Modifications in Therapeutic Oligonucleotides Improving the Delivery

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Abstract Oligonucleotides are increasingly used in clinical applications. RNA-based therapeutics include inhibitors of mRNA translation, agents of RNA interference, ribozymes, and aptamers targeting various molecular targets. Challenges with the delivery, specificity, and stability of these therapeutics have spawned the development of chemically modified oligonucleotides. In this chapter, we will describe modifications improving delivery and stability of RNA molecules in human cells. Because the most of the cell transfection methods using oligonucleotide complexes with cationic lipids revealed to be toxic, specific modifications and various conjugates have been recently developed to promote the carrier-free

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cellular uptake of therapeutic oligonucleotides. Nucleic acids (NA) are relatively unstable in cytosol due to a plethora of nucleases; therefore, various modifications can be introduced to design nuclease-resistant molecules. These modifications should not interfere with the therapeutic activity and intracellular localization of the oligonucleotides. The influence of nucleotide modifications on the siRNA efficiency and on the anti-replicative activity of therapeutic RNA imported into human mitochondria is discussed.

Keywords Delivery • Oligonucleotide therapeutics • Lipophilic conjugates • Modified oligonucleotides • Mitochondrial diseases • Anti-replicative RNA

1 Introduction: Nucleic Acids' Delivery Systems

Synthetic oligonucleotides and their conjugates are widely used in various fields of molecular biology, nanobiotechnology, and medicine as tools for fundamental and applied research, as well as promising drugs for diagnosis and treatment of viral and genetic diseases, cancer, and other diseases of humans and animals (Ginn et al. 2013; Tan et al. 2011). Development of oligonucleotides as potential therapeutic agents is limited by low efficiency of penetration into target cells due to their large size, negative charge, and low stability. Many systems of gene targeting have been developed to overcome these barriers. Delivery vectors can be divided into the two major groups: viral and nonviral systems (Kay 2011). Each type has its own advantages and disadvantages. Systems based on viral vectors have efficient mechanisms for entering the cell, escaping endosomal entrapment, and translocating gene cargo to the nucleus. Despite of a high efficiency of targeting, several limitations are associated with viral systems: insertional mutagenesis, immune response to viral proteins, tumorigenesis, and cytotoxic effects (Thomas et al. 2003; Walther and Stein 2000).

The shortcomings in viral vectors stimulated development of nonviral delivery carriers, which can be readily synthesized and modified to facilitate biocompatibility. Improving of nonviral delivery systems relies on the detailed understanding of the barriers associated with the nucleic acids targeting into cells. The successful system for nucleic acids' (NA) delivery should meet a number of requirements: biocompatibility and low cytotoxicity, resistance to nuclease activity, possibility of endosomal escape, and capability of entering the appropriate cellular compartment.

Mammalian cells internalize extracellular macromolecules by the endocytosis leading to formation of vesicle-like structures that fuse with early endosomes (De Haes et al. 2012). Thus, the efficacy of transfection and the expected effect depend on both the ability of a carrier to efficiently deliver the NA cargo with minimal toxicity and its potential to overcome the endosomal compartmentalization (Huotari and Helenius 2011). To facilitate NA escape into the cytosol, various compounds have been used in combination with the delivery vectors.

Endosomolytic agents vary in type (natural or synthetic compounds) as well as in their mechanisms of action, which include the endosomal membrane destabilization (TAT HIV, KALA, or GALA peptides), pore formation (e.g., listeriolysin O toxin produced by *Listeria monocytogenes,* gp41HIV protein), and endosomal disruption via the "proton sponge" mechanism (e.g., PEI, ammonium chloride, chloroquine, methylamine) (Varkouhi et al. 2011).

Nonviral delivery systems can be carrier-mediated or carrier-free. The carrier-mediated NA targeting systems can be further subdivided into the three main groups:

(a) Polymeric systems, in which NA form complex with a polymer through charge interactions between the positive groups of the polymer and the negatively charged NA (Oliveira et al. 2015)
(b) Lipidic systems, in which cationic lipids interact with negatively charged NA and condensate or encapsulate them (Balazs and Godbey 2011)
(c) Inorganic carriers involving various materials such as gold nanoparticles, silica, and carbon nanotubes, which can bind NA through different mechanisms (Dizaj et al. 2014)

In this chapter we will describe another approach for delivery of oligonucleotides, which consists in conjugation of NA molecules with targeting ligands.

