23 Small Interfering RNAs and RNA Therapeutics in Cardiovascular Diseases

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Abstract

Ribonucleic acid (RNA) is being exploited and understood in its many aspects of function and structure for development of valuable tools in the therapeutics of various diseases such as cardiovascular etc. The expanded knowledge regarding function of RNA in the genomics and inside the cell has dramatically changed the therapeutic strategies in the past few years. RNA has become a spotlight of attention for developing novel therapeutic schemes and hence variety of therapeutic strategies is being coming into the picture that includes RNA interference, use of aptamers, role of microRNA (miRNA) that can alter the complex gene expression patterns. It is due to the fact that RNA offers various advantages in disease management as it can be edited and modified in its various forms such as secondary and tertiary structures. Although scientists are in process of manufacturing RNA-targeting therapies using variety of endogenous gene silencing regulators, Small interfering RNAs (Si RNAs), aptamers and microRNA for cardiovascular diseases yet the development of a novel, risk free therapeutic strategy is a major challenge and need of the hour in cardiovascular medicine. In this regard these agents are required to overcome pleothra of barriers such as stability of drug targets, immunogenicity, adequate binding, targeted delivery etc. to become effective drugs. Recent years have witnessed the progress of RNA therapeutic strategies in cardiovascular diseases that are likely to significantly expand the cardiovascular therapeutic repertoire within the next decade. The present manuscript has been compiled to summarize various approaches of siRNA based therapies in cardiovascular diseases along with the advantages, outcomes and limitations if any in this regard. In addition, the future prospects of RNA therapeutic modalities in cardiovascular diseases are summarized.

Keywords

Cardiovascular disease (CVD) · Small interfering RNAs (Si RNAs) · Aptamer · microRNA

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1 Introduction

Cardiovascular disease (CVD) is becoming a leading cause of morbidity, mortality as well as disability across the globe despite of the advancements in therapeutics and risk management strategies [1].The disease has been reported to be chronic in nature and the symptoms of the disease deteriorate as time period increases. Besides noteworthy therapeutic achievements in CVD, there are multiple undesirable risk factors associated with therapeutic modalities such as drug toxicity, complexity, resistance and many more. Hence the development of a novel, risk free therapeutic strategy is a major challenge and need of the hour in cardiovascular medicine. As per the availability of current literature, several lines of evidences are available supporting the function of RNA specifically the application of small interfering RNAs in silencing the disease causing genes in cardiovascular diseases [2]. It has been considered that RNA plays dynamic and versatile role in regulating the gene expression by acting as an intermediate molecule between DNA and proteins [3, 4].

RNA can offer various advantage in disease management as it can be edited and modified in its various forms such as secondary and tertiary structures. Moreover it may undergo tight, dynamic and various post transcriptional regulatory modifications by using plenty of RNA binding proteins [5, 6]. Hence RNA interference (RNAi) can propose major advantage over pharmacological therapy as they can target specific pathogenic genes that are associated with CVD with low toxicity and high potency. In addition to it, it is pertinent to mention that various biotechnology companies are already in process of manufacturing RNA-targeting therapies using various drug- able targets. For the same purpose, variety of endogenous gene silencing regulators, Small interfering RNAs (Si RNAs), aptamers and microRNA are being exploited to investigate the potential therapeutic agents [7].

It has been observed that novel technologies involved multiple types of small RNA that include miRNA, siRNA, snRNA, piRNA and snoRNA etc. Recent studies are evidencing the extremely broad spectrum of RNA species. Amongst these classes, the expression of mRNA target is inhibited by using siRNA whereas the miRNA are either used for inhibiting other miRNA or to mimic as some other miRNA which will be antagonizing the function of endogenous miRNA due to mimic behaviour [8]. This hypothesis explains how subclasses/varieties of RNA talk to each other and respond differently that leads to altered functional genetic information playing a vital role in various pathological conditions such as CVD etc. To date, therapies involving RNA agents are being exploited to treat various diseases such as cancer [9], infectious [10] and neurodegenerative diseases [11] as well.

