RNA Targeting Small Molecules for Neurological Diseases

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Neurological diseases encompass a diverse range of disorders that present complex challenges to therapeutic interventions. In order to address the intricate molecular pathways underlying these illnesses, this paper explores the promising field of RNA-targeting small molecules—an emerging field in therapeutic innovation. Such therapeutics can be placed into two categories: RNA molecules that are used directly as drugs (considered large molecules) and RNA-targeted small molecule medications1. This paper will focus on the latter, providing a comprehensive summary of the current landscape while addressing the several methods used in the screening, development, and delivery of these molecules. The promise of these therapeutics, major barriers to their development, and strategies to overcome such challenges will be discussed. Key takeaways include the use of combination strategies to overcome individual limitations, vital considerations when designing small molecule therapeutics, and the use of cell-penetrating peptides for distribution efficacy.

Keywords: *RNA targeting, small molecules, ASOs, disorders, neurological, nucleic acids, miRNA, long coding RNA*

Introduction

Neurodegenerative diseases represent a large and diverse spectrum of illnesses caused by the progressive loss of neurons in the central nervous system. The prevalence of such disorders is on the rise as the global population's life expectancy continues to increase. According to the World Alzeimher's Report, which records Alzeimher's cases and makes estimates, there were over 55 million people worldwide with dementia in 2020, which will almost double every 20 years, reaching 139 million in 2050[1]. In addition, the number of deaths caused by Alzeimher's is now equal to the number of deaths caused by stroke, the third most common leading cause of death worldwide[2]. Although all neurological disorders have distinct characteristics and mechanisms, it has become increasingly clear that abnormalities in RNA represent a common cause among many neurodegenerative diseases[3]. For example, in Alzeimher's disease, the toxic gain of function is caused by RNAs with nucleotide repeat expansions and the formation of nuclear RNA foci3. The number of deaths caused by Alzeimher's is now equal to the number of deaths caused by stroke, the third most common leading cause of death worldwide[2]. Few fields of biology have been revolutionized as extensively as RNA molecular biology in recent years[4]. Accompanied by growing research, these RNA-based therapeutics hold great potential in the treatment of "incurable" diseases.

RNA as a Target for Small Molecules: In the past, proteins and DNA have been the predominant targets of medicine, making up the majority of the targets for therapeutics in clinical use[5]. However, only about 1.5% of the human genome encodes proteins, and of this percentage, only 10-15% are thought to be disease-related- substantially limiting their impact[6]. This means that, with only 700 approved protein targeted products, a mere 0.05% of the human genome has been interacted with by therapeutics. On the contrary, 90% of the human genome encodes RNA, proving that expanding the scope of small-molecule therapies to include RNA would significantly increase the number of treatable macromolecules[5] (Figure 1). The role of RNA in biology has only recently been discovered, as disturbances in RNA play a crucial role in the development of diseases of cellular dysfunction[7]. Unlike DNA, RNA is single stranded and minimizes free energy by folding into itself. Proper folding of RNA is crucial to proper function, and thus misfolding leads to disruption in cellular processes[8]. Through its canonical Watson-Crick base pairing, RNA can form complex three- dimensional structures as stable as proteins. This intricacy is vital for its recognition of small molecule ligands and nucleic acids with high affinity and specificity[9]. Additionally, studies have found that targeting RNA was more specific, led to fewer side effects, and made it possible to target "difficult to drug" proteins.

Types of RNA involved in Neurological Diseases: The diverse types of RNA molecules involved in neurological diseases play crucial roles in the intricate molecular mechanisms underlying these conditions. By identifying and understanding more RNAs involved in disease, the scope of potentially "druggable" RNAs has expanded- especially due to some of their well- defined, functional structures[10]. One major class of

RNA, called non-coding RNA (ncRNA), is essential to epigenetic regulation and gene expression control[11]. Around 70% of the human genome is transcribed into ncRNAs[5]. One study found that the number of long noncoding RNAs (lncRNAs) increased in relation to the complexity of the organism[6].

