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ILPO JÄÄSKELÄINEN

Cationic Lipids as Antisense Oligonucleotide Carriers into Cells

Studies on Complexation and Transfection

Doctoral dissertation

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ABSTRACT

Antisense oligonucleotides (ODNs) bind to a complementary sequence in mRNA of the selected target gene by Watson-Crick hydrogen bonds and thus can inhibit protein synthesis. The arrest of translation can be due to sterical blocking or by recruitment of the enzyme RNaseH to the binding site. ODNs can possibly be used as therapeutic agents for various diseases by blocking gene over rexpression, interfering with mutant gene and inhibiting viral infection. As most ODNs are polyanions they can not pass cellular membranes in adequate amounts for pharmacological activity.

The objective of this study was to evaluate widely used cationic lipids as ODN carriers into cells *in vitro*. Cationic lipids form micelles or liposomes in the water solutions and are able to bind negatively charged ODNs by their cationic charges on the polar head-groups, but the physical chemistry of this system is poorly understood. We studied the physicochemical properties such as aggregation, size distribution, lipid mixing (fusion) and morphology of the complexes in buffer and in cell culture medium. These properties were related to *in vitro* antisense effect in luciferase expressing cells. Finally, a novel cell line based on nuclear receptor CAR (constitutive androstane receptor) was developed, where luciferase activity can be switched off or on with some steroids or drugs, respectively. This cell line can be used in antisense experiments.

PS-ODNs (one non-bridging oxygen is replaced by sulphur) were complexed with cationic liposomes DDAB/DOPE or DOTAP at different ratios with apparent -/+ charge ratios of 0.03 - 5.6 in 20 mM Hepes, 150 mM NaCl buffer (pH 7.4). Mean size of the complexes increased with charge ratio (-/+) so that at charge ratios of 0.4 - 2.0, the size increased by at least an order of magnitude due to the ODN induced lipid aggregation. At lower and higher -/+ ratios the mean complex size was smaller (about 50 – 200 nm). FRET based lipid mixing experiments showed ODN induced fusion of cationic liposomes. The fusion was rate-controlled by the initial aggregation step. Importantly, DOPE containing liposomes showed at least 3-fold higher maximal lipid mixing.

Similar behaviour was found, when preformed complexes (in water) were added into buffer or DMEM. Freeze-fracture electron microscopy showed, that DOPE in DOTAP/PS-ODN complexes induced hexagonal lipid tubule formation that was most pronounced in cell culture medium, whereas without DOPE the complexes were mostly aggregates. Upon incubation (at pH 5, 6 and 7.1) with endosomal membrane mimicking liposomes ODN was partly released from the complexes and the amount of ODN released was significantly higher when the complexes contained DOPE, especially when incubated at acidic pH. These results indicate that DOPE may facilitate the release of ODN from the complexes and its escape from the endosomes.

Different ODN carriers were tested to assess the importance of physicochemical properties in respect to cellular delivery of active PS-ODN. Cationic polymers, polylysines (PLL), polyethyleneimines (PEI) and PAMAM dendrimer were compared with various lipid-based vehicles. Additionally a membrane active JTS-1 peptide (conformational change at endosomal pH) was used with some carriers. Significant antisense effect was seen only with lipid based carriers with a membrane active component (DOPE or JTS-1). The effect of the complexation medium was also studied. Complexes prepared in water were generally the most effective ones indicating that a membrane active component and small complex size are needed for an efficient cellular delivery of ODNs.

Finally, we generated a novel cell line expressing luciferase gene under the control of drug-responsive nuclear receptor CAR using the tetracycline-regulated system (tTA). The kinetics of luciferase expression and its inhibition by antisense ODNs in cells compare well with those in the tTA system and due to its wide ligand selectivity and transferable ligand binding domain, CAR responsive expression system is an alternative system for regulated gene expression. This system was found applicable for the studies on antisense effects with cationic carriers.

