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Review Article

Overview of pharmacology and clinical development of small interfering RNA

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Abstract

Nanocarrier-based delivery of small interfering RNA (siRNA) has been recognized as a promising approach in cancer treatment. To produce siRNA, short nucleotide sequences are generated exogenously. siRNA inhibits target gene expression in a sequence-specific manner and initiates RNA interference (RNAi) in cells. SiRNA is a recently popularized nucleic acid-based medication that shows unique promise in the treatment of cancer. Before clinical siRNA delivery devices are created, there are still a lot of challenges to be solved. This review covers the potential targets for siRNA drug design, elucidates the characteristics and advantages of siRNA drugs and provides a summary of the available clinical siRNA therapies for cancer treatment. Therapeutically complex siRNA chemical alterations and delivery systems are described, and bio-responsive materials for siRNA release have been classified. This study will support continued advancements in clinical applications of siRNA by acting as a resource for disseminating information for more accurate and effective targeted delivery systems.

Keywords: *Small interfering RNA (siRNA), Pharmacology, Delivery systems, Bio-responsive materials, Clinical development*

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INTRODUCTION

The RNA interference (RNAi) mechanism was identified in US institutions in 1998 and 2006, and RNAi studies resulted in the award of a Nobel Prize [1]. Plants have been found to harbor small interfering RNAs (siRNAs) that are involved in post-transcriptional transcription (PTGS) [2]. It is seen to be a breakthrough in the application of RNAi process in medication development, as other papers have demonstrated that chemically synthesized siRNAs induce RNAi in mammalian cells [3]. RNA interference is a potent strategy for focusing on and inhibiting certain genes and it has been used in antiviral treatments and gene function studies [4]. Since nucleic acid-based treatments like RNAi, are more versatile than conventional antiviral medications, they are used to treat newly emerging infectious diseases. Small interfering RNAs (siRNAs) are double-stranded snippets that induce gene silence by posttranscriptional regulation, and they are the mediating factor for RNA interference (RNAi) [5].

One of the most promising developments in precision medicine is the application of small interfering RNA (siRNA). Diseases may be treated selectively by binding to a specific

sequence, which is a nucleic acid-based medication, and silencing genes linked to the condition [6]. The accurate and efficient manufacture of siRNA at a lower cost than antibodies and small molecule medicines has been made possible by advances in nucleic acid synthesis technology [7]. Currently, siRNA-based medications are being utilized to treat a wide range of illnesses, such as cancer [8], viral infections [9], genetic disorders [10], and cardiovascular problems [11].

Personalized medicine has faced a significant problem in targeting genetic regions that were previously thought to be untreatable through the construction of siRNA sequences [12]. The US Food and Drug Administration (FDA) has approved four siRNA medicines, and over 20 siRNA therapeutics are undergoing clinical testing [13]. Many chemical changes have been made to siRNAs to improve their characteristics, such as lowering immunogenicity, decreasing off-target effects, and increasing stability [14]. These alterations are categorized inside siRNA duplex according to the modification sites that have been found. Base modification [15], backbone modification [16], ribose modification [17], and terminal modification [18] are a few examples. Many siRNA therapies are still awaiting approval in the pipeline, but recently, certain obstacles have been overcome and some have been approved for commercial usage [19].

Endogenous promoters of RNAi pathway

RNAi pathway's endogenous promoters include the following: Aberrant transcripts, including transposons and pre-microRNA (miRNA), which are made from repetitive sequences in the genome, as well as foreign double-stranded RNA (dsRNA) or DNA of viral origin (Figure 1).

Plants' primary defense against pathogens is virus-induced gene silencing, or VIGS. Studies on C. elegans have shown that the potential mechanism is the RNA machinery regulating endogenous genes. As long as (> 30 nt), dsRNAs typically trigger an Interferon response in mammalian cells (Figure 2).

A simplified model for RNAi pathway

Two processes that use the ribonuclease enzyme explain a basic concept of RNAi system. The first step involves the conversion of RNA (either dsRNA or miRNA main transcript) into a short interfering RNA (siRNA) by the RNase II enzymes Drosha and Dicer (Figure 3).

SiRNAs are added to the active RNA-induced silencing complex (RISC) in the second stage. which unites with the mRNA target after assembling with RSIC as a single strand RNA.

