RNA therapeutics – the next generation of drugs for cardiovascular diseases

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Abstract

Purpose of review:
RNA therapeutics are a new and rapidly expanding class of drugs to prevent or treat a wide spectrum of diseases. We discuss the defining characteristics of the diverse family of molecules under the RNA therapeutics umbrella.

Recent findings:
RNA therapeutics are designed to regulate gene expression in a transient manner. For example, depending upon the strategy employed, RNA therapies offer the versatility to replace, supplement, correct, suppress, or eliminate the expression of a targeted gene. RNA therapies include antisense nucleotides, microRNAs and small interfering RNAs, RNA aptamers, and messenger RNAs. Further, we discuss the mechanism(s) by which different RNA therapies either reduce or increase the expression of their targets.

Summary:
We review the RNA therapeutics approved (and those in trials) to treat cardiovascular indications. RNA-based therapeutics are a new, rapidly growing class of drugs that will offer new alternatives for an increasing array of cardiovascular conditions.

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Introduction

Nucleic acid-based therapies consist of exogenous sequences, either DNA or RNA, that are designed to generate a therapeutic effect *in vivo*. Although RNA therapeutics have only recently gained notoriety, they have been under development for several decades [1-5]. The initial proof of concept experiments for RNA therapies involved using messenger RNA (mRNA) to artificially express a protein in vivo were performed about three decades ago [6, 7]. This first work used intramuscular injection to deliver one of three in vitro transcribed mRNAs to mice [6]. Protein expression from the injected mRNAs was verified and proved just as effective as (as judged by the protein levels expressed from the injected nucleic acid) using DNA-encoded vectors [6]. The next key study used a lab-made vasopressin mRNA to transiently correct a rat model of diabetes insipidus [7]. Since these initial experiments with mRNA, a diverse family of molecules is now covered by the umbrella of RNA therapeutics. RNA therapeutics can contain a diverse mixture of nucleotides and can be single- or double-stranded [8, 9]. Currently, antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), microRNAs (miRNAs), RNA aptamers, and mRNAs are all grouped together as RNA therapies [1-10]

As evidenced by the two COVID-19 vaccines from Moderna and Pfizer/BioNTech, RNA therapies can be designed, developed, evaluated, manufactured, and distributed rapidly [11, 12]. A detailed overview covering the theory and functional aspects of different RNA therapies is beyond the scope of this review but is available here [8]. Instead, this review describes the major families of RNA therapies, summarizes the RNA therapeutics currently in use (or in development) for cardiovascular indications (Table 1), and describes the mechanism of action for selected RNA drugs (Figure 1). We also collect and group the different drug and company names with their respective clinical trial identifiers to track each RNA therapeutic over time (Table 1).

Antisense oligonucleotide (ASO) therapeutics

In this section, we briefly discuss ASOs (refer to [13, 14] for more thorough reviews) and highlight the technological aspects that expedited their clinical translation and enabled their development into cardiovascular disease-targeting therapeutics. ASOs are short (18 – 30 nucleotides in length) synthetic, single stranded nucleic acids whose sequences are complementary to a cellular RNA target [14]. Importantly, although considered RNA therapeutics, ASOs can be either homo- or mixed polymers consisting of RNA, DNA and/or LNA (locked nucleic acids) bases [14]. ASOs use base-pairing interactions to (1) disturb or correct the splicing and/or

