Antisense oligonucleotides as therapeutics and their delivery

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Antisense oligonucleotides are novel, highly selective inhibitors or modulators of gene expression. Attention is now being paid for the use of these agents not only in the treatment of genetic disorders but also for non-genetic disorders, where the treatment involves modulation of gene expression. However, antisense oligonucleotides face immense challenges for their use as therapeutics and successful application of these agents requires appropriate design and delivery strategies to increase their stability and intracellular uptake.

Keywords: Antisense oligonucleotides, delivery strategies, gene expression, modulators, therapeutics.

ANTISENSE OLIGONUCLEOTIDES (As-ODNs) are single-stranded molecules which are synthetically prepared. They contain strands of deoxynucleotide sequences comprising 18–21 nucleotides, complementary to the mRNA sequence of the target gene[1]. Most of the human diseases are caused due to inappropriate protein production or disordered protein formation. Conventional drugs target proteins (enzymes, receptors) usually follow an approach that involves screening of thousands of compounds to find a therapeutically active molecule, whereas As-ODNs comparatively follow a rational approach. As-ODNs target mRNA rather than targeting proteins[2]. As-ODNs are formed by manipulation or modification of DNA or RNA so that their nucleotide forms a complementary base pair with the target mRNA and binds via Watson–Crick hybridization[3]. As-ODNs offer potential advantages over traditional drugs as they are easy to manufacture, the target is often one-dimensional compared to multi-dimensional targets in case of proteins and inhibition of mRNA produces quicker and long-lasting effect compared to inhibition of formed proteins targeted by conventional drugs[4]. Gene inhibition based on antisense mechanism was first demonstrated by Zamecnik and Stephenson in 1978 on chicken fibroblast culture infected with Rous sarcoma virus[1]. Introduction of synthetically prepared 13mer ODNs complementary to the 3′-terminal sequence of the Rous sarcoma virus significantly inhibited viral production and prevented fibroblasts to form sarcoma cells[1]. The aim of antisense therapy is to downregulate the expression of disease-causing protein at the mRNA level[2]. As-ODNs selectively bind to target mRNA sequences and cause cleavage of the mRNA, thus inhibiting the expression of the target gene[1]. As-ODNs can also be used for restoring or modifying proteins by interfering with pre-mRNA splicing[5]. They are also being exploited for the treatment of various diseases where protein synthesis is dysregulated, e.g. cancer, HIV, hepatitis, genetic diseases, neurodegenerative diseases, asthma, allergic inflammatory diseases and autoimmune diseases[1,6]. As-ODNs have many potential applications, as they target mRNA which is ubiquitous and more accessible to manipulation compared to DNA. The mRNA of any gene can thus be theoretically inhibited due to the availability of gene sequence information from the human genome.

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Mechanism of antisense oligonucleotides

Normal process of protein synthesis

The normal processing of a genetic message to form proteins involves a series of steps. In the first step, the sense strand of the DNA is transcribed into a pre-mRNA. In the second step the pre-mRNA is converted into a mature mRNA by the action of three separate processes which involves 5′-capping, intron excision and poly-adenylation. In the final step the mRNA is transported into the cytoplasm from the nucleus to the ribosomes for translation into the appropriate poly-peptide chain to form proteins (Figure 1). All these steps are highly regulated and play a major role in the synthesis and functioning of mRNA[3].

Protein synthesis in presence of As-ODNs

As-ODNs are designed in such a way that they bind and interfere with any one of the processing steps of the target mRNA, thereby inhibiting protein synthesis (Figure 2). The following are the major mechanisms of As-ODNs.

RNase H-mediated degradation: As-ODNs can exhibit their action either in the nucleus or in the cytoplasm. In the nucleus they target various regions such as the 5′-region, 3′-region, splice site or any other region on the pre-mRNA. When an As-ODN binds to the target mRNA,

it forms mRNA–As-ODN heteroduplex. This leads to recruitment of an enzyme RNase H[7]. This is an endonuclease which is ubiquitous and is responsible for recognizing and hyrdrolysing the RNA strand of the mRNA–As-ODNs heteroduplex (Figure 2). It serves as a potent mechanism for inhibiting protein expression[8]. However, the cleavage allows As-ODN to be intact so that it can bind to other mRNA copies and cause their downregulation. This enables the As-ODN to be recycled, leading to a long-lasting effect; thus it can be used in micro or nanomolar concentrations[1]. Recognition of As-ODN by RNase H depends on the chemical structure of the oligonucleotide[5].