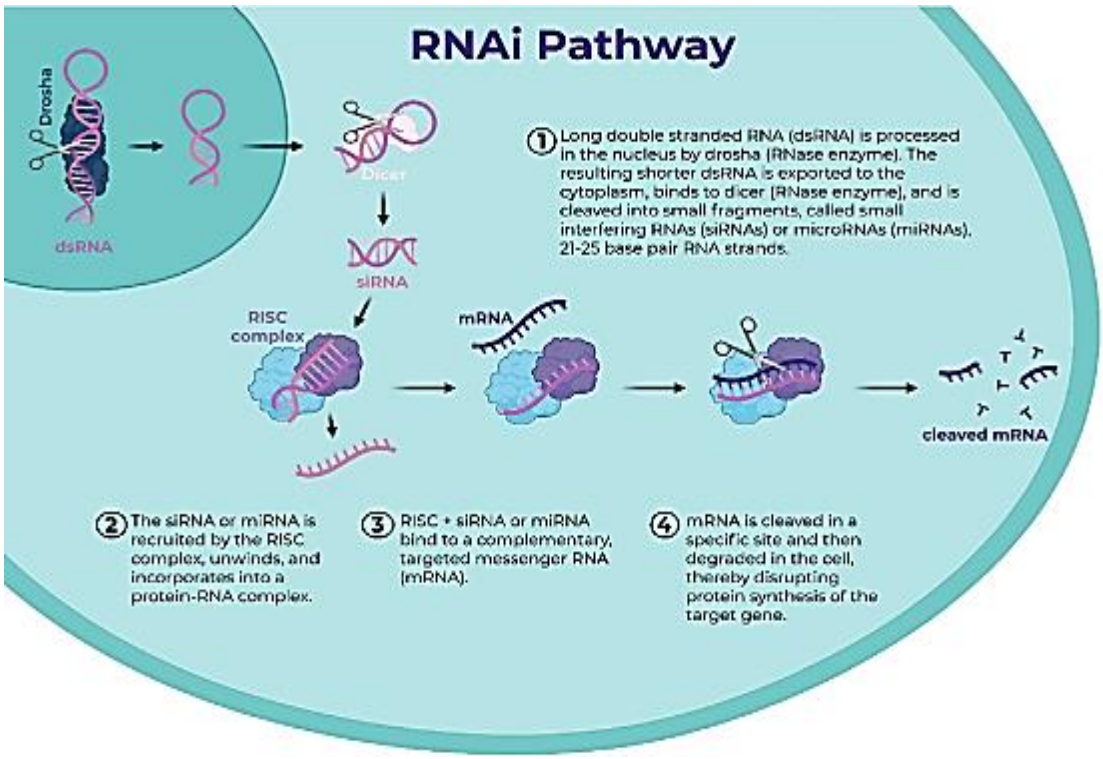

Figure 1: Endogenous promoters of RNAi pathway

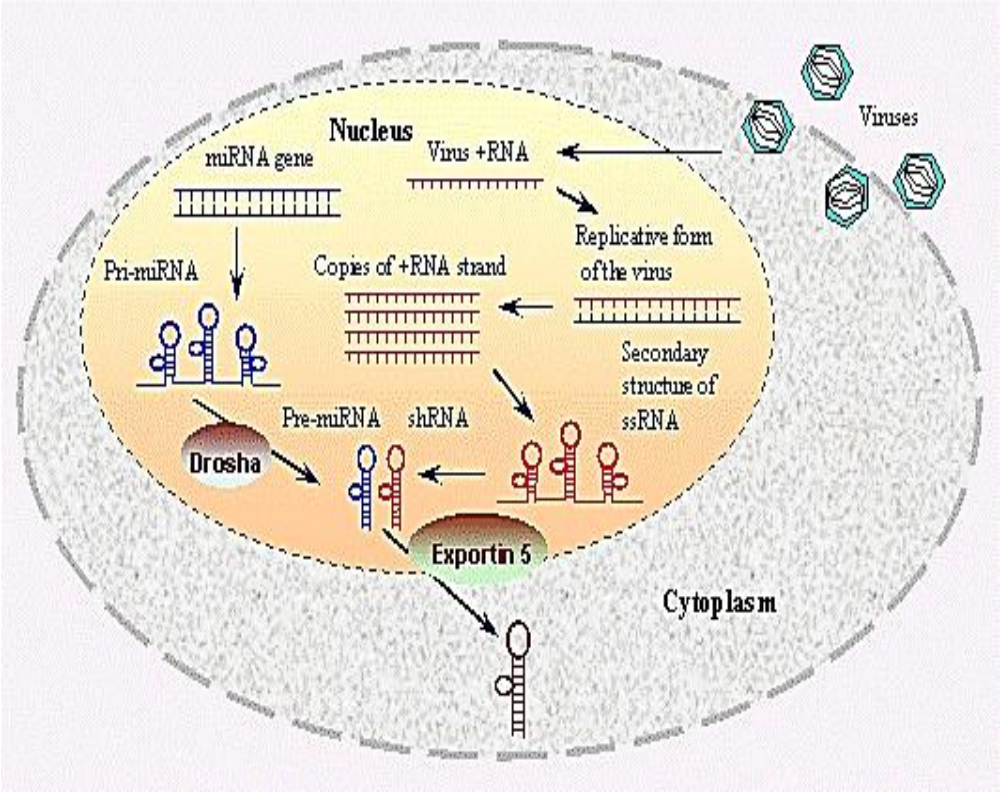

Figure 2: Endogenous promoters of RNAi pathway in plants

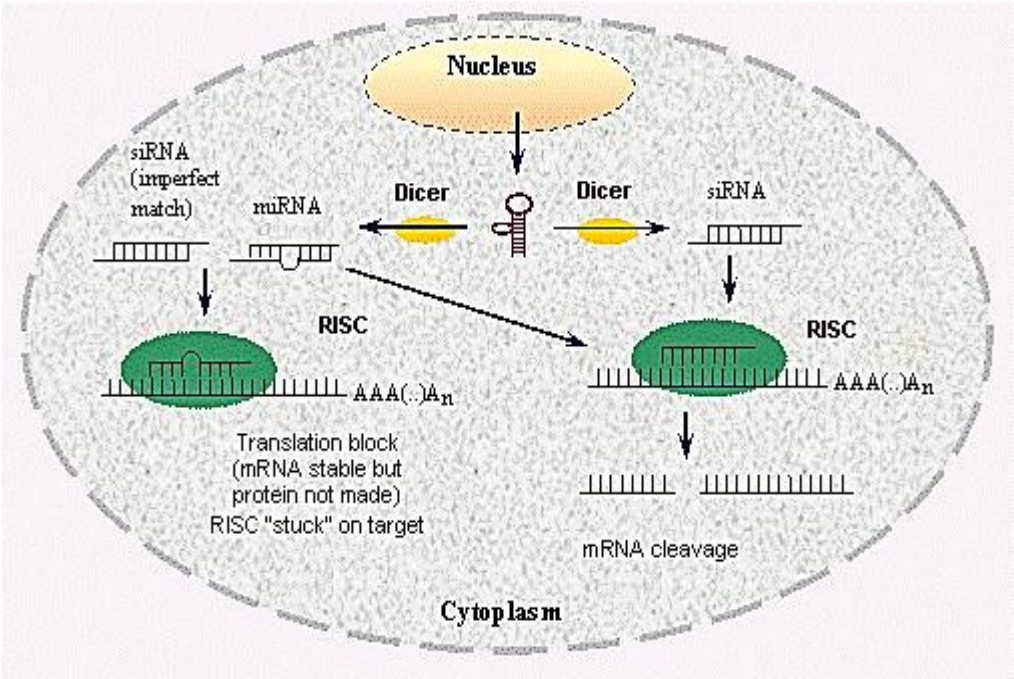

Figure 3: A simplified model for RNAi pathway

Gene silence is thus achieved by the RNase H enzyme Argonaute (Slicer) nucleolytically breaking down the targeted mRNA. When siRNA/mRNA duplexes are mismatched, translational inhibition results in the silence of genes because the mRNA is not cleaved.

RNAi in experiments and therapeutics

Exogenous injection of dsRNA or fragments of expressed shRNAs initiates RNA interference (RNAi) in an experimental setting. The high levels of efficiency and specificity of RNAi determine its primary benefits. As a result, RNA

interference (RNAi) is used in both functional genomics, which involves the systematic analysis of phenotypes that result from loss-of-function caused by RNAi triggers, and in the development of therapeutics for a variety of diseases, including viral infections, neurological disorders, dominant disorders, and many cancer types (via *in vivo* inactivation of gene products linked to human disease progression and pathology).

Mechanism of RNA Interference (RNAi)

One excellent defensive mechanism seen in eukaryotic cells is RNA interference (RNAi). These cells possess the ability to exogenously degrade any invasive genetic material, including viruses [20]. This RNAi mechanism is based on two primary RNA types, small interfering RNAs (siRNAs) and microRNAs (miRNAs), both of which are essential for the control of genes. Endogenous miRNAs are transcribed from