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processing of pre-mRNAs or to (2) suppress the translation or (3) induce the degradation of targeted mRNAs [1, 15, 16]. Each of these approaches ultimately modulates the levels of a targeted protein [14]. Many ASOs trigger endogenous RNA degradation pathways by recruiting RNase H1 which is recruited to and degrades the RNA strand of DNA:RNA duplexes [17]. The small size and well-understood principles underpinning ASO sequence design helps prevent potential toxicities associated with off-target binding and can be exploited to enhance binding specificity between ASOs and targets [18]. *In vivo*, ASOs with unmodified phosphodiester bond backbones are rapidly destroyed by serum nucleases and cleared from circulation by the kidneys [19]. Therefore, numerous chemical nucleotide modifications have been introduced to improve the pharmacokinetics and pharmacodynamics of ASOs [9]. For example, phosphodiester linkages of ASOs can be replaced by phosphorothiorate linkages to strengthen nuclease resistance and diminish hydrophilicity while maintaining robust RNase H1 activity [9, 20]. Unfortunately, these changes need to be made studiously as certain modified nucleotides were shown to induce a strong immune response and/or lower ASO to target binding affinities when compared to standard unmodified nucleotides [9]. Since it increased ASO stability without affecting ASO targeting, initial base modifications targeted the 2’ position of the ribose sugar. Common modifications include replacing the 2’-hydroxyl moiety with either a 2’-O-methyl, 2’-O-methoxyethyl, 2’-O-aminopropyl, or 2’-fluoro groups to prevent hydrolysis of the ASO [9]. Several other important base modifications or substitutions to alter nucleoside pairing interactions and the molecular conformation of ASO, including the incorporation of LNA bases, restrained ethyl nucleoside analogues, artificial amido-bridged nucleic acids, or other ASO backbone changes [9]. ASOs have also been coupled to ligands (GalNAc for example) to target their delivery to a particular tissue [21]. Finally, helping to reduce production-related costs, due to their heavily modified structures, many ASOs do not require specialized delivery vehicles [9].

Several targets such as proprotein convertase subtilsin kexin type 9 (PCSK9), lipoprotein(a) (Lp(a)), and ANGPTL3 have been genetically linked to cardiovascular and metabolic diseases [22-24]. In 2019, Pfizer partnered with Akcea Therapeutics (an affiliate of Ionis Pharmaceuticals) to investigate and license AKCEA-ANGPTL3-LRx, an ASO targeting ANGPTL3 [9]. At the same time, Norvartis also collaborated with Akcea and Ionis Pharmaceuticals to develop and license AKCEA-APO(a)-LRx, using Ionis’ ligand conjugated antisense technology platform [9]. Both of these ASOs have entered the Phase II clinical trials (Table 1) and have been showing potential to treat heterozygous familial hypercholesterolemia and atherosclerotic cardiovascular diseases.

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As mentioned above, the properties of ASOs and oligonucleotide therapeutics in general allow ASOs to reach every tissue, including the heart, effectively. As with many drugs, ASOs can be targeted to the liver with little or no assistance [9]. Although many ASOs have been approved by the FDA, since 2013 only Mipomersen (Kynamro, Figure 1, Top Left), has been approved by the FDA as a treatment for a cardiovascular indication (NCT00770146) [25]. Mipomersen is approved as a treatment for homozygous familial hypercholesterolaemia (HoFH), a rare genetic disorder where both low-density lipoprotein (LDL) receptor alleles are mutated [25]. Untreated, HoFH leads to reduced clearance of circulating LDL cholesterol in plasma [25]. The Kynamro compound is a “second generation” 2’-O-methoxyethyl chimeric ASO [25]. The ASO is built with phosphorothioate linkages rather than the phosphodiester linkages found in natural RNAs [26]. In addition, the ASO contains DNA nucleotides in the center of the molecule with 2’-O-methoxyethyl-modified RNA nucleotides at the ends [26]. In the liver, this drug initiates the degradation of the mRNA encoding apolipoprotein (Apo)B-100 (Figure 1, Top Left), a key structural element of LDL and its metabolic precursor, very-low-density lipoprotein [26]. Reduction of ApoB protein then helps reduce LDL cholesterol and lipoprotein (a) (Lp(a)) levels in the blood [26, 27]. A double-blind, randomized, placebo-controlled, Phase III clinical trial (NCT00607373) was completed in 2010 and showed that Mipomersen effectively inhibited ApoB protein production by ~25% and reduced LDL cholesterol level in HoFH patients who were already being treated with lipid-lowering drugs [28, 25]. However, several subsequent studies showed the adverse events of Mipomersen in treated patients, including serious injection site reactions and flu-like symptoms [28, 29]. Moreover, a severe risk of liver damage has also been reported. According to liver function tests, around one in three patients receiving Mipomersen exhibited measurable signs of liver toxicity [30-33]. Therefore, in April 2021, this drug was discontinued on the open market and can only be prescribed in the context of an FDA- approved Risk Evaluation and Mitigation Strategies program.