National Library of Medicine Classification: WB 340, QU 85, QU 57
Medical Subject Headings: drug delivery systems; drug carriers; oligonucleotides; antisense; lipids; liposomes; particle size; polymers; gene expression; receptors, cyoplasmic and nuclear

Dedicated to Maria Jääskeläinen (1905-1996)
and Joe Strummer (1952-2002)

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Kuopio, April 2003

Ilpo Jääskeläinen

ABBREVIATIONS

ANDR	5α-androst-16-en-3α-ol
CAR	constitutive androstane receptor
CE	capillary electrophoresis
Chol	cholesterol
CV-1	monkey kidney fibroblasts
D 407	retinal pigment epithelial cells
DPPG	1,2-dipalmitoyl-3-phoshatidylglycerol
EPC	egg phosphatidylcholine
FITC	fluorescein isothiocyanate
FBS	fetal bovine serum
FRET	fluorescence resonance energy transfer
β-gal	beta-galactosidase
G418	geneticin
Gang	gangliosides
HEK293	human embryonic kidney cells
Hepes	[N-2-hydroxyethylpiperazine-N′-2-ethane sulphonic acid]
HYG	hygromycin B
ITP	isotachophoresis
JTS-1 peptide	NH_2 -GLFEALLELLESLWELLLEA -COOH
LUC	luciferase
MES	2-N-morpholino ethane sulphonic acid
N-NBD-DOPE	[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-DOPE]
N-Rh-PE	[N-(lissamine rhodamine B sulfonyl)-PE]
ODN	oligo(deoxyribo)nucleotide
PAMAM	polyamidoamine
PBREM	phenobarbital-responsive enhancer
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEI	polyethyleneimine

PI	phosphatidylinositol
PLL	poly-(L-)lysine
PS	phosphatidylserine
PO-ODN	phosphodiester oligo(deoxyribo)nucleotide
PS-ODN	phosphorothioate oligo(deoxyribo)nucleotide
QELS	quasi-elastic light scattering
RET	resonance energy transfer
REV	reversed phase evaporation
RLU	relative luciferase unit
SAXS	small-angle x-ray scattering
SM	sphingomyelin
SUV	small unilamellar vesicle
TCPOBOP	1,4-*bis*[2-(3,5-dichloropyridyloxy)]benzene
TET	tetracycline
tTA	tetracycline-controlled transactivator

LIST OF ORIGINAL PUBLICATIONS

This doctoral dissertation is based on the following publications, referred in the text as Roman numerals **I-IV**. Some unpublished data are also included.

I I. Jääskeläinen, J. Mönkkönen, A. Urtti: Oligonucleotide-cationic liposome interactions. A physicochemical study.
Biochimica et Biophysica Acta 1195: 115-123, 1994.

II I. Jääskeläinen, B. Sternberg, J. Mönkkönen, A. Urtti: Physicochemical and morphological properties of complexes made of cationic liposomes and oligonucleotides.
International Journal of Pharmaceutics 167: 191-203, 1998.

III I. Jääskeläinen, S. Peltola, P. Honkakoski, J. Mönkkönen, A. Urtti: A lipid carrier with a membrane active component and a small complex size are required for efficient cellular delivery of anti-sense phosphorothioate oligonucleotides.
European Journal of Pharmaceutical Sciences 10: 187-193, 2000.

IV P. Honkakoski, I. Jääskeläinen, M. Kortelahti, A. Urtti: A novel drug-regulated gene expression system based on the nuclear receptor constitutive androstane receptor (CAR).
Pharmaceutical Research 18: 146-150, 2001.

CONTENTS

1. INTRODUCTION

Antisense oligonucleotides (ODNs) bind, in optimal conditions, to its complementary sequence in target mRNA by Watson-Crick hydrogen bonds and inhibit the protein synthesis. Thus, function of a target gene can be inhibited selectively. Theoretically, the selectivity of an ODN of about 15-mer is unique and should not have other targets than the selected one. ODNs are promising therapeutical agents for numerous diseases resulting from overexpression of genes, expression of mutant genes and viral infections. A dozen or so clinical trials are ongoing for treatment of various diseases (e.g. Crohn´s disease, non-Hodgkin´s lymphoma, HIV and cytomegalovirus infections) and FDA has approved Vitravene (fomivirsen sodium) for the treatment of cytomegalovirus retinitis in AIDS patients.