genes, and Drosha and DGCR8 generate their main transcripts (pri-miRNA) in the cell nucleus to make precursor miRNA (pre-miRNA). Following its transfer to the cytoplasm, the so-called hairpin structure is eliminated through further processing by the enzyme Dicer. Next, the pre-miRNA is inserted into the RNA-induced silencing complex (RISC), which is made up of or contains the proteins known as Argonaute 1–4 (Ago1–Ago4). The 3' untranslated region (UTR) of the target mRNA is where the RNA-induced silencing complex (RISC) attaches itself after separating from the complementary strand of RNA, ultimately leading to mRNA destruction or inhibition of mRNA translation [21].

Conversely, double-stranded RNA (dsRNA) is inserted into the cell at the start of siRNA-mediated RNA interference. This dsRNA may have originated from synthetic siRNAs or viruses (exogenous genetic material). As the dsRNA increases in length, Dicer recognizes it and breaks it up into smaller pieces, each measuring around 20 nucleotides. These fragments are subsequently transferred to the RISC, a protein complex. One dsRNA strand functions as the guide strand inside the RISC, guiding the complex to its target mRNA. Additionally, the complementary sequence of the target mRNA will be connected to the guide strand of the RISC through base pairing. Proteins like Argonaute 2 (Ago2) are recruited and activated as a result of this interaction, which makes the target mRNA easier to cleave or degrade. This effectively stops or greatly reduces the matching protein production. RNA interference (RNAi) is a useful tool for research and has potential therapeutic uses (Figure 4) [22].

