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Oligonucleotide conjugates for therapeutic applications

Insufficient pharmacokinetic properties and poor cellular uptake are the main hurdles for successful therapeutic development of oligonucleotide agents. The covalent attachment of various ligands designed to influence the biodistribution and cellular uptake or for targeting specific tissues is an attractive possibility to advance therapeutic applications and to expand development options. In contrast to advanced formulations, which often consist of multiple reagents and are sensitive to a variety of preparation conditions, oligonucleotide conjugates are defined molecules, enabling structure-based analytics and quality control techniques. This review gives an overview of current developments of oligonucleotide conjugates for therapeutic applications. Attached ligands comprise peptides, proteins, carbohydrates, aptamers and small molecules, including cholesterol, tocopherol and folic acid. Important linkage types and conjugation methods are summarized. The distinct ligands directly influence biochemical parameters, uptake mechanisms and pharmacokinetic properties.

Although oligonucleotides have been regarded as a new class of drugs for more than three decades, their therapeutic applications have so far failed to fully live up to the expectations [1,2]. Today, there are many different subclasses, which are mainly divided by different mechanisms of biological actions, and include antisense [3], splice-switching oligonucleotides [4,5], siRNA [6], miRNA [7], **aptamers** [8] and immunostimulatory oligonucleotides [9]. Structural differences are minimal, and consequently all nucleic acid-based drugs generally suffer from poor pharmacokinetics [10], the lack of cell membrane permeation [11], and often insufficient stability and sometimes relevant off-target effects [12]. Those properties are generally caused by their hydrophilicity, multiple anionic charges and ready interaction with degrading enzymes. Decades of therapeutic development have brought advances in enzymatic stability through chemical derivatization [3], and a multitude of distinct liposomal or polymeric delivery systems for improving their pharmacokinetic behavior [13]. Until 2012, one antisense agent (fomivirsen) and one aptamer (pegaptanib) had won market approval. Both are restricted to local application, circumventing the major problems. Mipomersen (Kynamro® [CA, USA]), an antisense agent targeted at apoB-100 for treatment of homozygous familial hypercholesterolemia [14–16], has been approved by the US FDA in January 2013, and could mark a major breakthrough for this type of biological therapeutic. Mipomersen, like other phosphorothioate drugs, accumulates in the

liver, the site of apoB production. Clinical safety profiling showed a risk of hepatotoxic effects, including elevation of liver enzymes alanine aminotransferase and aspartate aminotransferase, and hepatic steatosis [17]. As a consequence, the FDA is requiring a risk evaluation and mitigation strategy program for mipomersen, including an enhanced pharmacovigilance program and a long-term registry of patients to determine the long-term safety.

Contrastingly, the European Medicines Agency has rejected the submission for market approval due to safety concerns. In Europe, the marketing application included a broader patient population, encompassing not only homozygous, but also severe heterozygous familial hypercholesterolemia. However, mipomersen and other front-running oligonucleotide development candidates target the liver (**TABLE 1**), the organ most easily accessible after parenteral application. Other approaches for oligonucleotides intended for action in other tissues were ultimately unsuccessful in clinical evaluation. Prime examples are oblimersen and aprinocarsen, for which comprehensive Phase III trials were conducted, but ended with disappointing outcomes [18].

Drisapersen is a promising representative of splice-correcting oligonucleotides. Recently, encouraging clinical results have been reported against Duchenne muscular dystrophy (DMD) by inducing the expression of a truncated, but functional splicing pattern of dystrophin, mutations of which cause the disease [19]. Similar to

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Table 1. Clinical status of selected antisense and siRNA agents.

Compound	Application route	Formulation/modification	Target	Indication	Company	Clinical status
siRNA oligonucleotides						
Bevasiranib	Intravitreal	-	VEGF	AMD	Opko Health Inc.	Phase III, terminated
AGN-745 (siRNA027)	Intravitreal	-	VEGF	AMD	Allergan/Sirna	Phase II, terminated
SYL040012	Ophthalmic drops	-	β2 adrenoreceptor	Glaucoma	Sylentis	Phase II
ALN-RSV01	Inhalation	-	RSV nucleocapsid gene	RSV infection after lung transplantation	Alnylam Pharmaceuticals	Phase II
RX1109 [†]	Intradermal	Modified siRNA (phosphorothioate, lipophilic ligands) [†]	Connective tissue growth factor	Dermal scarring after surgery	RXi Pharmaceuticals	Phase I
QPI-1002	Intravenous	Modified siRNA (alternating 2'-O-Me)	p53	Delayed graft function and acute kidney injury	Quark Pharmaceuticals	Phase II
CALAA-01	Intravenous	RONDEL™ (cyclodextrin polymer, PEG and transferrin ligands)	M subunit of ribonucleotide reductase	Solid tumors	Arrowhead Research Corporation	Phase I
ALN-TTR02	Intravenous	SNALP	TTR	TTR-mediated amyloidosis	Alnylam Pharmaceuticals	Phase II
ALN-TTRsc [†]	Intravenous	GalNAc conjugate [†]	TTR	TTR-mediated amyloidosis	Alnylam Pharmaceuticals	Phase I
ARC-520 [†]	Intravenous	Dynamic polyconjugate [†]	Coagulation factor 7 (F7)	Hepatitis B virus infection	Arrowhead Research Corporation	Phase I (mid-2013)
Antisense oligonucleotides						
Mipomersen	Subcutaneous	PS-2'-MOE gapmer ODN	apo-B-100	Homozygous familial hypercholesterolemia	ISIS/Genzyme	Approved in USA
Alicaforsen	Intravenous, enema	PS-ODN	ICAM-1	Pouchitis, ulcerative colitis	ISIS/Atlantic	Phase III (orphan drug status)
Disapersen (PRO051, GSK-2402968)	Intramuscular	PS-2'-O-Me ASO	Dystrophin (splice correction)	Duchenne muscular dystrophy	Prosenza/GSK	Phase III
Custirsen (OGX-011)	Intravenous	PS-2'-MOE gapmer ODN	Clusterin	Prostate cancer, lung cancer	ISIS/OncogeneX	Phase III
Miravirsen	Intravenous, subcutaneous	PS-LNA gapmer ODN	miR-122 (Hepatitis C)	Hepatitis C virus infection	Santaris Pharma	Phase II
Eteplirsen (AVI-4658)	Intravenous	PMO ASO	Dystrophin (splice correction)	Duchenne muscular dystrophy	Sarepta Therapeutics	Phase II
ISIS-TTR02	Intravenous	PS-2'-MOE gapmer ODN, liposomal formulation	TTR	TTR-mediated amyloidosis	ISIS	Phase II
Cenersen (EL-625)	Intravenous	PS-ODN	p53	Acute myeloid leukemia	Eleos, Inc.	Phase II
GTI-2040	Intravenous	PS-ODN	RRM2 (ribonucleotide reductase M2)	Acute myeloid leukemia	Lorus Therapeutics	Phase II

[†]Oligonucleotide conjugates.