MicroRNA (miRNA), a class of ncRNA, is an already well-validated therapeutic target for small molecules[5]. These are short and single stranded RNAs that are partially complementary to messenger RNA (mRNA), and increasing evidence shows that miRNAs are dysregulated in several neurological disorders. The expression of target genes can be altered by using miRNA inhibitors or synthetic miRNA mimics. One downside to targeting miRNAs, since they regulate hundreds of targets and are highly abundant, is the likelihood of having side-effects unrelated to disease pathology[12]. Another class of RNA is mRNA. The proper translation of mRNA into proteins is essential to correct cell function. Growing evidence suggests that translational dysregulation is a key factor in the cause of several neurodegenerative disorders, such as Alzeihmer's and Parkinson's disease[13]. Although there are currently no mRNA therapeutics treating neurological disease, the success of the Covid-19 vaccine has spurred research into mRNA vaccines. For neurological diseases, administering mRNA as a therapeutic could allow the creation of native proteins without ever entering the nucleus[14]. As mRNA is transient and does not affect the DNA of the host cell, this decreases the chance of unintended consequence. Note that the RNA types included above are not all types, but those most commonly involved in disease or a target.

Mechanisms of RNA involvement in Disease Progression

The reason RNA is an attractive target is due to its role in the causation of pathological mechanisms; understanding such mechanisms can allow for the development of its therapeutic strategy. Disease mechanisms associated with RNA that result in pathology can be put into two categories- gain or loss in function. Loss of function usually refers to a mutation in sites that result in disruption for regulatory proteins[8]. The therapeutic strategy for a loss of function mutation aims at restoration of the affected protein, known as gene replacement[15]. As for gain of function disease mechanisms, aberrantly folded RNA in locations where it isn't normally present to represent the main cause. These are most commonly caused by genetic mutations that lead to the inclusion of pathogenic RNA fragments into gene transcripts, including downstream effects reliant on the location of the repeat expansions[8]. Also, insertions in coding sequences can lead to the translation of a toxic protein, like poly(Q) in Huntington's disease (HD). Insertions in non-coding sequences lead RNA to cut off regulatory proteins, leading to alterations in the pre-mRNA splicing pattern that result in changes to the mature mRNA, resulting in altered encoded proteins and expression levels[7]. Other downstream pathological mechanisms include the production of non-functional or toxic proteins and the sequestration of essential proteins[8]. Insight into such mechanisms has greatly aided the development of RNA- based therapeutics and has further advanced our understanding of the molecular underpinnings of these disorders.

Highlighting the current state of RNA targeting Small Molecules

An array of RNA-targeting small molecules has been developed as promising therapeutic agents for the treatment of neurological diseases, each designed to address specific RNA-related pathogenic mechanisms. Notably, antisense oligonucleotides (ASOs) have established their potential with success stories like Nusinersen[16]. They are single stranded oligonucleotides, usually 14-20 nucleotides long, designed to bind and inhibit the actions of complementary RNA transcripts[15]. A key characteristic of ASOs is their nucleic acid- based approach, making them mostly applicable to non-structured RNAs[8]. They alter gene expression by one of two mechanisms– degradation or steric blockade. Degradation is when the ASO binds to and causes the degradation of faulty mRNA, effectively silencing the gene. Steric blockade is when the ASO binds to the RNA and physically blocks interaction with proteins involved with splicing or translation, resulting in the selective inclusion/ exclusion of exons or introns[17]. However, due to several shortcomings, such as delivery challenges and adverse reactions, many favor the research of structure-based small molecule therapy[5,7]. Such therapeutics, conversely, have good absorption, blood- brain barrier penetration, and oral bioavailability. Also, their physicochemical properties make them more 'drug-like' than nucleic acids[7]. Another notable therapeutic strategy worth mentioning is small interfering RNAs (siRNAs). SiRNAs regulate the expression of genes by a process known as RNA interference (RNAi). The process starts when a gene's DNA is translated into mRNA in the nucleus. A siRNA, whose sequence matches that of the target mRNA, is then exported to the cytoplasm, where it joins the RNA-induced silencing complex (RISC). This complex searches for mRNA sequences that are complementary. When a match is discovered, the RISC-siRNA complex initiates the mRNA's cleavage, rendering the target non-functional. The cleaved mRNA fragments effectively downregulate gene expression and prevent the creation of a functional protein (Figure 2)[4,7]. The largest problem with siRNAs is delivery, specifically bypassing the blood-brain barrier, which is most effectively overcome by direct injection into the brain[14]. Finally, RNA aptamers are three-dimensional RNA molecules that have been optimized to bind a target with great affinity and