2 Carrier-Free Targeting Systems

2.1 Nucleic Acids' Conjugation with Targeting Ligands

The transporting molecule should be capable of binding to the cell surface or to specific receptors on it and inducing endocytosis (Juliano et al. 2013). The ligand molecule can be attached to NA directly or through a linker. Depending on the type of oligonucleotide and its purpose, the linker can be connected via the 2′-, 3′-, or 5′-terminus, the C5 atom of pyrimidine bases, the C8 atom of adenine, the exocyclic amino group of guanine, or an internucleoside phosphate (Winkler 2013). Conjugated NA cargo needs additional chemical modifications shielding it from nucleases (see Sect. 3 for details). For most carrier-free systems, the endosomal escape is passive, which reduces the efficiency of transfection; thus, there is a necessity to use additional compounds to promote the release of oligonucleotides from endosomes before they are degraded and recycled. However, the simplicity of design and the small size, in comparison with nanoparticles, ensure a lower toxicity and a better biodistribution of NA conjugates. Carriers with sizes larger than 5 nm can only be used for NA delivery to certain types of tumors and to normal tissues with fenestrated endothelia, such as the liver and spleen, whereas conjugates can also reach many other types of tissues (Juliano et al. 2009).

Various delivery systems through covalent attachment of addressing ligands to the NA cargo have been developed: carbohydrate–NA conjugates, peptide–NA conjugates, antibody–NA conjugates (Uckun et al. 2013), aptamer conjugates, and lipid–NA conjugates. Here, we will briefly characterize the main types of conjugates and provide a more detailed analysis of lipophilic molecules.

2.2 Conjugates with Carbohydrates

It had been demonstrated that the asialoglycoprotein receptor located on the surface of hepatocytes can bind diverse chemotherapeutic agents, including galactose glycoproteins, and helps their internalization by endocytosis (Stockert 1995). This permitted the use of carbohydrate-based vectors for addressed NA delivery. For instance, 5′-glycoconjugates of oligonucleotides have demonstrated excellent cell-type specificity and cellular uptake in the nanomolar concentration range (Biessen et al. 1999). Triantennary N-acetyl galactosamine conjugates (Fig. 1) facilitate the targeted delivery of siRNAs and antisense oligonucleotides to hepatocytes in vivo (Nair et al. 2014; Prakash et al. 2014).

2.3 Conjugates with Peptides and Aptamers

Peptides used for NA delivery can be divided into the two classes. The first group includes cell-targeting peptides, specific ligands for surface receptors overexpressed in diseased cells (Juliano et al. 2013; McGuire et al. 2014; Vives et al. 2008). For instance, bombesin peptide (a ligand for the gastrin-releasing peptide receptor) and a specific peptide for the IGF1R receptor overexpressed in

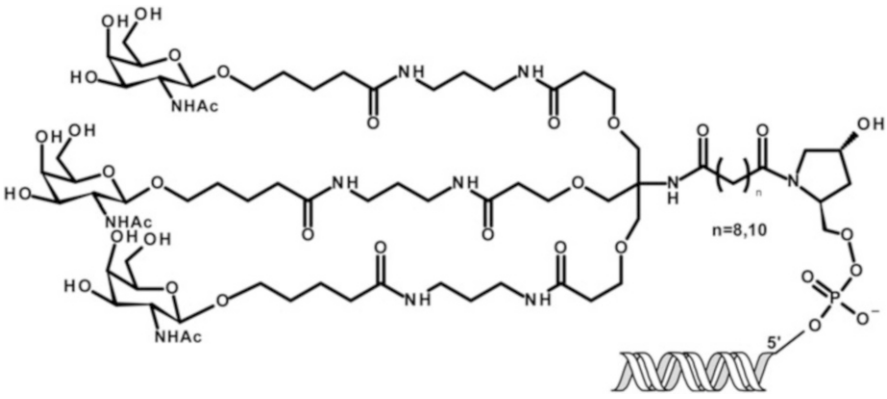


Fig. 1 Triantennary N-acetyl galactosamine-nucleic acid conjugate

breast cancer were conjugated with an siRNA and an antisense oligonucleotide, respectively, for targeted delivery (Cesarone et al. 2007; Ming et al. 2010). The second group comprises the cell-penetrating peptides (CPPs). These peptides are short, amphiphilic, and enriched with the basic amino acids. CPPs can enter cells by two ways, either via endocytosis through electrostatic interactions with negatively charged glycosaminoglycans, similar to cationic polymers (Juliano et al. 2008), or through membrane translocation. The choice of the entry pathway is dependent on the CPP sequence, concentration, and the temperature (Boisguerin et al. 2015). CPPs can also promote the endosomal escape.