AMD: Age-related macular degeneration; ASO: Antisense oligonucleotide; cEt-BNA: Constrained ethyl bicyclic nucleic acid; GalNAc: N-acetyl galactosamine; LNA: Locked nucleic acid; MOE: Methyl oxethyl; ODN: Oligodeoxynucleotide; PMO: Phosphorodiamidate morpholino oligomer; PS: phosphorothioate; RSV: Respiratory syncytial virus; SNALP: Stable nucleic acid lipid particles.

Table 1. Clinical status of selected antisense and siRNA agents (cont.).

Compound	Application route	Formulation/modification	Target	Indication	Company	Clinical status
Antisense oligonucleotides						
Imetelstat (GRN163L) [†]	Intravenous	N3'-P5' thio-phosphoramidate, palmitoyl conjugate [†]	Telomerase	Essential thrombocythemia, multiple myeloma, solid tumors	Geron	Phase II
ISIS-STAT3Rx, ISIS-481464	Intravenous	PS-cEt-BNA gapmer ODN	STAT3	Solid tumors, lymphoma	ISIS	Phase II
Liposomal Grb-2 (BP-100-1.01)	Intravenous	P-ethoxy ODN liposomal formulation	L-Grb-2	Leukemia	Bio-Path Holdings, Inc.	Phase I

[†]Oligonucleotide conjugates.

ASO: Antisense oligonucleotide; cEt-BNA: Constrained ethyl bicyclic nucleic acid; GalNAc: N-acetyl galactosamine; LNA: Locked nucleic acid; MOE: Methyl oxethyl; ODN: Oligodeoxynucleotide; PMO: Phosphorodiamidate morpholino oligomer; PS: phosphorothioate; RSV: Respiratory syncytial virus; SNALP: Stable nucleic acid lipid particles.

mipomersen, it exhibits a full phosphorothioate backbone, and 2'-O-methylated nucleosides. In contrast to antisense agents, RNase H activation is unwanted for splice correction, and consequently all of drisapersen's nucleosides are methylated. Eteplirsen is a similar agent, also targeted at dystrophin, but featuring a different chemical modification; it is a phosphorodiamidate morpholino oligomer (PMO) [20]. A Phase II trial reported significant clinical benefit for DMD patients, and biochemical analysis proved the expression of a functional dystrophin protein in skeletal muscles [20,21]. Both agents take advantage of increased accumulation in muscular tissue due to tissue damage caused by the disease. Eteplirsen is rapidly eliminated by renal filtration because PMOs lack substantial binding to plasma proteins. As a result, high doses are necessary, and splice correction was not detected in all muscle tissues in animal experiments and clinical trials [20]. Dystrophin expression in cardiac muscle fibers is still a major hurdle.

These examples illustrate that despite some progress, the challenges of therapeutic application of nucleic acid-derived compounds are not yet solved. Delivery to tissues and organs other than the liver has not been achieved in a satisfactory manner. Toxicology issues, which are linked to the chemical modifications, are not sufficiently understood and even after market approval a major cause for concern. In some cases, oligonucleotides are rapidly eliminated by renal filtration, demanding high doses. For full realization of the enormous potential of oligonucleotide therapeutics, the pharmacokinetic properties need to be improved. Besides structural optimization and the development of delivery systems, the chemical coupling of ligands that modulate the pharmacokinetic behavior or target specific receptors is an attractive strategy for improving oligonucleotide therapy.

Over the last decades, the chemical derivatization of antisense and siRNA oligonucleotides has seemingly been carried out exhaustively [3,22-24]. It is probable that thousands of different modifications have been prepared and many of those evaluated for their therapeutic use. The outcome of these efforts suggest that structural optimization is a viable option to improve stability, but is, on its own, not sufficient to achieve proper bioavailability. Careful structural evolution has resulted in second generation antisense molecules, consisting of terminal 2'-O-methyl (2'-O-Me) or 2'-O-methoxyethyl (2'-O-MOE) nucleosides and a full phosphorothioate

Key Term

Aptamers: Nucleic acids that bind specifically to molecular targets including proteins, other nucleic acids and small molecules. Aptamers are selected from libraries through repeat rounds of selection called systematic evolution of ligands by exponential enrichment.

backbone. The backbone modification seems to be essential for triggering plasma protein binding and at least some extent of cellular uptake, however, the mechanism is not yet fully understood [11,25]. While 2'-alkylation supports resistance against enzymatic degradation, an internal stretch of deoxynucleosides is essential for activation of RNase H. This nuclease is vital for degradation of the corresponding mRNA and a catalytic antisense effect.

There are also high hopes for RNA interference by siRNAs. Compared with antisense agents, they offer improved effects through activation of the RNA-induced silencing complex. On the downside, this mechanism tolerates a significantly lower number of structural modifications. Advanced delivery systems, such as lipid particles, are essential for adequate half-lives and effective cell membrane permeation [26–30]. For achieving sufficient stability, a limited number of 2'-O-Me nucleosides are usually distributed within the sequence, but no backbone modifications are employed. Therapeutic development of siRNA is still in its infancy and major Phase III clinical trials are set to begin within the next few years (TABLE I).

In addition, locked nucleic acids (LNAs) derivatives have been successful primarily in molecular biology techniques through their increased selective binding affinity to matching nucleic acids. Consequently, LNA modifications enable shorter nucleic acid sequences for effective hybridization [31]. This modification does not significantly influence the pharmacokinetic properties, and higher binding affinity is of minor importance for several therapeutic applications, including antisense and siRNA [31]. The use of LNAs is particularly promising for those clinical applications in which only short oligonucleotides sequences are used. Among those, miRNA antagonists are particularly attractive, because they are designed to bind to a short RNA, which leaves little space for sequence optimization. One promising substance is miravirsin, targeted at a short RNA, miRNA-122, which is currently being tested for the treatment of hepatitis C [32].