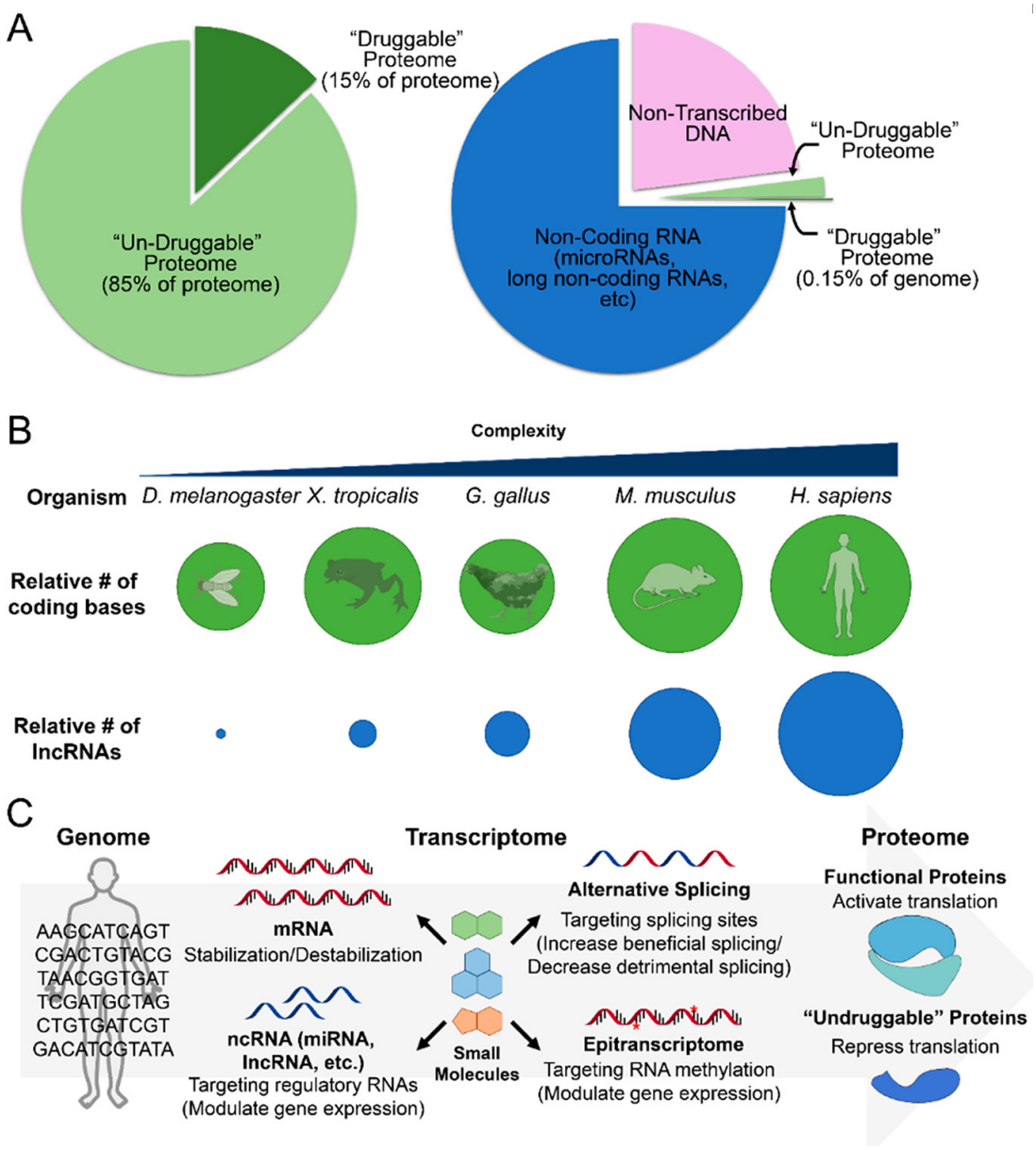

Fig. 1 (A) A large portion of the transcriptome is neglected as a therapeutic target because it only represents a small portion of the transcribed genome[6] (Modified).

specificity, making them an attractive therapeutic. The biggest challenge for RNA aptamers is entering cells without specific cell-penetrating components. Therefore, they are largely used for cell surface molecules. Additionally, their ability to effectively bind to cell-surface proteins specifically makes them a promising delivery agent for siRNA. In theory, designing an aptamer-siRNA chimera could allow the inhibition of a receptor function using the aptamer and the silencing of a specific mRNA using the siRNA[14]. In the last two decades, many of these therapeutics have entered clinical trial phases, and 13 oligonucleotide- based drugs have been approved by the FDA, with that number increasing each year[18]. One example of a current clinical trial is one investigating Survival Motor Neuron (SMN) Circular RNAS as new targets for potentially more "personalized therapies." This is for the treatment of Spinal Muscular Atrophy– aimed to discover ways to increase the efficacy of Nusinersen, an antisense oligonuceotide[19]. Note that the therapeutics included above are not all types, but those

most researched or reviewed.