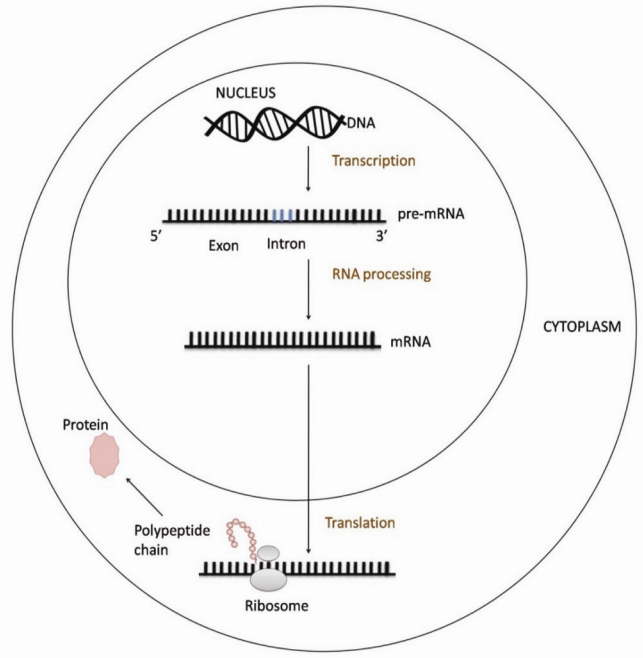

Figure 1. Normal process of protein synthesis.

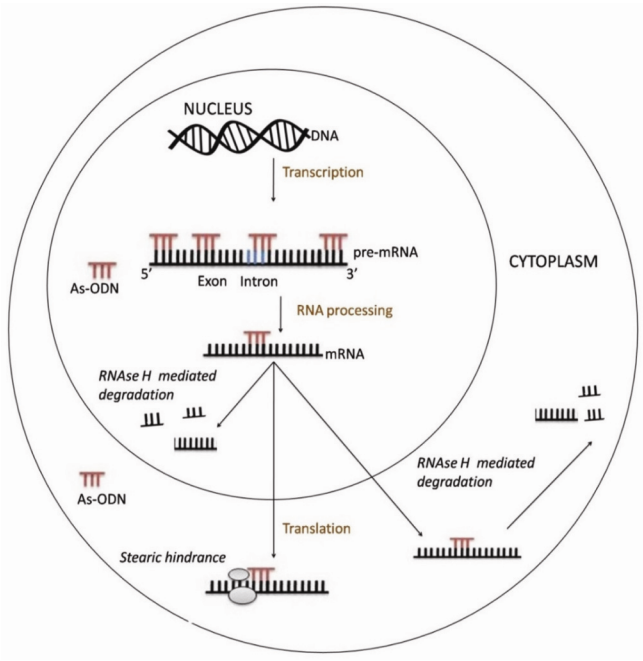

Figure 2. Protein synthesis in the presence of antisense oligonucleotides.

Stearic hindrance: Apart from RNase H-mediated degradation, protein levels can also be reduced by inhibiting translation which occurs in the cytoplasm (Figure 2). This type of mechanism is shown by those As-ODNs where the sugar group has been modified and thus they are not capable of activating RNase H. For RNase H to be activated, the 2′-region of the nucleotide should be unmodified. As-ODNs act by binding to the target mRNA at the translation start site and inhibits the translation process. It can also bind to any other region of the target mRNA providing stearic hindrance for the ribosomes. As a result, the ribosomes cannot traverse along the mRNA and hence protein synthesis is altered[5].

Challenges faced by As-ODNs as therapeutics

Despite having the ability to inhibit protein synthesis, As-ODNs face immense challenges to be used as therapeutic agents. The first major challenge is the action of nuclease enzyme that causes rapid degradation of oligonucleotides and hence reduces their efficacy. A 3′- to 5′-exonuclease degrades naked As-ODNs within 30 min in serum, while intracellular exonucleases and endonucleases degradade the oligonucleotide even in a shorter time span[5]. The second major challenge is limited cellular uptake. Since As-ODNs are negatively charged molecules (due to phosphate backbone), it renders them impermeable to the cell membrane which is also negatively charged[9]. This results in compromised biological activity.

Overcoming the challenges

For As-ODNs to be used as therapeutic agents, it is essential that they overcome the above challenges. This is possible by chemical modification of the structure of oligonucleotides which would make them nuclease-resistant and increase their stability, thus increasing their persistence. The cellular uptake of As-ODNs can be increased using a delivery system. This would also aid in targeted drug delivery or for achieving a sustained effect.