Aptamers are small nucleic acids that fold into a well-defined structure, which determines their affinity and specificity for target molecules. They can be selected from pools of randomized sequences by SELEX (Systematic Evolution of Ligands by Exponential Enrichment) approach (Tuerk and Gold 1990). Aptamers can be evolved to bind small molecules but also nucleic acids, carbohydrates, and soluble or membrane proteins. For instance, so-called escort aptamers recognize cell surface receptors specific for the certain cell type and can be used for the targeted delivery of therapeutic agents. One of the most known aptamers of this type is the 2′-F-RNA aptamer against prostate-specific membrane antigene (PSMA). After binding to its target, the anti-PSMA aptamer can be internalized; therefore, this escort aptamer is widely used now as a delivery vehicle for a number of antitumor drugs, including siRNAs and shRNAs. A comprehensive overview of the therapeutic nucleic acids delivery strategies using aptamers can be found in recently published reviews (Aaldering et al. 2015; Davydova et al. 2011; Ming and Laing 2015; Tan et al. 2011; Zhou and Rossi 2011).

2.4 Lipid-Containing Conjugates

One of the most popular methods of delivery of conjugated oligonucleotides is the use of lipids. Various lipophilic molecules have been conjugated to oligonucleotides, including phospholipids, fatty acids, bile acids (e.g., cholic acid), cholesterol, and fat-soluble vitamins (as α-tocopherol, folic acid) (Bhat et al. 1999; Guzaev et al. 2002; Raouane et al. 2012). The structures of these compounds are shown in Fig. 2. Among those, cholesterol, studied by various groups for the past 25 years since the pioneering work of Letsinger et al. (1989), is by far the most extensively characterized addressing agent.

Cholesterol is an essential lipid of cell membranes of many eukaryotes, which make it attractive for delivery of various therapeutic molecules. Intravenous administration of cholesterol-containing siRNA conjugates resulted in significant levels of their accumulation in liver, heart, kidney, adipose, and lung tissues (Soutschek et al. 2004). In another study, accumulation of a cholesterol-conjugated siRNA in brain cells upon intrastriatal injection has been demonstrated (DiFiglia et al. 2007). These and other studies show that cholesterol conjugation significantly improves delivery of NA. Cellular uptake of cholesterol-conjugated oligonucleotides in vivo

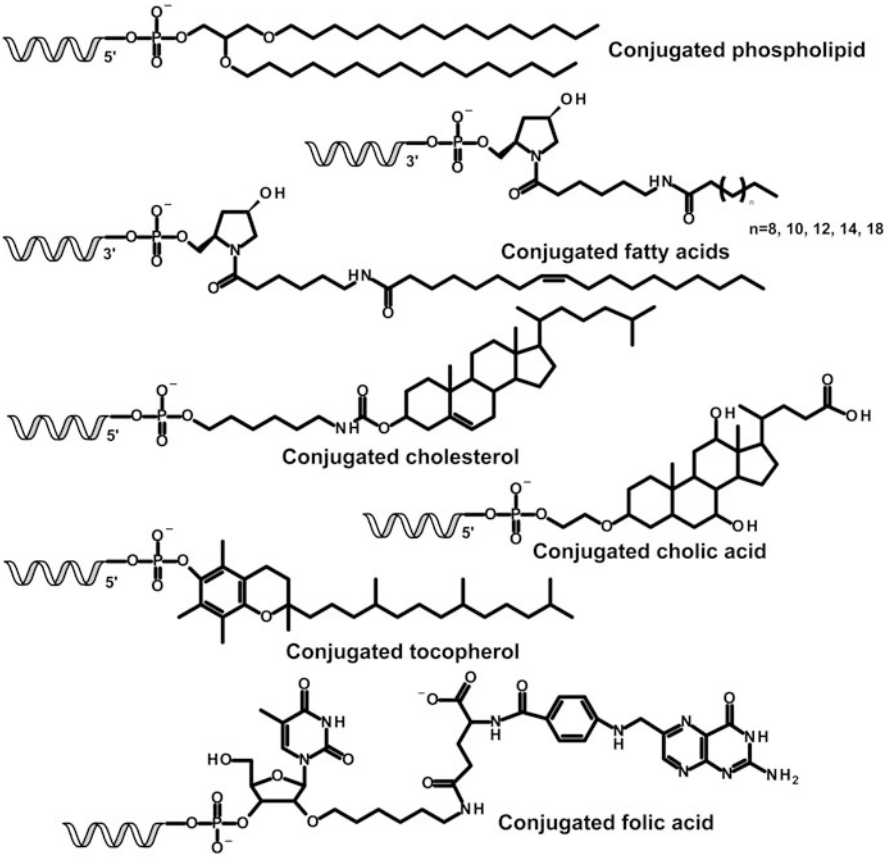


Fig. 2 Various lipophilic molecules used for conjugation with oligonucleotides

depends on the complex formation between the conjugates and the high-density or low-density lipoproteins circulating in the bloodstream. Binding of the complexes to the lipoprotein receptors leads to the uptake of the conjugates by the various tissues (Wolfrum et al. 2007).