Currently, many research programs focus on the generation of delivery systems, primarily for siRNA [28]. The nucleic acid cargo can be covalently attached to a carrier, or loaded, usually by charge complexation or absorption, to supramolecular delivery devices. The latter includes lipid particles, multi-componental liposomes, cationic polymers and nanoparticulate

systems of different classes. Without a doubt, the pharmacokinetic properties can be positively influenced by the use of these systems, but the successful therapeutic application in the clinical setting remains to be proven. In particular the challenges include reliable and reproducible constitution of the systems, which often consist of up to six different components, as well as immunogenicity, adequate stability with sufficient release and problems associated with large-scale manufacturing.

Conjugations of uptake-enhancing or targeting ligands to oligonucleotides offer the advantage of generating a defined molecule, which, in turn, enables traditional pharmaceutical quality assessment. Scientists are widely using oligonucleotide-dye conjugates as probes for qPCR [33], for nucleic acid sequencing and hybridization, as well as in microscopy applications such as fluorescence *in situ* hybridization and fluorescence resonance energy transfer [34]. A number of diverse molecules have been attached to therapeutic oligonucleotides in order to improve their delivery, biodistribution and cellular uptake [35,36]. Most conjugation strategies aim to simply improve biochemical properties and cellular uptake characteristics, but some attempts to combine tissue- or tumor-targeting functionalities with oligonucleotide effector molecules have also been described. Among the ligands primarily designed to increase the extent of uptake into the cytosol are the lipophilic compounds cholesterol and tocopherol (FIGURE 1), cell-penetrating peptides (CPPs) such as penetratin, and polyamines such as spermine. In terms of achieving tissue-specific accumulation, proteins for antigen-specific binding, as well as small-molecule-based targeting moieties such as the carbohydrate *N*-acetyl galactosamine (GalNAc), have been employed as oligonucleotide ligands.

Conjugates with uptake-enhancing ligands

For therapeutic applications, conjugates of antisense and siRNA oligonucleotides have primarily been investigated, as both need to reach intracellular sites of action. Stability and biodistribution of siRNA oligonucleotides can be easily improved by the attachment of small lipophilic molecules. Cholesterol was tethered to siRNA for one of the first reports of silencing of an endogenous gene *in vivo* under physiological conditions with a normal pressure injection in mice [37]. Cholesterol was attached to the controlled pore glass solid support prior to oligonucleotide synthesis, and an aminocaproic acid pyrrolidine

Figure 1. Selection of ligands for oligonucleotides for influencing pharmacokinetic behavior, membrane permeation or receptor-specific cellular uptake. Arrows indicate attachment sites.

phosphate linker was used between ligand and siRNA. Cholesterol–siRNA conjugates resulted in an mRNA reduction of the targeted apoB of around 50%, while unconjugated siRNA had no effect. Similar results were reported for the lipid docosanyl and stearyl ligands [38]. *In vivo* mRNA downregulation efficiency was found to be dependent on fatty acid length. Shorter compounds (<C18) were unable to reduce apoB mRNA levels, but stearyl (C18) and docosanyl (C22) resulted in 30 and 50% reductions, respectively. Circulation time is increased through association of the conjugates with lipoproteins. Uptake in liver, but also in the kidney and adrenal glands is dependent on binding to

lipoproteins and mediated by lipoprotein receptors [38]. Repeated applications of high doses were necessary for target downregulation, which limits the application, and the conjugation of cholesterol markedly increases accumulation in the liver. Thus, this strategy is all but confined to targeting hepatic diseases. Cholesterol–siRNA can be employed for noncovalent association to polymers, demonstrated by *in vivo* gene silencing in combination with a targeted engineered polymer [39]. Later developments focused on the improvement of lipid particles [26,27,40,41], which have similar characteristics, but are more effective and allows easy structural optimization for increased stability and effect.

Key Terms

PEGylation: Attachment of PEG chains. A versatile technique to increase the molecular mass and hydrodynamic size of a drug or antibody. It reduces immunogenicity (often referred to as 'stealth' properties), prolongs circulation time by lowering renal filtration rates, and the amphiphilicity increases solubility of hydrophobic substances.

Protein scaffolds:

Polypeptide sequences of a structured core with variable domains, which account for modulation of specific binding to antigens. Alternative (non-IgG) scaffolds are attractive developments for scientific, diagnostic and therapeutic applications, and include anticalins, avimers, DARPins and monobodies (adnectins). Some of those scaffolds are extremely stable, and offer easy and site-specific derivatization opportunities.

Imetelstat is a N3'-P5' thio-phosphoramidate oligonucleotide conjugated with a palmitic acid ligand tethered to the 5'-phosphate via an ethylene glycol linker [42]. The 13-mer oligonucleotide is not an antisense agent, but directly binds to the active site of the human telomerase enzyme, and the lipid conjugate is currently in Phase I/II clinical evaluation as an anticancer agent [43]. The chemical oligonucleotide modification induces sufficient stability, and the lipid ligand markedly increases cellular uptake and bioavailability. In cell lines, IC₅₀ values of the palmitoyl conjugates were five- to ten-times lower than those of the parent oligonucleotides. In a mouse xenograft tumor model, the lipid conjugate demonstrated significantly more uptake in the tumor and the liver, and inhibited telomerase activity by more than 50%, while the unconjugated siRNA was ineffective [42].

α -tocopherol (vitamin E) was attached to an apoB1-targeted siRNA at the 5'-position via a phosphate linkage [44]. Gene silencing in the liver was achieved at lower concentrations as needed for cholesterol conjugates, but uptake is similarly mainly limited to the liver. *In vitro* uptake relied on the presence of serum, indicating an interaction with tocopherol-binding proteins with subsequent uptake into the cytosol. Pre-absorption of the tocopherol conjugate to HDL allowed efficient gene silencing in neurons after intracerebroventricular infusion [45]. The LDL receptor was identified as an essential factor for successful uptake, which was absent for unconjugated siRNA.