Effective RNA Targeting Small Molecule requirements and Ideal Properties

To effectively target RNA molecules for therapeutic purposes, small molecules must possess a set of specific requirements and ideal properties, ensuring precision and efficacy. RNA, like proteins, is only considered druggable when it possesses clefts in which small molecules can bind[20]. Also, the RNA structural motif should be complex. One study revealed that the complexity of the RNA target increased along with the increased affinity for small molecules, as well as the number of emerging drug-like chemical compounds. These chemical compounds are likely effective for RNA targets as they have complex binding pockets, resembling riboswitches or proteins[11]. High drug-likeness also seems to be significant. Currently, the 'gold standard' in biologically effective small molecules is represented by linezolid, ribocil, branaplam, and SMA-C5. Each molecule possesses a high quantitative estimate of drug likeness (QED) score and targets a complex RNA structural motif. This is because drug-likeness is heavily influenced by bioavailability and human physiology. However, it's important to note that there are successful drugs that have low QED scores and that low drug-likeness does not necessarily rule out potential usefulness[5]. Along with being drug-like, RNA binders must be "RNA-like," having characteristics like being dipolar and hydrophobic, having positively charged functional groups, hydrogen bond acceptors, and base-like moieties[20]. Often, RNA possesses changing functional structures, and in order for small molecules to be effective, we must be informed of such changes. It is highly beneficial to utilize natural RNA cognate ligands as it allows for the identification of which RNA to target, at which location, and which shift in the conformational ensemble would result in a therapeutic outcome[20]. To summarize, RNA targeting small molecules should have bioavailability for cell permeability, high binding affinity, and have "RNA-like" binders.

Key Limitations

Screening limitations

Screening approaches are crucial for discovering effective compounds suitable to target RNA. However, without a known structure, it's difficult to assess the accuracy of predictions made by algorithms used to predict such RNA structures[11]. All three screening types (in silico, in vitro, and in vivo) possess flaws. In silico screening is attractive because of the atomic precision it allows, although it is relatively more expensive. Transient states could be utilized to provide more relevant ligand capture by the RNA, as well as offer insights into changing structures. However, in silico screening doesn't always recapitulate in vivo transitions, which happen according to precise kinetics and may require assistance absent from simulations. This is also true for in vitro screening, as it may also lead to compounds that act by means other than binding RNA. This is because proteins and other molecules that compete with small molecules in the natural environment are absent. Phenotypic screening, an in vivo strategy, monitors how a defined function within a specific biological pathway is affected and is powerful because the results account for natural circumstances. However, since it does not solely identify molecules that would bind to a particular RNA molecule, phenotypic screening requires complementing it with other methods that evaluate RNA binding[20]. Previously, cell-based high-throughput screening, which utilizes phenotypic readouts such as gene expression and cell survival rate has been used. For phenotypic readout, reporter systems are developed in cells and then treated with small molecules. Although phenotypic experiments are not used to identify small molecules that selectively target RNA, this method has been utilized to identify the majority of successful and well-known RNA-targeted medications, including ribocil and SMN-C[5]. Although initially identified through phenotypic screening, it wasn't until validation that it was revealed that they were bound to RNA targets. This approach is more effective than target-based approaches at locating small compounds that result in phenotypic alterations and aid in drug discovery. Although attempts are made to investigate the biological targets and mechanisms after their identification, the direct targets and molecular mechanisms of advantageous drugs remain unknown[20]. Another useful biochemical screening method is called small molecule microarray (SMM). It was initially developed to find "undruggable" protein inhibitors, but it has since been applied to find RNA-binding ligands. In comparison to cell-based screening, this method can cover a wide range of RNA structures and has a better throughput. It can recognize substances that directly bind targets. The main issue is that the small compounds discovered by in vitro screening typically have low cell permeability and bioactivity, and validation and modification are required for further drug development[20].