Chemical modification of the structure

Basic structure of oligonucleotides: The simplest and unmodified oligonucleotide is phosphodiester. An oligonucleotide constitutes three major components, viz. phosphate, sugar and base (Figure 3). The use of As-ODNs

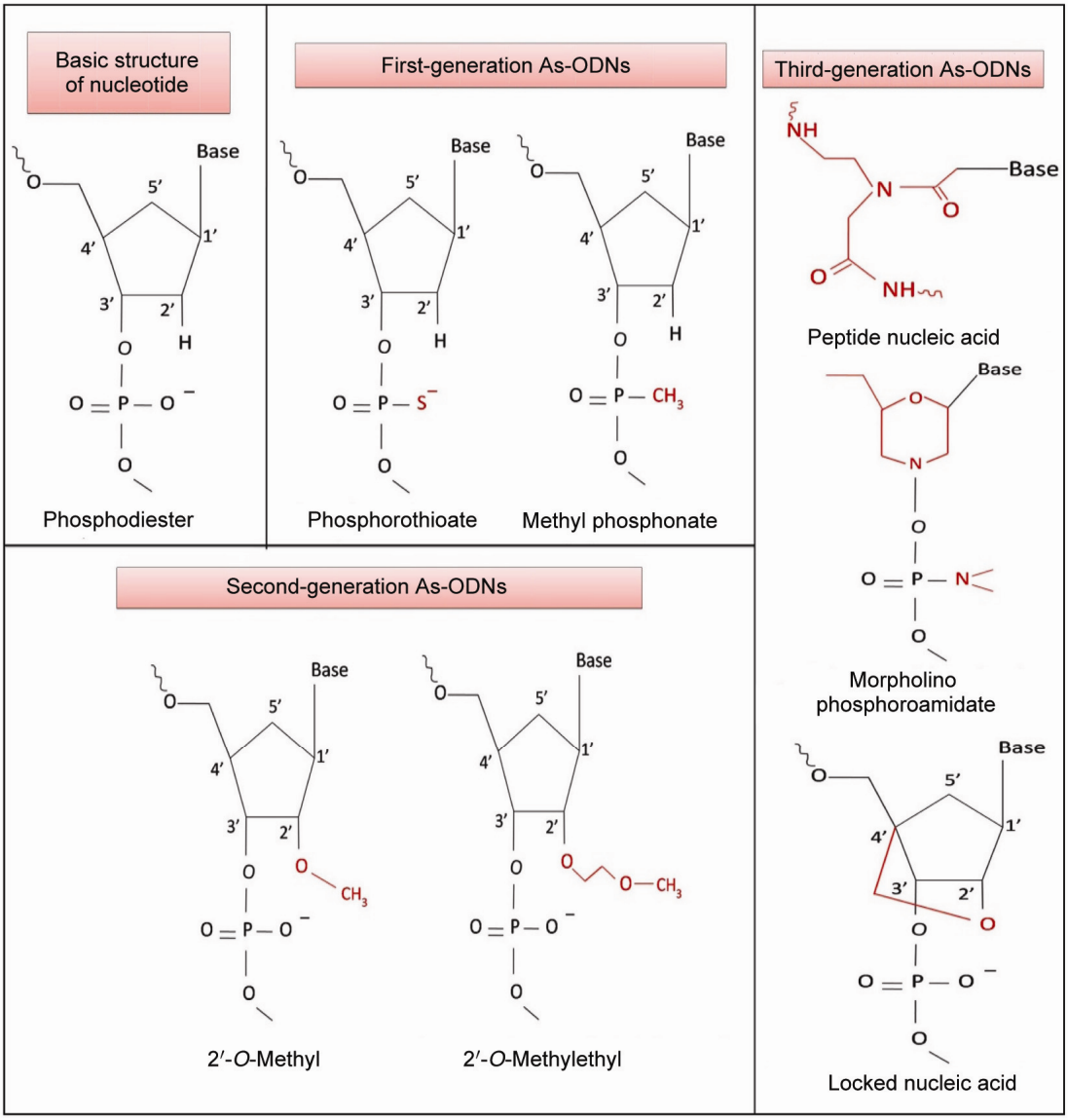

Figure 3. Chemical modification of the structure of oligonucleotides.

as therapeutics is hampered by factors, including poor solubility of oligonucleotides, poor permeation across the cell membrane and quick degradation by nucleases. Thus various chemical modifications have been introduced in the basic structure of As-ODN, in order to increase potency, bioavailability and their stability against nucleases.

Chemical modifications of oligonucleotides

On the basis of these modifications, As-ODNs are classified into three generations: As-ODNs with alteration of the phosphate group, those with sugar modification and those with altered backbone. In all the cases the base is not modified, since it is responsible for binding with complementary nucleotides of the target mRNA (Figure 3). Table 1 summarizes the comparative properties of various generations.

