalized by endocytosis and packed into a vesicle, termed endosome. Then, the endosomes fuse with lysosomes, organelles rich in hydrolases, which ultimately degrade a high portion of the internalized AONs. In fact, one strategy to improve delivery of AONs is the use of short cell-penetrating peptides (CPPs), sequences of short cationic and/or amphipathic peptides (fewer than 30 amino acids) that translocate small drugs/cargo across cell membranes. CPPs are attached to their cargo through covalent linkages or through the formation of noncovalent nanoparticle complexes [99] that can promote uptake of macromoleclues via endocytosis. CPPs covalently conjugated to AONs were already used for therapeutic purposes in DMD [100–105] Myotonic Dystrophy type I [106] and SMA [107, 108]. However, CPPs have a limited endosomal escape and overcoming the rate-limiting step of endosomal escape into the cytoplasm remains a major challenge to their successful use. Several studies tried to overcome this and some relevant results have been obtained. For instance, specific synthetic endosomal escape domains (EEDs) significantly enhanced cytoplasmic delivery in the absence of cytotoxicity [109] and a CPP-adaptor system capable of efficient intracellular delivery was also recently developed [110]. Another possibility is to incorporate AONs into nanoparticles (NPs) that based on their size and materials, will determine the AON biodistribution and interaction. In fact, the progress of nanotechnology has provided several nanosystems with the aim to increase the drug targeting efficacy. The most common types used for drug delivery are solid lipid nanoparticles (SLNs), polymer nanoparticles, lipid-based nanoparticles (LNPs) and carbon-based nanomaterials [110]. For example, cationic core-shell NPs named T1 and ZM2 (a type of polymer nanoparticles) were used to conjugate AONs for exon skipping application in preclinical studies in DMD mice [110, 111]. As these obstacles of delivery are overcome, the advantages of antisense technology will warrant that antisense oligonucleotide therapeutics will be one of the most promising clinical approaches to genetic diseases in the future.

7.3 U1 snRNA-Mediated Therapy

7.3.1 Brief Overview

Since its discovery in the early days of splicing research, U1 snRNA has been recognized as a crucial player in the first stages of the splicing process [112–114]. U1 snRNA is a 164 nucleotides long molecule with a well-defined structure consisting of four stem-loops, which primarily exerts its function in the form of a ribonucleoprotein (RNP) complex (termed U1 snRNP) containing seven Smith antigen (Sm) proteins and three U1-specific proteins U1A, U1C and U1-70K [115] (Fig. 7.4a). It is now well-established that U1 snRNP initiates spliceosome assembly by binding to the 5′ splice donor site (ss) through base pairing between the single stranded 5′ tail of the U1 snRNA molecule and the moderately conserved stretch of nucleotides at the 5′ss (CAG/GURAGU; R-purine) marking the exon-intron boundary [116]. However, not all base pairs at different 5′ss positions are equally important, and their contribution to splicing

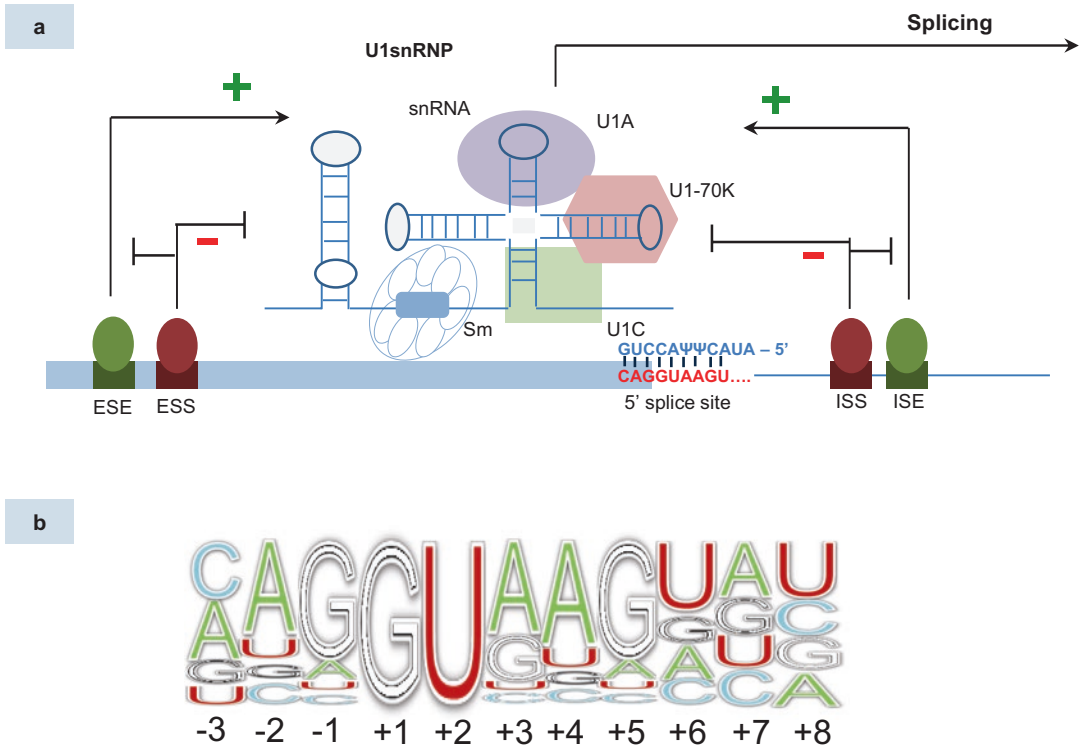

Fig. 7.4 Role of U1 small nuclear ribonucleoproteins (snRNPs) in splicing. (**a**) The 5′ end of U1 snRNA base pairs to the 5′ splice site (ss) in order to define a functional splice donor site. This process is positively and negatively modulated by different splicing factors, which bind to exonic and intronic splicing enhancer and silencer motifs (ESE, ISE, ESS and ISS, respectively). (**b**) The 5′ss motif. The height of each nucleotide corresponds to its conservation at the corresponding positon (−3 to −1 are exonic positions, while +1 to +8 correspond to intronic positions). The most conserved 5′ss positions are +1 and +2, which determine the 5′ss subtype: the GU subtype accounts for ~99% of 5'ss. Minoritary subtypes have a mismatch to U1 at either +1 or +2 and include the GC and the very rare AU 5′ss. The next most conserved 5′ ss positions are -1G and +5G, which form strong G-C base pairs with U1 through three hydrogen bonds. Consensus nucleotides −2A, +3A, +4A, and + 6U are also conserved but have a lesser although important contribution to 5'ss strength because their base pairing to U1 involves only the formation of two hydrogen bonds. The 5'ss positions +7 and +8 do not exhibit substantial conservation in humans, yet several lines of evidence indicate that these positions can base-pair to U1 and contribute to splicing

roughly correlates with their conservation (Fig. 7.4b). In the 9 nucleotides consensus sequence the most conserved 5′ss positions lie at the first two intronic nucleotides (+1 and +2), and the sequence GU at these positions accounts for ~99% of all 5′ss. The next most conserved 5′ss positions are −1G and +5G, which form strong G-C base pairing with U1 [117]. Once the donor site does not always conform to the consensus sequence, but can instead have a degenerate pattern feature, it is understandable that many other additional elements such as splicing silencer and enhancer motifs, the presence of alternative splice sites, secondary structures and regulatory proteins can influence the splice site selection (Fig. 7.4a) [117, 118].

U1 snRNA is classically known for its role in pre-mRNA splicing events. However, the finding that U1 snRNA levels far exceed other spliceosomal associated snRNA levels led to the notion that it may have additional roles in the cell apart from splicing regulation [115, 119]. Indeed, emerging evidence suggests that U1 snRNA plays a key role in transcription initiation and in the protection of pre-mRNAs from degradation, as also has a regulatory function in the 3′-end for-

