Cell Membranes As Barriers for The Use of Antisense Therapeutic Agents

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Abstract: Antisense oligonucleotides are promising therapeutical agents for numerous diseases resulting from overexpression of genes, expression of mutant genes and viral infections. As most oligonucleotides are polyanions they can not readily pass cellular membranes in adequate amounts to show activity. Therefore, different types of carrier systems and modifications have been developed to enhance absorption and distribution at the level of tissues and cells. The current state of delivery systems will be reviewed with a major part devoted to the commonly used cationic lipids.

I. INTRODUCTION

Antisense drugs utilize the ability of single-stranded oligonucleotides to hybridize with the target sequence in mRNA. In principle, this provides selective drug action only on the target, if the ODN consists of about 15 or more nucleotides. Hybridization results in the arrest of translation due to sterical blocking or by recruitment of the enzyme RNaseH to the binding site. Currently, a number of clinical trials are ongoing for the treatment of various diseases (e.g. Crohn´s disease, non-Hodgkin´s lymphoma, HIV and cytomegalovirus infections) with antisense ODNs and FDA have approved Vitravene (fomivirsen sodium) for the treatment of cytomegalovirus retinitis in AIDS patients.

Although ODNs have poor cell membrane permeability they do have some access to their target site *in vivo*. Therefore, the ongoing clinical trials are carried out using simple salt or buffer solutions of ODNs. Nevertheless, improved penetration into the target cells should provide antisense effect at lower doses, thereby improving the efficacy and reducing the risk of side-effects.

Due to the inefficient uptake of polar and large ODN molecules into most cells, and especially into cytosol and/or

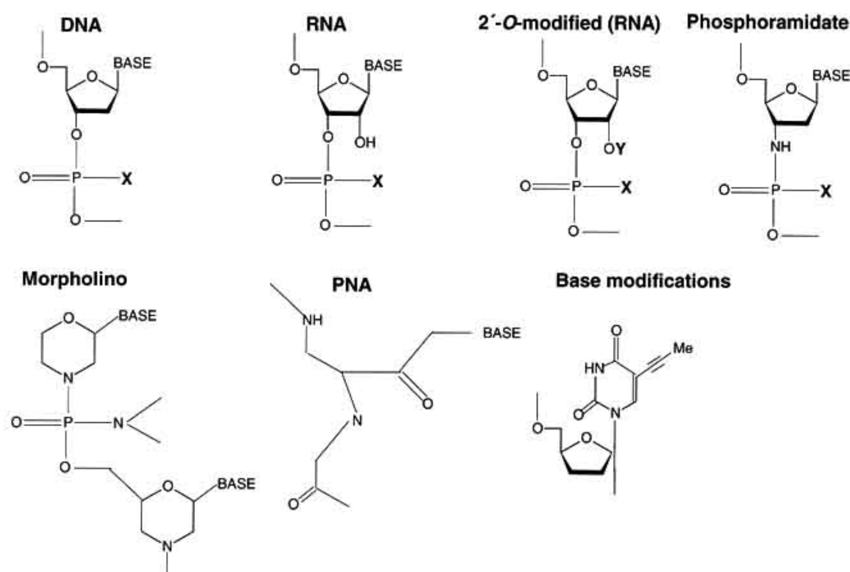

Fig (1). Commonly used oligonucleotides, modifications and analoques. BASE = adenine, cytosine, guanine, thymine, uracil, **X** = e.g. O^-, S^-, methyl; **Y** = e.g. methyl, methoxyethyl (2´-*O*-MOE);. Phosphoramidate (e. g. N3´-O5´ phosphoramidate); PNA = peptide nucleic acid; Base modifications (e. g. propynyl pyrimidine).

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nucleus for activity, various carrier compounds have been introduced. The delivery systems include liposomes, nanoparticles and peptides. In this review we update the current literature on cellular uptake of ODNs and particularly on the delivery systems. Various chemical modifications have been made to the oligonucleotides to optimize cell membrane permeability, hybridization, protein binding, and enzymatic stability. These aspects have been reviewed recently [1,2,3] and are not dealt in detail in this text.

II. OLIGONUCLEOTIDE STRUCTURE, MODIFICATIONS AND MECHANISMS OF ACTION

Commonly used ODN modifications or analoques are shown in **Fig. 1**. Basic single stranded DNA, i.e. oligodeoxyribonucleotide (PO-ODN, ssDNA) is sensitive to extracellular and intracellular nucleases and rapid degradation *in vivo* restricts their use in most cases. Their stability and activity can be increased by labeling, especially from 3´-end. Mechanism of action (**Fig. 2**) for PO-ODN is the formation of a duplex with mRNA that is then degraded enzymatically by the recruited RNase H. Protein synthesis is thereby prevented, and this effect is restricted to the target mRNA by the selective hybridization.

To avoid nuclease degradation, numerous modifications have been introduced. Replacement of one of the non-bridging oxygen atoms in the phosphate with sulphur leads to phosphorothioate oligonucleotides (PS-ODNs). These are easily prepared and most commonly used in antisense research due to their greatly enhanced stability against nucleases. Mechanism of action is similar with PO-ODNs (activation of RNase H). Disadvantages of PS-ODNs include non-specific effects, protein binding and inhibition of RNase H at high concentrations, especially with longer than 20-mer ODNs [4]. The side-effects can be reduced by replacing only some oxygens with sulphur.

To improve target binding and reduce the side-effects of PS-ODNs numerous other modifications have been introduced. Methylphosphonates, peptide nucleic acids (PNAs), morpholino-oligomers, phosphoramidates and 2´-O-sugar modified oligoribonucleotides represent compounds that act with RNase-independent mechanism. Steric blocking of RNA processing, and subsequent inhibition of translation initiation are the main mechanisms of action. Generally 5´ terminus or AUG region are effective targets, while no effect in the coding region is seen.