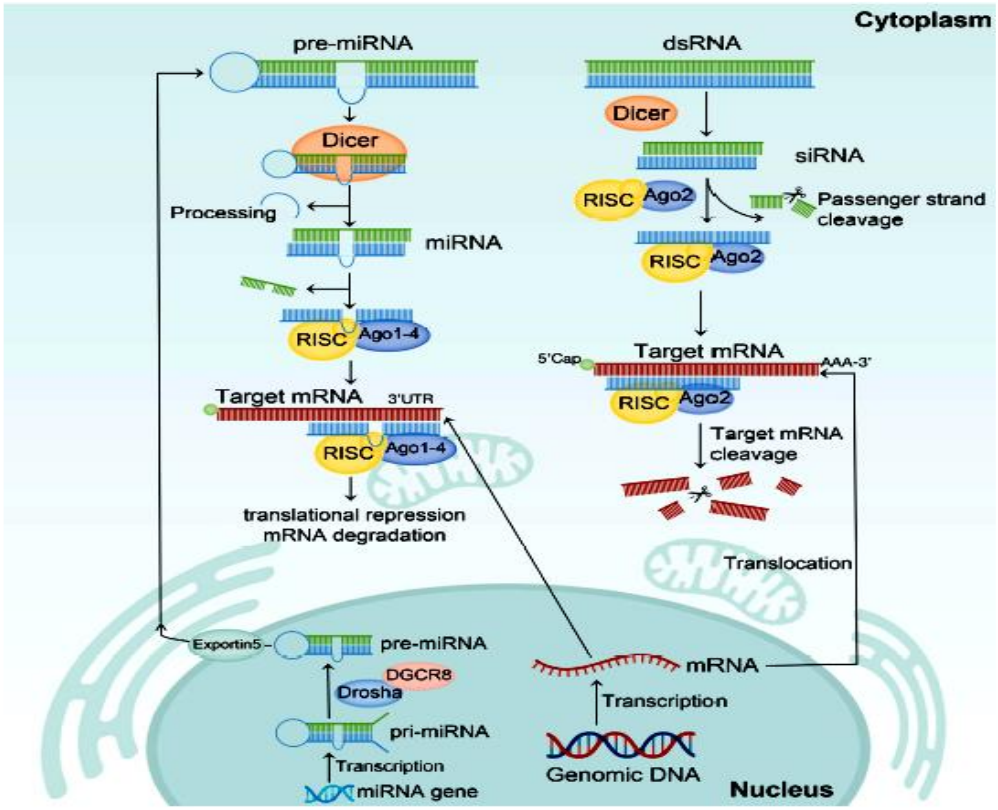

Figure 4: Mechanism of RNA interfering

miRNA

"In the cell nucleus, miRNA gene is translated into pri-mRNA, which is subsequently processed by Drosha and DGCR8 to generate pre-miRNA. Exporting 5 carries the pre-miRNA into the cytoplasm, where Dicer performs additional processing to eliminate the stem-loop structure, resulting in mature miRNA. Next, the passenger strand is disposed of and the miRNA is put into the RISC that contains Argonaute 1–4 (Ago1–Ago4). Translational repression or mRNA degradation results from the poorly complementary pairing of the miRNA-RISC with the target mRNA".

siRNA

Dicer is the first organelle to identify and break down double-stranded RNA (dsRNA), which is the progenitor of siRNA. The precursor molecules are broken up into short siRNA pieces by the dicer, which are usually 21 – 23 nucleotides long. The RNA-induced silencing complex (RISC) is then formed by combining these siRNA fragments. Once established, base matching between the mRNA sequence and siRNA allows siRNA-RISC complex to attach to a particular target mRNA molecule. The target mRNA is then cleaved at a particular location by siRNA-RISC complex, more especially by the Argonaute-2 endonuclease (Ago2) within RISC. The target protein expression is ultimately decreased as a result of this cleavage, which stops the target mRNA from being translated into protein.

Description of small interfering RNAs (siRNA)

Transposable genetic elements are silenced and viruses are warded off by siRNAs. Short RNA molecules known as siRNAs control and have the ability to silence genes post-transcriptionally and, in certain circumstances, at position 63 of the transcriptional level. Synthetic RNA duplexes, or siRNAs, are designed to specifically target and degrade a single target mRNA. In a wide range of cell lines, siRNA causes gene knockdown. Cells that are open to the transfection of synthetic oligonucleotides are treated by siRNA.

Suitability of siRNA as drugs

The following four key reasons highlight the superiority of siRNA drugs:

a) The specificity in silencing: The shortness of siRNAs (20 nucleotides) enables them to recognize any target gene and reduce off-target effects.

b) The exceptional safety profile: The post-transcriptional gene silencing effects are done by siRNA only in the cytoplasm, thus minimizing the risk of host gene mutations that are caused by genome integration and nuclear entry.

c) The remarkable efficiency: siRNAs induce significant gene silencing effects in cells.

d) The unlimited number of potential targets: The developments in whole genome and molecular biology sequencing produced a large number of cDNA libraries, human genomic databases and disease-causing gene databases. Thus, with the simple design of siRNAs based on the mRNA sequence for the target gene, it is possible to get siRNA that effectively silences any disease-causing gene [23].

"Since these RNA molecules (siRNA) achieve transient silencing, they are relatively short time frames on the order of 2 - 4 days. siRNAs are also employed for knockdown of non-protein coding genes, as same as long noncoding RNAs (lncRNA)".

How does siRNA work? Composition and formation

They consist of a duplex that is 19 to 25 bp long with 3' nucleotide overhangs, made up of two RNA strands: the antisense (or guide) strand and the sense (or passenger) strand (Figure 5).