First-generation antisense oligonucleotides

These are formed when one of the non-bridging oxygen atoms in the phosphate group is replaced by a sulphur atom to form phosphorothioates, or a methyl group to form methyl phosphonates respectively (Figure 3). When compared to phosphodiester oligonucleotides, they display ease of synthesis, long plasma half-life and are nuclease-resistant. They have the ability of recruiting RNase H and display appropriate pharmacokinetics. Phosphorothioate oligonucleotides (PS-ODNs) are the most widely used As-ODNs. The FDA-approved As-ODN drug, Vitravene (Formiversin given by intravitreal route

Table 1. Summary of comparative properties of various generations of antisense oligonucleotides

Generation	Examples	Advantages	Limitations	Mechanism
First generation (replacing non-bridging O)	Phosphorothioates, methyl phosphonates	Nuclease resistance than phosphodiester, high-affinity for mRNA	Immune stimulation at high concentration (toxic)	RNase H activity
Second generation (alkyl modification at 2′ position)	2′-O methyl oligonucleotides, 2′-O methoxy ethyl oligonucleotide, chimeric As-ODNs	High nuclease resistance, high affinity, better tissue uptake, less toxic than first generation	Incapable of activating RNase H	Stearic hindrance (chimeric As-ODN: RNase H activity)
Third generation	Locked nucleic acid, peptide nucleic acid, morpholino phosphoramidates	Higher nuclease resistance, higher binding affinity with mRNA, uncharged and so do not bind serum proteins	Rapid clearance, neutral backbones make solubility and uptake difficult (need delivery systems), incapable of activating RNase H	Stearic hindrance

for CMV infection), and majority of As-ODN drugs undergoing clinical trials are PS-ODNs. However, As-ODNs display a tendency of unspecific binding *in vivo* due to interaction with the cell surface and intracellular protein resulting in immune stimulation and complement activation[1,10,11].

Second-generation antisense oligonucleotides

They are formed by introducing alkyl modification at the 2′-position of the ribose sugar (Figure 3). This modification enhances nuclease resistance and improves the binding affinity towards target mRNA. Widely used second-generation As-ODNs are 2′-*O*-methyl (2′-OME) and 2′-*O*-methoxyethyl (2′-MOE) oligonucleotides[1]. Since the 2′-position is modified, these agents are unable to activate RNase H enzyme and thus they exert activity by stearic blocking. Chimeric As-ODN are synthesized to enable RNase H activation. This newly formed As-ODN consists of a central block (gapmer oligonulceotide) of phosphorothioate deoxynucleotide (which induces RNase H cleavage) surrounded by nuclease-resistant arms like 2′-OME or 2′-MOE on both sides (5′- and 3′-directions) thus preventing degradation by nucleases[3,7,12] (Figure 4). Compared to the first-generation, the second-generation As-ODNs are reported to have better tissue uptake, longer *in vivo* half-life and lower toxic effects.

Third-generation antisense oligonucleotides

These are formed by the chemical modification of the furanose ring of ODNs along with modification of phosphate linkages (Figure 3). The introduction of these changes in the structure displays enhanced nuclease stability, more affinity for target and better pharmacokinetic profiles of ODN. Peptide nucleic acid (PNA), morpholino phosphoroamidates (MFs) and locked nucleic acid (LNA) are the three most commonly used third-generation As-ODNs. PNA is formed by replacing the sugar–phosphate backbone with pseudo peptide polymer. MFs are non-ionic As-ODNs which are formed by replacing the ribose sugar with morpholino ring and replacing the phosphodiester bond by a phosphoroamidate linkage. LNA is a ribonucleotide formed by connecting the 2′-oxygen of the ribose with the 4′-carbon through a methylene bridge.

Third-generation As-ODNs have higher biological stability because of higher resistance to nucleases and peptidases, and show strong hybridization affinity with mRNA. However, third-generation As-ODNs do not have the ability to activate RNase H, and thus produce antisense effect by stearic hindrance to ribosomes, leading to translational arrest. Since they are uncharged, As-ODNs have no affinity for serum proteins, lowering the chances for unspecific interactions but, on the contrary, they exhibit rapid clearance from the body. Being electrostatically neutral in nature due to their backbone, As-ODNs face challenges regarding solubility and cellular uptake. Employment of delivery systems *in vitro* to increase their uptake would thus help circumvent this problem. PNA, MF and LNA have demonstrated favourable results in different *in vitro*, *ex vivo* studies. LNA can be incorporated in the DNA to form chimeric gapmers resulting in greater affinity, greater nuclease resistance and the ability to recruit RNase H[1,10].