Another well-known ASO candidate called Volanesorsen (Table 1), which targets the mRNA encoding hepatic apolipoprotein C-III (APOC3), has been shown to reduce plasma triglyceride levels, and has been submitted to the FDA for authorization to market [34, 35]. Ionis Pharmaceuticals and Akcea Therapeutics developed this drug and registered it under the brand name Waylivra. In patients with familial chylomicronemia syndrome (FCS), weekly doses of Volanesorsen markedly reduce triglycerides (1700 mg/dL vs 90 mg/dL compared to placebo treatment) [35]. In 2019, the Phase III APPROACH study also showed mean triglyceride levels decreased 77% in Volanesorsen-treated patients versus an 18% increase in patients in placebo group [36]. They also revealed that Volanesorsen lowered triglyceride levels below the risk threshold for triglyceride-induced acute pancreatitis [36]. However, since most ASOs can be

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distributed broadly and accumulate in the liver and kidneys, with half-lives of 2-4 weeks, Volanesorsen showed some evidence of adverse effects associated with thrombocytopenia and risk of bleeding [37, 38]. Despite these side effects, the significant reduction of plasma lipid levels led the European Commission to approve Volanesorsen as the only approved therapy for FCS in 2019 [39].

Numerous second- and third-generation ASOs are currently being developed to treat not only cardiovascular diseases (Table 1), but other life-threatening and rare genetic diseases including spinal muscular atrophy (Spinraza), Duchenne's muscular dystrophy (Vyondys 53), and hereditary transthyretin amyloidosis (Inotersen) [40-43]. Although the near-term safety of ASOs has been examined in preclinical and clinical trials, the potential consequences of long-term ASO administration still remain unclear [18]. Moreover, some possible adverse effects may happen due to ASO chemistry or downstream effects of target involvement. For these reasons, extended follow up of patients treated with ASO drugs is required to determine the long-term efficacy and side effects of these ASO therapies. Despite these unknowns, ASOs provide a new approach that have the versatility to improve the quality of life for patients with some previously untreatable diseases.

RNAi: RNA interference for gene silencing

The discovery of RNA interference (RNAi) entirely reshaped how gene expression and regulation was perceived [3, 44]. RNAi is a natural process by which mRNAs are regulated post-transcriptionally [44]. In addition to regulating the expression of endogenous genes, the RNAi pathway also protects an organism from foreign nucleic acids [44]. Targeted, sequence-specific gene silencing offers nearly limitless applications such as defining the function(s) of newly-discovered genes, identifying novel and therapeutically relevant genes, and targeting genes previously labeled as "undruggable". In mammals, RNAi is triggered by short double-stranded RNAs (dsRNAs) from endogenous or exogenous (synthetic RNAs, pathogens) origins. There are two main types of RNAi: small interfering RNAs (siRNA) and microRNAs (miRNA) [45]. They both target mRNAs using base-pair recognition and initiate mRNA degradation, which then decreases the levels of the corresponding protein [45]. However, key differences separate the two RNAi mechanisms. For example, siRNAs are perfectly complementary to the targeted mRNA and cause its cleavage and degradation [46]. Whereas miRNA sequences contain multiple mismatches to their targeted mRNA and initiate mRNA degradation by recruiting decapping enzymes and deadenylases [47]. As they are beyond the scope of this manuscript, other differences and similarities are thoroughly detailed in this review [46].