In addition to antisense effect (i.e. inhibition of translation), triplex forming ODNs can bind to double-stranded DNA by Hoogsteen base interactions (hydrogen bonding) preventing transcription. ODNs can bind to pre-mRNA to correct splicing and this may have potential, e.g., in treatment of thalassemia, where mutation prevents the formation of hemoglobin subunit β-globin (Sierakowska et al. 1996).

While antisense ODNs require enzymes for activity, ribozymes, are a class of enzymes themselves. They bind to substrate mRNA by Watson-Crick base pairing and optimally cause sequence-specific cleavage. To work effectively, they should dissociate from the cleavage product to act on further substrates (Opalinska and Gewirtz 2002).

Aptamers, often PS-ODNs due their known non-specific binding, may also have use as therapeutic agents. They act with non-antisense mechanism, binding to proteins (enzymes) thereby inhibiting their function in triggering gene transcription (Andreola et al. 2000).

Due to the inefficient uptake of naked ODNs into cells, various carrier compounds have been introduced to enhance antisense effect. In addition to the poor cellular uptake, also the enzymatic degradation of ODNs by nucleases decreases their activity. Cationic liposomes, developed in late 80´s for DNA delivery into cells (Felgner et al. 1987), were found to be effective also in ODN delivery into cells (Bennett et al. 1992) and they increased stability of ODNs against nucleases (Lappalainen et al. 1994). The cationic liposomes bind negatively charged DNA and ODNs by electrostatic interaction. Efficiency of delivery can be affected by modifying the lipid composition. *In vitro*, liposomes (or other carriers) are usually needed for sufficient delivery and suitable intra-cellular distribution. Thus the *in vitro* studies with liposomes give valuable information on the cellular delivery of ODNs and enable the use of ODNs as experimental tools in cell biology. *In vivo,* the delivery of ODNs by cationic liposomes

is further complicated by accumulation of liposomes in the lungs and liver (Bennett et al. 1996, Litzinger et al. 1996). Therefore, ongoing clinical trials do not use liposomes or other carriers, since at large doses ODNs do have some access to their target site even without carrier. Recent studies, however, indicate that appropriate modifications of liposome/ODN complexes can increase the circulation time and, subsequently, alter the tissue distribution of ODNs (Stuart et al. 2000). Despite their extensive use as DNA and ODN carriers, the properties and cell interactions of liposomal ODN complexes are poorly known.

The objective of this study was to gain mechanistic insight in the physical chemistry of ODN/cationic liposome complex formation and biological effects of ODN complexes.

2 REVIEW OF THE LITERATURE

2.1. Antisense oligonucleotides

2.1.1. Oligonucleotide structure and modifications

Phosphodiester oligonucleotides: Basic phosphodiester oligodeoxyribonucleotide (PO-ODN, ssDNA) structure is shown in Fig. 1A. Sensitivity of ODNs to extracellular and intracellular nucleases restricts their use in many cases. Degradation is due to exonucleases acting 3′ → 5′. Stability can be increased by protecting (e.g., fluorescent probes or various protective groups) the 3′-end (Dheur et al. 1999). Mechanism of PO-ODN action involves formation of a duplex with mRNA that is thus degraded enzymatically by RNase H. Subsequently, protein synthesis is prevented.

Phosphorothioate oligonucleotides: To avoid nuclease degradation, numerous modifications to ODNs have been introduced. Replacement of one of the non-bridging oxygen atoms with sulphur leads to phosphorothioate oligonucleotides (PS-ODNs, Fig. 1B). PS-ODNs are easily prepared and they are commonly used as antisense molecules due to their greatly enhanced, but not total, stability against nucleases. The common method of PS-ODN synthesis leads to a mixture of R_p (nuclease resistant) and S_p (nuclease sensitive) diastereomers in phosphorothioate linkages (Lebedeva and Stein 2001). Mechanism of action is the same as with PO-ODNs: hybridization and activation of RNase H. Disadvantage of PS-ODNs is their inhibitory activity against RNase H at high PS-ODN concentrations, especially with longer than 20-mer ODNs (Gao et al. 1992). PS-ODNs bind easily to proteins. This is a major drawback leading to many non-specific effects. This can be partly reduced by replacing only some of the oxygens with sulphur (i.e. PS-ODN, PO-ODN mixture).