Pharmacokinetics of antisense oligonucleotides

Various routes, including subcutaneous, intradermal and intravenous and topical are being studied to determine the pharmacokinetic properties of As-ODNs. The pharmacokinetic properties depend on the chemistry of the backbone and are not influenced by the length or sequence[1]. Tissue bioavailability is dependent on protein binding that reduces glomerular filtration and urinary excretion of oligonucleotides. Replacing oxygen by hydrophobic sulphur (PS-ODN) increases stability and binding to serum proteins like albumin and $\alpha 2$ macroglobulin, thus preventing rapid clearance by glomerular filteration. This causes increase in the plasma half-life of the drugs and

increases peripheral distribution[12]. Studies have shown that about 90% of PS-ODNs are plasma protein bounded. The binding affinity for serum proteins was evaluated and found to depend on species in the order: guinea pig > rat > rabbit > human. It was found that at higher concentration excretion of intact oligomers occurs due to saturation of binding sites.

PS-ODNs show a dose-dependent plasma clearance rate, having plasma half-life between 30 and 60 min (ref. 13). There is wide distribution of PS-ODNs in the body, showing peak concentrations in liver, kidney and spleen which are highly perfused. PS-ODNs have a much longer tissue half-life ranging from 1 to 5 days, but show only trace levels of full-length intact ODNs in the tissues after 48 h. They are metabolized by endonucleases and exonucleases, resulting in shorter nucleotides, and ultimately nucleosides that are further degraded by the body through normal metabolic pathways. The major route of elimination for PS-ODNs is through urine. A comparative study between a fully modified 2′-MOE phosphodiester As-ODN, a gapmer PS-ODN and a PS-ODN with 2′-MOE modification only at the 3′-end was performed to determine tissue distribution, protein binding and metabolism of As-ODNs following intravenous injection in monkeys. It was found that both PS-ODNs had a similar plasma half-life (~45 min). On the other hand, plasma half-life of the phosphodiester As-ODN was comparatively short (~14 min). Also, the tissue distribution of both PS-ODNs was almost the same, showing the highest concentrations in liver, kidney, spleen and lymph nodes. Interestingly, the tissue half-life of the gapmer was found significantly higher (~22 days) compared to the 3′-end modified 2′-MOE PS-ODN. Hence for better plasma half-life and efficacy, modification at both 2′-end and 3′-end is required[13,14]. The third-generation As-ODNs such as MFs and PNAs lack charge and are less extensively bound to plasma proteins. They undergo rapid clearance either due to their metabolism or excretion in urine. MFs when administered systemically are distributed rapidly within 1–4 h and have a plasma half-life of 1–9 h. They show rapid accumulation in the organs, including liver and kidney, and are eliminated by urine and faeces[1].

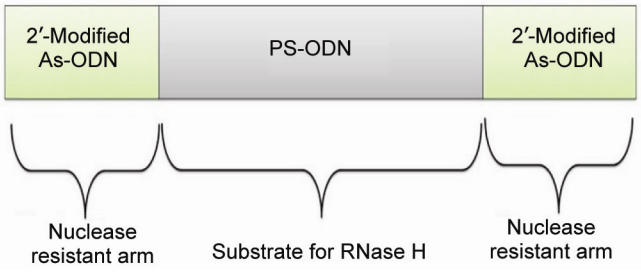

Figure 4. Chimeric antisense oligonucleotides.

Toxicology of antisense oligonucleotides

The toxicological data of As-ODNs obtained by studying their effects in different species, including mice, rats, monkeys and humans demonstrate their safety when used at normal therapeutic dose[1]. The toxicological effects are observed at a value above the normal dose used in clinical studies. Most common acute toxicities include activation of complement cascade, thrombocytopenia and elevation of serum transaminase. Comparatively PS-ODNs are negatively charged than phosphodiester and this results in affinity to various proteins like platelet-derived growth factor, epidermal growth factor receptor, heparin-binding proteins, etc., leading to non-specific interactions. PS-ODNs have the tendency to interact with factor H, a circulating negative regulatory factor causing complement cascade activation via an alternative pathway and subsequent cardiovascular events, such as hypotension. PS-ODNs bind to multiple coagulation factors, including VIIIa, IXa, X and II, affecting the clotting profile. PS-ODNs containing four or more contiguous guanosine residues lead to the formation of quadruple-stranded tetraplexes and other higher-order structures which are extremely biologically active because of their negative charge and produce non-specific side effects like inhibition of smooth muscle proliferation or bone marrow macrophage progenitor cells proliferation. As-ODNs comprising CpG motifs stimulate the immune system in humans. CG dinucleotide found in viral and bacterial DNA is mostly present in the unmethylated form, whereas in humans it is in present in the methylated form; therefore, when As-ODNs comprising unmethylated CpG motifs are delivered to humans, they activate the immune system leading to the release of cytokines, activation of NK and T helper cells, antibody production, and humoral responses leading to splenomegaly and lymphoid hyperplasia[13]. Therefore, newer generations of As-ODN are being designed to overcome this side-effect by removal of the CpG motif or by methylation of cytosine to decrease the immune stimulatory effects. As-ODNs show no teratogenic effects or changes in reproductive performance or fertility in primates and rodents. Various modifications in the chemical structure of As-ODNs have led to safer toxicity profiles, low immune stimulation and fewer nonspecific effects compared to the first-generation As-ODNs[1].