Recently, correction of aberrant splicing (**Fig. 3**) by oligonucleotides was demonstrated. This may have therapeutic use in the future e.g. in the treatment of thalassemia [5,6] or cystic fibrosis [7]. A single-point mutation in pre-mRNA, that prevents the formation of an active mRNA (excision of introns) and thus prevents normal

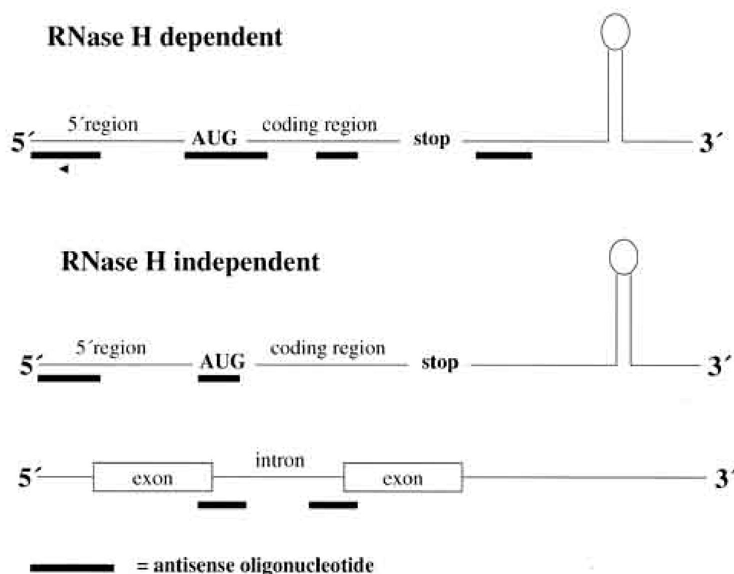

Fig (2). Mechanisms of action of antisense oligonucleotides. RNaseH dependent antisense can be targeted to various sites, while RNase independent mechanism is applicaple only at regulatory regions and at translation start site, Splicing inhibition should be targeted to the exon-intron junctions.

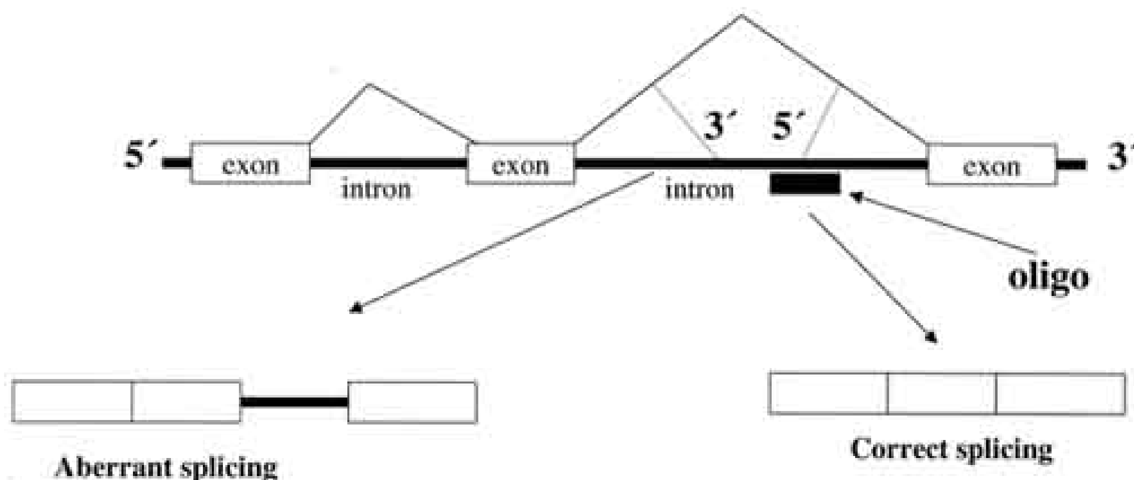

Fig (3). Correction of a mutation with an oligonucleotide leads to correct splicing and subsequent formation of an active mRNA.

protein synthesis can be corrected with an appropriate ODN. 2´-*O*-methyl-oligoribonucleotide (2´-*O*-methyl-ON) is mostly used for splicing correction studies, but other modifications, that do not activate RNase H, can be used as well [8].

III. CELLULAR UPTAKE OF OLIGONUCLEOTIDES

The targets for antisense drugs are intracellular. Therefore, antisense oligonucleotides must permeate into the cells for pharmacological activity.

III.1. In Cell Culture

When ODNs as such are incubated with cells, some cellular uptake is observed [9,10]. Uptake appears to be mediated by endocytosis. Mechanisms of uptake have been investigated but the picture is not clear yet. An 80-kDa surface protein was identified in myeloid cell line HL60 responsible for ODN binding and saturable uptake, that was inhibited by polynucleotides of any length possessing 5´-phosphate [11]. Two ODN binding proteins on the surface of L929 mouse fibroblast and Krebs 2 ascites carcinoma cells with more efficient uptake at low (< 1 μM) ODN concentration, indicating absorptive endocytosis predominant at low ODN concentration, and fluid phase endocytosis predominant at higher concentration, was shown by Yakubov *et al*. [12]. Many of the earlier studies about ODN receptors have been summarized by Vlassov *et al.* [13]. On human HL60, HepG2 and KB cells two ODN binding proteins of about 100-110 kDa were identified by Yao *et al*. [14] and additional 40-58 kDa proteins on HepG2 cells [15]. Benimetskaya *et al.* [16] defined heparin-binding integrin Mac-1 (CD11b/CD18; M 2) as a receptor for ODNs. It is found predominantly on polymorphonuclear leukocytes, monocytes, macrophages and natural killer cells. This study was the first to characterize structurally and functionally an ODN-binding protein. Hanss *et al.* [17] identified a 45-kDa protein from rat renal brush border membrane that, when reconstituted, formed a gated channel that allowed the passage of both PS- and PO-ODNs. Recently, a 66 kDa protein was identified by two methods and purified and partly characterized in HepG2 cell membranes. From the total amount about half was resistant to extensive surface proteolysis suggesting localization both at plasma membrane and cytoplasmic vesicles [18]. Although endocytosis is considered to account almost exclusively for ODN internalization, discrepancies and inconsistencies in results as well as the still unknown mechanism of escape from endosomes has led to the suggestions of multiple mechanisms of uptake [19]. There is also a putative binding site and internalization of DNA in cells. It is still unclear whether this is similar or different from ODN binding proteins. Plasmid DNA is not taken up by the cells *in vitro*, but *in vivo* naked DNA can transfect various cell types at low levels [20]. Also, the physiological role of such mechanisms remains to be elucidated.