The therapeutic potential of RNA based agents are being explored in multiple clinical trials in context to cardiovascular diseases. This manuscript focuses to summarize three such approaches that include siRNA, miRNA and aptamers as well along with the clinical trials conducted in this regard. Further the future directions of RNA therapeutics alongwith their outcomes and limitations in regard to cardiovascular diseases are critically summarized.

2 RNA and Its World: Traditional Concept vs. Current Concept

2.1 Traditional Concept of RNA World

As per the description of various classical studies, it has been observed that a large amount of RNA is transcribed into the cell. The structure and function of variety of RNA transcribed in the cell is becoming better understood with respect to their role in molecular biology as well as their therapeutic potential. Most of the RNA transcribed in the cell do not encode for proteins rather only a part of it (such as tRNA and rRNA) is involved in the process of translation and its regulation. As per the traditional knowledge, RNAs were considered to be transmitters of genetic information i.e. from DNA to RNA and then to the ribosome for proteins synthesis, and

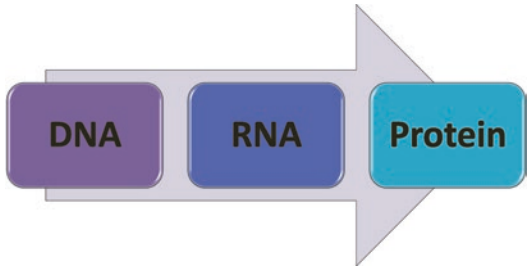

Fig. 23.1 Representation of traditional classical central dogma of molecular biology

hence considered to be the regulators of protein synthesis/Gene expression [12]. The traditional concept of RNA and its role in gene expression was dictated in central dogma which is represented as follows in Fig. 23.1.

2.2 Current Concept of RNA World

Since last decade the multiple studies have expended the role of RNA within the cell and as other catalytic RNAs [13, 14]. As per current status, it is obvious that RNA is not only the intermediary molecule to encode proteins from genes rather some of its types may act as functional end products to control the gene expression in a different but strategic manner. Moreover, recent studies are evidencing the use of new classes of small RNAs such as miRNA as well as siRNA which are generated as a product of some novel biosynthetic pathways and are helpful to mediate regulatory functions [15]. Earlier it was considered that RNA are of two types that include coding RNA (that codes for proteins) and Non coding RNA (which do not encode for proteins). The various types of RNA include rRNA, tRNA, mRNA, snRNA, siRNA and snoRNA etc. The availability of different types of RNA along with their percentage is shown in Fig. 23.2.

Although a variety of RNAs are known to the scientist still the current understanding of RNA molecules and its function is only the tip of the iceberg. The RNA research is gaining momentum on a fast pace due to the rapid development of the molecular biotechnology as well as the various classes of RNA have attracted considerable attention of the scientists to unfold their role in gene regulation and in developing novel drug discovery and development targets. Amongst the above mentioned classes, microRNAs (miRNAs) as well as small interfering RNAs (siRNAs) are being highly exploited for their therapeutic potential and has been depicted by number of scientists against various deadly diseases such as cancer and others [16, 17]. Moreover these small molecules have a potential to become non-druggable target to cure plethora of diseases without undergoing drug induced side effects [18].

3 Small Interfering RNAs

SiRNAs are small non-coding RNA; a novel class of RNA based therapeutic agent with an important role in gene expression. siRNAs have a well-defined structure usually varying in its length from 20 to 24-bp with phosphorylation on 5′ end and hydroxylation on 3′ end [19]. In addition to it, siRNAs exists with overhanging nucleotides on both the sides. In the cell itself, the Dicer enzyme is responsible for the production of siRNAs from either long dsRNAs or small hairpin RNAs. Although these molecules are only of length 20–25 base pairs yet they work to inhibit the gene expression. These are the key molecules to direct post transcriptional gene silencing process called RNA interference (RNAi) rather their existence was first defined by the participation of siRNA in RNAi. It has been demonstrated that the siRNA are derived from the longer transcripts and the processing involves an enzyme named as DICER [20]. The general properties of the siRNA are shown in Table 23.1.