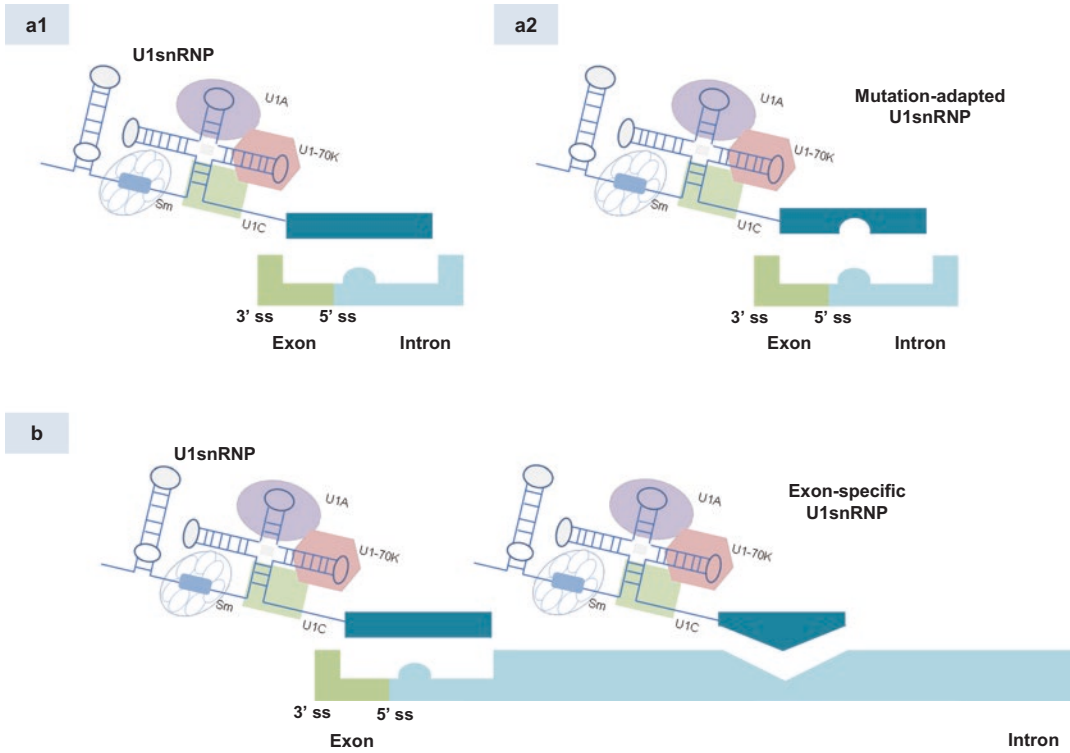

Fig. 7.5 U1 snRNA-mediated therapy for mutations affecting 5′ splice site (ss). (**a1**) A wild-type endogenous U1 small nuclear ribonucleoprotein (snRNP) does not bind to 5′ss due to the presence of a 5′ss mutation. (**a2**) An exogenous U1 snRNP (first generation particle) is modified in 5′ tail with a compensatory alteration (semi-circle) that allows for base-pairing with the mutated 5′ss and the restoration of exon recognition and inclusion. (**b**) The presence of a 5′ss mutation does not allow the correct 5′ss recognition by U1 snRNP but an exon-specific U1 snRNP (second generation particle) with an engineered 5′ tail which binds a downstream non-conserved intronic region can activate the mutated 5′ss, through a mechanism not yet fully understood, allowing the correct exon recognition and inclusion

mation, protecting pre-mRNA transcripts against premature polyadenylation and contributing to the regulation of alternative polyadenylation [115, 119–121].

Splicing mutations at the 5′ss, which are frequent among defects that cause human disease, compromise U1 snRNA binding and can prevent spliceosome assembly and subsequent splicing, which results in exon skipping, intron retention or activation of cryptic splice sites [117, 122]. The most deleterious mutations at a 5′ss are those affecting the nearly invariant GU dinucleotide at the positions +1 and +2. For the remaining nine positions the effects on splicing are less understood. Indeed, nucleotide substitutions in the less conserved positions can cause splicing defects in several but not all 5′ss, suggesting that this 5′ss positions and/or the general context define at what level splicing is changed [117, 123].

7.3.2 Two Generations of Engineered U1 snRNAs to Correct Splicing Defects

As donor splice site mutations disrupt the complementarity of the donor site with the endogenous U1 snRNA, restoring the complementarity through engineered modification of the U1 snRNA represents a valuable approach (Fig. 7.5a1, a2). In fact, in the mid-80s, Zhuang and Weiner [124] demonstrated for the first time that modified U1 snRNAs were able to suppress 5′ss mutations. Since then, the physiological role

of the U1 snRNA to promote exon inclusion in the presence of 5′ss mutations affecting different positions of the donor site, has been extensively exploited as a possible therapy for numerous diseases. Significant correction levels have been achieved for mutations located in less conserved 5′ss positions in diseases like Neurofibromatosis type 1 [125], Coagulation factor VII deficiency [126, 127], Retinitis pigmentosa [128, 129], Propionic acidemia [130], Phenylketonuria [131] and Bardet-Biedl syndrome [132, 133]. Furthermore, recent studies have also demonstrated the feasibility of this approach in the more conserved GU region. In fact, partial correction of splicing defects caused by mutations in the +1 position of 5′ss was observed, not only in a Fanconi anemia case [134], but also in Sanfilippo C disease patient cells [135]. Also, for a mutation in the +2 position causing Hemophilia B, the treatment with a modified U1 snRNA led to an increase in the proportion of correct transcripts (~20%) [136]. In general, though, results of U1 snRNA therapeutic approaches can vary depending on the nature of the mutation and on the overall genomic context.

Until now, modified U1s effects *in vivo* were only addressed in two studies. In the first one, Balestra and co-workers [137] showed the rescue of the expression of a splicing-defective human factor VII (hFVII) mutant by a mutation-adapted U1 snRNA which improved hFVII circulating levels in mice, highlighting the potential of this strategy as a therapy for FVII coagulation deficiency. In the second study, Lee et al. [138] demonstrated the therapeutic effect of a mutation-adapted U1 snRNA in a knock-in mouse model of Aromatic L-amino acid decarboxylase (AADC) deficiency.

In common with other rescue strategies based on targeting RNA by complementarity, modified U1 snRNAs have to deal with potential off-target effects. This is particularly dangerous for modified U1 snRNAs with only one base change from the natural U1 snRNA, which might activate normally silent cryptic donor splice sites and induce aberrant splicing in other genes [139]. The consequences of such unwanted side reactions are hard to predict and depend on the function of the spliced transcript. However, the binding site sequence screening and mapping against the human genome to rule out sequence homologies should extensively decrease nonspecific events although their total exclusion cannot be guaranteed [140]. Therefore, experimental analysis should be performed whenever possible to test the effect of the U1 treatment on non-target transcripts. In mutation-adapted U1 snRNA *in vitro* approaches to correct 5′ splicing defects in Retinitis pigmentosa [129] and Bardet-Biedl syndrome [133], this type of test was performed and no missplicing events were found in the non-target transcripts. Also, in the *in vivo* U1 snRNA therapeutic strategy for AADC deficiency, the treatment was well tolerated and no toxic effects were seen within the study period [138]. However, in the *in vivo* study for hFVII deficiency [137], the authors observed hepatotoxicity, most probably caused by the binding of the engineered U1 to similar consensus 5′ss in other genes.

It was previously shown that U1 snRNAs do not necessarily have to bind at the 5′ss to promote exon definition. Some atypical 5′ss are recognized by U1 snRNA shifted by one nucleotide [141] and U1 snRNAs complementary to intronic sequences downstream of the 5′ss were originally reported to enhance the recognition of 5′ss in model gene systems [142, 143]. Given this, to reduce the possible interaction of modified U1 snRNAs with non-target 5′ss, a second generation of engineered U1s called Exon-Specific U1 snRNAs (ExSpeU1s) was developed. The ExSpeU1s have engineered 5′ tails that direct their loading into non-conserved intronic regions downstream of the 5′ss of a specific exon, and are expected to improve specificity and reduce potential off-target events [139, 144] (Fig. 7.5b). In different cellular models (i.e. minigene assays, patient's cells or iPSC's), a number of ExSpeU1s has been successfully applied, allowing an efficient rescue of exon skipping caused by various types of splicing mutations in Hemophilia B [144, 145], Cystic Fibrosis [144], SMA [144, 146, 147], Fanconi anemia [148] and Netherton syndrome [149]. The ExSpeU1 strategy has also been investigated in mouse models. For SMA, Dal Mas et al. [146], reported that AAV-mediated

delivery of ExSpeU1 corrects splicing, increasing the inclusion of *SMN2* exon 7 in different tissues/organs. In another study, Rogalska and colleagues [150] created a mouse expressing a particular ExSpeU1 and, after crossing it with a severely affected SMA mouse, observed increased inclusion of the missing exon, followed by SMN protein production and increased mice lifespan. Possible gene expression side effects were also addressed and, from a panel of 12,414 analysed genes, only 12 had altered expression after treatment.

ExSpeU1 molecules have also successfully rescued splicing in a transgenic mouse model of Familial Dysautonomia, a rare genetic disease with no treatment [151]. For Hemophilia B, another ExSpeU1 was explored in mice expressing two natural *F9* splicing defective variants at 5′ss or 3′ss, and efficiently rescued human *F9* splicing in liver resulting in an increase of the target protein and coagulation activity [152]. This study, as the pivotal one developed by Fernandez Alanis et al. with the ExSpeU1 strategy [144], interestingly showed that a single ExSpeU1 can be used to correct exon-skipping mutations at the consensus 5′ss (apart from canonical GU dinucleotide), the polypyrimidine tract, and even at exonic regulatory elements, thus extending the applicability of ExSpeU1s to panels of mutations and cohorts of patients with the same genetic disorder.

Despite the promising results obtained with ExSpeU1s in different studies, its precise mechanism of action for splicing correction is not totally clear. Pagani and co-workers demonstrated that ExSpeU1s are assembled as U1-like particles and that their splicing rescue activity is dependent on the U1-70K protein and on the loop IV structure of the U1 snRNA; not on the recruitment of endogenous U1 snRNP to the upstream 5′ss [150]. This may indicate that ExSpeU1s promote correct exon recognition through the recruitment of splicing factors that subsequently activate the mutated 5′ss [144, 146, 149–152]. However, it is important to stress that the splicing stimulator activity of ExSpeU1s was also responsible for the activation of a cryptic 5′ss in an approach attempted to correct a splicing defect causing Intrahepatic Cholestasis, which resulted in the production of an additional splice transcript with intron retention [153].

Globally, both mutation-adapted U1 snRNA and exon-specific U1 snRNA constitute a novel therapeutic strategy to correct splicing defects associated to defective exon definition in several human disorders. Once the U1 snRNA-mediated approaches act at pre-mRNA level, they have the main advantage of maintaining the regulated expression of the targeted gene in the normal chromosomal context [139, 154]. Also, given that the U1 snRNA gene used for splicing rescue includes promoter and regulatory sequences, it has the capability of guaranteeing long term correction of the genetic defect [139]. Despite these advantages, U1 snRNA-mediated therapies may also face some problems such as the presence of off-target effects and low efficacies. Therefore, in a near future, it will be imperative not only to develop a specific method or tool to search for off-target effects, but also to adjust the expression levels of U1 snRNA therapeutic particles in preclinical *in vivo* studies [154].