After administration of antisense ODN without carrier to cells in culture, free ODN has been found in clathrin coated pits, endosomes, lysosomes, cytoplasm and even in the nucleus by a supposed diffusion driven import process [10]. Nuclear delivery of less than 10 % of total intracellular accumulation for PS-ODN was found by Thierry and Dritschilo [9]. ODNs can readily diffuse from cytoplasm into the nucleus [21] because the molecular sizes of ODNs are smaller than the nuclear pores.

In order to study and compare the efficiences and cellular effects of various ODN modifications or analogues (excluding the ability to penetrate cell membranes) methods like electroporation, scrape-loading and Streptolysin-O (SLO) permeabilization have been used. With SLO the antisense efficacy (KYO-1, chronic myeloid leukaemia cells) has been shown to increase with reduced phosphorothioate content by, for e.g., partial replacement of PS-linkages with 2´-methoxyethoxy (2´-MOE)-PO-ODNs and especially if PO-ODN is protected by 2´-MOE modifications from both 3´ and 5´ends of the ODN [22]. With scrape-loading (HeLa

cells), neutral morpholino oligomers, although ineffective with SLO-permeabilization [23], have been shown to be 3 times more active than 2´-methyl-PS/PO-ONs and 6-9 times more active than 2´-methyl-PS-ONs for splicing correction. Additionally, free uptake from culture medium was about 20 times that of PS-ONs [24]. Recently, cationic PNAs (lysine tail) and morpholino oligomers have been shown to be taken up in free form (HeLa cells, splicing correction) in much higher extent than 2´-*O*-Me or 2´-*O*-MOE PS-ODNs with morpholino oligomers concentrating mainly into the nuclei and PS-ODNs mainly in the cytoplasm. With scrape-loading all oligomers showed similar nuclear accumulation, except 2´-*O*-Me oligomer, that showed poor nuclear uptake indicating possible lower resistance to nucleases [25].

III.2. *In vivo*

Systemic pharmacokinetics of oligonucleotides is a difficult topic of research for several reasons. Firstly, the amount of ODN needed for *in vivo* pharmacokinetic studies, especially in larger animals, like monkeys, is beyond the synthetic capacity of academic laboratories. Therefore, most *in vivo* studies have been carried out in the industry. Secondly, quantitative determination of full length ODN requires capillary electrophoresis analysis or combination of labeled compounds and electrophoretic techniques. Furthermore, reliable extraction of ODN from the tissue samples is labour intensive.

Most *in vivo* studies with ODNs have been carried out in rodents (mice, rats) but also the monkeys have been used [26]. Although extrapolation to humans is difficult, some major features are observed in the animal data. For basic pharmacokinetic profiling the intravenous administration is the starting point and, in the case of ODNs, it is also the current route of administration in most clinical trials [27].

After intravenous bolus injection PS-ODN distributes rapidly to many tissues [26, 28, 29]. ODN concentration in mouse and rat plasma follows two compartment kinetics with rapid distribution phase (half life minutes) to the tissues followed by slow elimination phase (half life several hours or more). There are conflicting values for the half-life of elimination phase [26, 27, 28]. This is due to the analytical differences: the metabolic products of ODNs retain longer in the body and therefore the apparent half-life is prolonged. Species differences and dosing differences contribute as well. However, the sequence differences do not cause kinetic changes after *i.v.* injection [30].

PS-ODNs are distributed especially to the liver and kidney, but also to the muscle, spleen and lungs after intravenous administration (**Fig. 4**) [26,28]. Bijsterbosch *et al*. [31] showed saturable uptake of PS-ODNs (liver, spleen, bone marrow, kidneys) with major uptake by the scavenger receptors of the liver endothelial cells (EC). Similar results were obtained with PO-ODNs showing that the liver accounted for most of the elimination. Interestingly, in this study the clearance of ODN was somewhat variable depending on the sequence. Binding studies indicated saturable uptake mediated by moderate affinity membrane protein [32]. Similar scavenger receptor mediated uptake was suggested by using isolated rat perfused kidney [33]. Distribution to the skin, eye and brain from the blood is very low (**Fig. 4**). Peng *et al*. [29] have generated physiologically based pharmacokinetic model for PS-ODN in rats after *i.v*. administration. Due to the large molecular weight of PS-ODN the distribution to the tissues is limited by the vascular permeability, not by blood flow (**Fig. 4**). However, in addition to the access to the tissues also distribution coefficient Kp (tissue/blood) affects the distribution. Some tissues have surprisingly high Kp values suggesting strong binding of PS-ODN in tissue components. The strorage capacity of each tissue is best estimated on the basis of partial steady state distribution volumes of each tissue. The whole volume of distribution is the sum of all partial volumes of distribution. The largest factions of the total PS-ODN is located in the muscle (24.6 %), liver (26.8 %) and kidney (17.1 %). PS-ODNs do not permeate across the blood brain barrier: the fraction in the brain is 0.3 %.