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siRNAs

A standard siRNA drug is a 21 to 25 nucleotide dsRNA. *In vivo*, these exogenous dsRNAs are trimmed into siRNA precursors by an enzyme called Dicer which leaves a 3'overhang [48, 49]. The processed siRNA precursor is then loaded into the RNA-induced silencing complex (RISC) which preferentially retains the targeting RNA strand to make the active siRNA [50]. siRNA-mediated gene silencing occurs when a perfectly complimentary siRNA sequence triggers the endonuclease 'slicing' activity of AGO2 which cleaves the targeted mRNA leading to its degradation thereby reducing protein levels [51]. Therapeutic siRNAs initially faced multiple challenges like immunogenicity, specificity, and instability; however, many studies were performed to optimize the structure and delivery of siRNA drugs. Today, numerous siRNAs drugs have obtained FDA approval, while others are currently being tested in clinical trials [8]. Most cardiovascular system-focused siRNA therapeutics or candidate drugs are designed to treat conditions via liver-specific delivery. Inclisiran, sold as Leqvio, (Figure 1, top right) is approved in the EU and is currently under review by the FDA as a treatment for primary hypercholesterolaemia or mixed dyslipidaemia [52]. Inclisiran is an artificial siRNA conjugated with GalNAc on the sense strand to allow for liver-specific delivery [53]. Upon absorption by hepatocytes, Inclisiran targets the mRNA encoding PCSK9 thereby reducing the expression of PCSK9 protein [54]. In so doing, Inclisiran increases cell surface levels of LDL receptor by reducing its turnover which ultimately reduces bloodborne LDL-C levels by increasing the uptake of LDL-C by the liver [52].

miRNAs

MicroRNAs are short, naturally occurring non-coding RNAs that have vital roles in cellular function via post-transcriptional gene regulation [55, 56]. As mentioned above, miRNAs contain sequence mismatches which can be disadvantageous since they can lead to unwanted off-target effects. However, mismatches can also be beneficial as they can allow for the simultaneous targeting of multiple distinct mRNAs. miRNAs typically bind to the 3'UTR (untranslated region) of mRNAs, and represses their translation or recruit deadenylases and/or decapping enzymes to facilitate the degradation of targeted mRNA(s) [57]. Notably, offering another possible treatment avenue, a small minority of miRNAs have also been reported to up-regulate gene expression as reviewed in [58]. Currently, there are no marketed miRNA therapeutics. However, patents and clinical trials for miRNA inhibitors (anti-miRs) and miRNA mimics are on the rise.

miRNA blockers (Anti-miRs)

Since miRNAs can simultaneously target multiple disease-linked mRNAs and misregulation of miRNAs has been linked to many diseases, repressing well-described miRNAs quickly became an attractive therapeutic approach. Anti-miRs are designed to specifically

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recognize and inhibit naturally occurring miRNAs. This can be accomplished by targeting miRNAs for degradation or by sequestering the miRNA so it could no longer bind its targets. Both mechanisms can prevent miRNAs from acting on their mRNA targets. Multiple approaches to target miRNAs exist, including antagomiRs (cholesterol-conjugated anti-miRs), Locked Nucleic Acids, and ASOs [59-61] . All of these molecules are designed to bind and sequester miRNAs, thus preventing miRNA-mRNA interactions [62].

miRNA mimics

miRNA mimics are synthetic RNAs that are patterned after endogenous miRNAs. Unlike anti-miRs which aim at inhibiting miRNAs that are overexpressed in disease, miRNA mimics are designed to replace or supplement the levels of beneficial miRNAs. The therapeutic miRNA mimics are processed similarly to endogenous microRNAs and will reduce the level of specific genes [63]. Current miRNAs mimics in clinical trials are mainly for Hepatitis C and different cancers [64].