Because the high anionic charge density is a major factor for the poor pharmacokinetics of oligonucleotides, complexation or covalent attachment of cationic molecules has been extensively performed and evaluated. In particular, basic peptides have been tethered to oligonucleotides in a large variety of ways. Several, usually highly cationic, peptides have the ability to cross cellular membranes [46]. Those peptides are known as CPPs or protein transduction domains [47,48]. Although the exact mechanism of uptake is not completely understood, it has been established that CPPs interact with several anionic cell-surface molecules. Proteoglycans such as syndecans are negatively charged anchoring groups on cellular membranes, and CPPs, including their cargo, are subsequently internalized into the endosome [49]. Depending on poorly characterized properties including size and structure of the internalized complex, the effectiveness of cytosolic delivery is variable [36]. Peptide-oligonucleotide conjugates have been investigated extensively [50], and

a considerable number of different possibilities of chemical conjugation have been developed. Overall, uncharged oligonucleotides seem to give better results in conjugates with CPPs than their charged counterparts. Interaction of lysine and arginine side chains with phosphates or phosphorothioates lead to aggregation into larger particles, which, in turn, seems to hinder effective cytoplasmic delivery. Initially, CPP-mediated uptake was believed to be a nonendocytotic mechanism, but recent evidence strongly points to cellular uptake via clathrin-mediated endocytosis [51]. The observed higher efficiency of CPP-mediated transfection is probably attributable to a higher rate of endosomal escape. While a considerably high number of different CPP-oligonucleotide conjugates have demonstrated successful uptake *in vitro* and *in vivo*, no clinical trials have been undertaken so far.

Morpholino oligonucleotides, which particularly suffer from poor cellular uptake, have been conjugated to arginine-rich CPPs [52-54]. Lacking any charged backbone functionalities, they especially profit from CPP conjugation. Preclinical data reports that by conjugation of arginine-rich peptides to the parent PMO, significantly lower doses are sufficient for exon skipping in mouse models of DMD [54]. Dystrophin expression was found in all tested muscle tissue, including cardiac muscle [55]. Conjugations increased cellular uptake, but the majority of the dose ends up in liver and kidney. Attempts to increase muscle selectivity by fusing a muscle-specific peptide selected by phage display illustrated lower effects, but combining this peptide with a basic peptide for enhancing cellular uptake resulted in an enhanced effect [56]. However, little tissue selectivity was achieved [54]. These data indicate that conjugations of short basic peptides may be a viable option for overcoming some of the limitations of uncharged PMOs. In a comparison of different CPPs conjugated to either the 3'- or the 5'-end of PMOs in Ebola virus-infected mice, a correlation of virus replication inhibition with a number of arginines in the peptide ligand was found. Efficient replication inhibition was observed with a peptide containing eight arginine residues [57], while PMOs lacking any positive charge had no effect at all.

Similar to basic peptides, polyamines can be used to alleviate the negative consequences of the anionic charges of oligonucleotides. Fusing several spermine molecules to an oligonucleotide results in increased binding affinity to its target counter strand, mediated by a

reduction of electrostatic repulsion between the two strands [58,59]. No data on biological activity of these conjugates have been reported. In a similar approach, an attachment of polyamines at the 2'-position of antisense oligonucleotides demonstrated a dependency on binding affinity and *in vitro* gene silencing effect on the length of the polyamine, and, thus, the number of cationic charges [60]. Short polyamines all but abolished the gene-silencing activity, but conjugates with spermidine and an artificial pentaamine ligand had a higher effect on the gene target than the unmodified antisense agent. Shorter ligands hinder the hybridization to the mRNA due to steric effects, but with longer polyamine chains, the charge interaction prevails. In both approaches, an aggregation towards nanoparticulate structures can be assumed, which can be speculated to increase cellular uptake.

Pegaptanib is one of only three oligonucleotide drugs having been approved for therapy. It is a PEGylated aptamer targeted at VEGF and applied locally for treatment of age-dependent macular degeneration [61]. The branched, 40 kDa PEG chain attached at the 5'-end prolongs half-life and increases retention in the eye [62]. *In vitro*, a conjugate of PEG 5000 and an anti-VEGF siRNA was transfected into cultured cells using polyethylene imine, resulting in reduction of VEGF protein release [63]. The **PEGylation** slightly prolonged serum stability, however there is no indication of uptake enhancement in the absence of a transfection agent.

Conjugates with ligands for receptor-specific targeting

Antibodies, antibody fragments [64,65] and alternative binding **protein scaffolds** [66] promise antigen-dependent and cell-type specific delivery of drug cargoes. This is certainly an attractive option for antisense and particularly siRNA agents, which rely on delivery enhancers. While a number of approaches using complexes of oligonucleotides and antibody derivatives [30,67–69], biotin–streptavidin coupling [70] and antibodies fused to liposomes [71,72] have been reported, little research has been undertaken on direct covalent conjugation of protein binders to oligonucleotides. Site-specific derivatization of antibodies is a challenging task because of the protein structure. Linkers for oligonucleotide attachment can be tethered at lysine-amines [73] or cysteine-thiols [74], usually rendering mixtures of multiple-loaded antibodies, but not defined chemical structures. Together with high costs,

these challenges and concerns of immunogenicity have limited the approach [75,76]. Alternative protein scaffolds generally offer easier derivatization [77–79], but their use for oligonucleotide targeting remains to be evaluated.

A recent example of antibody–oligonucleotide conjugate employed an α -CD19 monoclonal antibody and a phosphorothioate antisense oligonucleotide targeted at the fusion gene *E2A-PBX1* [80]. α -CD19 is a B-lineage-specific surface receptor and a biomarker for acute lymphoblastic leukemia (ALL), and the *E2A-PBX1* fusion gene is a transcriptional activator, and the result of a common chromosomal translocation in ALL. A disulfide linker was used, and a 15-fold surplus of antisense oligonucleotide was added to the antibody, but only a fraction successfully reacted [80]. A high extent of specific *in vitro* and *in vivo* silencing of the fusion gene was reported, with the pharmacological effect depending on both active antibody binding and the functional antisense sequence. A near complete reduction of clonogenic activity of ALL cell lines as well as significant increase in the survival of mice with xenograft tumors was found.