Other oligonucleotide modifications: To improve stability and target binding and to reduce the side effects of especially PS-ODNs numerous modifications have been introduced (Fig. 1.), including sugar 2′-*O*-modifications, base modifications, phosphoramidates, morpholino-oligomers and peptide nucleic acids. It should be noted that the latter two are non-ionic, and thus can not be delivered complexed with cationic lipids, allthough they can delivered with cationic liposomes using other entrapment methods. Therefore, they are not dealt later to any great extent.

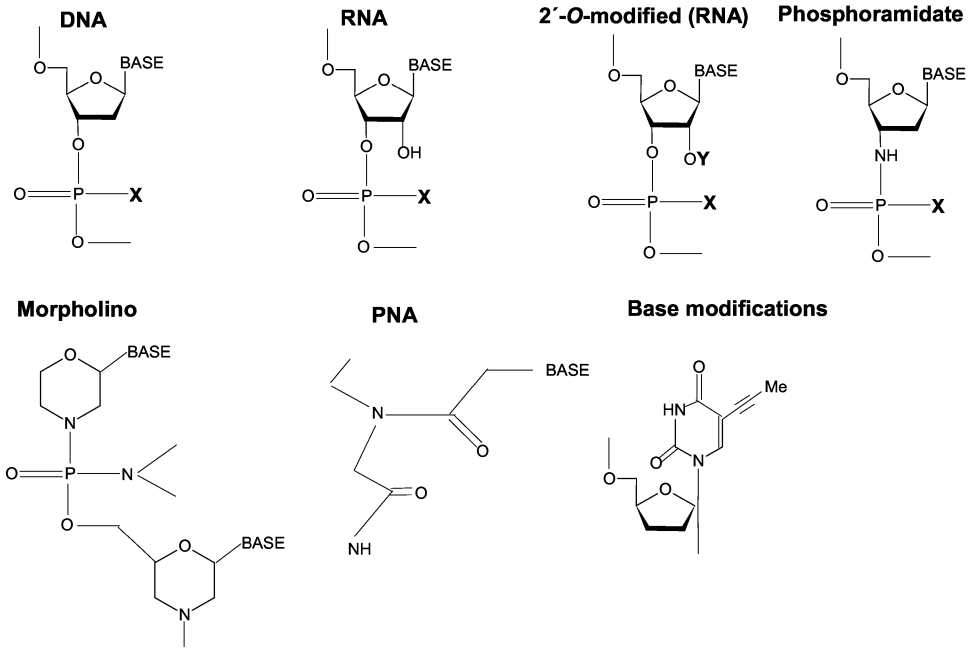


Figure 1. Commonly used oligonucleotides, modifications and analoques. BASE = adenine, cytosine, guanine, thymine, uracil, X= e.g. O^-, S^- , methyl; Y= e.g. methyl, methoxy ethyl (2′-MOE);. Phosphoramidate (e. g. N3′-O5′ phosphoramidate); PNA = peptide nucleic acid; Base modifications (e. g. propynyl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

2.1.2. Mechanism of action

RNase H dependent: Most studies in antisense field, due to predominant use of phosphodiester or phosphorothioate oligonucleotides, thus far have utilized RNase H dependent mechanism of action (Giles et al. 1995), where degradation of target RNA from RNA/DNA duplex is achieved efficiently. The enzymes are present in most cells and primarily located in the nucleus. Most antisense oligonucleotides in clinical trials, and also Vitravene utilize RNase H mechanism (Baker and Monia 1999). Advantage of this mechanism is the wide range in target selection (Fig. 2), since target sequences can be selected from any part of the RNA transcript, whereas RNase H independent mechanisms usually require target to be selected from 5′cap or AUG region (Baker and Monia 1999).