Figure 5: General structure of siRNA

Double-stranded RNA from endogenous DNA transcription or an external source, such as a virus, is converted into siRNA in the cytoplasm. The ATP-dependent riboendonuclease and the catalytic protein Dicer subsequently cleave this double-stranded RNA into fragments that are 21–23 poly-nucleotides long. Such a fragment is then placed onto Argonaute, another protein, in the following step. The four distinct domains that make up the Argonaute are N-terminal, Mid, PAZ, and PIWI. Argonaute's RNase activity in the PIWI domain allows it to cleave target mRNA. The RNA-induced silencing complex (RISC) is created when the Argonaute-siRNA complex interacts with other proteins and a helicase enzyme. The guide strand directs RISC toward a complementary target mRNA whereas the sense

strand in RISC degenerates in the cytoplasm. The target mRNA's fate is determined by the guide strand's base-pairing, whether it is ideal or suboptimal. When the guide strand's bases are optimally paired, the Argonaute will cleave the target mRNA, and the RISC complex will target a different mRNA. Conversely, if the target mRNA and guide strand base-pairing are not perfect, resulting in translational block, Argonaute will not cleave the mRNA because the RISC complex will obstruct ribosome engagement and translocation. Silencing transposable DNA elements in the nucleus prevents their unwanted and hazardous random insertions into the genome.

Dicer-homolog protein molecule from *Giardia intestinalis* acts as an RNase that cuts off long double-stranded RNA molecules producing short interfering RNAs (siRNAs; Figure 6). This is the first step in the RNA interference process and also starts the production of the RNA-induced silencing complex (RISC).

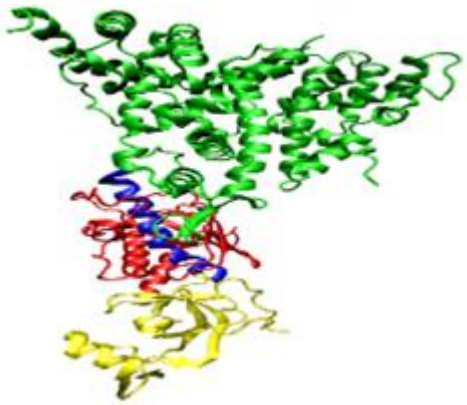

Figure 6: The dicer protein from Giardia intestinalis colored by domain (PAZ domain yellow, platform domain red, connector helix blue, RNase and bridge domains green

siRNAs applications

The delineation of the distinct contributions of genes toward a diverse range of cellular characteristics, such as insulin signaling [24], cytokinesis [25], apoptosis [26], and cell, is one common application of siRNAs. In order to validate targets and discover novel pathways for various diseases such as hepatitis [27], HIV infection [28], cancer [29], and other cellular processes, siRNA screens are employed. Lastly, the *in vivo* RNAi has been used for target validation in animal illness models. and in developing the potential to be utilized therapeutically, particularly for diseases caused by genes, wherein these genes are specifically targeted and repressed [30].

siRNA function

In order to achieve a tolerable level of functional knockdown in any given situation, early work on siRNA design established well-known steps for siRNA structural characteristics [3]. To produce strong siRNAs with desirable qualities, extensive functional studies on thermodynamics and sequence-based guidelines is done [31]. These investigations made it possible to create algorithms that significantly enhanced the capacity to identify powerful siRNA sequences. Ongoing studies aim to find compounds with improved activity, and specificity, and obtain appropriate siRNAs devised techniques.

siRNA obstacles or challenges

The off-target effect and how to overcome

Genes that are not specifically targeted by siRNA may experience unintentional knockdown due to its sequence complementarity-based approach. The so-called "off-target" effect results from this. Numerous strategies have been used to steer clear of these and guarantee on-target activity. One of these strategies, which has been successfully applied to both lower sense strand loading and activity [32,33] and induces the preferential loading of targeted antisense strand into RISC complex [34,35], is the chemical alteration of siRNA. Moreover, chemical alterations result in a decreased likelihood of off-target effects from siRNA guide strand seed area. Numerous filters that remove high-frequency seed sequences from known mammalian microRNAs are obtained by using design algorithms [36]. To prevent unwanted interactions, siRNA seed area alternatively be modified chemically or by taking thermodynamic-based design concerns into account [37]. Ultimately, combining multiple separate siRNAs to target a single gene has been shown to reduce the overall count of non-specific gene targets while simultaneously lowering the frequency of off-target symptoms, maintaining strong target gene knockdown [38].

By combining all aforementioned strategies, off-targeting is reduced and targeted silence for RNA interference experiment is achieved. Although siRNAs hold great promise for therapeutic development, their use *in vivo* is restricted by several extracellular and intracellular obstacles [39], particularly in systemic administration. The naked siRNAs are degraded by endogenous nucleases in serum [40], and the tiny size and low molecular weight of siRNAs cause them to be rapidly cleared by the kidneys [41]. Through either Toll-like receptor

(TLR)-dependent or - independent pathways, siRNAs activate the innate immune system [41]. The reticuloendothelial system (RES) sequesters and entraps plasma protein [41], and the negative charge and high hydrophilicity of siRNAs result in membrane impermeability [42]. One of the primary intracellular obstacles is endosomal entrapment. The endosomal-lysosomal pathway may pose difficulties for siRNAs once they have been internalized by target cells. This could impede their capacity to reach the intended intracellular target mRNA and reduce their effectiveness; off-target effects have been linked to undesirable toxicities [43] and off-target effects [44]. “To overcome these barriers, delivery systems and/or chemical modifications are necessary to enhance the bioavailability of siRNA at therapeutic sites and target regions”.