7.3.3 Engineered U1 snRNAs Delivery

U1 snRNA-mediated therapies also have to deal with the challenge of an efficient delivery to a target tissue. In the *in vivo* studies already developed, one of the most successful gene therapy systems available nowadays – AAV vectors – has been chosen as the method for U1 snRNA-engineered particles delivery into mice [137, 138, 146, 150, 151]. AAV vectors allow a highly efficient delivery to various tissues following systemic injection, even though dependent on the viral serotype used [90, 155]. Also, the low packaging capacity of AAV vectors is quite adequate for U1 snRNA-based approaches given the small cassette size to package [90]. However, despite the modifications that have been introduced in viruses, the potential for antiviral immunity and phenotoxicity of the transgene are still major limitations to the use of viral vectors for therapy.

Possible alternatives to viruses are liposomes and nanoparticle delivery [155].

Among the several RNA tools enabling the rescue of splicing, both mutation-adapted U1 snRNA and ExSpeU1 snRNA therapeutic strategies have already shown their efficacy to repair different types of splicing defects at least in animal models of disease. Still, further developments will be necessary for this therapeutic approach to be translated to human trials.

7.4 siRNA-Based Drugs

7.4.1 Brief Overview

The last decade of the twentieth century has also witnessed the discovery of a new mechanism of gene regulation whose therapeutic potential is still being unveiled: RNA interference (RNAi). Interestingly, the first experimental observation of this mechanism came up from a failed genetic experiment aimed at developing more attractive petunia flowers. In fact, in 1990, Jorgensen and co-workers attempted to genetically engineer flower pigmentation genes, to be inserted into the target plant genome. To their surprise, however, instead of generating more colorful flowers, they ended up producing a generation of plants that had virtually lost all pigmentation, thus becoming white. This observation prompted additional studies to check the expression levels of endogenous genes involved in the natural pigmentation biosynthetic pathway and most of them were strongly reduced. Thus, a concept of co-suppression, whereby sequence-related genes could negatively regulate each other, was born [156, 157]. Still, little was known on its underlying mechanism. The first major breakthrough came from the pivotal studies by Andrew Fire and Craig Mello. By introducing various forms of long RNA molecules into *C. elegans*, their team observed that those with a double-stranded presentation (double stranded RNAs, dsRNAs) were the actual inducers of the silencing phenomenon, which was then coined RNAi [158]. Thus, the work by Fire and Mello, which earned them the 2006 Nobel Prize of Medicine, has not only represented a major advance in the understanding of RNAi basic mechanism, but also provided a simple and reproducible method by which long dsRNAs could be used to induce specific gene silencing in lower organisms commonly used in genetic research, such as *C. elegans* [159] and *D. melanogaster* [160]. In the meantime, other teams kept their focus in plant systems, aiming at a better understanding of the role that RNAi and additional silencing processes assume in plant homeostasis. Soon it became clear that gene silencing operating at the RNA level has roles in adaptative protection against viruses [161], genome defense against mobile DNA elements [162, 163] and developmental regulation of gene expression (reviewed in [164]). A second component of RNA silencing, in addition to dsRNAs, was then identified and coined short interfering RNAs, which resulted from the processing of dsRNAs into 21–26 nt counterparts [165]. Interestingly, those short interfering RNA molecules could be sorted into two classes depending on their size, and soon it became clear that each of those classes assumed different functions. The long ones (24–26 nt) were dispensable for sequence specific mRNA degradation, but essential for systemic silencing and methylation of homologous DNA [164]. Another interesting contribution to the deeper understanding of the overall RNA silencing process came from a work of Cogoni and co-workers, who described a new biological function for RNA silencing in *Neurospora* called quelling, which can be activated upon the introduction of transgenic DNA. These authors observed that quelling targets preferentially transgenes arranged in large tandem arrays and its effectors are also short interfering RNAs [166], reviewed in [167]. Altogether, these works unveiled an unexpected complexity in the RNA silencing process in plants, prompting additional studies to check whether the same would also apply in animals. By this time, however, no one foresaw that the RNAi mechanism would also work in mammalian systems because long dsRNAs were already known to induce a strong interferon response. The first demonstrations that RNAi also works in humans came from the work of two

independent groups in Germany, one operating at the Max Planck Institute, and the other at the University of Bayreuth, and a third one in the United States, operating at the NIH, Bethesda. The team at the Max Plank Institute showed that synthetic versions of short dsRNA molecules were able to trigger a strong gene silencing effect in mammalian cells without inducing the interferon response. Moreover, they tested a series of design features for those short dsRNAs including length, blunt or sticky ends and chemical modifications, finding that structurally defined 21–23 base-pair small RNAs, with 2 nucleotide unpaired overhangs at the 3′ ends, were the most efficient mediators of RNAi [168, 169]. This fundamental work was published in Nature in 2001, and became the scientific content for a key patent in the field called "Tuschl II". In parallel, the NIH team came up with another demonstration that synthetic siRNAs can induce gene-specific inhibition of expression in *C. elegans* and in cell lines from humans and mice. They did it by systematically comparing the level of gene expression decrease caused by siRNAs *versus* that caused by single stranded AONs [170]. Their work, published in PNAS, was another step to open a path toward the use of siRNAs as a reverse genetic and therapeutic tool in mammalian cells, as the authors themselves have stated. Around the same time, at the University of Bayreuth, Kreutzer and Limmer had also reasoned that short fragments of dsRNA would putatively mediate a RNAi response similar to the one originally described by Fire and Mello and, even though their findings have never been published, they did file key patents around the discovery. Additional studies on the subject ended up unveiling the endogenous RNA silencing pathway that was being fed by small dsRNAs, from now on called small interfering RNAs (siRNAs). It also became evident that the same pathway is also able to process microRNAs (miRNAs), as previously seen (Fig. 7.1c). Here, we will focus solely on the RNAi process triggered by siRNAs.

The siRNA pathway starts with the cytoplasmic cleavage of long dsRNAs by an enzyme called Dicer. As a result, short dsRNA duplexes are formed. Then, those dsRNAs are incorporated into the RNA-induced silencing complex (RISC), where the strands are separated, and one strand guides RISC to the complementary region of target mRNA (Fig. 7.6). The heart of RISC is the Argonaute (AGO) proteins. In humans there are 8 AGO proteins, 4 from AGO clade (AGO1-4) and 4 from P-element induced wimpy testis (PIWI) clade (PIWI1-4; [171]). Still, not all AGO proteins are cleavage competent. In fact, AGO2 is the sole executer that accomplishes siRNA-induced silencing. Thus, whenever the siRNA strand loaded into RISC has complete sequence complementarity with its target mRNA sequence, it triggers site-specific mRNA cleavage, which ultimately results in a reduced expression of that mRNA and of the target protein (Fig. 7.4; reviewed in [172]). This exact same process can also be induced by direct exogenous supply of synthetic siRNAs. Over the years, a series of empirical and rational guidelines started accumulating from the analysis of hundreds of functional siRNAs. There are now a number of guidelines one should follow in order to design an effective siRNA, which have been well reviewed elsewhere [173]. There are also many websites and companies that either offer reliable methods for the design of effective siRNAs or even design them on demand. Because of their small size, the chemical synthesis of siRNAs is relatively easy and nowadays, several companies offer them delivered in ready-to-transfect format. This is, therefore, a simple, easy-to-handle RNAi-effector for virtually every lab need.

Since the half-life of siRNA is short, an alternative RNAi-effector molecule has also been developed: short hairpin RNAs (shRNAs), which are not directly transfected into their target cells. Instead, shRNAs are transcribed in the nucleus from an exogenous DNA expression vector bearing a palindromic sequence with a spacer in between, whose transcript folds into a short dsRNA with a terminal loop. The shRNA transcript is processed by Drosha, an RNase III endonuclease. The resulting pre-shRNA is exported to the cytoplasm, where it can then be processed by another RNase III, called Dicer, and incorporated into RISC, thus triggering the same RNAi process previously described (reviewed in [172]). In

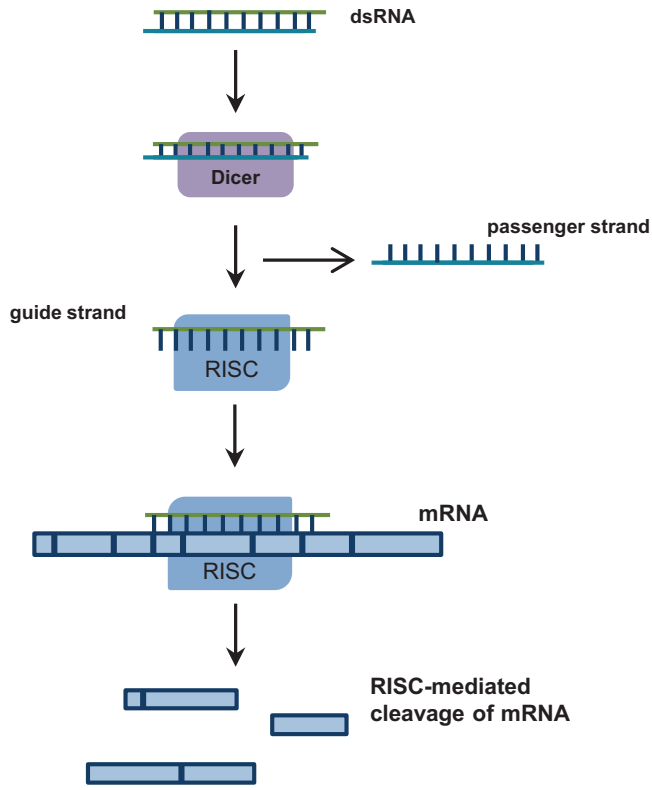

Fig. 7.6 The RNA interference (RNAi) mechanism. Entry of double-stranded RNA (dsRNA) into eukaryotic cells results in targeted RNA-induced silencing complex (RISC)-mediated cleavage of messenger RNA (mRNA) through activation of the endogenous RNAi mechanism: dsRNAs are recognized and cleaved into shorter fragments by Dicer, and subsequently loaded into a multiprotein conglomerate called RISC, which facilitates the separation of the two RNA strands. Once the double-stranded RNA is separated, one strand gets degraded while the other associated with RISC acts as a template for RISC-mediated cleavage of complementary RNA, thus reducing protein translation

general, shRNAs are harder to complex/internalize. Still, by delivering DNA instead of the effector RNA molecule, they take advantage of the cell's transcription machinery to produce specific shRNA transcripts, they allow for high potency sustainable effects using low-copy numbers. One such approach results in less off-target effects, putatively ensuring greater safety. Additionally, a shRNA expression vector does also cost less than the bulk manufacturing of siRNAs (reviewed in [174]).