RNase H independent: Methylphosphonates, PNAs (Larsen et al. 1999), morpholino oligomers, N3′-P5′ phosphoramidates, α-anomeric oligonucleotides and 2′-

O-alkyl modified oligonucleotides are representative of compounds acting with RNase-independent mechanism (Fig. 2). Steric blocking of RNA processing and subsequent inhibition of translation initiation are the main mechanisms of action. Generally 5′ terminus or AUG region are used as targets, while antisense ODNs against the coding region are ineffective, unless they are made functional by RNA cross-linking agents or other methods (Baker and Monia 1999). Although active by mechanisms using enzymes, like RNase H, these ODNs can not inhibit translation beyond the initiation codon.

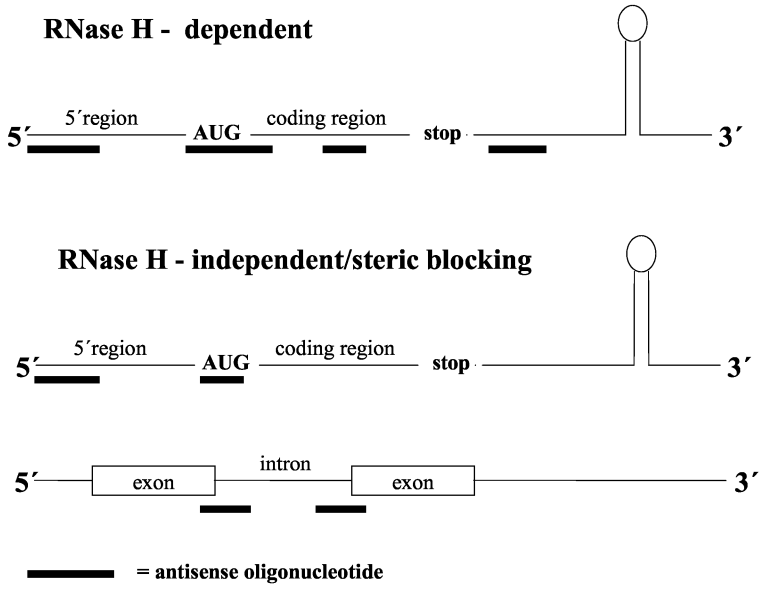


Figure 2. Mechanisms of action of antisense oligonucleotides. RNase H -dependent mechanism is used by PO-ODNs, PS-ODNs or mixed backbone modifications (e.g. partly modified with RNase H –resistant sequences). Steric blocking of RNA processing is the main mechanism of action for RNase H resistant modifications (target 5′ terminus, AUG region or exon-intron junctions).

Correction of aberrant splicing (Fig. 3) may have therapeutic use in the future, e.g. in the treatment of thalassemia (Sierakowska et al. 1996, Lacerra et al. 2000) or cystic fibrosis (Friedman et al. 1999). Moreover, a system utilizing luciferase gene interrupted by a mutated human β-globin intron 2 has been developed (Kang et al. 1998). This mutation causes aberrant splicing of luciferase pre-mRNA. This system can be used to compare the efficacy of various ODNs and delivery with different carriers. It has been used to compare plasmid DNA and 2′-*O*-methyl-oligoribonucleotide delivery

efficiencies with different carriers as both DNA and ODN need to have access into nucleus in order to have therapeutic effect (Kang et al.1999).

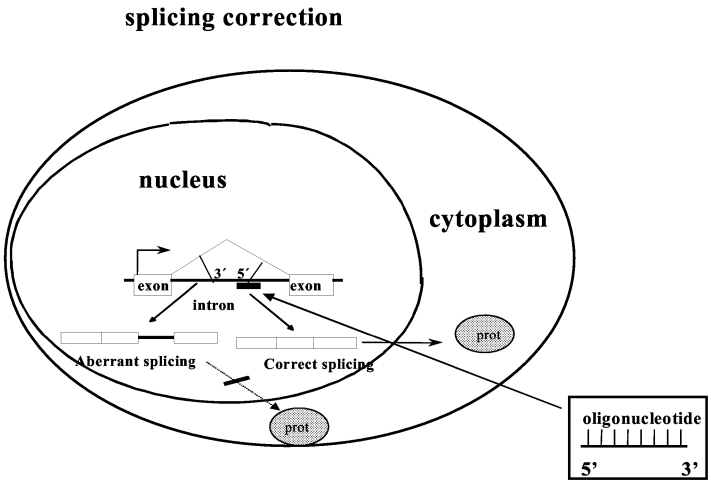


Figure 3. Correction of a mutation with an oligonucleotide leads to correct splicing and subsequent formation of an active mRNA.