RNA aptamers

Unlike other RNA therapeutics, RNA aptamers use their 3D conformation rather than sequence-specific base-pairing to recognize their targets [65]. Similar to an antibody, aptamers (DNA, RNA or protein-based) bind a desired ligand with very high affinity and selectivity [65]. Although similar in function to protein-based antibodies, RNA aptamer manufacturing is more straightforward, performed entirely *in vitro,* and more cost-effective compared to protein-based antibodies [66]. RNA aptamers are single-stranded molecules that are isolated using systematic evolution of ligands by exponential enrichment (SELEX) [4, 5]. In SELEX, a pool of RNA is generated, and those binding to desired targets with high specificity are isolated and enriched [4, 5, 66]. RNA aptamers exhibit flexible targeting and have been shown to bind specific molecules, cells, and tissues [66]. In contrast with other RNA therapeutics, RNA aptamers are not restricted to an intracellular target, and they can be designed to bind virtually any molecule in any cell compartment [4, 5]. The binding properties of RNA aptamers also allow them to be conjugated to other therapeutics or delivery vehicles for a tissue-specific delivery [67].

Currently, one RNA aptamer (Pegaptanib) has been approved by the FDA for age-related macular degeneration in 2004 [68]. Pegaptanib is a 28 nucleotide RNA aptamer and functions via binding vascular endothelial growth factor (VEGF) protein and blocking its pro-inflammatory activities in AMD patients, thus preventing serious vision complications [69, 68]. Other candidate RNA aptamer drugs are currently being evaluated in clinical trials. For example, BT200 (Table 1, Figure 1, bottom left) is a pegylated RNA aptamer candidate currently in Phase I or II trials for the

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treatment of Hemophilia A, Atherosclerosis, and stroke. BT200 shows promising results and acts as an antithrombotic agent by binding the A1 domain of the von Willebrand factor (VWF), a factor critical for thrombus generation [70].

Messenger RNA (mRNA) therapeutics

The proof of concept experiment for mRNA-encoded therapeutics was performed over three decades ago when Wolff *et al* showed that administering *in vitro* transcribed (IVT) mRNA into mouse skeletal muscle resulted in the expression of the protein of interest *in vivo* [6]. During the 1990s, preclinical trials of IVT mRNA were examined a variety of applications including protein replacement and vaccine-based designs to treat or prevent cancer and infectious diseases [6, 71, 10]. Numerous studies quickly discovered several major drawbacks of mRNA therapies, including short RNA half-life and non-specific immunogenicity. The intervening decades have seen the resolution of many of these issues and therapeutic mRNAs are becoming a favored approach. Several universities and pharmaceutical companies including Moderna, BioNTech, Novartis, CureVac, Sanofi Pasteur, Glaxo Smith Kline, AstraZeneca, and Alexion are developing mRNA-based therapeutics [8].

Conceptually, the numerous advantages of IVT mRNA-based therapeutic approaches make them just as or more versatile than other nucleic acid-based therapies. IVT mRNAs are fully functional in the cytoplasm and are rapidly translated to produce the desired proteins [8-10]. In addition, IVT mRNA-based therapeutics have a better safety profile. Simply, unlike plasmid DNA and certain viral vectors, mRNA therapeutics are incapable of integrating into the genome, and thus eliminate the risk of insertional mutagenesis [10]. Furthermore, IVT mRNA production is relatively manageable and inexpensive; therefore, it has sparked a broad interest in developing this new class of drugs for treatments in oncology, cardiology, endocrinology, hematology, pulmonary medicine, and as vaccines for infectious diseases [8-10].

Currently, IVT mRNA can be delivered via two approaches. The IVT mRNA can be transferred into the patient's cells *ex vivo*, then these modified cells can be delivered back to the patient. The direct delivery of the IVT mRNA to the host using different delivery vehicles is the other alternative [8]. Substantial energy has been invested with the goal of improving the translatability and the *in vivo* lifespan of IVT mRNA drugs. This includes improvements to optimize structural components of the IVT mRNA including the 5' cap, 5'- and 3'-untranslated regions, the coding sequences, and the polyadenylated tail of the mRNA. The immune-stimulatory profile of IVT mRNA can be altered and customized based on therapeutics purposes. As an example, for an mRNA-based vaccination strategy, the immune-stimulatory effect associated with IVT mRNA

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could be considered a benefit as it could help drive antigen-specific cellular and humoral immune responses. However, innate immune activation is a major hurdle for protein-replacement therapies; therefore, several approaches aim to create “de-immunized” mRNA have been, and continue to be, developed to overcome this problem [72].