Small-molecule ligands offer a much easier chemical conjugation methodology. Among the receptor-binding molecules that have been used for shuttling covalently attached oligonucleotide cargo into cells, folate [29,81] and anisamide [82] have been used most extensively. Folic acid (vitamin B₉) binds with high affinity to the folate-receptor protein and triggers cellular uptake via an endosomal pathway. The presence of the folate receptor on many cancer types has prompted the use for targeted therapy [83]. Unlike the use of folate on liposomes or polyplexes, tethering folate to siRNA has resulted in specific uptake, but not silencing of reporter genes [81,84]. It was concluded that the conjugates end up in endosomes with insufficient escape to the cytosol for gene silencing. Complexing the constructs with a polycationic transfection enhancer resulted in effective, and targeted downregulation of the reporter [81]. The gene-silencing effect was dependent on the presence of the folate, as unconjugated siRNA absorbed to the transfection enhancer was inactive.

The monosaccharide GalNAc is able to induce targeting to hepatocytes by binding to the asialoglycoprotein receptor (**FIGURE 2**) [85]. It has been used both as a targeting functionality on lipid particles and as a component in siRNA conjugates [86]. Alnylam Inc. (MA, USA) is advancing its GalNAc conjugates for subcutaneous

Figure 2. *N*-acetyl galactosamine–siRNA oligonucleotide conjugates.

injection towards clinical trials. For increasing binding avidity through multivalency, three GalNAc molecules are attached via a tridentate linker to the 3'-terminus of the sense strand of an siRNA. Animal experiments demonstrated encouraging efficacy data after subcutaneous injection of ALN–TTRsc, targeted at transthyretin for the treatment of amyloidosis [201]. In a similar drug candidate, a GalNAc conjugate is tested against hemophilia A and B by siRNA-mediated silencing of antithrombin III, which is an important regulatory protein for maintaining hemostasis. No comprehensive report on pre-clinical data has been published, but preliminary results have been presented at several scientific meetings and on the company's homepage [201].

Arrowhead Research Corporation (CA, USA) is developing **dynamic polyconjugates** for siRNA delivery (Figure 3). Herein, GalNAc is also used for hepatocyte targeting via the asialoglycoprotein receptor. Instead of directly attaching GalNAc to the siRNA, the cargo is tethered to the polybutyl aminovinyl ether polymer via a disulfide linkage [87]. The endosomal polymer is consequently masked by attachment of PEG chains and GalNAc with a dialkylmaleic acid linker [88]. After successful cellular uptake, the delivery system is designed to be cleaved into the single components, because the disulfide and acid-labile dialkylmaleic acid tether are unstable in the reducing and acidic endosomal

environment [29]. The siRNA is either directly linked to the polymer via a disulfide tether [88], or a cholesterol–siRNA conjugate is co-injected with the dynamic polyconjugate [89]. Clinical testing of a corresponding agent, ARC-520, is scheduled to begin in 2013.

Anisamide is a high-affinity ligand for sigma receptors, and has been used in targeted delivery approaches on lipid drug and siRNA nanocarriers [90], as well as a ligand covalently attached to a splice switching oligonucleotide [82]. Both uptake and splice switching was increased compared with an unconjugated oligonucleotides. A trivalent construct demonstrated an increase in uptake more than twofold compared with the unconjugated oligonucleotides, while a monovalent compound had only approximately 1.5-fold higher intracellular accumulations. In terms of splice switching, the trivalent conjugate was three- to four-times more effective than the naked siRNA and the monovalent conjugate, which resulted in no increase compared with the siRNA [82].

The RGD tripeptide is an attractive, small peptide for integrin targeting [91]. Integrins are cell-surface receptors with high expression in various cancer types like breast cancers, melanoma and glioblastoma [92]. Cyclic RGD peptides (cRGD) are ligands for $\alpha_v\beta_3$ integrin. Bivalent attachment of cRGD via a lysine to a splice-switching oligonucleotide [93] or siRNA [94] markedly increased uptake. Interestingly, when comparing bi-, tri-,

Key Term

Dynamic polyconjugates:

Macromolecules that undergo structural modification after application. Typically, disulfide and acid-labile linkages are cleaved after cellular uptake in the endosome, releasing cationic groups, which result in endosomal destabilization.

and tetra-valent cRGD–siRNA conjugates, the uptake efficiencies of fluorescently tagged oligonucleotides were very similar, but gene silencing effect was correlated to the number of cRGD peptides [94]. Obviously, effects after the initial uptake are responsible for the divergent pharmacological results, however the detailed mechanisms of uptake and trafficking are not yet completely understood.

Another recent example of a ligand introduced for receptor-mediated endocytosis involves

anandamide, designed for binding to the cannabinoid receptor [95]. In a leukemia cell line reporter gene system, the anandamide conjugate was more potent than isosequential cholesterol and folate conjugates [96]. High concentrations of conjugates of up to 1 μM were necessary to achieve up to 50% reduction of the reporter gene.

The cyclic insulin-like growth factor receptor-binding peptide D-(CSLC) was tethered to an siRNA to enable specific cellular uptake [97]. Relatively high doses were necessary for efficient

Figure 3. Dynamic polyconjugates with covalent attachment of siRNA.

Key Terms**Splice-switching oligonucleotide:**

Designed for inducing functional protein expression by influencing aberrant splicing. They block their corresponding sequence in pre-mRNA, and thus redirect mRNA splicing. In a therapeutic strategy, the splicing of dystrophin, which is expressed in a non-functional manner in Duchenne muscular dystrophy, is reconstituted to produce a truncated, but functional protein.

Solid-phase synthesis:

Technology for chemical synthesis of short nucleic acid sequences. The process has been highly optimized over several decades and is carried out in automated synthesizers on controlled pore glass resin, which facilitates reagent removal and purification.

target downregulation in the breast cancer cell line MCF-7, and the uptake and gene silencing efficiency of approximately 50% was very similar to that of cholesterol–siRNA conjugates.

The gastrin-releasing peptide receptor is a G-protein coupled receptor that regulates a number of functions in the gastrointestinal system. It is up regulated in many human cancers, and the 14 amino acid-peptide bombesin is a specific ligand. Maleimide–thiol coupling of a **splice-switching oligonucleotide** to bombesin improved cellular uptake in gastrin-releasing peptide receptor-expressing cells approximately twofold compared with unconjugated siRNA [98]. The splice-switching effect was successfully competed with free bombesin. Compared with lipofectamine transfection, uptake efficiency and pharmacological response were rather low. The maximal biological effect was detected after 3 days, indicating slow intracellular trafficking.