Conjugates of oligonucleotides with cholesterol have been developed by many research groups, wherein attachment to an oligonucleotide was performed mainly through either the 5′- or the 3′-termini. Cholesterol can be attached through the unique hydroxyl group of the steroid either directly (MacKellar et al. 1992; Seo et al. 2006) or via various aliphatic linkers. Examples of linear linkers are molecules based on diamines (Letsinger et al. 1989), amino alcohols with various lengths of aliphatic chain (Lorenz et al. 2004; Petrova et al. 2011), and polyethylene glycol (Kubo et al. 2007) (Fig. 3).

Another approach to the synthesis of cholesterol-conjugated oligonucleotides is introducing the steroid residue at the 5′- or 3′-terminus of the oligonucleotide chain through branched linkers containing several reactive groups. The compounds used

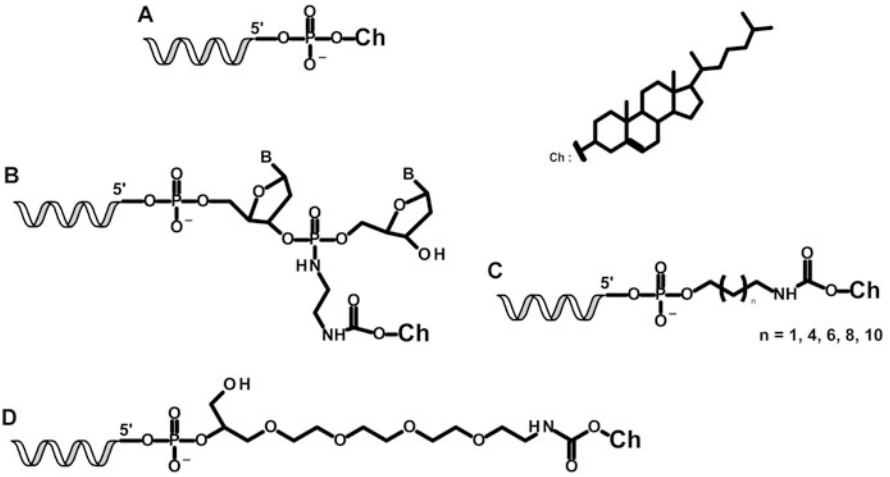


Fig. 3 Cholesterol conjugated with oligonucleotides directly (**a**), through linear linkers based on ethylenediamine (**b**), amino alcohols with various lengths of aliphatic chain (**c**), or polyethylene glycol (**d**)

as the branched linkers include glycerol (Ueno et al. 2008; Vu et al. 1993), 2-aminobutyl-1,3-propanediol (Rump et al. 1998), L-hydroxyproline (Manoharan et al. 2005; Reed et al. 1991), lysine (Stetsenko and Gait 2001), serine (Chaltin et al. 2005), and serinol (Manoharan et al. 2005) (Fig. 4).

A cholesterol residue may negatively affect the therapeutic effect by anchoring the NA cargo to the lipid bilayer membrane or by reducing the efficiency of annealing with the target molecule. To alleviate these effects, the cholesterol residue can be conjugated to the sense chain in siRNA, or it can be added through a cleavable arm, usually containing a disulfide bond (Boutorine and Kostina 1993; Chen et al. 2010; Manoharan et al. 2005; Moschos et al. 2007; Oberhauser and Wagner 1992) (Fig. 5).

The length of the linker can influence the cellular uptake; the optimal efficiency had been achieved for the RNA chain and the cholesterol residue spaced by 6–10 methylene units (Petrova et al. 2011). After penetration of the siRNA conjugates into cells, they affect the target gene expression, suggesting that they are able to escape from endosomes. So far, the mechanism of their endosomal release is still not understood, although it was hypothesized to be related to intracellular traffic of cholesterol (Maxfield and Wustner 2013).