Once all cells have the RNAi machinery and, in principle, any gene can be knocked down, soon siRNAs became invaluable tools in the lab, enabling the easy genetic knockdown of any sequence. RNAi was rapidly exploited as a tool to promote unbiased genome-wide screening to search for relevant genes involved in specific biological processes, first in invertebrate cells [170, 175–177] and latter in mammalian cells [178–182]. In fact, this knockdown technique provides a valuable tool for the functional annotation of mammalian genes [183, 184], for the creation of knockout animals [185] and for the identification of new drug targets (reviewed in [186]), but these are far from being the major application of this technology. In fact, RNAi has been regarded as one of the major breakthroughs in the field of molecular medicine, and its potential as a therapeutic effector has been largely tested over the last decades.

The need to optimize the technique and take it from bench to clinic is also prompting extra research efforts to gain a deeper understanding of

the overall RNAi mechanism. For example, in order to function, siRNAs need to escape to the cytosol, where the RISC works. Thus, release from the endosome is an important barrier. Understanding the mechanism(s) that promotes and limits endosomal release may help to optimize this limiting step. This remains, though, a major area of investigation for all nucleic acid therapeutics [11].

7.4.2 Recent Successful Applications of siRNA-Based Drugs

Being a naturally occurring post-transcriptional gene silencing process, this mechanism has several advantages when compared to other AON technologies, and that recognition triggered major investments in RNAi-based drug development by large pharmaceutical and biotechnological companies [187]. The potential of siRNA therapeutics was first demonstrated by Song and co-workers 15 years ago, when injection of *Fas* siRNAs protected mice from autoimmune hepatitis. Fas-mediated apoptosis is implicated in a broad spectrum of liver diseases, where inhibiting hepatocyte death can be life-saving. These authors investigated the silencing effect of siRNA duplexes targeting the gene encoding the Fas receptor (*Fas*), to protect mice from liver failure and fibrosis in two models of autoimmune hepatitis. Intravenous injection of *Fas* siRNA specifically reduced both *Fas* mRNA and Fas protein expression levels in mouse hepatocytes, and the effects persisted without diminution for 10 days [188]. This pioneer work has not only shown that siRNA-directed Fas silencing could work *in vivo* and be of therapeutic effect for preventing and/or treating acute and chronic liver injury [188], but also provided the proof-of-principle on the potential of the overall RNAi technology to treat or prevent disease (reviewed in [189]). Since then, drug development has been rapid, with siRNAs facing virtually the same obstacles as AONs. Fortunately, some of the AON strategies could be adapted to siRNA therapeutics, thus accelerating siRNA preclinical drug development and clinical evaluation. In general, RNAi clinical trials are progressing well. Clinical Phase I and II studies of siRNA therapeutics have demonstrated potent (as high as 98%) and persistent (lasting for weeks) gene knockdown effects, especially in liver, with some signs of clinical improvement and without unacceptable toxicity (reviewed in [189]). There are also several trials in Phase III development (Table 7.2; reviewed in [11]).

Early this year Alnylam has announced U.S. FDA acceptance of New Drug Application (NDA) and Priority Review Status for Patisiran, an investigational RNAi therapeutic for the treatment of hereditary transthyretin amyloidosis (hATTR) [225]. Almost at the same time, the company presented new clinical results from the APOLLO Phase III study of this drug at the 16th International Symposium on Amyloidosis. The APOLLO Phase III trial was a randomized, double-blind, placebo-controlled, global study designed to evaluate the efficacy and safety of Patisiran in hATTR amyloidosis patients with polyneuropathy. The primary endpoint of the study was the change from baseline in modified Neurologic Impairment Score +7 (mNIS+7) relative to placebo at 18 months. According to the general manager of the transthyretin (TTR) program at Alnylam, "*the clinical results presented further highlight the robust profile of Patisiran and provide evidence supporting Patisiran as a potentially transformative treatment approach for patients with hATTR amyloidosis*". Also the results obtained in the cardiac subpopulation, which corresponded to approximately 50% of the patients enrolled in the APOLLO study, revealed significant improvements in measures of cardiomyopathy, the leading cause of death in patients with hATTR amyloidosis, relative to placebo [201]. Finally, in August 2018, the drug got its U.S. FDA approval, and is now commercialized under the designation Onpattro™ [226].

Hopefully, the approval of the first RNAi therapeutic will pave the way for approval of other targets (reviewed in [227]), especially if we take into account that there are several other siRNA drugs under evaluation, which have recently advanced for phase III development (Table 7.2; reviewed in [11]). The most relevant examples

Table 7.2 Summary table of clinical trials for Small interfering RNAs (siRNAs)

Chemistry	Drug	Target/organ	Dose/route	Indication	Key observations	References
Phase 3						
PS, 2′-OMe, 2′-F (LNP)	Patisiran (ALN-TTR02)	*TTR*, Liver	0.3 mg/kg once every 3 weeks, IV (Pretreatment w/ glucocorticoids, antihistamines and analgesics)	Hereditary ATTR	Phase 3 findings included (1) significant improvement with patisiran compared to placebo in neuropathy and quality of life metrics, (2) infusion-related reactions, and (3) peripheral edema	NCT01960348 (Coelho [190, 191]) (Alnylam Pharmaceuticals, press release, Sept 20, 2017)
2′-MOE (None)	QPI-1002 (15NP)	*TP53*, Kidney	0.5–10 mg/kg, IV	Delayed graft function; AKI following cardiac surgery	Phase 2 findings in renal transplant patients included improved (1) dialysis-free survival time, and (2) reduced number dialysis in first 30 days	NCT02610296 NCT02610283 [192, 193]
Unmodified (None)	SYL1001	*TRPV1*, Eye	11.25 g/mL/drop, Eye drops	Ocular pain; dry eye syndrome	Phase 2 findings included analgesic trend that was not significant compared to placebo	NCT03108664 [194]
2′-MOE (None)	QPI-1007	*CASP2*, Eye	1.5 mg, IVT	Acute NAION, acute primary angle closure glaucoma	Phase 1 findings included conjunctival hemorrhage	NCT02341560 [195, 196]
PS, 2′-OMe, 2′-F, GalNAc (None)	Fitusiran (ALN-AT3SC)	*SERPINC1*, Liver	80 mg once monthly, SC	Severe Hemophilia A or B	Phase 1/2 open-label and open-label extension findings in patients included (1) mean 80% target reduction and reduced bleeding, and (2) 1 death due to thromboembolic event	NCT03417245 NCT03417102 NCT02035605 NCT02554773 [197, 198]
PS, 2′-OMe, 2′-F,GalNAc (None)	Inclisiran (ALN-PCSSC)	*PCSK9*, Liver	300 mg once every 3–6 months, SC	ASCVD, HC, HoFH, HeFH	Phase 2 findings included (1) mean 53% reduction of LDL-C, (2) injection site reactions, and (3) ALT elevations	NCT03400800 NCT03399370 NCT03397121 [199, 200]

(continued)

Table 7.2 (continued)

Chemistry	Drug	Target/organ	Dose/route	Indication	Key observations	References
PS, 2′-OMe, 2′-F, GalNAc (None)	Givosiran, (ALN-AS1)	*ALAS1*, Liver	2.5 mg/kg once monthly, SC	Acute hepatic porphyrias	Phase 1 findings in ASHE and AIP patients included (1) dose-dependent reduction of target, (2) 63% reduction in annualized AIP attack rate, (3) injection site reactions, and (4) myalgia	NCT03338816 [201], press release, Nov 7, 2017) NCT02452372
Phase 2						
Undisclosed (PLGA polymer)	siG12D-LODER	mutant *KRAS*, Tumor	0.025-3 mg, Surgical implant	Pancreatic ductal adenocarcinoma	Phase 2 findings included (1) suggestion of improved median survival, and (2) cholangitis and neutropenia	NCT01676259 NCT01188785 [202, 203]
Unmodified (None)	Bamosiran (SYL 040012)	*ADRB2*, Eye	300-900 mg/eye/day, Eye drops	Ocular hypertension; open angle glaucoma	Phase 1/2 findings included (1) reduction of intraocular pressure, and (2) no drug related adverse events	NCT02250612 [204, 205]
Undisclosed (LNP)	TKM-080301 (TKM-PLK1)	*PLK1*, Liver	0.15-0.9 mg/kg, IV	Solid tumors with liver involvement; neuroendocrine tumors; adrenocortical carcinoma	Phase 1/2 findings included (1) some evidence of antitumor activity, (2) infusion reaction-like inflammatory response, (3) ALT elevations, and (4) acute respiratory failure	NCT01437007 NCT01262235 NCT02191878 [206]
Undisclosed (None)	RXI-109	*CTGF*, Skin/ Eye	1-10 mg, ID/IVT	Hypertrophic scars; keloids, and subretinal fibrosis	Phase 1 findings included modest target reduction	NCT02246465 NCT02079168 NCT02599064 [207]
2′-OMe (cationic lipoplex)	Atu027	*PKN3*, Vascular endothelium	0.001-0.336 mg/kg, IV (administered w/ gemcitabine)	Advanced solid tumors; pancreatic ductal carcinoma	Phase 1/2 findings included possible anti-tumor effects	NCT00938574 NCT01808638
2′-OMe (None)	PF-655	*DDIT4*, Eye	0.05-3 mg/eye, IVT	AMD	Phase 1/2 findings included (1) no evidence of benefit in AMD patients, and (2) increased intraocular pressure	NCT01445899 [208]