Aptamers can be engineered to target virtually any protein. Aptamers binding to extracellular proteins are an obvious choice for the use as the cell-recognition proportion of conjugates with antisense or siRNA agents. Aptamers are typically 20–60 base long oligonucleotides and mediate selective ligand binding through their characteristic 3D shape [99]. Aptamers against specific antigens can be developed by selection and evolution of a library by the systematic evolution of ligands by exponential enrichment process. In addition, aptamers specific for cell-types, without prior identification of a molecular target, can be engineered using cell-systematic evolution of ligands by exponential enrichment [100]. Aptamer–siRNA or aptamer–antisense chimera, that is oligonucleotides with an aptamer and an antisense or siRNA part, can easily be prepared by chemical synthesis of long oligonucleotides [101,102]. Other options for aptamer-mediated delivery are post-synthetic linkages, which afford endosomal cleavage by using appropriate tethers, and noncovalent linkage by partial sequence overlaps [103].

Conjugation reactions & linkage types

There are several positional options for ligand attachment to oligonucleotides, which can have an influence on the biophysical properties (**FIGURE 4**). For antisense agents, tethering ligands to the 3′- or 5′-hydroxyl terminal groups is the most popular method, because little interference with base pairing, even when sterically large ligands are used, can be expected. Furthermore, these positions are technically easy to access, and

even enable online conjugation methodologies that prepare the final bioconjugates on a single solid support [104]. Similarly, aptamer chimeras are synthesized using solid-phase chemistry for long-chain oligonucleotides [102]. In the case of double-stranded siRNA, the sense strand is typically preferred for ligand tethering over the antisense strand, because, although the duplex is recruited in the RNA-induced silencing complex, only the antisense strand is needed for mRNA hybridization and cleavage [105]. Consequently, less influence on RNAi activity is expected for sense-modified siRNAs.

Small-molecule ligands, such as cholesterol, tocopherol, GalNAc and fatty acids, have been directly added to controlled pore glass resins for use in solid-phase oligonucleotides synthesis. By appropriate design of the structure, including protecting groups, DMT-containing linker for nucleotide coupling, and a cleavable site, such resins are available for standard oligonucleotides synthesis [104]. This elegant approach avoids the need for separate ligand coupling and elaborate purification of the conjugate from a mixture of educts.

The generation of peptide–oligonucleotide conjugates is possible via post-synthetic coupling, that is separate assembly of peptide and oligonucleotides using their respective automated **solid-phase syntheses**, and stepwise (online) solid-phase assembly in succession on the same solid support. While the latter holds obvious advantages in terms of purification, the noncompatible standard syntheses of the two components have so far limited the development of general and feasible procedures [106]. Postsynthetic protein or peptide coupling yields strongly depend on structure and solubility of the protein or peptide. Highly basic compounds typically interact with anionic oligonucleotides, impeding efficient conjugation. Workup and purification of the final conjugate can be tedious.

The 2′-*O*-hydroxy function of RNA–oligonucleotides offers the possibility to attach one or multiple ligands at internal positions [23,24,107,108]. Since groups attached there are localized in the minor groove of a duplex, interference of counter-strand hybridization is limited. Except for a change towards the A-form of nucleic acids of DNA-based oligonucleotides, the impact on helical structure can be expected to be negligible. The attractiveness of 2′-*O*-conjugations is adequately reflected in the importance of 2′-modification for structural stability of antisense and siRNA oligonucleotides,

Figure 4. Selection of ligand attachment sites in oligonucleotides. Most linkers can be used at the 3'- and 5'-ends, which are technically the easiest to access, followed by 2'-hydroxy groups and nucleobases.

including 2'-*O*-Me, 2'-*O*-MOE and 2'-fluor chemistries [1].

Other options for ligand attachment include the nucleobases [96], especially the 5-C-atom of pyrimidine bases cytosine, thymine, uracil, the 8-C of adenine, and the exocyclic amino group (2-N) of guanine, as well as the linker tethering at the backbone phosphate. Using these anchoring groups will generally result in a more profound impact on base pairing. By incorporation at a terminal position within the oligonucleotide sequence, this detrimental effect can be minimized [96]. Ligand attachment to nucleobases can either be afforded by using prederivatized nucleoside building blocks [104], or by incorporating functional linkers for post-synthetic reactions.

The employed linker holds great importance for biophysical properties and can play a role in the pharmacokinetic behavior of the conjugate. A large number of different ligation methods have been used for oligonucleotide conjugations [109]. Among the most important ones are

disulfides, amide bonds and click chemistry (**FIGURE 5**). Disulfides offer linkages that are sufficiently stable for characterization, storage and handling, but are designed to be cleaved in the reductive environment in endosomes after successful cellular uptake [110]. The generation of disulfide linkages can be achieved by reaction of two free thiol groups – necessitating the removal of homodimeric byproducts – or by prior activation of one of the reacting thiols. Disulfide bridges are particularly attractive for peptide–oligonucleotide conjugates, for which a cysteine amino acid can be used as a handle. Thiol linkers are commercially available for attachment to oligonucleotides at the 5'-end during solid-phase synthesis, but can also be attached to the more widely used amino linkers. A common example is the use of thiol-amine heterobifunctional crosslinkers such as succinimidyl 3-(2-pyridyldithio)propionate [80].

Thiol functions can also be used for preparing maleimide-type linkages. This reaction, widely

Figure 5. Ligand attachments to oligonucleotides. (A) Amide coupling via active ester method. (B) Disulfide linkages with pyridyldithiol activated ligand. (C) Thiol-maleimide coupling resulting in stable covalent linkages. (D) Copper-catalyzed click chemistry by coupling an azide-modified linker to an alkyne-oligonucleotide. (E) Copper-free click chemistry using dibenzo-cyclooctyne-azide reaction, proceeding in aqueous buffers without catalysts.

applied for labeling proteins, proceeds without a catalyst in aqueous buffers, and yields covalent stable linkages [98].

Due to the highly specific, efficient and fast reaction time, click chemistry is an attractive methodology for the generation of bioconjugates. Azide-alkyne additions with or without copper catalysts can be carried out in aqueous buffers at room temperature [111]. Copper-based catalysts are difficult to remove after the reaction and due to their cytotoxicity are prone to influence biological assays. Copper-free click chemistry is available by using cyclooctyne-based educts, which react without catalysts with azides. Linkers for introducing an alkyne- or dibenzocyclooctyne group during oligonucleotide synthesis are commercially available, and many ligands can be easily derivatized for click chemistry reactions [96]. Azides and alkynes can be attached to terminal hydroxyl groups with phosphoramidite reagents or by coupling to amine linkers. Alkyne handles tethered to C5 of uridine can be employed for click reactions at the nucleobase. Procedures for introducing azido or propargyl anchors into proteins have been established [112], and can be achieved by metabolically engineering an unnatural amino acid, such as

azidohomoalanine or homopropargylglycine into the protein sequence.