Design limitations

It is still unknown how to create these therapeutics in a repeatable and scalable way[5]. Considering the dynamics of small molecule activity will likely improve design. It is necessary to find the optimal interplay of interactions between the ligand and RNA, and this is complex because it requires the determination of the precise combination of interactions that will result in effective binding. Additionally, the ligand's electronic state can also change during the binding process, such as through protonation or tautomerization, adding an-

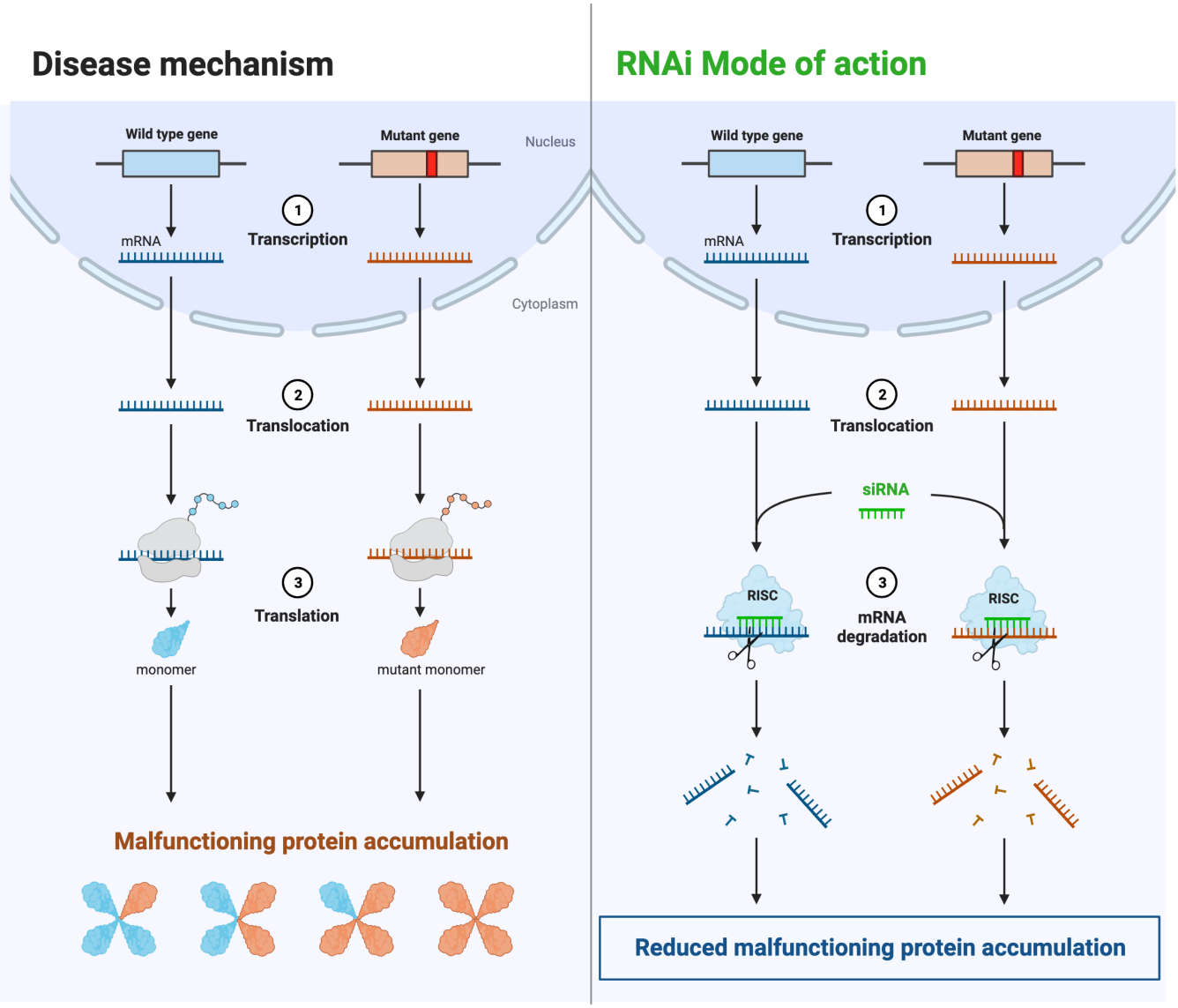

Fig. 2 . The figure presents the essential steps involved in RNA interference (RNAi) and the action of small interfering RNA (siRNA). (created by BioRender)

other layer of complexity to the binding mechanism. Ligands need to slide into cavities by taking advantage of correlated motions in the RNA and this is complex to anticipate. Also, both the RNA and ligands are surrounded by ions and water molecules, and the effects of water on stability are difficult to understand. Depending on the particular substance and its mechanism of binding, the ligand atoms' displacement of the water molecules may vary[20].