Distribution into the tissues and subsequent metabolism is considered to be the major factor in ODN clearance from blood circulation (**Fig. 4**). Metabolism takes place from the 3' end of ODN as very little endonuclease activity is seen *in vivo* [34]. Metabolism by nucleases starts rapidly after injection: already at 5 min some chain shortening is seen and the full length ODN remains as majority species for 4 hours [34]. Half life in the tissues varies typically between 20 and 120 hours [34].

Protein binding of PS-ODN in the plasma is a major determinant of their pharmacokinetics (**Fig. 4**). The protein binding varies in mice from 87 –98 % [35]. The major proteins that bind ODNs are albumin and alpha-2-macroglobulin. The protein binding prevents the otherwise rapid excretion by the kidney, since proteins are too large for glomerular filtration (**Fig. 4**). PO-ODNs bind less to the plasma proteins and therefore they are excreted more through the urine than PS-ODNs [34, 35]. Since protein binding extends the half-life, the ODNs have more chances for tissue distribution. Only small fraction of the excreted drug is secreted unmetabolized as full length PS-ODN [35]. Despite its relative stability against exonucleases and endonucleases PS-ODNs retain in the tissues long enough to be eventually degraded. Good inverse correlation has been seen between the clearance of oligonucleotides and their protein binding in the plasma [35]. The higher the protein binding, the slower is the clearance.

PS-ODNs have been modified with 2’-MOE substitution. This improves the metabolic stability, but otherwise 2’-MOE substitution does not affect pharmacokinetics (protein binding, tissue distribution, clearance) after i.v. injection [35]. On the contrary, the nature of the bridge between the nucleotides (phosphodiester or phosphorothioate) affects the pharmacokinetics clearly. PO-ODNs have less protein binding and, therefore, they are cleared more rapidly from the body.

Pharmacokinetics of ODNs is important determinant of the pharmacological activity. This was confirmed recently in *in vivo* study that showed the clear dependence between the ODN concentration in the hepatocytes and mRNA level decrease [36].

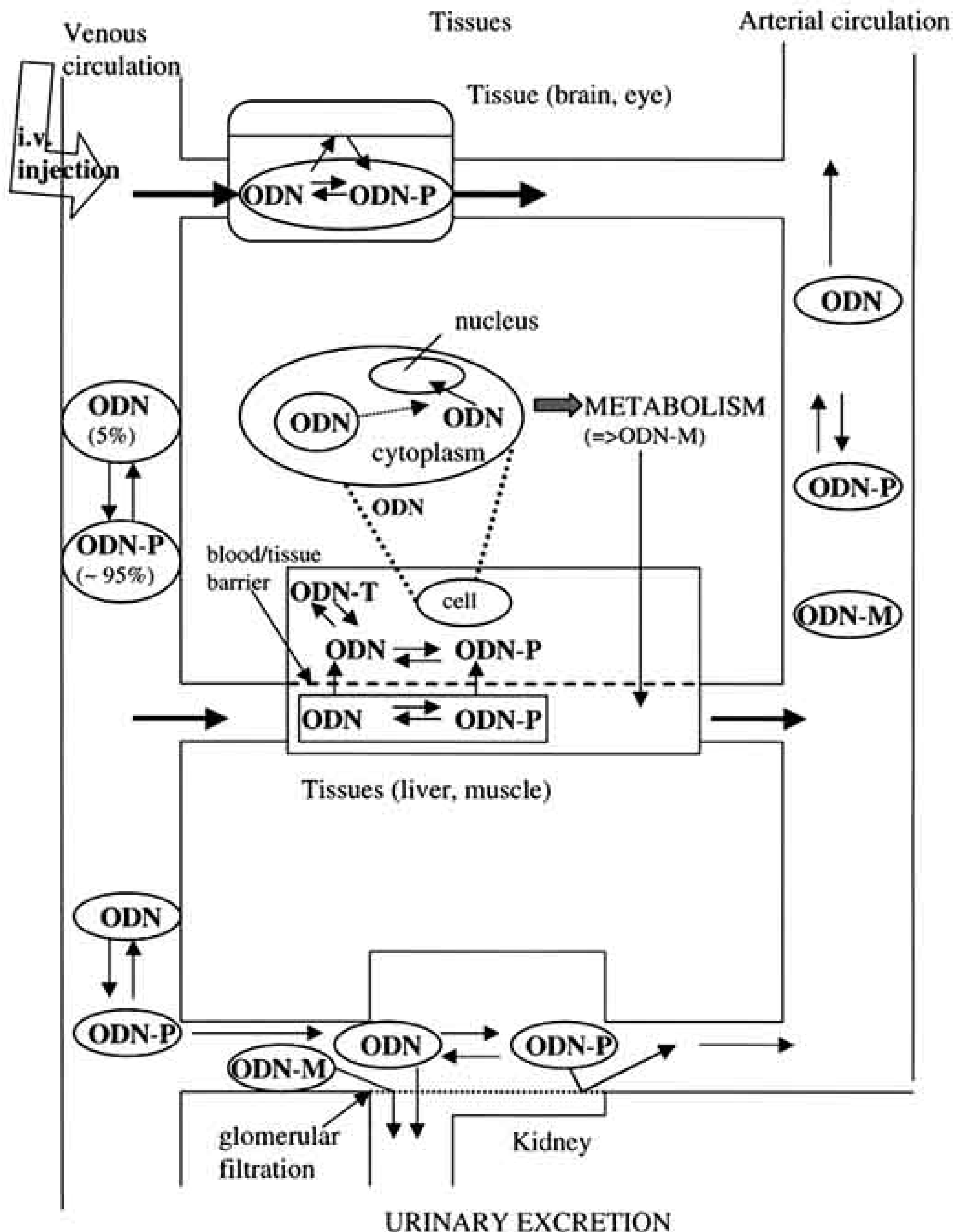

Fig (4). Scheme of phosphorothioate oligonucleotide pharmacokinetics after intravenous administration. ODN = oligonucleotide, ODN-P = protein bound oligonucleotide, ODN-T = tissue bound oligonucleotide, and ODN-M = metabolites of oligonucleotides.