Chemical modification

Although chemical modification affects the effectiveness of siRNA distribution, it does not function as a vehicle for siRNA transport. The latent characteristics of siRNAs were improved by chemical changes before to the development of siRNA delivery vectors. Significant advancements have been made in serum stability, immunological evasion capacity, and siRNA interaction with RISC [45]. SiRNAs generally include a variety of locations that are amenable to chemical modification, including the backbone, bases, terminal, and ribose moieties (Figure 7).

Phosphorothioate (PS) alteration at the terminal improves the modified material's pharmacokinetic characteristics, nuclease resistance, and serum stability. However, excessive PS alteration has harmful side effects [46].

On the Backbone: Locking nucleic acids (LNAs) were inserted at the 2' and 4' positions of methylene-linked ribose, and phosphonothioate was substituted for phosphodiester nucleotides at the 3' end of the RNA backbone [22].

Furthermore, when siRNA is altered with a tiny chemical like 2.4-dinitrophenol (DNP), it increases cell membrane crossing and promotes cellular uptake in addition to inhibiting nuclease breakdown [47].

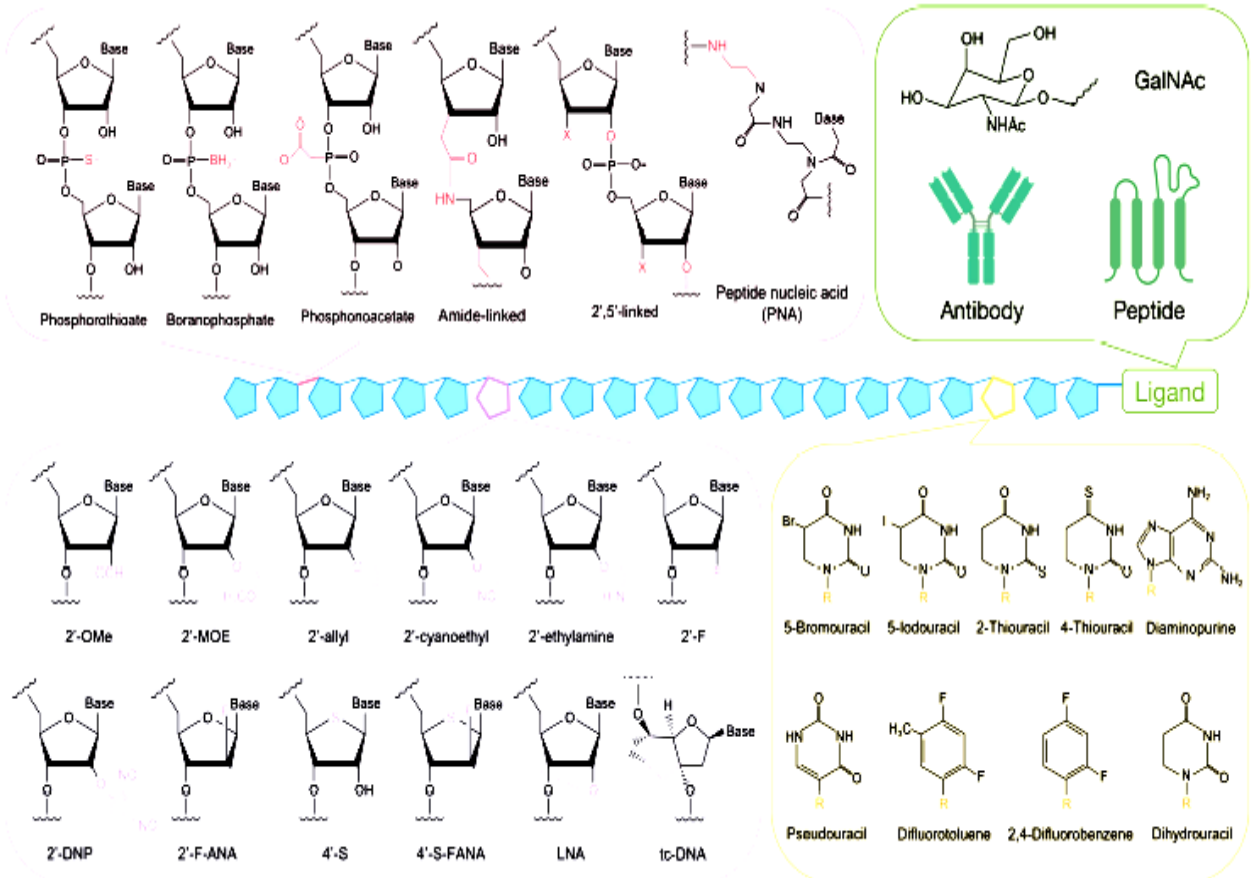

Figure 7: Several commonly used methods for siRNA modification

Table 1: Advantages and limitations of each delivery method

Process	Delivery Mode	Advantages	Disadvantages
Transfection	Cationic liposomes or polymer-based	Delivery of siRNA, microRNAs, and shRNA into most cell types	Not all cell types amenable to transfection reagents
Electroporation	Electrical pulse	Effective for difficult-to-transfect cells	Cell death often increased
Viral-mediated Delivery	Lentivirus Retrovirus Adeno-associated virus	Effective for difficult-to-transfect cells For use in stable selection *In vivo* application	Requires BSL2 facilities Matrigger antiviral response in some cell types
Modified siRNA	Modified siRNA (Accell) To enable passive uptake by many cell types	Effective for difficult-to-transfect cells Repeated dosing is possible for longer-term silencing *In vivo* application	Delivery efficiency is inhibited by the presence of >3% serum during the application