Delivery Challenges

One of the biggest challenges to the development of therapeutics for central nervous system disorders is adequate drug delivery. The drug needs to enter the brain parenchyma to be effective, protected by the blood-brain barrier (BBB). Without assistance, the uptake of RNA drugs is impossible due to the tight junctions between the endothelial cells. Many drug targets have been deemed undruggable due to such issues, making delivery an important aspect of development. Mod-

ifications such as increased target affinity and increased resistance to nucleases have not been able to sufficiently enhance delivery across the BBB. Therefore, the most common strategy is intrathecal (IT), intracerebroventricular (IVC), or intraparenchymal administration of the drugs. IT injection is currently the most common administration route for RNA- based therapeutics for neurological diseases. In the context of spinal muscular atrophy, a subcutaneous catheter pump has been utilized. However, chronically implanted pumps are more attractive due to decreased physical discomfort and lower malfunction rates. The downsides must also be addressed, however, including adverse events, catheter tip granulomas, infections, and hygroma[15].

Distribution challenges

In order for a drug to be effective, it must reach the affected cell population. In many neurological disorders, including Parkinson's and Alzheimer's, only a specific neuronal or glial cell population in a selective brain area is affected. For illustration, Parkinson's disease affects 600,000 midbrain dopaminergic neurons of the substantia nigra and ventral tegmental area. The entire human brain is composed of about 80 billion neurons and 80 billion glial cells, highlighting the importance of proper drug distribution[21]. One recommended apprach to efficient ASO delivery is the use of receptor- ligand systems, with advantages including increased potency, delivery to the targeted tissue, and a longer ASO half-life. Currently, a well established system using the asialoglyoprotein receptor (ASGP-R) is demonstrating it's clinical importance. When tested in vivo and in vitro, triantennary N-acetylgalactosamine (GN3) conjugated ASOs had increased uptake and efficacy in U87 cells[22]. Another approach is lipid nanoparticles (LNPs)- used by the pharmaceutical industry to deliver poorly soluble drugs. For example, a catonic lipid: mRNA complex was used to carry the mRNA in the SARS-CoV2 vaccine. Also, recent reports of simplified synthesis of LNP:RNA suggest it's usefulness for small molecules[23].

Cellular uptake challenges

The last step for a drug to elicit its effect is cellular uptake. However, even with improved delivery mechanisms, issues still remain with the endocytic pathway. Agents such as siRNA and ASOs get entrapped in endosomes and thus require a way to facilitate the endosomal escape[24]. With limited understanding of intracellular trafficking and the low rate of RNA therapeutics' endosomal escape, development has been slowed down[25]. To increase the rate of endosomal escape, an RNA drug needs a proportionally larger drug uptake, requiring the development of carrier systems to enhance BBB transport, increase CNS distribution, and increase cellular drug uptake. One promising solution is a class of bioconjugates called cell penetrating peptides (CPP), which have the ability to get to the cytoplasm either through the membrane or through endocytosis[26]. The first step of a CPP is attaching to the cell membrane while linked to a cargo molecule. The CPP then engages in membrane interaction and partially inserts itself into the lipid bilayer. This insertion triggers the beginning of endocytosis by causing the creation of an endocytic vesicle that contains the CPP-cargo complex. It then circulates within the cell and releases the cargo molecule into the cytoplasm, making it possible to facilitate its biological effects (Figure 3)[26]. CPPs are able to do this without using chiral receptors or causing significant damage to the membrane while serving as delivery vectors for large molecules, such as ASOs[27]. Also, they can be conjugated with other peptides to ensure specific cell delivery[15].

Conclusion

In conclusion, while extensive research has been conducted on RNA-targeting small molecules, the majority has been focused on the identification of RNA with disease- related functions and the development of screening methods. However, the after-effects of an RNA-based drug, such as the contribution of the solvent, cellular uptake, and distribution, must be further considered. Also, although we have developed a multitude of small molecule therapeutics with a variety of mechanisms, many individual strategies possess limitations. This is why it is recommended that future research be focused on small molecule drug conjugates and/ or chimeras in the context of neurological disease[28]. This research is in the early stages for anticancer drugs, but similar principles could be applied to RNA-targeting small molecules[29]. Finally, investigating the long-term effects and potential resistance mechanisms associated with these therapies will be crucial for advancing their clinical use and ensuring their sustained efficacy.