The linker length can be crucial for successful attachment of larger molecules to oligonucleotides. To avoid a negative steric impact, longer linkers often need to be used. Because alkyl chains are exceptionally lipophilic, poorly soluble in aqueous buffers and have a tendency for aggregation, PEG linkers are preferred for tethers exceeding about eight to ten atoms [113]. In addition to playing a role during conjugate preparations, linker structure and length can also influence the pharmacokinetic behavior.

Pharmacokinetics of oligonucleotides conjugates

Short and rigid linkers can impede efficient ligand binding to the receptor, especially in multivalent approaches. Biochemical data suggest that six to ten atom spacers are sufficient to allow binding of small-molecule ligands to several receptors on the cell surface [82,85]. Long PEG tethers not only improve the solubility and reaction yields, but can also increase the hydrodynamic size, in turn decreasing renal filtration rates, and shielding the conjugate from degrading enzymes and plasma-protein interactions [114].

There is a general consensus that the bottleneck of cellular uptake of oligonucleotides is not the association to membrane components and initial uptake into endosomes, but rather the transition from those endomembrane compartments to the cytosol. The uptake mechanisms are of enormous complexity and we are only at the beginning of dissecting the distinct pathways (**FIGURE 6**) [36,115]. Most receptor-mediated uptake events including transferrin-, LDL-, and many G-protein coupled receptor-mediated endocytoses function via the clathrin-coated pit pathway [116]. Clathrin forms triskeleons of heterodimers, and assembles in a lattice-like structure. After receptor binding, the membrane is coated with clathrin and associated adaptor proteins followed by invagination and subsequent decoating of the vesicle [115]. Clathrin-coated pits are of between 100 and 200 nm in size. Another important pathway is caveolar-dependent uptake, often associated with protein toxins. Caveolar structures are generally smaller, below 100 nm, than other vesicles. For both clathrin- and caveolin-mediated uptake, dynamin is a key protein for scission of the newly formed vesicles. Several other clathrin- and caveolin-independent processes have been

described recently, some of which seem to be independent of dynamin [117,118]. In addition, uptake can take place in the form of macropinocytosis, and phagocytosis, restricted to certain cell types such as macrophages, granulocytes and Kupffer cells.

Free unmodified oligonucleotides are not readily capable of passing cellular membranes. One of the most widely used antisense modification, phosphorothioates, induce cellular uptake through a slow and ineffective process [10,11,25]. The precise mechanisms is equally unclear as is the proposed existence of an oligonucleotides receptor [119]. By conjugating receptor ligands, the uptake process clearly shifts toward receptor-mediated endocytosis [120]. Consequently, uptake efficiency is mainly dependent on the choice and properties of the ligand, but also has to eventually rival unspecific uptake induced by the phosphorothioate backbone. Cholesterol, transferrin and anisamide, among others, bind to their respective receptor before being internalized by receptor-mediated endocytosis. For efficient binding, high affinity to the respective receptor is desirable. Multivalency is an easy strategy for improving affinity or exceeding a certain threshold. In that regard, multivalent

Figure 6. Uptake routes of oligonucleotides conjugates. The different endocytotic vesicles all converge at the formation of early endosomes, which are transported along the cytoskeleton to the target organelle and are eventually fused with membranes to deliver the contents or mature into lysosomes, ending in degradation of the cargo.

linkers can be attached to the 5'- or 3'-end of oligonucleotides during solid-phase synthesis.

The relevance of the distinct uptake mechanisms for oligonucleotide delivery and the eventual possibilities for guidance uptake towards one or several of those are yet not well understood. There seem to be productive and unproductive pathways [120,121], that is, some result in a pharmacodynamic effect while others do not, despite cellular uptake, because of degradation in lysosomes. The exact mechanism of uptake is probably dependent on ligand, targeted receptor and tissue, and potentially on the chemical structure of the oligonucleotides. Some cargoes change the type of uptake dependent on the concentration. For example, EGF is internalized via clathrin-dependent endocytosis at low concentrations, but by a clathrin-independent mechanism in higher concentrations [122].

Most of the different endocytosis pathways seem to converge at the stage of early endosomes [36]. Subsequently, intracellular trafficking involves the formation of late and recycling endosomes, lysosomes, as well as the endoplasmic reticulum and the golgi apparatus. After pinching off from the membrane and uncoating, the vesicle eventually wanders along actin- and tubulin-based cytoskeletal structures to its destination compartment, where it delivers its content by membrane fusion. These processes are regulated by Rab proteins [36]. Several reports have indicated enhanced activity of conjugates over naked oligonucleotides, despite only minor changes in cellular accumulation being found using fluorescently labeled cargoes [93,98,120]. These data indicate a principal dependence of the intracellular trafficking and ultimately the pharmacological result on the initial uptake mechanism. However, the underlying biological basics are not sufficiently understood for rational direction towards a productive uptake.

Mechanisms of transition from the initially formed vesicles (endosomal escape) to the cytosol or target compartment are generally inefficient. Strategies for enhancing endosomal escape include basic functionalities, which are supposed to lead to endosome destabilization by osmotic swelling through protonation in the acidic milieu (the proton sponge effect), and fusogenic peptides, which undergo conformational changes inducing fusion to the endosomal membrane [122]. The addition of respective components, either by chemical conjugation or complexation, is an approach for improving

uptake efficiency of oligonucleotides conjugates. Amine-rich polymers or peptides induce the proton sponge effect by becoming protonated in the acidic endosomal environment. In addition to protons, chloride anions enter the endosome to maintain a neutral charge, followed by a volume increase caused by osmotic effects. Finally, the endosome ruptures, setting free its contents into the cytosol.