The primary considerations in selecting a delivery method for siRNA are the suitability of the method to the cells and the assay requirements for duration of silencing

How is siRNA delivered to a cell or its cytoplasm?

In order to facilitate cell absorption, siRNA needs to be transfected into cells using cationic lipid or polymer-based transfection reagents, electroporation (physical delivery through pores in the plasma membrane produced by an electrical field), or insertion of chemical changes to the duplex (Table 1).

Challenges in siRNA drug development

In addition to the various benefits that siRNA therapy provides, targeting several cancer-related genes is a good use of this strategy. However, there are several challenges with using siRNA medications for clinical cancer treatment. The latent instability of siRNAs in physiological circumstances is the first barrier. This causes them to be cleared quickly. Numerous nucleases found in the physiological milieu, as shown in Figure 8, degrade siRNAs as they enter the circulation [48]. Additionally, tissues and organs like the liver, spleen, and blood have large numbers of phagocytes. As siRNAs reach the body, they are engulfed by the mononuclear phagocytic system (MPS)/reticuloendothelial system (RES), which is made up of phagocytic cells, neutrophils, and mononuclear macrophages [49]. The unmodified siRNAs have a brief half-life in systemic circulation blood, lasting from a few minutes to an hour [48]. Significant quantities of siRNA are readily removed from the kidneys during circulation by glomerular filtration [49]. Additionally, siRNA that successfully enters target cells may be broken down by the nucleases found in lysosomes and endosomes, which have pH range of 4.5 to 6 (acidic) [50].

The lysosomes and endosomes that siRNA first comes into contact with provide an acidic environment with a pH range of 5 to 6, and an even lower pH of 4.5 within the lysosomes, even if siRNA is effective in reaching the target cells. The second barrier appears when unbound siRNAs are absorbed by cells. Due to siRNA's hydrophilic nature and negative charge, they are unable to pass through and penetrate cancer cells and negatively charged hydrophobic cell membranes, which results in negative charge repulsion and impermeability [49]. The escape of siRNAs from endosomes is the third barrier. In the cytoplasm, siRNA encounters barriers when trying to pass through biological membranes freely during the development of siRNA-RISC complex, necessitating the use of different techniques to achieve effective endosomal escape and release into the cytoplasm. This puts up a barrier that hinders the transport of siRNA [31]. The immunogenicity of siRNAs is the fourth barrier. Significant cytokine production occurs upon siRNA injection into the body, activating the innate immune system [51]. However, significant amounts of cytokines are generated as a result of Toll-like receptors (TLRs), pattern recognition receptors expressed on immune cells that particularly recognize nucleotide sequences in siRNAs. [51].

The off-target effects of siRNAs represent the sixth challenge. It is well known that cells contain a vast number of long-stranded mRNAs and miRNAs and that siRNAs, which are 20 nucleotides long, have a high affinity for base-complementary pairing. Thus, unwanted mRNAs and miRNAs may be degraded as a result of siRNAs' limited specificity, which has a non-specific impact on gene regulation. As a result, siRNAs degrade target miRNAs in addition to target mRNAs, resulting in erratic (off-target)

changes in gene expression [52]. Since siRNA holds enormous potential in cancer therapy, it is important to define efficient and safe delivery strategies that fully use the benefits of siRNA drugs in cancer therapy. The following requirements must be fulfilled in siRNA delivery systems:

i. Securing the serum stability of siRNA.

ii. Assisting siRNA immune evasion.

iii. Attenuation of siRNA interactions with plasma proteins and phagocytes.

iv. Prevention of renal clearance.

v. Increasing the ability of siRNA to penetrate the vasculature and reach cancerous tissues.

vi. Assisting cellular uptake of siRNA.

vii. Enhancing siRNA escape from endosomes.

viii. High biocompatibility and non-toxicity.

When the above-mentioned conditions are fulfilled, an efficient delivery of siRNA drugs is obtained [22].

First siRNA approved drugs for clinical applications

Many large pharmaceutical companies, including Amgen, AstraZeneca, Alnylam, Regeneron, Eli Lilly Company, Arrowhead, Johnson and Johnson, and others, made significant investments in this field because they believed that siRNA therapies could be successful as biological drugs [53]. These companies have also had great success developing siRNA therapy pipelines. Successful collaboration between Alnylam and Regeneron Pharmaceuticals has improved treatments for liver, central nervous system (CNS), and eye conditions [54]. Commencing in 2020, ONPATTRO and GIVLAARI, two siRNA drugs, have been marketed. Furthermore, the FDA has approved the use of two additional siRNA treatments, lumasiran (ALN-GO1) and inclisiran, as new pharmaceuticals. Work on an RNAi drug to treat severe COVID-19 cases started in 2020 as a result of a partnership between Alnylam and Vir Pharmaceuticals [55]. Phase 3 clinical trials are presently being conducted on some siRNAs, and many of the candidates are just beginning their developmental stages [56].