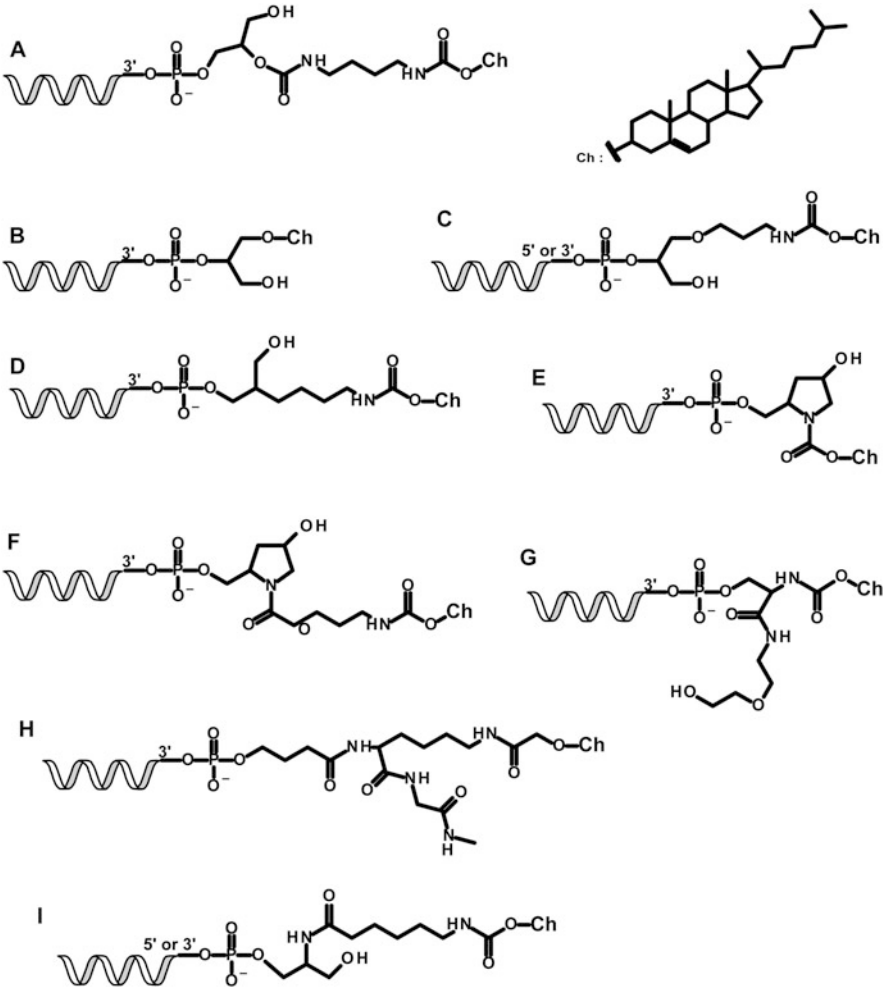


Fig. 4 Cholesterol conjugated with oligonucleotides through branched linkers containing the following compounds: (**a**–**c**) glycerol, (**d**) 2-aminobutyl-1,3-propanediol, (**e**, **f**) L-hydroxyproline, (**g**) lysine, (**h**) serine, (**i**) serinol

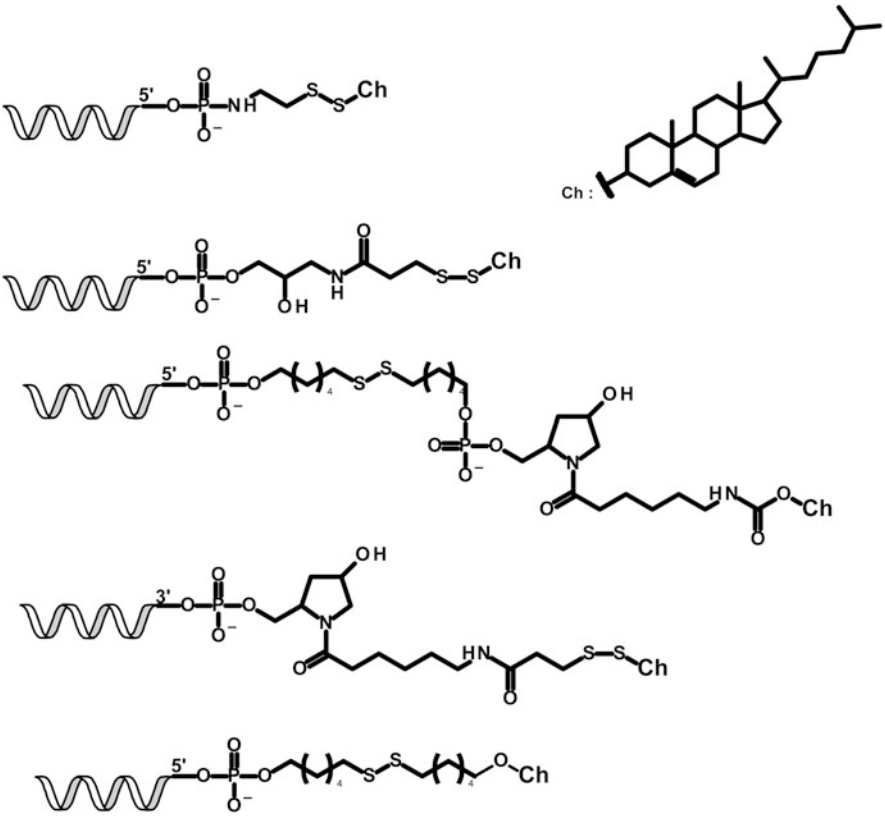


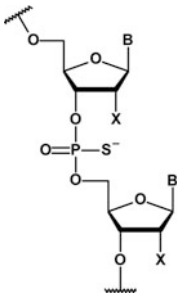
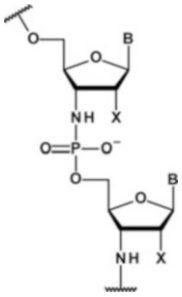
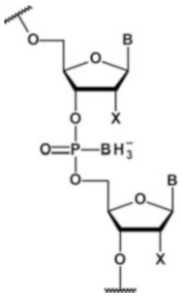
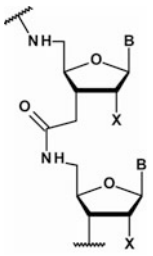
Fig. 5 Cholesterol conjugated with oligonucleotides through various cleavable linkers containing disulfide bond