4 Mechanism of Action of siRNA

siRNAs are double stranded RNAs that direct post transcriptional gene silencing process called RNAi. These dsRNAs can be introduced into the cell with the help of transfection method. With the help of complementary siRNA sequence any

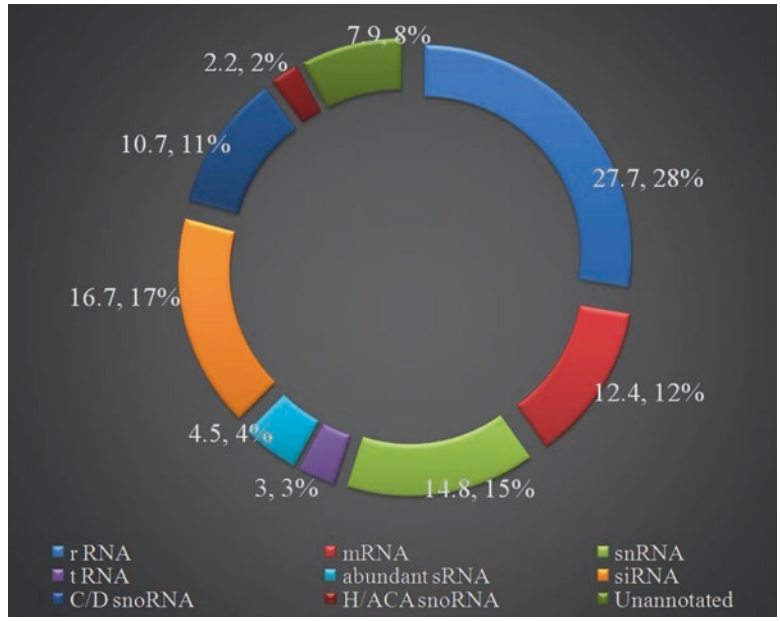

Fig. 23.2 Representation of various types of RNA and its availability in eukaryotic cell

of the gene can be knocked down and hence this RNA class is becoming an important tool for validating the gene functions as well as drug targeting. RNAi is a normal process that took place in almost all the eukaryotic cells. In this highly conserved process, dsRNA molecules i.e. siRNAs silence the post transcriptional effect of any gene of interest [7, 21]. Now a day, synthetic siRNAs are being synthesized by various biotechnology companies that are used to silence the effect of some pre-decided target genes. The knockdown of the target genes is entirely based upon the complementarily between a siRNA and the target gene [22]. Although, synthetic siRNAs are designed to silence targeted gene, but sometime unintended genes may also get knock down, due to imperfect complementarily to non-targeted mRNAs [22].

Table 23.1 Representation of general properties of siRNA

S. no.	Features	Property of siRNA
1.	Before processing DICER	Double-stranded RNA having nucleotides from30–100
2.	Structure	RNA duplex having 21–23 nucleotide with 2 nucleotides overhanging on 3′termial
3.	Complementary	Fully complementary to mRNA
4.	mRNA target	Single
5.	Gene regulation mechanism	mRNA having Endonucleolytic cleavage
6.	Clinical applications	Therapeutic agent

The steps involved in the RNA interference are as follows:

1. **Synthesis of siRNA**: The synthesis of siRNA is the main stage of RNAi. To achieve the synthesis of siRNA, long dsRNAs are transfected inside the cell followed by cleavage of the ds DNA by an endo-ribonuclease enzyme. The enzyme used for the cleavage of the dsRNA is known as Dicer. This enzyme helps to cut a long piece of dsRNA into smaller pieces of 21–25 bp dsRNAs having 2 nucleotides on the 3′ terminus alongwith phosphate groups on 5′ terminus. The smaller dsRNA produced in this process are known either as silencing RNA or short interfering RNA [23].
2. **Incorporation of siRNA to RISC**: In this step, siRNA duplexes are inserted into the RNA-induced silencing complex (RISC). RISC complex plays a crucial role in RNAi. It is a RNA/protein nuclease complex that ini-

tially binds to the siRNA duplexes [24]. For the incorporation of siRNA to RISC, 5′- terminal phosphorylation of the siRNA is compulsory. RISC has two domains named as PAZ and PIWI that help to recognize the 5′-terminus and 3′-terminus of the actual guiding strand followed by targeting the homologous regions in the guiding strand [25].