PS, 2′-OMe, 2′-F, GalNAc	Cemdisiran (ALN-CC5)	*C5*, Liver	600 mg once every 4 weeks, SC	Complement mediated diseases (aHUS, PNH)	Open-label phase 1 findings included (1) sustained control of hemolysis with up to 67% reduction in eculizumab dose, (2) 99% reduction of C5, (3) injection site reactions, and (4) 1/6 (17%) Gr 3 LFT	NCT03303313 NCT02352493 [209]
PS, 2′-OMe, 2′-F, GalNAc (None)	Lumisiran (ALN-GO1)	*HAO1*, Liver	1.0 mg/kg, SC	1st Hyperoxaluria type 1	Phase 1 findings in healthy volunteers included (1) increased plasma and urine glycolate, and (2) injection site pain	NCT02706886 [210]
3 siRNAs Undisclosed (LNP)	ARB-1467 (TKM-HBV)	*HBV*, Liver	2 mg/mL, IV in combination w/ nucleoside analogues (Pretreatment w/ medication to mitigate infusion-related reactions)	HBV infection	Phase 1 findings in patients included dose-dependent additive HBsAg reduction	NCT02631096 [211]
Phase 1						
PS, 2′-OMe, 2′-F, GalNAc (None)	ALN-TTR$_{SC}$02	*TTR*, Liver	5-300 mg, SC	Hereditary ATTR	Phase 1 findings in healthy volunteers included (1) dose dependent reduction in plasma TTR	NCT02797847 (Alnylam Pharmaceuticals, press release Dec 16, 2016)
Undisclosed (LNP, vitamin A)	ND-L02-s0201	*SERPINH1* Liver	0.03-0.6 mg/kg/wk, IV (Pretreat w/ antihistamines)	Hepatic fibrosis	Phase 1b findings included (1) no doselimiting toxicities, and (2) infusion reactions.	NCT02227459 [212]
Undisclosed (siRNAelectroporated PBMCs)	APN401	*CBLB*, Tumors	5-50 3 10^5 cells /kg, IV	Melanoma, pancreatic cancer, renal cell cancer	(1) Immune modulation shown by in vitro increase in IFN-g and IL-2 with CD3/CD28 stimulation, and (2) infusion reaction like adverse events	NCT02166255 NCT03087591 [213, 214]

(continued)

Table 7.2 (continued)

Chemistry	Drug	Target/organ	Dose/route	Indication	Key observations	References
Undisclosed (liposome)	siRNA-EphA2-DOPC	*EPHA2*, Tumors	IV	Advanced cancers	Not published	NCT01591356
2 siRNAs, Undisclosed (LNP)	ALN-VSP02	KIF11 and *VEGFA*, Liver/Tumors	0.1-1.5 mg/kg, IV (Pretreatment w/ glucocorticoids, antihistamines, and analgesics)	Advanced solid tumors with liver involvement	Phase 1/2 findings included (1) evidence of antitumor activity, and (2) infusion reactions, dose-limiting thrombocytopenia, and complement split product Bb increase	NCT01158079 [215, 216]
Undisclosed (LNP)	ALN-PCS02	*PCSK9*, Liver	0.4 mg /kg, IV (Pretreatment w/ glucocorticoids, antihistamines, and analgesics)	HC	Phase 1 findings included (1) reductions in PCSK9 of up to 84% and LDL cholesterol plasma levels of up to 50% after a single IV dose, and (2) infusion reactions	NCT01437059 [217]
Undisclosed (None)	TD101	*KRT6A*, Nails/Skin	0.1-17 mg, Intra-lesion	PC	Limited data from one patient	NCT00716014 [218]
Undisclosed (gold NP)	NU-0129	*BCL2L12*, Cancer	IV	Glioblastoma multiforme	Not published	NCT03020017
Discontinued						
Unmodified (None)	Bevasiranib (Cand5)	*VEGFA*, Eye	IVT	Macular degeneration, diabetic macular edema	Terminated in Phase 3 due to lack of efficacy.	NCT00499590 (Health, press release Mar 2009)
Unmodified (None)	AGN211745	*FLT1*, Eye	0.100-1 mg, IVT	AMD w/choroidal neo-vascularization	Terminated after Phase 2 due to lack of efficacy	NCT00395057
Undisclosed, GalNAc (None)	ALN-HBV	*HBV*, Liver	0.1-3.0 mg/kg, SC	Non-cirrhotic chronic HBV	Replaced by more potente compound	NCT02826018

2′-OMe, 2′-F, GalNAc (None)	Revusiran (ALN-TTRSC)	*TTR*, Liver	500 mg once weekly, SC	TTR-mediated cardiac amyloidosis	Terminated due to imbalance of mortality in the drug arm compared to placebo, adverse events included ISRs and LFT elevations	NCT02319005 [219, 220]
Unmodified (SNALP)	PRO-040201 (TKM-ApoB)	*APOB*, Liver	IV	HC	Terminated after phase 1due to infusion reactions	NCT00927459 [221]
Undisclosed (RONDEL™ cyclodextrin insert polymer, adamantane-PEG-transferrin)	CALAA-01	*RRM2*, Tumors	3-30 mg/m^2, IV	Solid tumors	Terminated in phase 1due to dose-limiting toxicities, including infusion reaction-like adverse events, elevated creatinine and LFTs, and dose-dependent thrombocytopenia	NCT00689065 [222]
Undisclosed (LNP)	DCR-MYC	*MYC*, Tumors	0.1-0.85 mg/kg, IV	Multiple cancers	No evidence of clinical activity	NCT02110563 NCT02314052 (Dicerna Pharmaceuticals, press release Sept 26, 2016)
Undisclosed (LNP)	DCR-PH1	*AGXT*, Liver	0.005-0.05 mg/kg, IV	Primary hyperoxaluria type 1		NCT02795325
PS, 2′-OMe, 2′-F, GalNAc (None)	ALN-AAT	*SERPINA1*, Liver	0.1-6.0 mg/kg, SC	Alpha-1 Antitrypsin deficiency	Terminated after phase 1/2 due to LFT elevations.	NCT02503683
Undisclosed, cholesterol (MLPCDM-GalNAc)	ARC-520	*HBV*, Liver	4 mg/kg, IV (Pretreatment w/ antihistamine and acetaminophen)	Chronic HBV	Terminated due to toxicities in NHPs, but had favorable tolerability in clinic	NCT02349126 (Arrowhead Pharmaceuticals, press release Nov 29, 2016)
Undisclosed, cholesterol (MLPCDM-GalNAc)	ARC-521	*HBV*, Liver	0.6-6.0 mg/kg, IV (Pretreatment w/ antihistamine and acetaminophen)	Chronic HBV	Terminated due to toxicities in NHPs, but had favorable tolerability in clinic.	NCT02797522 [223] (Arrowhead Pharmaceuticals, press release Nov 29, 2016)

(continued)

Table 7.2 (continued)

Chemistry	Drug	Target/organ	Dose/route	Indication	Key observations	References
UNA, cholesterol (MLP-CDM-GalNAc)	ARC-AAT	*SERPINA1*, Liver	0.38-8.0 mg/kg, IV (Pretreatment not disclosed, but assumed same as other ARC drugs)	Alpha-1 antitrypsin deficiency	Terminated due to toxicities in NHPs, but had favorable tolerability in clinic and dose-dependent reductions in sérum AAT up to 90%	NCT02363946 [224] (Arrowhead Pharmaceuticals, press release Nov 29, 2016)
Undisclosed (LNP)	ALN-TTR01	*TTR*, Liver	1 mg/kg, IV (Pretreatment w/ glucocorticoids, anti-histamines and analgesics)	Hereditary ATTR	Terminated due to greater potency of patisiran (ALN-TTR02)	NCT01148953 [190]