3 Modifications Improving the Stability of Nucleic Acids

After penetration in the cell and release into the cytoplasm, NA become vulnerable to nuclease attack. This complication can be circumvented if the oligonucleotide cargo is chemically modified to improve its stability. Chemical modifications of nucleic acids can be classified into three distinct categories: internucleoside linkage modifications, sugar modifications, and base modifications (the latter type can affect the thermal stability of the duplex but is not used for NA stabilization and is not considered here). The different types of modifications may be used within the same molecule, depending on the desired effect. We present the structure and the properties of the most used and promising modifications (Table 1).

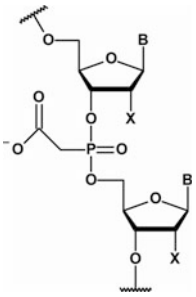
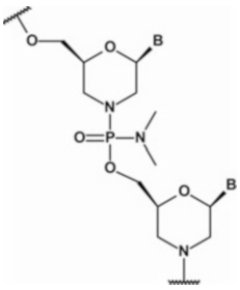
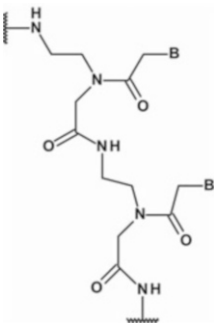
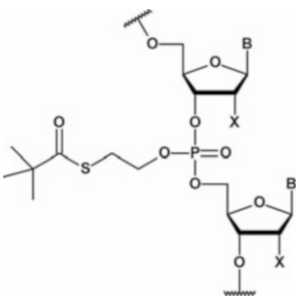
Among oligonucleotide derivatives listed in Table 1, there are modifications, which are well known and have been recommended for therapeutic use; one can mention phosphorothioates, phosphorodiamidate morpholino oligomers (PMO), peptide nucleic acids (PNA), and different types of 2′-modified oligonucleotides

Table 1 Chemical modifications improving nucleic acids' stability

Modification	Structure	Properties	References
Modification of internucleoside link			
Phosphorothioate		Highly resistant to nuclease cleavage; decreases the duplex stability; binds to serum albumin; toxic	Bennett and Swayze (2010), Milligan et al. (1993)
N3′ → P5′ Phosphorodiamidate morpholino oligomers (PMO)		Confers resistance to nucleases; increases T_m of duplex by 2 °C per residue for an RNA target	Gryaznov et al. (1995), Heidenreich et al. (1997)
Boranophosphate		Confers high nuclease resistance	Hall et al. (2004)
Amide-internucleosidic linkage		Confers nuclease resistance; leads to the duplex T_m change from −4 to +0.9 °C per residue for an RNA target	De Mesmaeker et al. (1994), Mutisya et al. (2014)

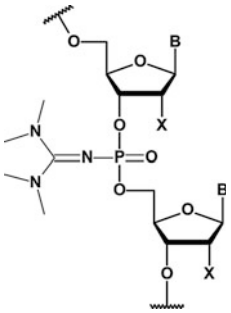
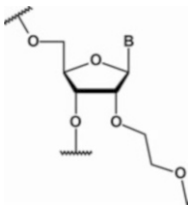
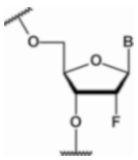
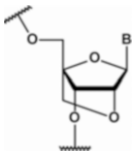
(continued)

Table 1 (continued)

Modification	Structure	Properties	References
Phosphonoacetate		Confers nuclease resistance; uncharged; decreases T_m of duplex by 1.2 °C per residue for an RNA target	Sheehan et al. (2003)
Morpholino		Highly resistant to nuclease cleavage; uncharged	Summerton and Weller (1997)
Peptide nucleic acid (PNA)		Highly resistant to nuclease cleavage; uncharged; increases T_m of duplex by 1 °C per residue for a DNA target	Shabi et al. (2006)
SATE (S-acyl-2-thioethyl-phosphotriesters)		Highly resistant to nuclease cleavage; uncharged; reversible protecting group (cleaved off by thioesterase in the cytoplasm)	Meade et al. (2014)

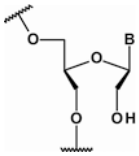
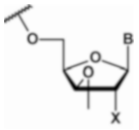
(continued)

Table 1 (continued)