3. **Gene silencing**: After incorporation into RISC complex, siRNAs are identified by RNA-induced silencing complex (RISC) as well as Argonaute 2 (AGO2) followed by uncoiling of the dssiRNAs into single strands [26–28]. Single stranded siRNA are recognized by their complementary mRNA and after binding with perfect complementary strands target mRNA is finally degraded by exonucleases and hence mRNA cleavage is induced. After the mRNA cleavage, the same will not be recognized by the cell as it is recognized as abnormal mRNA which will in turn silence the gene due to no translation [29, 30]. The step to step process of RNA interference is shown in Fig. 23.3.

There are several advantages of siRNA over conventional drugs [19, 31]. Detection of target sites is pretty easier with high flexibility since both the target siRNA and mRNA are sequences-specific. The inhibitory effect of the siRNA may be realized by targeting particular region of the mRNA [32]. In the process of gene silencing only a small amount of siRNA is required to reduce the concentration of homologous mRNA drastically within a period of 24 h. Physiological impact of cells is not altered by siRNA [33]. The transcripts of interest are destroyed selectively by high level homology of siRNA to the target region of cognate transcription. In the absence of the target sequences, the siRNA remain dormant in the cells and in the presence of the target sequence, the genes are silenced stably. siRNAs displays long term biological effects [34].

5 Chronological Representation of Discoveries in the Area of RNAi

The field of RNAi is not new to us. As far as the discovery of RNAi is concerned, geneticists Craig Mello and Andrew Fire discovered that upon injecting double-stranded RNA (dsRNA) into small worms some of the corresponding genes get switched off and hence the concept of the RNAi came into picture [35]. Finally research-