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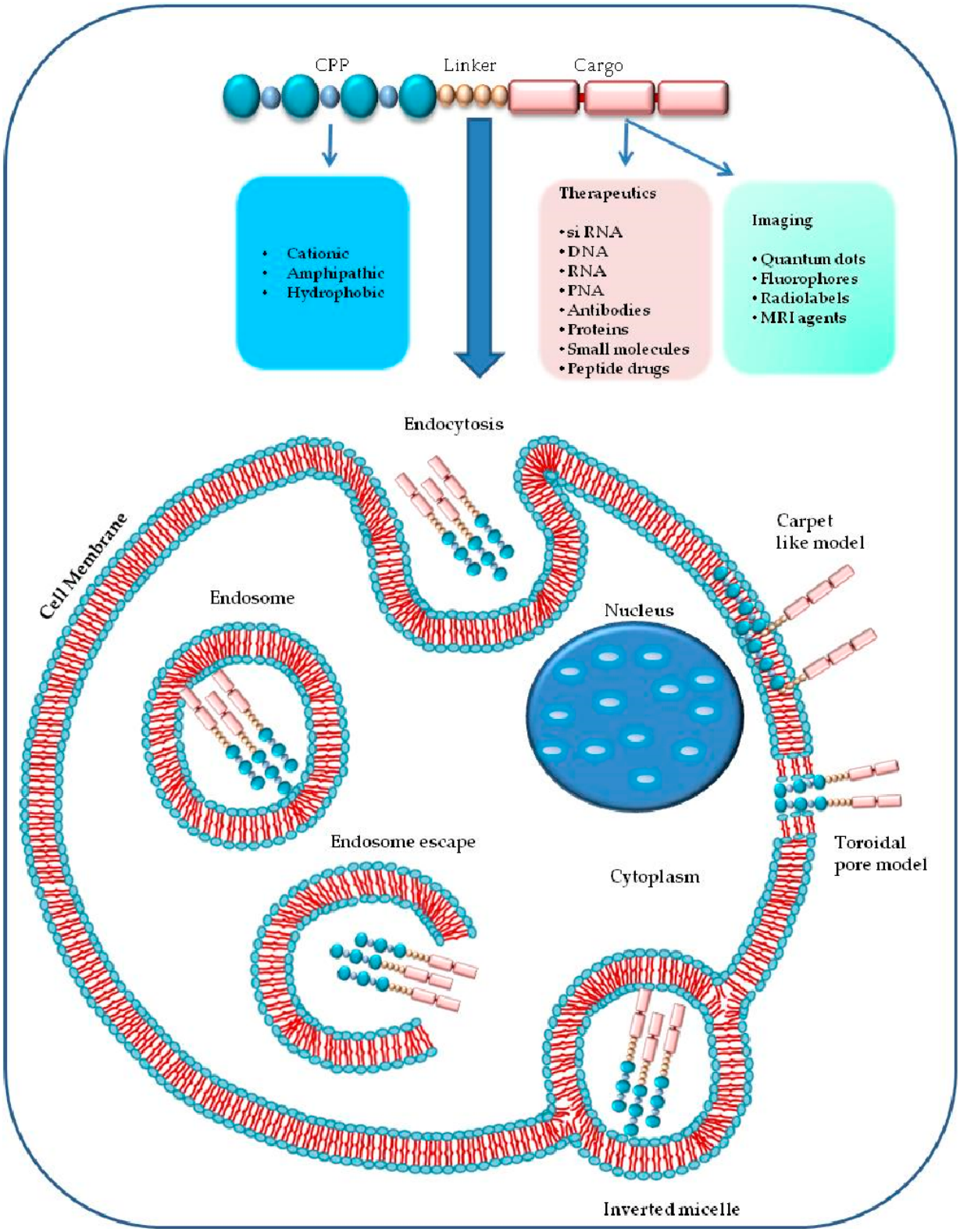

Fig. 3 The steps in which cell-penetrating peptides (CPP) carry various therapeutics across the cell membrane to elicit their therapeutic effects[26].

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