Extravascular administration of ODNs would widen the possibilities of ODNs as therapeutic agents for obvious reasons. Per oral administration is the most desirable route of administration. Despite some high values for bioavailability of ODNs after oral administration, it appears that the absolute bioavailability of ODNs after p.o.

administration is less than 1 % [37]. This is in line with very low permeabilities in the CaCo-2 cell line (1 x 10 $^{-8}$ cm/s). Recently, improved oral bioavailability with PS 2'MOE derivatives was reported [38]. The reported biovailability of 5 % may be adequate for therapeutic purposes (many other marketed drugs show such oral bioavailabilities). However, the variability of drug absorption is high at low bioavailabilites and, furthermore, there may be economical constraints in the case of ODNs (i.e. the dose of the drug must be 20 times higher than actually needed).

Other routes of extravascular administration have also been tested in animals. For example, intraperitoneal and subcutaneous injections yield bioavailabilities of about 30 % in rats [37]. Intratracheal administration resulted in variable systemic ODN absorption. Depending on the dose the bioavailability was 3 – 40 % [37]. In the same study oral bioavailability was less than 1 %. From the drug delivery point of view it is important to realise that the allowed ODN concentration in the plasma is limited by toxicities. The dose limiting toxicities are hemodynamic (drop of blood pressure and complement activation). For safer use of ODNs controlled drug release systems or extravascular administration may have advantages. For many localized applications ODNs do not have to absorb into the systemic circulation for activity. Examples are inhalations, intraocular injections, topical dermal application and direct application to the blood vessel walls or brain during surgical procedures [39, 40, 41].

Interestingly, the first commercial antisense product Vitravene (fomivirsen) is administered by intravitreal injection into the eye. Fomivirsen is a 21-mer PS-ODN in saline solution. The target tissue is retina (cytomegalovirus infection) and the ODN stays in the vitreous and retina (66 µg injection) of the rabbit for about 3 weeks before its elimination. In the vitreous, fomivirsen is eliminated with a $t_{1/2}$ of 62 hr so that 0.17 µM of intact PS-ODN remains after 10 days. In the retina there was accumulation for 5 days (maximally 3.5 µM). Thereafter, elimination with $t_{1/2}$ of 79 hr occurred and the concentration of intact PS-ODN was 1.6 µM after 10 days [42]. This provides adequate driving force so that anti-viral drug concentrations are achieved in the CMV infected in the cells.

IV. OLIGONUCLEOTIDE DELIVERY SYSTEMS

Chemical modifications have been used to improve the properties of ODNs. However, these modifications change the properties in several ways, not limited to the cellular delivery. Carrier systems can be used for improved delivery without changes in the hybridization and other properties.

IV. 1. Liposomes

Cationic liposomes are the most commonly used *in vitro* transfection agents for ODNs. They bind negatively charged ODNs by electrostatic interactions. Upon complexation at +/- charge ratios (cationic lipid/ODN phosphates) above unity the surface charge (zeta potential) will be positive. The size of these complexes varies from about 50 nm to µm scale depending on buffer species, charge ratio and concentration. Each complex contains large number of ODN molecules and their dimensions are substantially greater than those of ODNs as such. The cationic lipids enhance the uptake of ODNs (or plasmid DNA) by binding to the negatively charged cell membranes, presumably to glycosaminoglycans [43]. Enhanced ODN uptake and activity was first shown by Bennett *et al*. [44] and increase in stability against nucleases by Lappalainen *et al* [45]. Structures of the most commonly used cationic lipids for ODN transfections and helper lipid DOPE are shown in **Fig. 5.** After the introduction of these carriers, a large amount of novel transfection reagents [46, 47] have been introduced, but the data is mostly about DNA transfections, and much less information is available about ODN delivery with various liposomal carriers.

IV. 1. 1. Cationic lipid/ODN Complexes In vitro

Cationic lipid DNA complexes (lipoplexes) bind to the cell surface proteoglycans and mediate the transfection *in vitro* [43] and *in vivo* [48]. Lipoplexes bind to the negatively charged membrane-associated proteoglycans (e.g. heparan sulfate), which is followed by endocytosis. Transfection activity diminishes dramatically, e.g., after enzymatic removal of proteoglycans or after inhibition of their sulfation. Additionally, transfection of a mutant, proteoglycan deficient cell line led to 50-fold decrease in transfection activity [43]. On the other hand, extracellular proteoglycans (secreted by the cells) have been shown to decrease the transfection activity of the complexes by impairing the uptake or intracellular behavior of the complexes [49], although the effect on cellular uptake and activity is highly dependent on the carrier used [50, 51]. This may also be an important factor in *in vivo* transfer of the complexes of DNA and ODNs.

A significant increase in cell association and activity was found for PS-ODNs used with DOTMA/DOPE compared to ODNs alone and the enhancement was attributed to increased cellular uptake and, especially, to the altered intracellular distribution [44]. Similar results were reported, when DOTAP or DDAB/DOPE were used as transfecting agents [45,52]. They also showed that the cationic lipids can protect ODN from degradation in the cells. From positively charged DOTAP complexes, fluorescently labeled PS-ODN has been shown to redistribute from punctate cytoplasmic regions into the nucleus and cytoplasmic delivery at an early stage of endocytotic pathway was proposed [53]. ODNs have been shown to dissociate from cationic lipids before entering the nucleus [54, 55] and from the cytoplasm they diffuse readily into the nucleus [21]. A mechanism for ODN release from cationic liposomes was proposed, where a flip-flop of anionic lipids from cytoplasmic facing monolayer occurs and anionic lipids diffuse into the complex leading to ODN release from cationic lipid and subsequent release into the cytoplasm [54]. Similarly it has been shown, that various anionic vesicular structures lead to DNA release from the complexes if the negative charges in these structures were equal or in excess to negative charges in DNA [56]. Negatively charged micellar or vesicular or polymeric aggregates (negatively charged compounds alone are usually