Abbreviations: **Chemistry:** *PS* phosphorothioate, *2′ –Ome* 2′ –methoxy, *2′ – F* 2′ – fluoro, *2′ – MOE* 2′ –O- methoxy ethyl, *GalNAc* N-Acetilgalactosamine, *PLGA* poly(lactic-co-glycolic) acid, *LNP* lipid nanoparticle, *NP* nanoparticle, *PEG* polyethylene glycol, *SNALPs* stable nucleic acid-lipid paricles, *MLP* melittin-like protein, *CDM* carboxy-dimethylmaleamide, *UNA* unlocked nucleic acid. **Target/ organ:** *TTR* transthyretin, *TP53* tumor protein 53, *TRPV1* transient receptor potential cation chanel subfamily V member 1, *CASP2* caspase2, *SERPINC1* serpin family C member 1, *PCSK9* proprotein convertase subtilisin/kexin type 9, *ALAS1* 5′-aminolevulinate synthase 1, *KRAS* KRAS proto-oncogene, *ADRB2* adrenoreceptor beta 2, *PLK1* Serine/threonine-protein kinase, *CTGF* connective tissue growth factor, *PKN3* protein kinase N3, *DDIT4* DNA damage inducible transcript 4, *C5* complement C5, *HAO1* hydroxyacid oxidase 1, *HBV* Hepatitis B Virus, *SERPINH1* serpin family H member 1, *CBLB* Cbl proto-oncogene B, *EPHA2* ephrin receptor A2, *KIFF11* kinesin family member 11, *VEGFA* vascular endothelial growth factor, *KRT6A* keratin 6A, *BCL2L 12* BCL2 like 12, *FLT1* fms related tyrosine kinase 1, *APOB* apolipoprotein B, *RRM2* ribonucleotide reductase regulatory subunit M2, *MYC* MYC proto-oncogene, *AGXT* alanine-glyoxylate and serine-pyruvate aminotransferase, *SERPINA1* serpin family A member 1. **Dose/route:** *IV* intravenous, *IVT* intravitreal, *SC* subcutaneous, *ID* intradermal. **Indication:** *ATTR* transthyretin amyloidosis, *AKI* acute kidney injury, *NAION* non-arteritic anterior ischemic optic neuropathy, *ASCVD* atherosclerotic cardiovascular disease, *HC* hypercholesterolemia, *HoFH* homozygous familial hypercholesterolemia, *HeFH* heterozygous familial hypercholesterolemia, *AMD* age-related macular degeneration, *aHUS* atypical hemolytic-uremic syndrome, *PNH* paroxysmal nocturnal hemoglobinuria, *HBV* Hepatitis B Virus, *ATTR* transthyretin amyloidosis, *HC* hypercholesterolemia, *PC* pachyonychia congenital, *AMD* age-related macular degeneration. **Key observations:** *ALT* alanine aminotransferase, *ASHE* asymptomatic high excreters, *AIP* acute intermittent porphyria, *AMD* age-related macular degeneration, *C5* complement C5, *LFT* liver function tests, *TTR* transthyretin amyloidosis, *IFN-g* interferon gamma, *IL-2* interleukin 2, *PCSK9* Proprotein convertase subtilisin/kexin type 9, *LDL* Low-density lipoprotein, *ISR* injection site reaction, *NHP* non-human primate, *AAT* Alpha-1-antitrypsin

include Revusiran, which is a second siRNA drug under evaluation as a treatment option for patients with familial amyloid cardiomyopathy by reducing plasma TTR levels [220]; QPI-1002, which is being developed for the treatment of delayed graft function for kidney transplants [192]; Fitusiran, a potent siRNA drug under study for patients with hemophilia, which aims at ameliorating the disorder by reducing the plasma levels of anti-thrombin [198], and Inclisiran, which targets proprotein convertase subtilisin/kexin type 9 (PCK9) to reduce the risk of cardiovascular disease [200], as reviewed in [11].

7.4.3 Delivery of siRNA-Based Drugs

Intracellular delivery of double-stranded siRNAs is more challenging than delivery of single stranded AONs [186]. Still, it is also worth mentioning that, as suggested by the best estimates, only a few hundred cytosolic siRNAs per cell are needed for efficient and sustained gene knockdown [228, 229]. This happens because the guide strand of the siRNA remains stable within the RISC for weeks, even though it gets diluted with every cell division [230]. Thus, the same siRNA molecule can target multiple transcripts, knocking down gene expression in slowly dividing or non-dividing cells over the same period. Overall, as noticed by several authors, this actually contributes to turn the delivery obstacle into a less formidable one than that faced by other antisense mechanisms, which act on a one-to-one basis [230] (reviewed in [229]).

Still, knowing that the translation of siRNAs from the bench to the clinic would be hindered by their limited cellular uptake, low biological stability and unfavorable pharmacokinetics, the development of appropriate delivery methods became mandatory to proceed with preclinical studies. Therefore, different approaches have been (and are being) attempted to ensure safer and long-lasting delivery methods for siRNA-based drugs, for both systemic and targeted delivery. Most of these developments were made in parallel with siRNA drug development and only through the combined efforts of several independent teams were these drugs modified in ways that allowed their clinical evaluation, with the promising results highlighted in the previous section. Whatever the case, an effective delivery system must fulfill a series of criteria, which have already been listed by Tatiparti et al. amongst other author: be stable at the body temperature and pH variations, have an endocytosis promoting shape, cannot be toxic, must exhibit high siRNA loading abilities and have a size that avoids rapid renal and hepatic clearance [231]. In general, all the delivery systems developed for gene therapy may also be adapted for siRNA delivery [232].

7.4.4 Non-targeted Delivery

Early strategies for solving the dual problems of intracellular delivery and rapid excretion involved incorporating siRNAs into LNPs – smaller, more homogeneous analogues of lipoplexes used for laboratory transfection [233–235], (reviewed in [189]). LPNs were first shown to be effective in targeting the hepatitis B virus (HBV) in mice, where the LPN-formulated siRNA was given in 3 daily injections of 3 mg/Kg/day. This treatment regimen resulted in a decrease of HBV levels by 1–2 orders of magnitude [236], as reviewed in [237]. Nevertheless, these complexes (and other nanoparticle strategies for siRNA delivery) accumulate in the liver and other filtering organs, which limits their effectiveness in penetrating other tissues [235, 238] (reviewed in [189]). Furthermore, the administration of siRNAs with LNP delivery vehicles is quite pro-inflammatory. In fact, lipid-based vehicles can become entrapped in endosomes [237], where the Toll-like receptors (TLR) will recognize various moieties in dsRNAs, modified siRNAs or even from their degradation products [239], eliciting an undesirable innate inflammatory response. So, in most circumstances the siRNAs require pretreatment regimens including antihistamines, non-steroidal anti-inflammatories and even relatively high doses of glucocorticoids [190, 191, 240] (reviewed in [11]).

Still, recent developments by several independent teams have demonstrated the feasibility of systemic administration of either chemically modified or complexed siRNAs. In fact, even though unmodified siRNAs do not distribute broadly to tissues after systemic administration (reviewed in [11]), simple chemical modifications of the 2′-position of the ribose and substitution of phosphorothioate linkages, such as the ones described for AONs in the first section of this chapter (2′-OMe and 2′-MOE), and 2′-fluoro (2′-F), protect siRNAs from nuclease digestion, thus prolonging their half-lives, both in serum and other body fluids [236, 241] (reviewed in [189]). 2′-modifications can also prevent recognition by innate immune receptors by blocking the binding to TLR [242–244] and reduce off-target effects that could arise from the suppression of partially complementary sequences [245] (reviewed in [189]). As already referred, these modifications had been previously designed for use in AONs and not siRNAs and, even though they did show effective in improving stability, specificity and immunogenic properties, they do not improve potency. Recently, however, two novel, siRNA-optimized 2′-O modifications, were shown to increase *in vivo* activity of siRNAs, not only by increasing their potency but also their *in vivo* duration compared to their unmodified counterparts when delivered using LNPs: 2′-O-benzyl and 2′-O-methyl-4-pyrimidine (2′-O-CH2Py(4); [246]). Several teams have also been assessing different complexation methods with functional peptides [247] and/or different vectors: exosomes [248] including lipid nanocarriers such as pegylated immunoliposomes (PILs; [249]), stable-nucleic-acid-lipid-particles (SNALPs; [250]), or polyhydroxyalkanoate-based nanovehicles [251], reviewed in [252]. Also under consideration are siRNA delivery strategies that use viral particles. The viral delivery of siRNAs is composed of two main strategies: siRNAs are either chemically synthesized and loaded into a viral capsule, or they can be expressed from the DNA of a recombinant virus (reviewed in [253]).

Meanwhile, second generation LPNs were also developed. Constructed with the anionic lipid dilinol eylmethyl-4-dimethylaminoburyate (DLin-MC3-DMA), they mediate potent gene knockdown at reduced doses compared with first generation LNPs, while improving delivery [190, 217]. Partisiran (previously termed ALN-TTR02; ChemIDplus-Partisiran), for example, is exemplary of a minimally chemically modified siRNA delivered primarily to the liver in a second generation liposome formulation.

7.4.5 Targeted Delivery

Overall, there has been a huge progress over the last decade concerning not only non-targeted but also targeted delivery of siRNA drugs. In fact, siRNAs can also be targeted for uptake in selected tissues or cell types by taking advantage of high-affinity antibody or antibody fragments [254–256], aptamers (nucleic acids selected for high-affinity binding; [257–259] or receptor ligands [260–264], which bind to specific cell surface receptors and mediate cell-specific uptake. The targeting moieties can be either directly conjugated to siRNAs (bound non-covalently) or incorporated into LPNs or other nanoparticles (reviewed in [189]). In general, targeted uptake has the advantages of being effective at a lower dose while exhibiting lower toxicity, which may potentially occur from knockdown effects in unintended tissues. It is also a tool of great advantage for the treatment of non-systemic diseases. The easiest organ to target is the liver, which is a filtering organ that traps nanoparticles. It is also the primary site of synthesis of many circulating proteins. That is why it has been the target organ in most early clinical attempts at translating RNAi (reviewed in [186]). Furthermore, several diseases, which directly affect this organ may benefit from a straightforward liver targeting method. The most successful possibility under study includes a series of more drastic chemical modifications, where siRNAs have a trivalent N-acetylgalactosamine (GalNAc) moiety conjugated to the 3′ terminus of one of the strands [220, 264] (reviewed in [11]). GalNAc mediates hepatocyte uptake through the hepatocyte-