Despite the potential of mRNA therapies to treat cardiovascular diseases, currently only one, named AZD8601 (Figure 1, Bottom Right), which encodes vascular endothelial growth factor-A (VEGF-A) is being jointly developed by AstraZeneca and Moderna [73]. When given to patients after a heart attack, or those with heart failure, diabetic wound healing problems, or other ischemic vascular diseases, AZD8601 could prove to be a regenerative treatment option [74]. AZD8601 is VEGF-A_{165} mRNA in buffered saline [74]. This drug was optimized to overexpress VEGF-A while minimizing innate immune activation [74]. AZD8601 is currently being evaluated in the EPICCURE (NCT03370887) Phase II clinical trial, a double-blind, randomized, placebo controlled, multicenter, 6-month trial, with 24 patients scheduled for elective bypass surgery, including 3 groups of 8 patients who were randomized to receive either 3 mg AZD8601 (low dose), 30 mg AZD8601 (high dose), or placebo injection [74]. During the first-in-human Phase I trial, the expression of functional VEGF-A was validated after AZD8601 administration [71]. AZD8601 also induced new blood vessel formation without an elevated innate immune responses in human volunteers [74]. The EPICCURE trial is designed to use quantitative ^{15}O-water PET imaging to map ischemic but viable myocardium [74]. However, this trial was limited to patients that undergo coronary artery bypass grafting; therefore, it is more difficult to assess the adverse events during drug administration or surgery [74]. In summary, EPICCURE integrated innovative VEGF-A mRNA delivery with novel ischemia-guided administration to assess the safety and potentially beneficial angiogenic effects of AZD8601 on cardiac function and myocardial perfusion in patients with coronary artery dysfunction that require bypass surgery [74].

Conclusions

The unquestionable success of the two mRNA vaccines for COVID-19 has shown the world the power and versatility of RNA-based therapeutics. This increased awareness has translated into an unparalleled surge of resources to develop new RNA medicines. However, with fewer than 40 ongoing or completed clinical trials evaluating different cardiovascular disease-targeting RNA drugs, RNA therapeutics remain a comparatively untapped source of treatments for these indications. The mRNA-based technologies described here amount to one of the most promising approaches for future drug development and can be applied to a broad range of potential applications.

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Conflicts of Interest, Ethics & Compliance

Dr. Kiss runs an externally funded lab (American Heart Association and NIH) that is actively designing and testing different candidate RNA therapeutics, including some with possible applications in cardiovascular disease. All authors anticipate seeking appropriate intellectual property protection for promising candidates that emerge from the lab's work. Further, Dr. Kiss serves as a consultant for the RNA Core at the Houston Methodist Research Institute. Dr. Bejar is named as a co-inventor on a provisional patent filing for a candidate RNA therapeutic.