Delivery system

Naked As-ODNs penetrate into the cells by adsoptive endocytosis and fluid phase pinocytosis which depend on the temperature, concentration and structure of the As-ODNs[10]. Unmodified oligonucleotides are negatively charged and barely penetrate the cell membrane. PS-ODN promotes adsorption into cell membrane, whereas PNA and MFs do not possess charge and therefore find it

difficult to interact with cell-surface proteins; as a result, adsorptive endocytosis becomes difficult[11]. Various mechanical techniques, including electroporation and microinjection have shown efficacious results of As-ODN *in vitro* cell cultures; however, their use is limited for *in vivo* application. Therefore, various delivery vehicles are studied in order to increase the cellular uptake of As-ODNs. The carrier system must have the ability to protect the As-ODNs from the extracellular and intracellular degrading enzymes till they reach their target. Also, the carrier system should exhibit prolonged circulation time so that As-ODNs remain for a longer time in the target region. The carrier system must have the ability to interact well with the cell membrane to hasten the uptake of As-ODNs, the carrier must easily escape from endocytic vesicles and dissociate from the As-ODN so that As-ODN can exert its action on the target mRNA[15]. Carriers such as liposomes, dendrimers, polymeric microspheres, nanoparticles, cell-penetrating peptides are employed for delivery of As-ODNs and are discussed below.

Liposomes

These are small microscopic spheres comprising one or more concentric, closed phospholipid bilayers which enclose an internal aqueous compartment. Polar drugs such as first- and second-generation As-ODNs can be incorporated in the internal aqueous compartment. Anionic lipids (negatively charged) have poor encapsulation for nucleic acids (negatively charged) and poor transfection efficiency in cells. Cationic lipids (positively charged) have the highest encapsulation efficiency for As-ODNs (negatively charged) and low transfection efficiency into the cells. Neutral lipids (no charge) have poor encapsulation but highest transfection efficiency. Therefore, usually cationic lipids are favoured along with some amount of neutral lipid to aid transfection. The most common type of cationic lipids are *N*-(2,3-(dioleyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) which is a component of Lipofectin® (commercially available transfection agent) and *N*,*N*-dioleyl-*N*,*N*-dimethylammonium chloride (DODAC) which are quarternary ammonium salts, 3β-[*N*-(*N*′,*N*′-(dimethylaminoethane)carbamoyl]-cholesterol (DC-chol) which is a derivative of cholesterol, dioctadecyldimethylammonium chloride (DOGS) which is commercially available as Transfectam® and has multivalent head group[16,17]. Cationic liposomes are commonly used for delivery of nucleic acids because they are capable of interacting with polyanionic nucleic acids to form lipoplexes[18]. The lipoplexes then enter the cell via the endocytic pathway and form endosome[19]. The release of As-ODNs from endosomes occurs in several steps: (i) Interaction between the positively charged cationic lipids and negatively charged endosomal membrane allowing the flip-flop of anionic lipids present on the cytoplasmic side of the cell membrane. (ii) Formation of ion-pairs with cationic lipids due to the diffusion of anionic lipids of the cell membrane into the cationic lipoplexes. (iii) Displacement by anionic lipids and release of As-ODNs into the cytoplasm[20,21]. *In vitro* studies were performed on G6 glioma cell line. Ultrastructural studies revealed that PS-ODN in the absence of delivery system was found to be sequestered within the endosome and less visible in the nucleus and cytoplasm. Using cationic lipid DOSPA (2′-(1″,2″-dioleoyloxypropyldimethyl-ammonium bromide)-*N*-ethyl-6-amidospermine tetra trifluoroacetic acid) and helper lipid DOPE (dioleoylphosphatidylethanolamine) complex in the ratio of 3 : 1 as a delivery system for ODN, it was found that the extent of ODN cellular association was markedly increased by 10–12-fold with an increase in sub-cellular distribution of PS-ODN[21,22]. Various *in vivo* studies indicated that cationic liposomes show fast serum clearance and adsorption of anionic serum proteins on them, which cause early release of therapeutics. Surface coating of cationic liposomes with PEG (polyethylene glycol) demonstrated better colloidal stability and extended blood circulation half-life[20]. Although cationic lipids have higher encapsulation efficiency, their use as a delivery vehicle is limited because they are cytotoxic in nature. Biodegradable lipids which are easily metabolized by enzymes (esterases and peptidases) can be used to decrease the cytotoxity caused by cationic lipids without affecting biological activity. Cationic lipids containing stable linker groups like ether linkage (e.g. DOTMA) were found to be more toxic compared to lipids with less stable linker groups like ester linkage (e.g. DOTAP). Less stable linkers lead reduction of biological activity and therefore, linkers of the cationic lipids should be such that they display a balance between biodegradability and stability[23]. Another type of liposome is pH-sensitive fusogenic liposome consisting of lipids such as DOPE and a titrable acidic amphiphile like oleic acid (OA) or cholesterolhemisuccinate (CHEMS). The amphile maintains the liposomal structure at pH 7, but as it moves through endosomes the pH decreases and the amphiphile becomes protonated. As a result, the liposome collapses and fuses with the endosomal membrane which then leads to release of liposome contents into the cytoplasm[2]. Cellular uptake of HSA (human serum albumin)-coated liposome–ODN complexes used for Bcl-2 mRNA downregulation in KB (HeLa derivative) human oral carcinoma cells was found to be about three-fold higher compared to uncoated liposomes, and six-fold higher than naked As-ODNs[24]. Targeted action towards specific cells can be achieved by conjugating folate or transferrin to the liposome to assist receptor-mediated endocytosis.