restricted asialoglycoprotein receptor (ASGPR), thus being a suitable mediator for whole-liver delivery [265] (reviewed in [189]). Uptake by this receptor is primarily through clathrin-dependent endocytosis [97]. Examples of GalNAc-modified siRNAs include Revusiran and Fitusiran (ChemIDplus-Revusiran; ChemIDplus-Fitusiran (reviewed in [11]). Also, local delivery to the CNS, a region that is difficult to deliver drugs to due to the BBB, is being addressed, with promising results. First preliminary evidence that *in vivo* downregulation of specific genes by RNAi could work at the CNS level came from studies in rats and mice using invasive local delivery methods (reviewed in [266]). Lately, however, evidence is accumulating on the successful brain delivery of si/shRNAs using specifically designed vectors and/or modifications that include the use of enzyme-sensitive LPNs [267], carbosilane dendrimers [268], cholesterol modifications [269], and recombinant fusion proteins [270]. Also, the pharmaceutical industry is investing in developing BBB-directed vectors. One of those examples is a family of vectors that take advantage on the existence of specific receptors and transport systems, which are highly expressed at the BBB to provide essential substances to brain cells. These vectors comprise a full-length protein (Melanotransferrin) and may be used to facilitate receptor mediated drug delivery into the brain to treat CNS disorders [271]. Recently, the application of this new peptide vector to siRNA and ongoing studies addressing the brain delivery of Iduronate 2-sulfatase (I2S) for the treatment of Hunter Syndrome, a rare X-linked lysosomal storage disorder, was discussed and its results in knockout mice were quite promising [272].

7.5 CRISPR-Cas Gene Editing

In addition to the most well known RNA-based therapeutics (antisense drugs and siRNA-based drugs) several other mechanisms of action are also potential strategies. Recently, a new gene editing technology, Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) and the CRISPR-associated protein 9 (Cas9) (CRISPR-Cas 9) system, has received unprecedented acceptance in the scientific community for a variety of genetic applications (reviewed in [273]) (Fig. 7.7a). Even though this technology lies beyond the scope of this chapter, it does deserve some attention, as it may become a leading method for gene editing and even RNA-based therapeutics, in the long term.

Similarly to what had already happened with RNAi, the CRISPR-Cas system was not specifically developed as a method for gene editing. Instead, it is a naturally occurring prokaryotic immune defense strategy against non-self DNA based invasions (e.g., viruses, plasmids), which was recently discovered in bacteria and archaea [273–276], and latter adapted for bench applications [277, 278]. Also like RNAi, the specificity of CRISPR-Cas relies on the antisense pairing of RNAs (here termed single guide RNA, sgRNA) to specific genes but instead of binding directly to RNA, sgRNAs bind to chromosomal DNA. Another relevant difference between the RNAi and CRISPR technologies has to do with the transiency of their effect. In fact, unlike siRNAs, sgRNAs induce stable changes in gene expression, which are invaluable for *in vivo* gene screening. Thus, genomic targeting through CRISPR-Cas creates indels that can be adapted for stable eukaryotic genome engineering, namely Cas-mediated gene knockdown (reviewed in [186, 273]). In general, the application of CRISPR/Cas9 for DNA editing as well as for mammalian gene editing was established in the 2012–2013 period and, in just 3 years, this technique has revolutionized the entire gene editing field. Currently, CRISPR-Cas gene knockdown in zygotes provides a fast method for the development of different animal models, when compared to homologous recombination. Nevertheless, it does hold a series of drawbacks and raises a number of concerns, particularly when its therapeutic potential is considered. In fact, since this technique has the ability to modify the genome, its ethical and safe concerns are enormous. Furthermore (and like every other

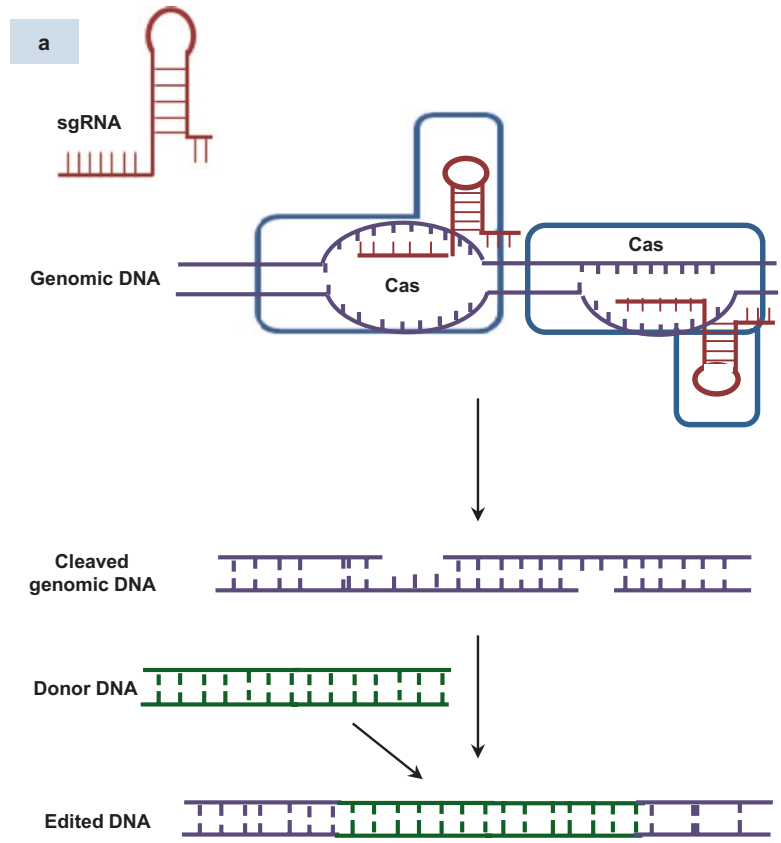

Fig. 7.7 Additional RNA-based drug mechanisms. **(a)** CRISPR/Cas9: CRISPR/Cas9 induces double strand breaks (DBS) when targeted to a specific genomic site by an appropriate guide RNA (sgRNA). This property may be used for mutation correction, by adding a donor DNA sequence that has homologous overlaps to the DBS (typically 100–1000 bp of overlap is used), thus promoting homologous repair of the cleaved genomic DNA; **(b)** Modified mRNAs: This approach consists in introducing chemically modified, stabilized mRNAs into cells to be translated to protein. Once internalized, sense-RNA drugs can be used for transient *in vivo* transcription (IVT) of mRNAs to replace mutated proteins or for vaccination without the risk of genomic alteration; **(c)** Aptamers: Aptamers take advantage of their selection for high-affinity binding to molecular ligands, often in the nanomolar or subnanomolar range. They can be compared to nucleic acid antibodies, having many of the advantages of conventional protein antibodies. They can be either agonists or antagonists, linked for bifunctional targeting and conjugated to other RNAs, small-molecule drugs, toxins or peptides. However, unless modified, they are rapidly excreted and do not activate immune functions, as other antibodies do

antisense technology), CRISPR-Cas holds potential for both on- and off-target effects. Moreover, by creating double-stranded DNA breaks, the Cas endonuclease can also lead to oncogenic gene translocations and trigger a DNA damage response, ultimately causing cell-cycle arrest or even cell death. Finally, depending on the repair pathway which is activated, gene editing may be imprecise [186]. Still, it must be noticed that in the 5 years following the publication of the method, several improvements to reduce off-target effects and provide a better control of the whole mechanism, while enhancing its efficiency have been developed and reported (reviewed in [186, 279]). It should also be mentioned that CRISPR/Cas9 is certainly a more versatile technique than RNAi, has it may not only induce indels but also repress or activate gene expression and cause both heritable and non-heritable genomic changes [280–

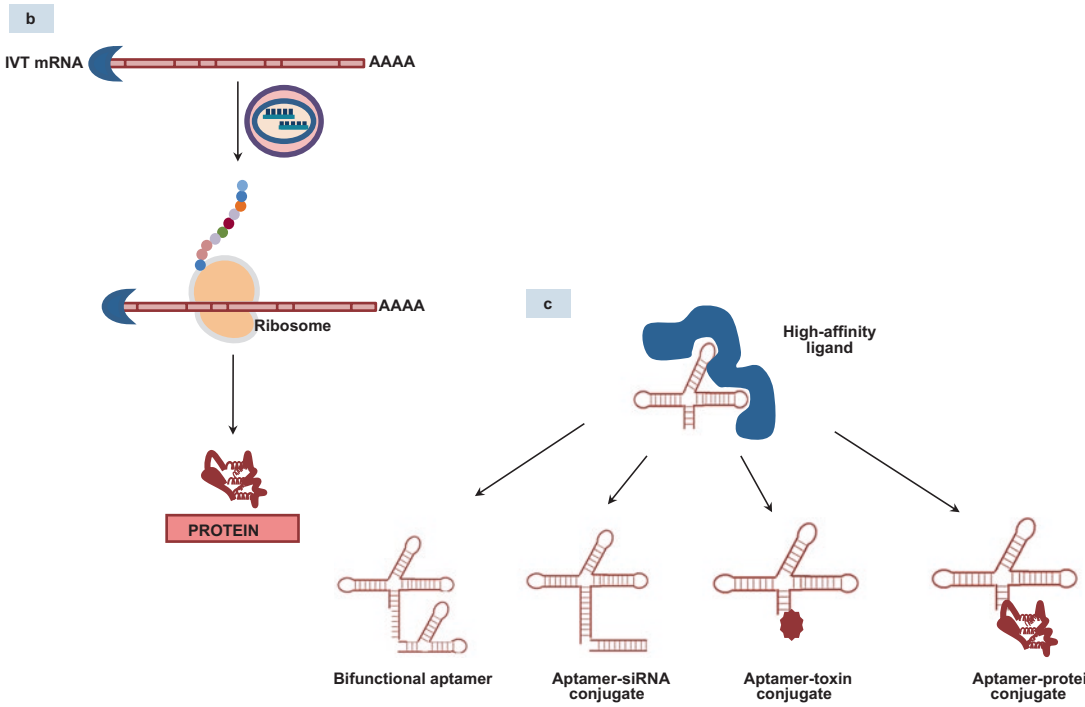

Fig. 7.7 (continued)