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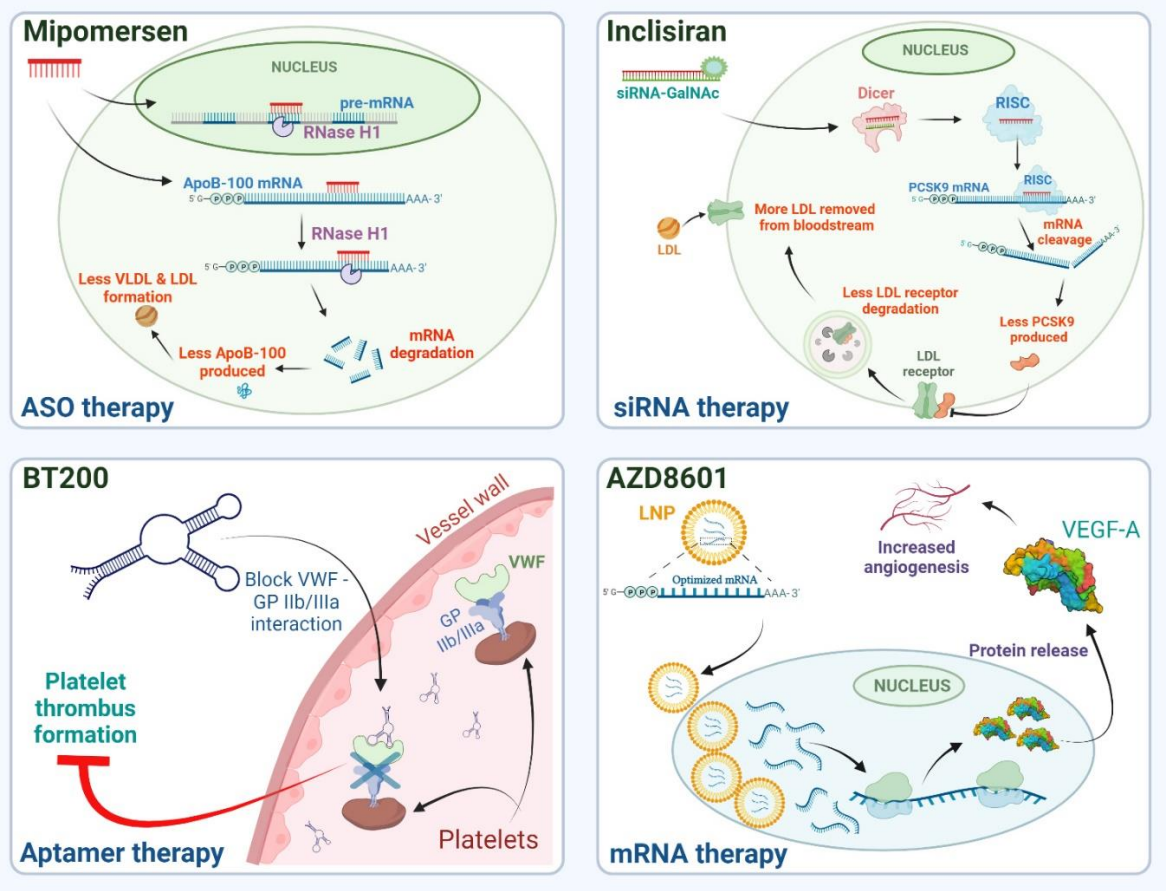

Figure 1. Mechanisms of action for selected RNA-based drugs to treat cardiovascular diseases. Top Left: Mipomersen is an example of an ASO drug (red) which hybridizes to ApoB-100 mRNA and recruits RNase H1 to cleave the targeted mRNA, preventing apolipoprotein B production, which then reduces the synthesis of VLDL and LDL. **Top Right:** Inclisiran as an siRNA therapy that targets PCSK9 mRNA. The targeting strand is incorporated into RISC complexes which then recognize the targeted mRNA and initiates its cleavage and degradation. This decreases the amount of PCSK9 protein produced, blocking the PCSK9-driven internalization and degradation of LDL receptors. The increased numbers of cell surface LDL receptors then remove more LDL from circulation thereby reducing bloodborne LDL levels. **Bottom Left:** BT200 is an RNA aptamer designed to inhibit aberrant thrombus formation. This aptamer blocks the interaction between VWF (von Willebrand factor) and GP IIb/IIIa receptors on platelet membranes, triggering the blockage of platelet thrombus formation. **Bottom Right:** AZD8601 is an mRNA therapy designed to increase angiogenesis. Optimized VEGF-A mRNAs are packaged in lipid nano-particles (LNP) which are endocytosed by the targeted host cells. The mRNA is then translated into VEGF-A protein which increases angiogenesis. Figure generated using Biorender.com.

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Table 1: RNA Therapeutics in Clinical Trials for Cardiovascular disease