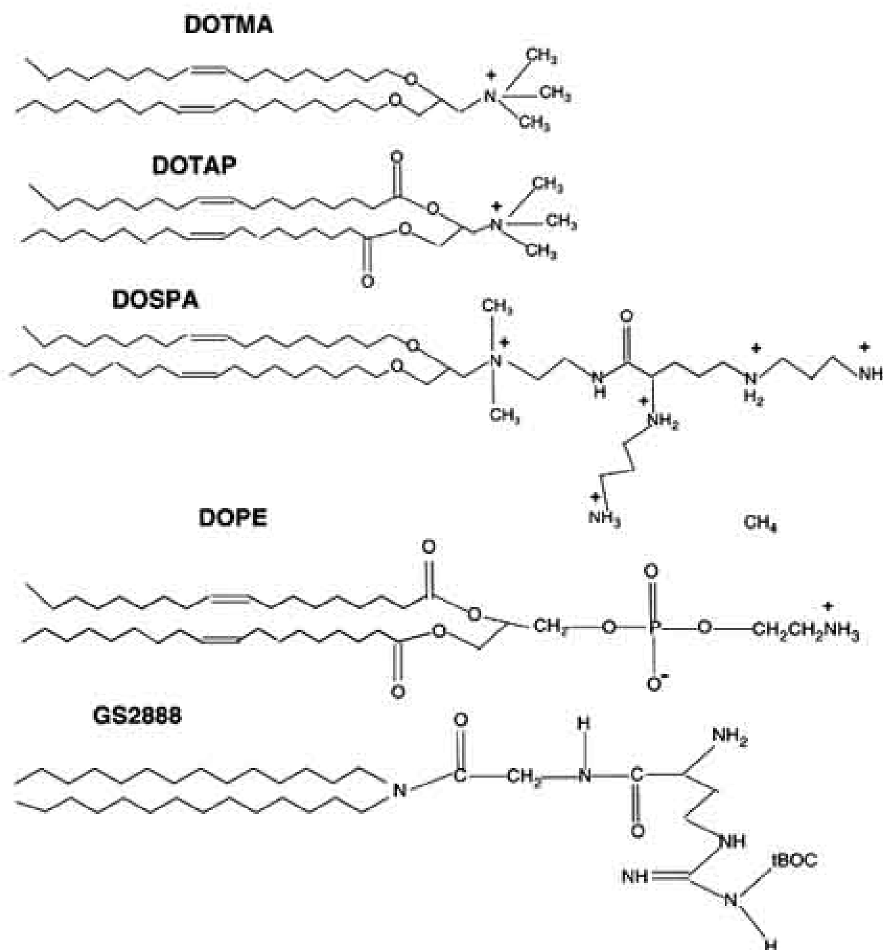

Fig (5). Structures of some commonly used liposomal ODN transfection agents and helper lipid DOPE.. DOTMA = *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium (chloride); DOTAP = *N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium (propane); DOSPA = (2,3-dioleoyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate); DOPE = 1,2-Dioleoyl-3-phoshatidylethanolamine; GS2888 = Dimyristylamidoglycyl-N^{w}-isopropoxycarbonyl-arginine dihydrochloride (=Cytofectin GS in combination with DOPE).

not efficient) are needed for DNA release and the release requires electrostatic as well as hydrophobic interactions [57]. Negatively charged compounds alone were usually not efficient, although GAGs (e.g. heparan sulfate) were not studied.

IV. 1.2. Helper lipids

In order to facilitate membrane fusion at endosomal level fusogenic helper lipids may be added to the liposomes. DOPE (dioleoylphosphatidylethanolamine) is widely used transfection enhancing component with cationic lipids. Due to its small polar headgroup and neutral net charge it is less hydrated than many other phospholipids with larger and/or charged headgroups. In addition, unsaturated acyl chains give DOPE a cone-like structure and at physiological pH it tends to form nonlamellar structures. PS-ODNs have been shown to be displaced from DOTAP liposomes when they are incubated with negatively charged fluid state liposomes but not with neutral or negative fluid/solid state liposomes [53]. Complexes that include DOPE release ODNs even in contact with model membranes containing solid state lipids [58] indicating higher fusogenic activity and enhanced release of ODN upon contact with lipid bilayers at endosomal level [58,59]. Antisense effect against luciferase expression in CV-1 fibroblasts and D 407 retinal pigment epithelial cells was higher with DOTAP/DOPE than with DOTAP liposomes [60]. DOTAP alone destabilizes purified lysosomal membranes but the effect is more pronounced at pH 7.4 than 5 [61], whereas release from DOPE –containing complexes may actually increase at lower pH [58]. Mui *et al*. [59] found about 10-fold enhancement in antisense

activity against EGFR (epidermal growth factor receptor) with PS-ODNs. They also concluded, that fusion was not the mechanism of the enhancing effect but more the ability to disrupt membrane integrity. In a study with 9 different cationic lipids, it was found that DOPE was required for maximal activity of all lipids (including DOTMA). The only exception was a cationic lipid containing myristoyl (C14, fluid state lipid at RT) acyl chains [62], that are also present in highly active Cytofectin [60, 63, 64]. In the case of plasmid transfections, C12:0 or C14:0 containing carriers (vectamidine, DMRIE-C, 1,4-dihydropyridine amphiphiles) were more active than C18:1 lipids [46, 65, 66]. This can partly be explained by inefficient DNA interaction due to lesser flexibility of solid state lipids (C16:0, C18:0) at room temperature and subsequently fluid state lipids are more efficient in transfections [57].