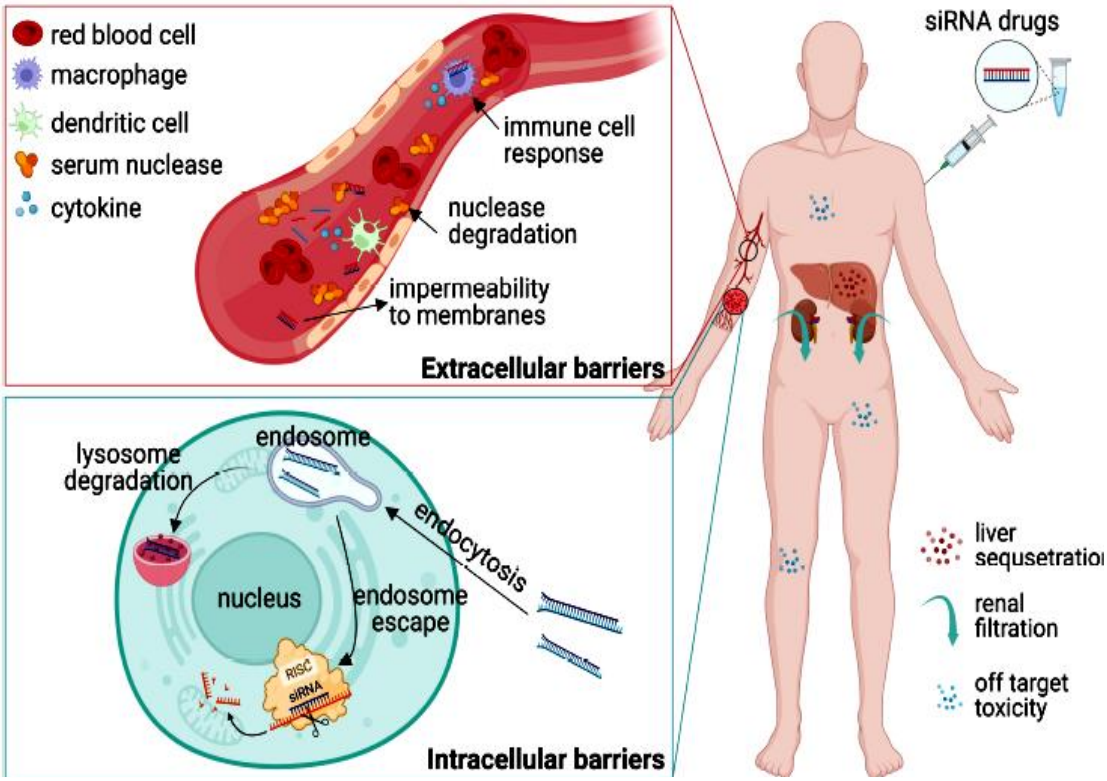

Figure 8: Challenges in siRNA drugs development

The Future: Other siRNAs in other diseases

Metabolic diseases

a. For the treatment of primary hyperoxaluria (PH). Nedosiran (RIVFLOZA™) is being developed by Dicerna Pharmaceuticals and is a once-monthly small interfering RNA (siRNA) treatment administered subcutaneously. The other hyperoxaluria subtypes, i.e., PH2 and PH3, are efficiently treated by the LDH knockdown which may be considered more potent than GO targeting by lumasiran. Such as GalNAc siRNA conjugate, administrated once monthly through subcutaneous injection [57].

b. Cemdisiran is a GalNAc-siRNA drug that targets the liver- producing the knockdown of the complement 5 (C5) protein. Such drug is also under test for the treatment of rare complement-mediated diseases which is life-threatening [58].

Hematology

Fitusiran (ALNAT3), an additional siRNA drug, has been evaluated for the treatment of hemophilia A and B. Fitusiran (ALNAT3), a GalNAc-siRNA conjugate, attacks the SERPINC1 Mrna, causing a decrease in antithrombin synthesis and an increase in thrombin generation. Fitusiran also corrects the imbalance in coagulation and halts the bleeding phenotype [59]. Fitusiran shows a very significant dose-dependent reduction in antithrombin in phase I and phase II trials in patients with or without inhibitors, however, phase III trials are still in progress [10].

CONCLUSION

Since the discovery of RNA interference (RNAi) more than 20 years ago, siRNA medications have opened up new pathways for novel therapeutics to be investigated and tested for a variety of disorders. Due to issues with transport, side effects, instability, and off-target behavior, siRNAs as drugs appear to be less beneficial clinically than other novel classes of pharmacological molecules. Several siRNA medications have been approved for use in clinical settings, and several more siRNAs are now undergoing clinical study. Appropriate delivery procedure strategies are still required for the future generalization of siRNA as medicines. The fact that siRNAs are directed towards any target gene further emphasizes the necessity of choosing the best targets.

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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