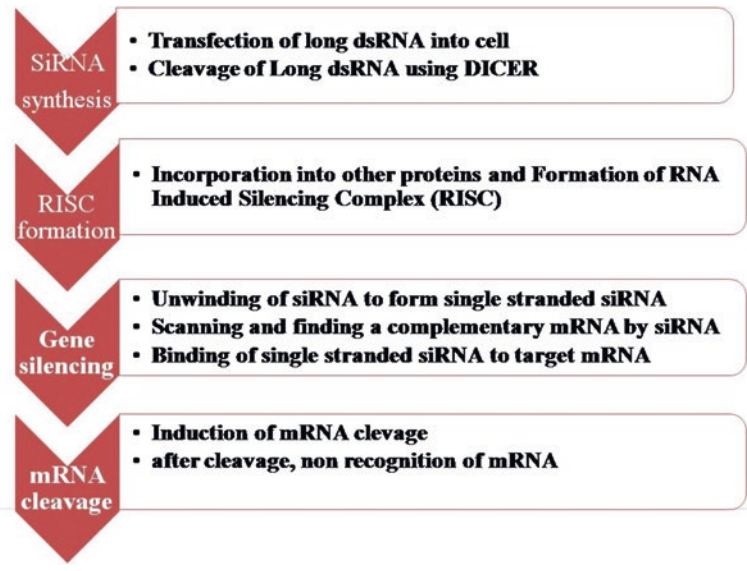

Fig. 23.3 Representation of step to step process of RNA interference

Table 23.2 Representation of chronological discoveries in the area of RNAi

Year	Discoveries in the area of RNAi	References
1998	When double stranded RNA (dsRNA), called small interfering RNA (siRNA), is injected into worms, it switches off the corresponding gene, which leads to the identification of RNA interference (RNAi) as a whole new cellular pathway.	[36]
2002	RNAi is a pathway common to mammals.	[37]
	One of the biggest RNAi companies *i.e.* Alnylam was established.	
2004	First clinical trial of an RNAi was conducted in patients having age-related macular degeneration.	[38, 39]
2005	Novartis agreed to purchase almost 20% of Alnylam's stock	[40]
2006	Mello and Fire won the Nobel Prize for Medicine and Physiology for their work	[41, 42]
	Merck purchases biotechnology company Sirna for US$1.1bn	
2007	Roche purchases just under 5% of Alnylam's stock and RNAi company, Dicerna, was founded	[43]
2008	A phase III trial of RNAi in wet age-related macular degeneration is terminated because of a lack of efficacy	[44]
2012	Alnylam discovers that a sugar molecule called N-acetylgalactosamine is key to getting RNAi into liver cells	[45]
2016	Alnylam reports an "imbalance of deaths" in a phase III study for a drug called revusiran, designed to treat hereditary transthyretin amyloidosis, and development is subsequently discontinued.	[46]
	Dicerna scraps the first two of its drugs to make it to clinical trials because preliminary results do not meet the company's expectations, USFDA refused to grant approval for further clinical trials of any of Arrowhead's RNAi drugs because of unexplained deaths in chimpanzees.	
2017	Alnylam's drug patisiran is found to be effective and safe in a phase III trial for treating hereditary transthyretin amyloidosis (pictured) with neuropathy	[47]
2018	Several of the key companies expect to start numerous clinical trials of new RNAi therapeutics.	[48]
	First ever gene-silencing drug won FDA approval	

ers were awarded with Noble Prize for Medicine and Physiology in 2006. Some of the chronological discoveries in the area of RNAi are shown in Table 23.2.

6 RNA Interference in Cardiovascular Diseases

The technique is used to down regulate the desired gene expression using easily formulated small interfering RNA fragments. The method is quickly evolving against various diseases since its discovery and is becoming a routine application in many of the laboratories. There are number of studies that explain the success of RNA interference in treatment of human diseases.

RNAi induced by siRNA has emerged as a crucial technique for screening new therapeutic targets and searching molecular mechanisms of cardiovascular diseases [49]. siRNAs can be used to inhibit the overproduction of proteins that are associated with cardiovascular disease. Proprotein convertase subtilisin/kexin type 9 (PCSK9) has been reported to increase the levels of cholesterol in plasma; inhibition of the enzyme decreases the chances of hypercholesterolemia [50]. Studies have been carried out on the monkeys and it has been concluded that the special siRNA delivery by lipidoid nanoparticles against PCSK9 can decrease the cholesterol effectively [51]. siRNA have been found to silence the CCR2 i.e. chemokine receptor that results in enhanced recovery from myocardial infarction as it reduces the penetration of inflammatory cells into the infracted area [52].

Apart from inflammation, cardiomyocyte apoptosis is also observed during myocardial in fraction. Cardiomyocyte apoptosis results in reduced cardiac contractility as observed in mouse models with myocardial infarction (MI). Post myocardial infarction, apoptosis is mediated by tyrosine phosphatase Src homology region 2 domain-containing phosphatase 1 (SHP-1) [53].

It has been reported that RNAi against SHP-1 rescues cardiomyocytes from apoptosis among mouse MI model [54]. Addition, a hurdle in RNA therapeutics has been observed in mouse MI model but it resulted in non-specific gene silencing because of the off-target binding of siRNAs [55]. To reduce off-target binding of siRNA is also very important in RNA therapy. Tough decoy (TuD) RNA has been introduced which is meant to silence the sense strand of the siRNA duplex [56]. Recently, RNAi-based gene silencing methods are being established in humans and varieties of clinical trials are ongoing which may act as a promising therapy for the fatal diseases such as cardiovascular, neurological diseases etc.

It has been observed that upcoming cardiovascular RNA drugs are used to address multiple organ systems. It is not always mandatory to address heart or vasculature directly rather the disease can be managed by targeting liver RNA drugs. Liver targeted RNA drugs are gaining success and are developed in the field of cardiovascular diseases. Another system such as immune system involving monocytes or macrophages can be targeted for delivering RNA drug to its tissue target [57]. Various studies involving the role of RNAi specifically in cardiovascular diseases are shown in Table 23.3.