RNA interference (RNAi): RNA- dependent RNA polymerases (RdRps) copy RNA from RNA and they probably have had significant role in early evolution. They do take part in RNA silencing, where short double-stranded RNAs (dsRNA) cause sequence specific gene repression (Ahlquist 2002, Carmell et. al 2002, Cullen 2002). Ribonucleic acid interference (RNAi) is based on dsRNAs to be cleaved to fragments about 19 to 23 base pairs (bp) by RNase III- like dicer and then the siRNAs associate with specific multiprotein complex (RNA-induced silencing complex, RISC). This ribonucleotideprotein complex subsequently binds to the target mRNA causing its degradation. The complex is then recycled for further activity (Capodi et al. 2002, Klahre et al. 2002, Yu et al. 2002).

2.1.3. Cellular uptake of oligonucleotides

In order to be active in cells, ODNs should reach at least cytoplasm to hybridize with mRNA for antisense activity. The high molecular weight, and the negative charge associated with ODNs, makes passive diffusion through the cell membrane impossible. Major pathway for internalization is active adsorptive or fluid phase endocytosis for charged ODNs. Neutral (e.g. methylphosphonates, PNAs, morpholino oligomers) do not adsorb well to the cell surface. Usually less than 4 % of the ODNs are taken up by cultured cells (Zelphati and Szoka 1996a). From the endosomes, a very small part is then released into the cytoplasm. The rest are transferred to lysosomes, where they are subsequently degraded relatively rapidly. Degradation of PS-ODNs with isolated lysosomal enzymes ($t_{50\%}$ = 50 min) is quite similar to PO-ODNs ($t_{50\%}$ = 30-40 min) (Hudson et al. 1996).

Intracellular distribution of oligonucleotides: Free ODN has been found in clathrin coated pits, endosomes, lysosomes, cytoplasm and even in the nucleus by a supposed diffusion driven import process (Beltinger et al. 1995). Nuclear delivery of less than 10 % of total intracellular accumulation for PS-ODN was found by Thierry and Dritschilo (1992). From this amount a very small portion is released to the cytoplasm, but from there ODNs can readily diffuse from cytoplasm into the nucleus (Chin et al. 1990, Leonetti et al. 1991, Fisher et al. 1993) because the molecular sizes of ODNs are smaller than the nuclear pores. Diffusion in aqueous solution and cytoplasm has been studied by Lukacs et al. (2000) for DNAs from 21 to 6000 base pairs. Diffusion decreased about 50 times for 6000 pb compared to 21 bp (near that of ODN size) DNA in water. In all cases diffusion in cytoplasm was drastically lower than in water. They also found that diffusion of comparably sized dextrans was much faster. Binding of DNA to immobile obstacles in cytoplasm may be a significant rate-limiting barrier in transfections.

Oligonucleotide binding proteins on cell surface: An 80-kDa surface protein was identified in myeloid cell line HL 60 as a receptor for ODN. This protein is responsible for ODN binding via a saturable uptake, since it was inhibited by polynucleotides of any length possessing 5′-phosphate (Loke et al. 1989). Yakubov et al. (1989) detected two ODN binding proteins on the surface of L929 mouse fibroblast and Krebs 2 ascites carcinoma cells. Uptake was more efficient at low ODN concentration (<1 μM) indicating absorptive endocytosis predominant at low ODN concentration and fluid phase endocytosis predominant at higher concentration. The binding could be inhibited by other ODNs, DNA and RNA. Sulfated polyanions, heparin and chondroitin, did not inhibit the binding. Similar concentration dependent