282]. In fact, CRISPR/Cas9 can be adapted to upregulate gene expression in different ways: the first, most obvious approach consists in using this technology to stably introduce constitutive active promoter elements to a gene, thus stably enhancing its expression. Alternatively, a modified Cas9 (fused to a transcriptor activator protein) may be targeted to any gene of interest, driving a transient enhancement in gene expression known as CRISPR activation (CRISPRa; [273]). In addition, the emergence of newer gene editing tools such as the Cpf1 enzyme, which is a single RNA-guided endonuclease, will eventually strengthen the portfolio of applications that may be achieved by CRISPR mediated genome engineering [283]. Therefore, it is becoming clear that clinical CRISPR-Cas studies will also be a trend in research over the next years, starting with *ex vivo* editing of differentiated cells, which may then be infused into patients. Furthermore, as other authors have already stated, it will also greatly benefit from the accumulated knowledge on other non-RNA-based gene editing tools, such as zinc-finger nucleases and on the delivery methods previously developed for AON- and siRNA-based drugs.

7.6 Messenger RNA as a Novel Therapeutic Approach

Another RNA-based approach is to introduce chemically modified stabilized mRNAs into cells, where those exogenous mRNAs will eventually be translated to protein (Fig. 7.7b). In fact, *in vitro* transcribed (IVT) mRNA has recently come into focus as a potential new drug class to deliver genetic information. Such synthetic mRNAs can be engineered to transiently express proteins by structurally resembling natural mRNAs [186, 284]. One advantage of mRNA-based therapy over viral gene delivery is that mRNA does not transit to the nucleus, thereby mitigating insertional mutagenesis risks. Moreover, mRNA provides transient, half-life-

dependent protein expression, while avoiding constitutive gene activation and maintaining dose responsiveness. Because of these advantages, IVT mRNA treatment is an emerging class of therapy, with multiple mRNA-based cancer immunotherapies and vaccines currently in clinical trials [284–286]. However, the fact that IVT mRNA, despite its strong resemblance to naturally occurring mRNA, can be recognized by the innate immune system may play an important part in its applicability. For vaccination approaches, the inflammatory cytokine production resulting from mRNA-induced immune stimulation might add to the effectiveness of the evoked immune response. For non-immunotherapy approaches, however, the story is different and so far, cancer immunotherapy is the only field in which mRNA based therapeutics have reached clinical trials [287–294]. Nevertheless, the potential of IVT mRNA is currently being explored for a variety of applications, ranging from inherited or acquired disorders to regenerative medicine, all of which remain at the preclinical stage [295, 296]. In fact, an increasing number of preclinical studies has evaluated mRNA-based therapy for a wide range of diseases such as surfactant B deficiency, myocardial infarction [297] sensory nerve disorders [298], fulminant hepatitis [299] hemophilia B [300, 301], congenital lung disease [302], cancer [303], liver and lung fibrosis [304], and methylmalonic acidemia [305]. However, the main hurdle in implementation of mRNA for therapeutics, the systemic delivery of mRNA molecules to target cells, remains a challenge. Better understanding of the factors that determine translational efficiency as well as RNA recognition by innate immune receptors, has improved the intracellular stability and functionality of mRNA transfected to cells. Still, when aiming to harness mRNA molecules for gene therapy purposes, this progress was insufficient. The need for mRNA protection from degradation in extracellular compartments, as well as for enabling its entry to the cell, has raised the demand for suitable delivery platforms [285, 295]. A possible solution for this challenge relies in the rapidly evolving field of nucleic acid-loaded NPs. In fact, the progress in the field of NPs-mediated RNAi-based therapy, has led to similar development of nanocarriers for mRNA. Particularly, the widely investigated family of LNPs was proposed to be such appropriate mRNA nanocarriers [296, 305, 306]. Moreover, the use of polyplex nanomicelles has also been explored [298, 299]. In order to achieve high efficacy *in vivo* some IVT mRNA specific formulation adjustments should be done in a near future. These adjustments are more important when systemic administration is required. Moreover, in order to expand the variety of mRNA-based therapies, cell specific targeted delivery systems are also needed especially in diseases involving a certain organ, which is inaccessible by standard LNPs, as well as in many types of solid tumors [296]. In conclusion, innovative design of nanocarriers for IVT mRNAs delivery will help to increase their potential and turn them into a valid therapeutic approach.

7.7 Aptamer-Based Drugs

Another potential class of RNA therapeutics are oligonucleotide aptamers (see Fig. 7.7c). The term aptamer comes from the Latin word "*aptus*", which means "to fix", as a clear reference to the lock and key relationship of aptamers and their targets [307, 308].

Aptamers are short (20–70 bases) single stranded oligonucleotides (ssRNA/ssDNA), which bind to their targets through 3D conformational complementarities with high affinity and specificity. Unlike the previously referred strategies, aptamers can be tailored selectively against a variety of targets, from nucleotides to amino acids, proteins, small molecules or even live cells [309]. Still, proteins are the major targets in aptamer research (reviewed in [310]). Oligonucleotide aptamers have affinity and specificity capacities, which are comparable to those of monoclonal antibodies, whilst having minimal immunogenicity, high production, low cost and high stability. These oligonucleotides can be selected trough an *in vivo* process called Systematic Evolution of Ligands by Exponential

enrichment (SELEX), which dates back to 1990. This method was originally described and performed by Szostak and Gold [307, 308]. The whole process starts with the synthesis of a screening library formed by a large number of randomly combinatorial ssDNA and/or ssRNAs. Each one of those random ssDNA/ssRNAs has one conserved sequence at each end. That sequence allows primer binding and amplification. The random library is then incubated with the target proteins, under proper conditions. Then, through a partition step, the sequences that had bind to target proteins are separated from those that did not bind. In the third step, the binding sequences are eluted and amplified with primers complementary to their conserved sequences, either by PCR (for ssDNA) or RT-PCR (for ssRNA). All these steps form a single SELEX cycle. This selection process is then repeated for about 7–20 rounds of incubation, partitioning and amplification. Ultimately, this results in the identification of a small number of binding sequences with high affinity and specificity for further processing and optimization. Generally, the binding sequences are then transformed into bacteria (*E. coli*) for further sequencing and characterization (reviewed in [310]). Naturally, in the post-SELEX process, the synthesized aptamers (as every other AON) can be chemically modified for therapeutic purposes, to stabilize and protect them against nucleases *in vivo*. Recent advances in SELEX technology, with the introduction of chemically modified bases and the use of deep sequencing to analyze enriched RNAs in early rounds of selection, have greatly reduced the time needed and the likelihood of identifying high-affinity aptamers (reviewed in [186]).

Over approximately 10 years, starting in 2005, when the first aptamer Pegaptanib (PubChem, Pegaptanib) was approved for wet age-related macular degeneration (AMD) therapy by U.S. FDA, oligonucleotide aptamers were growing more and more popular. Until 2016, when the last estimates were published online, there had been over 900 aptamers developed against various targets for diagnostic and therapeutic purposes [311]. Nevertheless, drug development of aptamers is currently not very active, with big pharmacological companies being much more focused on the technologies reviewed in the previous sections of this chapter. Still, it is worth mentioning that these oligonucleotides could substitute for some applications of therapeutic antibodies, with lower risk of developing immunological responses. They could also be used for targeted intracellular delivery of other molecules, including RNA-based drugs.

7.8 Conclusion

Over the last decades, an exceptional increase on the understanding of the versatile roles of RNAs has sparked the development of new classes of RNA-based drugs. Therapeutic RNA-based applications are emerging, in different fields, from inherited genetic diseases, oncology, viral infections and diabetes to neurological, cardiovascular, bone-related and ocular diseases. Over the last years in particular, much effort has been focused on the development of RNA-based therapeutics. Currently, even though there are a number of RNA-based therapeutic strategies, which may be attempted in order to either correct or modulate gene expression, there has been a clear prevalence of studies focused on splicing modification and gene expression inhibition using different types of AONs. Actually, the first AON-based drugs were recently approved, closely followed by the first siRNA-based therapeutic drug, which was approved last year. Still, there is a strong need to optimize the delivery steps of RNA-based technologies and to improve the drug-like properties of therapeutic nucleic acids. Expanding the range of targeted cells and tissues will require the development of robust strategies for cytosolic delivery, thus overcoming the two major hurdles of getting across the plasma membrane and out of the endosome.

In conclusion, as the first generation of nucleic acid therapeutics become drugs, the barrier for investing in RNA-based therapeutics will be lowered, and more resources will become available for exploring other mechanisms of action for RNA-based drugs apart from splicing modulation and single-gene knockdown. As already

pointed out by other authors, the flexibility of RNA design should allow for the facile construction of potent multifunctional drugs that have more than one mode of action and disrupt multiple targets. One such multifunctional drug may hold the promise of substituting for drug cocktails in a future not so distant. There is also the largely unexplored potential of targeting other RNA species and disrupting their functions. Therefore, in the near future, RNA-based drugs may become an increasing component of the pharmacopoeia, greatly expanding the universe of druggable targets and providing affordable treatment options for previously untreatable diseases. Ultimately, this kind of drugs may hold potential to actually cure genetic diseases [186].

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