Modification	Structure	Properties	References
Phosphoryl guanidine (PG)		Highly resistant to nuclease cleavage; uncharged when fully modified	Kupryushkin et al. (2014), Lebedeva et al. (2015)
Sugar modifications			
2′-OMe-RNA		Confers nuclease resistance; increases the duplex stability; decreases the innate immune response induction	Bennett and Swayze (2010)
2′-OMOE-RNA		Confers nuclease resistance; increases T_m of duplex by 2 °C per residue for an RNA target	
2′F-RNA		Confers nuclease resistance; increases T_m of duplex by 2.2 °C per residue for an RNA target	Kawasaki et al. (1993)
Locked nucleic acid (LNA)		Confers nuclease resistance; increases T_m of duplex by 5.1 °C per residue for an RNA target and by 4 °C per residue for a DNA target	Koshkin et al. (1998)

(continued)

Table 1 (continued)

Modification	Structure	Properties	References
Unlocked nucleic acid (UNA)		Decreases T_m of duplex by 5–10 °C per residue for an RNA target and by 7–10 °C per residue for a DNA target	Campbell and Wengel (2011)
Xylo nucleic acid (XNA)		Confers nuclease resistance; decreases duplex stability	Poopeiko et al. (2003)
X—H (DNA) or OH (RNA); B—bases: A, C, G, T, U			

(Wickstrom 2015). Another group consists of recently developed promising modifications of oligonucleotide structure whose potential should be studied in details in nearest future. Such modifications include amide-internucleosidic linkage, S-acyl-2-thioethyl-phosphotriesters (SATE), or phosphoryl guanidines (PG) incorporated instead of parent phosphodiester moieties. The derivatives and analogues listed in Table 1 can allow to design oligomers characterized by the electroneutral backbone, drastically increased nuclease resistance, and, therefore, enhanced therapeutic potential.

Modifications should be introduced with caution as they may change NA properties, such as toxicity and binding affinity for RNA/DNA targets. For instance, it was found that the increased amount of modified nucleotides enhanced the stability of siRNA in the presence of serum, but reduced its silencing activity. The targeted modification of nuclease-sensitive sites (mostly UpA, CpA, and UpG sites) improved the stability of siRNA and prolonged the silencing effect with minimal loss of silencing efficiency (Volkov et al. 2009). Moreover, 2′-O-methyl analogues of ribonucleotides introduced in the nuclease-sensitive sites of long dsRNA prevented the activation of innate immunity response without the loss of silencing efficiency and specificity (Gvozdeva et al. 2014).

4 Modifications of the Therapeutic RNA Imported into Human Mitochondria

Defects in human mitochondrial genome can cause a wide range of clinical disorders, mainly neuromuscular diseases. Most of deleterious mitochondrial mutations are heteroplasmic, meaning that wild-type and mutated forms of mtDNA coexist in the same cell (Pinto and Moraes 2014). Therefore, a shift in the proportion between mutant- and wild-type molecules could restore mitochondrial

functions. The anti-replicative strategy aims to induce such a shift in heteroplasmy by mitochondrial targeting specifically designed molecules in order to inhibit replication of mutant mtDNA. Recently, we developed mitochondrial RNA vectors (Kolesnikova et al. 2011) that can be used to address anti-replicative oligoribonucleotides into human mitochondria and impact heteroplasmy level. The observed effect was however transient, probably due to a rapid degradation of RNA molecules (Comte et al. 2013). Various chemically modified nucleotides have then been introduced in anti-replicative oligoribonucleotides to improve their stability, namely, nucleotides substituted at the 2′-hydroxyl group with 2′-OMe, 2′-F, and 2′-deoxy in combination with terminus capping chemistry. The most important increase of anti-replicative molecules' lifetime can be achieved by using synthetic RNA–DNA chimeric molecules or by ribose 2′-O-methylation in nuclease-sensitive sites. The presence of "inverted" 3′-3′ thymidine at the 3′-terminus and modifications of 2′-OH ribose moiety did not prevent the mitochondrial uptake of the recombinant molecules. Nevertheless, the modified oligonucleotides did not cause a significant effect on the heteroplasmy level in transfected *trans*mitochondrial cybrid cells bearing a pathogenic mtDNA deletion, proving to be less efficient than nonmodified RNA molecules (Tonin et al. 2014). One can hypothesize that the C3′-endo sugar conformation and 3′-3′ inverted nucleotides might be recognized by the replisome or by other mitochondrial nucleoid proteins as nonnatural and quickly eliminated.