mechanism for binding and uptake was found by Beltinger et al. (1995). They identified five 20-143 kDa proteins in K562 (human leukemia) cells. In spleen cells binding affinity of PS-ODNs was much greater than that of PO-ODNs (Krieg 1993). On human HL60, HepG2 and KB cells two ODN binding proteins (K_d 60 nM for 21 mer PO-ODN) of about 100-110 kDa were identified by Yao et al. (1996), and additional 40-58 kDa proteins on HepG2 cells (Corrias and Cheng 1998). Binding kinetics indicated different binding sites for thymine and cytosine, which were essential for high affinity. Benimetskaya et al. (1997) defined heparin-binding integrin Mac-1 (CD11b/CD18; αMβ2) 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. (1998) 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 (de Diesbach et al. 2000). Although endocytosis is considered to account almost exclusively for ODN internalization (Stein 1997), discrepancies and inconsistencies in results as well as the still unknown mechanism of escape from endosomes has led to suggestions of multiple mechanisms of uptake (Wu-Pong 2000). Although *in vitro* the uptake of ODNs (at least with reasonable concentrations) is insufficient for activity, the active *in vivo* uptake of some tissues or cells within certain tissues may be sufficient for pharmacological activity indicating the lack of *in vitro/in vivo* correlation (Bennett and Cowsert 1999).

2.1.4. *In vivo* pharmacokinetics of free oligonucleotides

***I.v.* injections:** After intravenous bolus injection, PS-ODN distributes rapidly to many tissues (Yu et al. 2001, Raynaud et al. 1997, Peng et al. 2001). ODN concentration in mouse and rat plasma follows two compartment kinetics: rapid distrubition phase ($t_{1/2}$ ~ min.) to the tissues followed by slow elimination phase ($t_{1/2}$ ~ hrs.). There are conflicting values for the half-life of elimination phase (Yu et al. 2001; Akhtar and Agrawal 1997; Raynaud et al. 1997) due to the analytical, species and dosing differences. However the sequence differences do not cause kinetic changes after *i.v.* injection (Levin 1999).

PS-ODNs are distributed mainly to the liver and kidney, but also to the muscle, spleen and lungs after intravenous administration (Yu et al. 2001; Raynaud et al. 1997).

Bijsterbosch et al. (1997) 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, and interestingly, the clearance was variable depending on the sequence. Binding studies indicated saturable, moderate affinity membrane protein mediated uptake (Biessen et al. 1998). Crooke et al. (1996) showed that modifiyng PS-ODNs e.g. by modifying their lipophilicity or increasing stability can somewhat alter the tissue distribution and elimination of PS-ODNs.

Peng et al. (2001) have generated a physiologically based pharmacokinetic model for PS-ODN in rats after i.v. administration. Due to the large molecular weight, the distribution is limited by the vascular permeability. Additionally, also distribution coefficient Kp (tissue/blood) affects the distribution. High Kp values in some tissues suggest strong binding of PS-ODN in tissue components. The storage capacity of each tissue is 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 fractions 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 a major factor in ODN clearance from blood circulation. The ODN is predominantly degraded by exonucleases from the 3' end, as little endonuclease activity is seen. Degradation starts rapidly after injection (about 5 mins) and the full length ODN remains as majority species for 4 hours and the half life in the tissues varies between 20 and 120 hours (Levin 1999).

Protein binding: Protein binding in plasma is the main determinant of PS-ODN pharmacokinetics (in mice 87 –98 %), the major proteins being albumin and alpha-2-macroglobulin (Geary et al. 2001). The protein binding prevents the otherwise rapid excretion of PS-ODNs. PO-ODNs bind less to proteins and are excreted more rapidly to the urine than PS-ODNs (Levin 1999; Geary et al. 2001). Protein binding extends the half-life, and subsequently there are more chances for tissue distribution and only small fraction of the excreted drug is secreted unmetabolized (Geary et al. 2001). Despite their relative good stability against degradation PS-ODNs retain in the tissues long enough to be eventually degraded. Good inverse correlation has been seen between the clearance of ODNs and their protein binding (Geary et al. 2001).