Dendrimers

These are highly branched, tree-like polymeric structures having a core molecule, with the monomer units attached to it. Compared to liposomes, the dendrimer–As-ODNs

Table 2. Antisense oligonulceotides in clinical trials

As-ODNs	Company-partner	Target mRNA	Clinical trial phase	Indication
Alicarforsen	Ionis Pharmaceuticals–Atlantic	ICAM-1	Phase III (completed)	Pouchitis
Oblimersen	Genta	Bcl-2	Phase III	Malignant melanoma, chronic lymphocytic leukaemia, multiple myeloma, acute myeloid leukaemia
IONIS-TTR$_{Rx}$	Ionis Pharmaceuticals–GSK	TTR	Phase III	Familial amyloid poylneuropathy
Nusinersen	Ionis Pharmaceuticals–Biogen	SMN2	Phase III	Children with spinal muscular atrophy
Custirsen	Ionis Pharmaceuticals–OncoGenex	Clusterin	Phase III	Metastatic castrate-resistant prostate carcinoma, non-small cell lung carcinoma
IONIS-HTT$_{Rx}$	Ionis Pharmaceuticals–Roche	HTT	Phase II	Huntington's disease
IONIS-SOD1$_{Rx}$	Ionis Pharmaceuticals–Biogen	SOD1	Phase II	Amyotrophic lateral sclerosis
Apatorsen	Ionis Pharmaceuticals–OncoGenex	Hsp27	Phase II	Cancer
IONIS-PTB1B$_{Rx}$	Ionic Pharmaceuticals	PTP-1B	Phase II	Type-2 diabetes
RESTEN-MP	AVI BioPharma	c-myc	Phase II	Restenosis
GEM 92	Hybridon	HIV-1 gag	Phase II	Human immuno deficiency virus infection
EPI-2010	EpiGenesis	Adenosine A1R	Phase II	Asthma
LErafAON-ETU	NeoPharm	Raf kinase	Phase I	Advanced cancer
LR3001	Genta	c-myb	Phase I	Chronic myelogenous leukemia

As-ODNs, Antisense oligonucleotides; ICAM-1, Intercellular cell adhesion molecule-1; TTR, Transthyretin; SMN2, Survival motor neuron 2; HTT, Hungtitin gene; SOD1, Superoxide dismutase; HsP27, Heat shock protein 27; PTP-1B, Protein tyrosine phosphatase 1B; HIV, Human immunodeficiency virus 1; Adenosine A1R, Adenosine A1 receptor.

complex is more stable; it remains active in the presence of serum and has limited cytotoxicity[10]. It helps in increasing cellular delivery both in the cytosol as well as the nucleus, and thus increases the retention time of As-ODNs in the target cells. The most commonly studied dendrimer for delivery of As-ODN is the PAMAM (polyamidoamine) dendrimer containing amino groups on its surface. It is positively charged at physiologic pH, enabling complexing with oligonucleotides[2]. PAMAM dendrimers have been shown to effectively deliver As-ODNs to correct splicing in HeLa cells which were transfected with plasmid pLuc/705 containing luciferase gene that was interrupted by a human beta-globin intron mutated at nucleotide 705 (responsible for incorrect splicing), thus allowing luciferase expression[25].