7 SiRNA Based Drugs: FDA Approval

Though the idea of the gene silencing has been revealed two decades ago, yet, the researchers are desperate and making critical steps for seeking approval for newly manufactured/to be manufactured new siRNA based medicines from US Food and Drug Administration (FDA). It has been observed that there is entry of FDA approved drugs into the market and amongst those drugs the drugs for various diseases are enlisted. The FDA approval for few of the siRNA has led the field of gene silencing to the new heights due to the fact that RNAi has the flexibility to design as many as novel targets against many different genes/diseases with high specificity. Few such drugs are enlisted in Table 23.4.

8 Future Perspective

The flexibility of RNAs makes them crucial therapeutics modality for number of human diseases. Exploring new classes of RNA that possess therapeutic potential will help in its successful translation to the clinic. Understanding the mode of action of various RNAs including long-noncoding RNAs (lncRNAs), miRNA, siRNA, etc. in CVD will help in improved therapeutics among patients. lncRNAs are distinct form of short noncoding RNAs that binds the targets through complimentary base pairing. lncRNAsfold into specific tertiary structures and interact with its proteins targets and the function of lncRNAs in MI have revealed [85]. The role of lncRNAs is only partially known and recently unfolded in cardiac development and the progression of MI [86]. Hence studies relating to regulation of long-noncoding RNAs for reversal of the diseased state and triggering the endogenous regenerative process can be a promising therapeutic tool for CVD patients [87, 88]. Many diseases result from multigene dysregulation; so administration of more than one therapeutic target will be beneficial for good outcome. Combination of RNA therapeutics with protein or small molecule drugs may also become promising therapy [89]. Delivery of RNAs will improve cell-based therapies. Cell based therapies will be promising approach for rejuvenation of ischemic myocardium since adult cardiomyocytes have restricted regenerative capacity [90]. However, this therapeutic approach has some obstacles like less cell retention and poor cell survival in infracted regions. These therapeutic challenges can be improved by incorporating few RNA agents. Delivery of mRNA encoding angiogenic factors along with RNAi that is responsible for decreased inflammation and fibrosis in the host tissue may improve survival [91]. For implementation of this approach few new strategies needs to be designed for effective cell and RNAs delivery. To achieve this goal, a new strategy should be designed for effective deliver of cells and RNAs to infracted area. Role of RNA therapeutics in treating MI has been successfully demonstrated in small animals however preclinical studies on large animals and

Table 23.3 Representation of studies involving RNAi as therapeutic agents in cardiovascular diseases

Clinical trials identification number	Recruiting status	Conditions of diseases	No. phases	Treatment	No of enrolments	Study completion date	References
NCT03060577 (ORION-3)	Not recruiting, active	Atherosclerotic cardiovascular disease	2	Inclisiran (ALN-PCSSC)	490	2022	[58]
		Symptomatic atherosclerosis		Evolocumab (REPATHA)			
		Familial hypercholesterolemia					
		Type2 diabetes					
NCT03792607	Recruiting	Type 2 diabetes mellitus			50	March 5, 2019	[59]
		Cardiovascular disease					
NCT03705234	Recruiting	Atherosclerotic cardiovascular disease	3	Inclisiran	15,000	March 25, 2019	[60]
				Placebo			
NCT03399370	Active, not recruiting	ASCVD	3	Inclisiran sodium	1561	April 17, 2019	[61]
		Elevated cholesterol		Placebo			
NCT03400800	Active, not recruiting	ASCVD	3	Inclisiran sodium	1617	April 17, 2019	[62]
		Elevated cholesterol		Placebo			
NCT03515772	Recruiting	HIV infections		Co-administration of darunavir with a cardiovascular	60	October 31, 2019	[63]
		Drug interactions					
		Cardiovascular diseases					
NCT03672994	Recruiting	Asthma			650	April 30, 2019	[64]
		COPD					
		Heart failure					
		Community-acquired PneumoniaHealthy					
		Breathlessness					
NCT02527343 (BROADEN study)	Active, not recruiting	Familial partial Lipodystrophy	2 and 3	Volanesorsen (ISIS 304801, IONIS-APOCIIIRx)	60	September, 2021	[65]
				Placebo			
NCT03371355	Recruiting	NAFLD	2	ISIS 703802 (AKCEA-ANGPTL3-LRx, IONIS-ANGPTL3-LRx)	144	September 26, 2018	[66]
		Diabetes mellitus,					
		Type 2Hypertriglyceridemia					
		Fatty liver, nonalcoholic		Placebo (sterile Normal saline (0.9% NaCl))			