To decrease the toxicity of the cell transfection procedure and create an approach of carrier-free targeting of various anti-replicative RNAs into living human cells, we designed conjugates containing a cholesterol residue. Because cholesterol could stall the mitochondrial import of therapeutic anti-replicative RNA due to attachment to the mitochondrial membranes, we developed the protocol of chemical synthesis of oligoribonucleotides conjugated with cholesterol residue through cleavable covalent bonds. Conjugates containing pH-triggered hydrazone bond (Fig. 6a) were shown to be stable during the cell transfection procedure and rapidly cleaved in acidic endosomal cellular compartments. RNAs conjugated to cholesterol through a hydrazone bond were characterized by efficient carrier-free cellular uptake and partial co-localization with mitochondrial network. Moreover, the imported oligoribonucleotide designed to target a pathogenic point mutation in mitochondrial DNA was able to induce a decrease in the proportion of mutant mitochondrial genomes (Dovydenko et al. 2015).

We suppose that anti-replicative RNA conjugated to cholesterol can be internalized by the endocytosis pathway (Fig. 6b). Thereafter, the hydrazone bond between RNA and cholesterol moieties would be cleaved in the acidic conditions of the late endosomes, and the endosomal escape can be induced by destabilization of endosomal lipid bilayer, due to the positively charged hydrazide group which is formed by the conjugate hydrolysis. Released RNA molecules can be partially degraded in the cytoplasm, but still partially targeted into mitochondria due to the presence of a structural determinant for mitochondrial import.

To improve the in vivo delivery of cholesterol-RNA conjugates, we are planning to design and synthesize conjugated molecules containing various nucleotide and

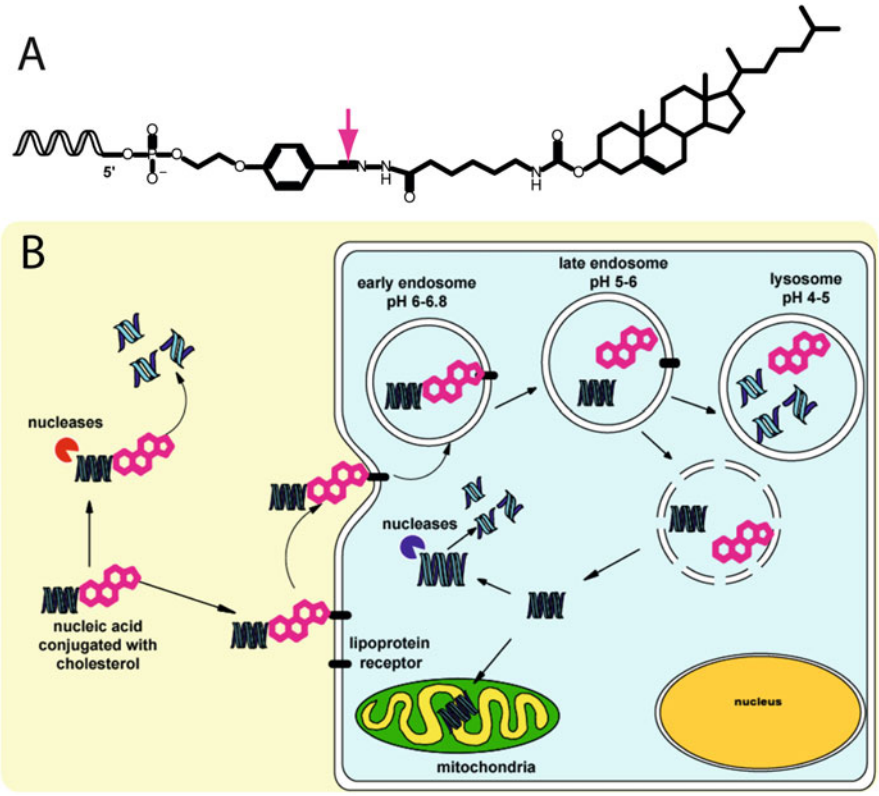


Fig. 6 Cell delivery of RNA conjugated to cholesterol through cleavable linker. (**a**) Oligoribonucleotide conjugated with cholesterol residue through pH-triggered hydrazone bond. The point of the cleavage in acidic conditions is shown by an *arrow*. (**b**) Schematic representation of the cell delivery of RNA conjugated to cholesterol through cleavable linker. RNA is represented by a *helix*; cholesterol residue is highlighted in *purple*. See text for the details

internucleotide bond modifications, which can improve the stability of anti-replicative RNA moieties and promote their tissue distribution and cellular uptake.

5 Conclusion and Future Prospects

As one can deduce from the examples discussed above, there are various nucleotide modifications that can protect RNA molecules introduced into human cells against nucleolytic degradation. Another type of modification, the conjugation of oligonucleotides to the ligands, which can be internalized into the cell by natural transport mechanisms, is a promising approach to overcome the problem of their inefficient delivery to target cells and tissues. Further development of the oligonucleotide

modification technology will allow creating novel therapeutic molecules characterized by high stability, low toxicity, and efficient delivery to various human tissues.

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