Release from bulky carriers is often necessary for endosomal escape and, particularly for siRNA agents, for induction of the biological effect through the cytosolic effector molecules [88,123]. Click chemistry and the formation of amide bonds, for example through the active ester method, yield covalent conjugates that will not be readily cleaved. For many small ligands, such as short peptides, cholesterol or defined PEG chains, this is not an impediment. The corresponding conjugates have been demonstrated to be active as antisense, splice-switching, or siRNA agents [82,94,96,98]. In covalent conjugates using larger ligands for which carrier release is beneficial, the use of disulfide linkages is thus particularly attractive, because of the reducing environment in the endosome [80,81,110]. Despite the oxidative environment, the presence of cysteine and glutathione in systemic circulation account for a certain extent of disulfide cleavage after prolonged circulation time. Consequently, a more stable linkage is preferred in cases where no dissociation from the carrier is necessary after successful intracellular uptake. Other approaches include the incorporation of linkages that are cleaved under mild acidic conditions, or peptide motifs such as furin, which are substrates for endosomal proteases [124]. Dynamic carrier systems are designed to be structurally modified into endosomolytic agents. Triggered by the acidic and reducing endosomal environment, ligands and masking groups are cleaved to produce polycationic polymers, which cause endosomal disruption. While this approach has resulted in increased gene silencing effects in model systems, the clinical benefit remains to be proven.

Contrasting delivery strategies in which the oligonucleotides are packaged into liposomes or nanoparticles, chemical conjugation alone does generally not protect from degradation by nucleases. Consequently, suitable chemical modifications need to be employed to ensure sufficient stability in biological fluids. For single-stranded antisense agents, the phosphorothioate backbone modification confers adequate stability,

but may, in turn, mediate unspecific (plasma) protein binding, and result in a competing cell membrane permeation mechanism. Off-target effects have been demonstrated for phosphorothioates, undermining their therapeutic value [10,12,125]. Other possibilities to shield from degradation include 2'-modifications (2'-O-Me, 2'-O-MOE or LNAs). Antisense oligonucleotides with phosphodiester backbones and 2'-modifications seem to be the optimal choice for ligand conjugations. In the case of siRNA, the molecular effectors demonstrate lower tolerance for chemical modifications, aside from 2'-methylation, 2'-fluoridation and isolated phosphorothioate bonds. Respective compounds are usually used for the preparation of siRNA conjugates.

For adequate bioavailability, biologics need to be above the renal glomerular filtration limit to prevent rapid elimination from the bloodstream. In the absence of plasma protein binding, oligonucleotides, with a size of approximately <50 kDa, are rapidly lost in the urine [8,10]. Conjugates with small-molecule ligands usually fall below this limit. An increase in apparent mass can be achieved with PEGylation, or by using modifications that mediate plasma protein binding.

In conclusion, tethering ligands to antisense or siRNA oligonucleotides is a viable way to alter their biophysical properties and result in increased membrane permeation or specific, receptor-mediated uptake. Compared with the more widely used encapsulation into liposomal or nanoparticulate delivery systems, they offer the advantage of being distinct molecules with defined structures, enabling analytical techniques and standard methods of quality assurance. Additionally, problems associated with dispersion of particle size and composition, as well as batch variations are eliminated. However, despite recent progress, the extent of uptake mediated by the distinct ligand conjugation generally seems to be inferior to what has been achieved with lipid nanoparticles [126,127]. Further development and optimization are needed for ligand-specific oligonucleotide uptake to be applied in the therapeutic setting.

Future perspective

After several years of setbacks to gene-silencing technology, the prospect for therapeutic gene silencing mediated by oligonucleotides is currently looking brighter. Stimulated by the approval of mipomersen, and encouraging clinical results from splice-switching agents

eteplirsen and drisapersen against DMD, as well as the preclinical advancement of siRNA agents, the enthusiasm for this class of biological has increased. The gained experience now seems to allow a fair assessment of the potential therapeutic value, and has highlighted the necessity for careful selection of molecular target and disease. The main challenges are identified and include improvement of pharmacokinetic behavior and increase of cellular uptake. Bioconjugates of oligonucleotides with ligands for enhancing uptake or for specific receptor binding are valuable means for tackling those challenges. The next few years will bring further preclinical and clinical evaluation of these compounds, finally leading to a clearer picture of their therapeutic potential. In particular, the GalNAc conjugate ALN-TTRsc is expected to be the first siRNA bioconjugate to be evaluated clinically for efficacy, pharmacokinetics and toxicity. If successful, its subcutaneous application could be an advantage over intravenous injections of stable nucleic acid lipid particle-delivered siRNA and naked phosphorothioate antisense oligonucleotides. It seems unlikely that an effective oligonucleotide bioconjugate will reach the market in less than 5 years from now, and further clinical development will depend on the rivalry with other delivery systems, especially lipid nanoparticles and related advanced nanoparticle formulations.

For more efficient targeting beyond the liver, superior binding ligands will have to be developed. The current preclinical approaches primarily rely on small molecules and peptides. Ligands for specific high-affinity binding, such as antibody derivatives and alternative protein scaffolds, could enable tissue-selective delivery. A better understanding of uptake mechanisms is required to facilitate distinction between functional and nonfunctional uptake pathways and consequently design delivery systems with preference for effective intracytoplasmatic oligonucleotide delivery.

Financial & competing interests disclosure

The work of the author is supported by the Austrian Science Fund (FWF): I519-B11 and the Johanna Mahlke Obermann foundation. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Oligonucleotide conjugates for improving cellular uptake and specific targeting

- Small-molecule ligands and short peptides are technically easy to attach to oligonucleotides, while the attachment of large proteins is more demanding.
- Specific receptor binding is possible by folic acid, GalNAc, RGD peptide and antibody derivatives.
- GalNAc conjugates show promise for subcutaneous application, and increase bioavailability in hepatocytes by binding to the asialoglycoprotein receptor.

Conjugation reactions and linkage types

- Labile linkers, such as disulfide or those cleavable in weak acids, release the oligonucleotides cargo from their carrier after uptake in endosomes.
- A variety of conjugation sites are available in oligonucleotides. The 3'- and 5'-hydroxy groups are preferred sites because of easy chemical conjugation, and minimal interference with oligonucleotides base pairing.

Pharmacokinetics

- Reaching a minimal hydrodynamic size prevents rapid elimination. Choosing larger molecules or adding PEG chains can avoid renal filtration.
- The relevance of different uptake mechanisms – clathrin- and caveolin-dependent, as well as clathrin- and caveolin-independent pathways – are not completely understood and not all are productive.
- Endosomal escape is often the limiting factor for a successful gene silencing effect. Strategies to enhance functional uptake include cleavage in the endosome, and addition of endosomolytic agents.

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