NCT03455777	Withdrawn (study withdrawn due to lack of available patients meeting entry criteria)	Homozygous familial Hypercholesterolemia	2	AKCEA-ANGPTL3-LRX (ISIS 703802)	0	December 3, 2018	[67]
NCT02900027	Completed	Elevated triglycerides (TG)	1	Placebo comparator APOC-III-L-Rx	56	May 22, 2018	[68]
NCT02963311 (ORION-2)	Completed	Lipid metabolism disorders dyslipidemias hypercholesterolemia Hyperlipidemias Hyperlipoproteinemia type II	2	ALN-PCSSC (PCSK9) Standard of care/low density lipoprotein-cholesterol (LDL-C)	4	December 21, 2018	[69]
NCT03159416 (ORION-7)	Completed	Renal insufficiency Kidney diseases Urologic diseases	1	Inclisiran (ALN-PCSSC)	31	November 9, 2018	[70]
NCT03360747	Completed	Lipoprotein lipase Deficiency Hyperlipoproteinemia type 1 Familial Chylomicronemia syndrome	2	AKCEA-ANGPTL3-LRX (ISIS 703802)	3	April 25, 2019	[71]
NCT03070782	Completed	Elevated lipoprotein(a) Cardiovascular disease	2	Placebo (sterile Normal saline (0.9% NaCl) ISIS 681257	286	December 13, 2018	[72]
NCT02824003	Completed	Type 2 diabetes	2	ISIS-GCGRRx Placebo	15	May 8, 2018	[73]
NCT02583919	Completed	Type 2 diabetes	2	ISIS-GCGRRx (Isis 449884) Placebo	79	June 25, 2018	[74]

Table 23.4 List of FDA approved siRNA drugs used for treatment of various diseases

Drugs	Disease treatment
ONPATTRO (Patisiran)	Used for the treatment of polyneuropathy in people with hereditary transthyretin-mediated amyloidosis (fatal rare disease) [75].
Givosiran (ALN-AS1)	It is available in a dosage form for subcutaneous administration for treating acute hepatic porphyria (AHP) and other porphyrias like acute intermittent, variegate and hereditary coproporphyria. It is also employed in ALAD-deficiency porphyria (ADP) [76].
Fitusiran (ALN-AT3)	Subcutaneous dosage form targeting antithrombin used for treatment of hemophilia and rare bleeding disorders (RBDs) [77].
Inclisiran (ALN-PCSsc)	Subcutaneous dosage form targeting proprotein convertase subtilisin kexin type 9 (PCSK9) used for the treatment of hypercholesterolemia [78].
Lumasiran (ALN-GO1)	Subcutaneous dosage form targeting glycolate oxidase (GO) used for the treatment of Primary Hyperoxaluria Type 1 (PH1) [79].
Vutrisiran (ALN-TTRsc02)	Subcutaneous dosage form targeting transthyretin (TTR) used for the treatment of transthyretin-mediated (ATTR) amyloidosis [80].
Cemdisiran (ALN-CC5)	Subcutaneous dosage form targeting the C5 component of the complement pathway and being used for various complement-mediated diseases [81].
ALN-AAT02	Subcutaneous dosage form targeting alpha-1 antitrypsin (AAT) used for the treatment of AAT deficiency-associated liver disease [82, 83].
ALN-HBV02 (VIR-2218)	Subcutaneous dosage form targeting the hepatitis B virus (HBV) genome used for the treatment of chronic HBV infection [82, 83].
ALN-AGT	Subcutaneous dosage form targeting angiotensinogen (AGT) used for the treatment of hypertension [82, 84].

patients will further explore/establish their efficacy in future. To maximize benefits and to avoid adverse effects, there is a need to draw stringent standard operative procedures for delivery strategies, development and improvement of RNA-based MI therapeutics.

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