Polymeric microspheres

Various *in vivo* and *in vitro* studies of ODNs showed that their biological effects are short-lived and therefore, repeat administration is required for sustained effect. In order to improve both the pharmacokinetics and pharmacodynamics of ODNs different sustained-release polymers are being studied. Microspheres made from biodegradable polymers afford protection to As-ODNs, increase the loading capacity of As-ODNs and also provide site-specific administration with minimum toxicity. The release from these microspheres can be controlled by altering the size and length of As-ODNs, and molecular weight of polymers. The most widely studied polymers for As-ODNs are polylactides and co-polymers of lactic acid and glycolic acid (PLGA). PLGA microspheres containing As-ODNs were prepared by double emulsion method. *In vivo* studies in neostriatum of the rat brain using PLGA microspheres loaded with fluorescently labelled As-ODNs revealed that the latter were significantly visible in the cells after 48 h of administration. In contrast, free fluorescently labelled As-ODNs showed distribution in the cell after 24 h with little or none remaining in the neostriatum after 48 h (ref. 26).

Nanoparticles

Polymerized nanoparticles form a colloidal suspension constituting As-ODNs entrapped, encapsulated or adsorbed to the surface of nanoparticles[19]. Nanogels

consisting of hydrophilic polymeric network formed by the cross-linking of polyethyleneimine (PEI) and PEG were loaded with PS-ODN. They showed higher accumulation in the MDR (multidrug resistant) human oral epidermoid carcinoma cells (KBv) in contrast to free PS-ODN, thereby inhibiting the expression of *P*-glycoprotein efflux pump[27]. Polymers such as PEI and chitosan are being studied for efficient delivery of antisense oligonucleotides[21]. PNA (third-generation As-ODN) which otherwise has low solubility and low cellular uptake has been modified to Mini-PEG-based γ-PNA (increased solubility of PNA) and Mini-PEG guanidium γ-PNA (increased uptake by mamalian cells). *In vitro* studies of these agents demonstrated efficient delivery and reduction in protein levels when loaded in PLGA nanoparticles targetted at CCR5 mRNA in TPH1 cell line[28].

Cell-penetrating peptides

Cell-penetrating peptides (CPPs) are relatively short (9–30 amino acids) polycationic peptides rich in arginine and lysine, with net positive charge. Commonly used CPPs include HIV-1 Tat protein, transportan, antennapedia protein of *Drosophila* and synthetic Pep-1 peptide[10]. To increase the cellular uptake, As-ODNs can be covalently conjugated to any of these CPPs via formation of disulphide bridge. PNA is the most commonly studied As-ODN for CPP-mediated antisense activity[11].

Applications of antisense oligonucleotides

The number of As-ODNs that are being studied is increasing profoundly. The first As-ODN to be marketed was Formiversin (Vitravene) by Ionis Pharmaceuticals in 1998 (ref. 29). This is used in the treatment of cytomegalovirus (CMV)-induced retinitis in AIDs patients and is given by the intravitreal route. It is a PS-ODN (21 nucleotides) and targets the major immediate early regions 1 and 2 (IE1 and IE2) of mRNA of the virus. Another drug that has been approved by the FDA in 2013 is Mipomersen (Kynamro^{TM}) developed by Ionis Pharmaceuticals and Genzyme[30]. This is used to reduce LDL-cholesterol and lipoproteins in patients with homozygous familial hypercholesterolemia and is given by subcutaneous route[31]. It is a chimeric As-ODN having PS-ODN in the middle flanked by 2′-methyl modification on both ends, and targets the mRNA of Apo-B100. Currently, there are many As-ODNs undergoing clinical trials, some of which are shown in Table 2 (refs 4, 15).

Conclusion

Use of As-ODNs serves as a promising and evolving approach for management of numerous diseases. However, clinical use of these agents would require circumvention of the challenges relating to their appropriate design, higher biological stability and targeted delivery. Various *in vitro* studies have revealed increase in cellular uptake of these agents by employing a delivery system[32–34]. However, delivery strategies need to be more refined and *in vivo* studies needs to be employed to evaluate the safety and toxicity associated with the delivery system[33]. Thus with the resolution of these hurdles, As-ODNs would serve as a breakthrough for various life-threatening diseases in the near future.

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