DOPE facilitates membrane fusion, PS-ODN release from the lipid complexes and antisense activity, although complexation and cell growth medium affects the complex size, morphology, and subsequently the transfection activity [58, 60]. Eventually, DOPE containing complexes are more sensitive to the effects of the medium than the other complexes. Similarly to DOPE, a biodegradable pH-sensitive surfactant DIP [(2-1´-imidazolyl) propionate] in PS-ODN/DOTAP complexes increases the cytosolic delivery of ODN, although total cell delivery is not affected, whereas no effect on plasmid DNA distribution was seen [67]. Inclusion of DOPE in liposomes enhances ODN release from the complexes [58] and this may explain the superior ODN transfection with DOPE containing complexes [58, 60]. An interesting hypothesis that DOPE facilitates DNA release suggests that the negative charge in DNA phosphate group actually binds to the positive charge in PE instead of the cationic lipid and this binding is much weaker thereby facilitating enhanced DNA release in contact with negatively charged membranes [68]. On the other hand, the morphology of ODN complexes with DOPE liposomes is also different (tubular hexagonal) compared to the complexes of ODNs without DOPE (´sandwich´ type structures) [58, 69, 70].

IV. 1. 3. Liposomes In vivo

ODN complexation with cationic lipids generates complexes with various sizes but always bigger than free ODN. These complexes may also have cationic surface charge. These factors change the *in vivo* pharmacokinetics of ODNs as expected. Litzinger *et al.* [71] found that DC-Chol/DOPE liposomes (*i.v.* injection to mice via the tail vein) accumulation was highest (about 70%) in liver, with about 10-fold lower accumulation into spleen and skin. PS-ODN/cationic lipid complexes, on the contrary, showed high transient accumulation in the lung in 15 minutes followed by rapid redistribution to the liver (Kupffer cells). This suggested embolism caused by large aggregates in pulmonary capillaries at early stages. Additionally, no nuclear delivery of ODN was observed. Bennett *et al.* [72] showed a broad distribution of a plain PS-ODN to many tissues, main locations being liver, kidney, skeletal muscle and skin. Complexing with DMRIE/DOPE led to increased distribution to liver, lung and spleen indicating that the biodistribution is altered by complexing with cationic lipids. Several studies with plasmid DNA/cationic lipid complexes have shown similar tissue distribution, i.e., accumulation into the liver, lung, and spleen. This is accompanied by short half-life in plasma.

Rapid accumulation of liposomes and lipid complexes in the liver and spleen is not optimal, e.g., if ODN is targeted to the tumors. Furthermore, the mobility and stability of lipoplexes in the extracellular matrix is not optimal due to the negative charges in the matrix [Pitkänen *et al.*, unpublished]. Therefore, the lipid complexes have been modified in order to prolong the half-life in circulation. Semple *et al.* [73, 74] have developed so called ´stabilized antisense-lipid particles´ (SALP) utilizing ionizable aminolipid (DODAP) that is positively charged at acidic pH and binds ODNs. Additional components are phosphatidylcholine, cholesterol and a pegylated lipid. High encapsulation efficiency (about 70 %) and greatly enhanced circulation times as well as the delivery to model sites of inflammation and tumor [73] were reported. Encapsulation of PS-ODN (5-50 mg/kg) in liposomes consisting of egg PC and Chol showed antisense activity against ICAM-1 (mice) in the same inflammation model [75]. A similar system to [73] utilizing cationic lipid (DOTAP)/ODN complexes coated with neutral lipids [76] to decrease aggregation and liver/lung accumulation, showed over 10-fold increase in blood half-life of ODN [77]. In addition to prolongation of half-life liposomal formulations also protect ODN from nuclease mediated degradation *in vivo* [76, 78].

IV. 2. Polymers

Similarly the cationic lipids, cationic polymers bind to ODNs via electrostatic interactions and the complexes are taken up by the cells endocytotically, apparently by a process mediated by glycosaminoglycans [50].

Polyethyleneimine (PEI) was introduced as a transfection agent due to its proposed lysosome buffering capacity, that prevents DNA from degradation and may facilitate escape from the endosomes due to swelling and consequent membrane rupture. A PO-ODN uptake and nuclear delivery into chicken embryonic neurons was shown by Boussif *et al.* [79], but PEI seems to be inefficient in the delivery of PS-ODN [60, 80]. Very stable complex formation between PEI (25 kDa) was shown to inhibit PS-ODN release from the complex and, subsequently, antisense activity, as opposed to a 15-mer 3´-capped (for nuclease protection) PO-ODN [80].

Starburst polyamidoamino polymers (PAMAM) are prepared by stepwise polymerization of a core molecule and depending on the extent of polymerization different generations (G, increase in molecular weight) of spherical polymers with molecular weight distribution can be achieved. PAMAM dendrimers contain positively charged amino groups on their surface. Dendrimers with defective branching have been shown to be more active in DNA transfections [49, 81].

The results of ODN delivery experiments with dendrimers are somewhat contradictory. PO-ODNs complexed with G7 dendrimers caused modest decrease of

about 25 % of luciferase activity in D5 cells [82]. In splicing correction experiment (HeLa cells) 2´-O-methyl PS-ODNs complexed with dendrimer showed activity in serum-containing medium [83] and resistance to 10 % FBS of G3 dendrimer and Superfect (a commercial dendrimer) has also been claimed [84, 85]. Yoo and Juliano [86] also showed 10-fold increase in efficacy for splicing correction with G5 dendrimers conjugated with Oregon green 488 suggesting that the hydrophobicity of the dye improved transfection efficiency. In comparative studies with different carriers in 2´-O-methyl PS-ODN and PS-ODN transfections, fractured dendrimer complexes showed poor activity compared to liposomal [60, 87]. Splicing correction system is more sensitive than antisense efficacy. Therefore, splicing correction does not necessarily mean that antisense efficacy is detected. Despite their high activity in DNA transfections, for unknown reason, dendrimer and PEI are relatively poor systems for ODNs, especially for PS-ODNs.

Polylysine (PLL) complexation enhances ODN uptake, but for antisense effect conjugation of ODN to PLL or adding enhancing moieties to PLL is needed. Using glycosylated PLL inhibition (IC_{50}=500 nM) of ICAM-1 expression with PS-ODN in A 549 cells was achieved, while non-glycosylated PLL failed to show any activity, although the cellular association enhancement was similar (10-15 –fold) [88]. *In vivo* (*i.v.*) studies with net negatively charged glycosylated-PLL complexes with PO-ODN and PS-ODN led to increased hepatic uptake and decreased urinary clearance with galactosylated PLL leading to greater parenchymal cell (PC) accumulation compared to mannosylated PLL with equal PC and non-parenchymal (NPC) distribution [89].

Experiments with polymeric complexes *in vivo* with ODNs are still sparse and their efficiency as *in vivo* delivery system is unclear.

IV. 3. Nanoparticles

Nanoparticles (nanospheres or nanocapsules) are submicrometre scale colloidal systems that are generally prepared from polymers. They have been studied as ODN carriers [90, 91]. Particularly studies have been done using polyhexylcyanoacrylate (PACA) polymer particles in combination with cationic copolymers or cationic hydrophobic detergents (e.g. cetyltrimethylammonium bromide, CTAB) to enable ODN binding by ion pairing [92]. PO-ODN complexed to nanoparticles was stable against phosphodiesterase for 5 hours (half-life of free ODN was 2 minutes), and half-lives in cell growth medium were 130-195 minutes (4 and 90 min for free ODN) depending on the nuclease activity when adsorbed with polyisohexylcyanoacrylate (PIHCA) nanoparticles. About 8-fold cell uptake (24 h) was found for PIHCA/ODN nanoparticles compared to free ODN and 20 % of both were found in the nucleus. Intact ODN was only found in the nuclear fraction of cells when incubated with free ODN, whereas it was also found in the extranuclear fraction after incubation of ODN with nanoparticles indicating probable entrapment in lysosomes/phagosomes after endocytosis, that was established as likely mechanism of uptake [93].

In order to improve serum stability and suitability for *i.v.* administration, PIBCA nanocapsules with an aqueous core containing ODN were developed, but no antisense effect was shown [94]. Polymethylmethacrylate (PMMA) core shell nanospheres with quaternary ammonium groups on the surface binding *c-myb* antisense PO-ODNs increased cell uptake 50-fold as well as significantly decreased *c-myb* protein levels after 4 days treatment [95]. A similar inhibition of ecto-5´-nucleotidase in PC12 cells for both PS-ODNs and PO-ODNs has been established using cationic monomethylaminoethylmethacrylate (MMAEMA) copolymer nanoparticles [96]. Other particles shown to enhance *in vitro* delivery of ODNs include e.g. systems based on poly (D,L) lactic acid [97] or biodegradable poly(lactide-co-glycolide) [98]. A simple nanoparticle/peptide complex prepared simply by mixing of ODNs with cationic 4000 Da protamine has been shown to internalize ODNs into the cytoplasm and nucleus, although the antisense activity remains to be evaluated [99].

Polyisobutylcyanoacrylate (PIBCA) nanoparticles loaded with PO-ODN increased initially the distribution into the liver at the expense of kidney and bone with some protection against nucleases in plasma [100].

IV. 4. Peptides

Peptides are recognized by receptors on the cell surface and amphiphatic peptides are able to destabilize cell membranes [101]. Therefore, peptides are potential targeting ligands and delivery enhancers for ODNs. Even when interesting peptide sequence has been identified the peptide can be utilized in different ways (e.g. complexed or conjugated with ODN or attached to liposomal or polymeric delivery system. Peptide-ODN conjugates have been reviewed recently [102]. Therefore, we do not review that literature.

In addition to the conjugation, peptides like JTS-1 have been used as components in liposomal ODN complexes to augment ODN delivery [60]. Likewise, hydrophobic and hydrophilic domains containing peptide (MPG), complexed with ODN showed greatly enhanced uptake and nuclear delivery, even in the presence of serum, with proposed non-endosomal pathway of internalization [103]. Conjugation of peptide to ODN yields lower molecular weight system than supramolecular complexation. Furthermore, upon complexation it is often difficult to get monodisperse particles with predefined size. Therefore, conjugates may have advantages *in vivo* in terms of wider body distribution (i.e. higher volume of distribution). Complexed systems may be suitable in localized applications and for *in vitro* delivery of antisense ODNs. However, the effects of peptides on *in vivo* delivery are not well known.

IV. 5. Lipid-ODN conjugates

Multiple drug resistance (MDR) mRNA levels in mouse 3T3 fibroblasts were inhibited to a similar extent (50-60%) by free 5´-cholesterol conjugated PS-ODNs and unconjugated PS-ODNs complexed with Lipofectin with a

more homogenous cell distribution of conjugated PS-ODNs [104]. Cholesteryl–conjugated PS-ODNs have been shown to bind high molecular weight plasma proteins more extensively than unconjugated PS-ODNs, leading to an increase in circulation half-life and high accumulation in different cell types in the liver [105].

V. CONCLUSIONS

Oligonucleotides are an interesting group of potential drugs acting with various mechanisms intracellularly. In the post-genomic era there will be a rapidly growing precise information about the mechanisms of diseases and mutations behind them. In this context there will be plenty of potential uses of ODNs. Poor cell membrane permeability remains as a factor that limits the usefulness of ODNs as drugs. Emerging knowledge about the delivery mechanisms of ODNs and novel delivery systems shall widen the usefulness of ODNs as drugs of the post-genomic era.

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