PS-ODNs have been modified in order to increase their stability. For example, 2′-MOE substitution improves the metabolic stability, but does not affect pharmacokinetics after *i.v.* injection (Geary et al. 2001). The nature of the bridge between the nucleotides (PO or PS) is the major factor to affect the pharmacokinetics.

Extravascular administration: Per oral administration is the most desirable route of drug administration. Despite some really high values (60-80 %) reported for bioavailability, commonly the absolute bioavailability of ODNs after p.o. administration is less than 1 % (Nicklin et al. 1998), which is in accordance with very low permeabilities in the CaCo-2 cell cultures (P_{app} 1 x 10^{-8} cm/s). Improved oral bioavailability may achieved with derivatized ODNs, e.g. PS-2'MOE derivatives (Geary et al. 2001). The reported biovailability of 5 % may be adequate, but the high variability of drug absorption at low bioavailabilites and expense of ODNs complicates their use as orally administered therapeutic agents.

Intraperitoneal and subcutaneous injections yield bioavailabilities of about 30 % in rats, while intratracheal administration results in variable systemic ODN absorption (3 - 40 % bioavailability) depending on the dose (Nicklin et al. 1998). The hemodynamic effects (drop of blood pressure and complement activation) due to toxicity limit the allowed plasma concentrations. In many localized applications ODNs do not have to absorb into the systemic circulation for activity. Thus dosing methods like inhalations, intraocular injections, topical dermal application and direct application to the blood vessel walls or brain during surgical procedures (Wu-Pong and Byron 1996, Ali et al. 2001, Roy et al. 1999) may have advantages from the safety point of view.

The first commercial antisense product Vitravene (fomivirsen, a 21-mer PS-ODN) is administered by injection into the eye vitreous of the target tissue being the retina. ODN stays in the vitreous and retina 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 intact PS-ODN remains after 10 days. In the retina there was accumulation for 5 days. Thereafter, elimination with $t_{1/}$ of 79 hr occurred and the concentration of intact PS-ODN was 1.6 μM after 10 days. These results indicate sufficient amounts of ODN are retained for therapeutic activity (Leeds et al. 1997).

2.2. Oligonucleotide delivery systems

2.2.1. Cationic liposomes

Cationic liposomes bind negatively charged ODNs by electrostatic interactions and enhance uptake, and especially cytoplasmic and nuclear delivery of ODNs (for reviews see Zelphati and Szoka 1996a, Liang et al. 1999, Garcia-Chaumont et al. 2000, Lebedeva et al. 2000).

Cationic lipid DOTMA, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride, was introduced in late 1980´s as a DNA (Felgner et al. 1987, Felgner and Ringold 1989, Felgner et al. 1989) and RNA (Malone et al. 1989) transfection reagent. It was used as 1:1 combination with dioleoylphosphatidylethanolamine (DOPE) and is commercially available as Lipofectin. It was shown later to enhance ODN uptake and activity (Bennett et al. 1992) and is still widely used as an in vitro ODN transfecting agent. A cationic lipid DOTAP, 1,2-bis(oleoyloxy)-3-(trimethylammonium) propane, was developed at the same time with DOTMA (Stamatatos et al. 1988) and was shown to transfect DNA (Leventis and Silvius 1990) and ODNs (Cappaccioli et al. 1993, Lappalainen et al. 1994, Ollikainen et al. 1996). Cationic liposomes composed of dimethyldioctadecylammonium bromide and dioleoylphosphatidylethanolamine at 8/15 molar ratio (DDAB/DOPE, LipofectACE™) were introduced for DNA (Rose et al. 1992) and ODN (Lappalainen et al. 1994, Ollikainen et al. 1996, Wielbo et al. 1997) delivery. Lipofectamine, a mixture of lipospermine DOSPA (2,3-dioleoyloxy- *N* -[2(sperminecarboxamido)ethyl] -*N*,*N*-dimethyl-1-propanaminium trifluoroacetate) and DOPE (3/1, w/w) containing 5 positive charges per molecule was found to be relatively efficient, albeit less than DDAB/DOPE, ODN delivery vehicle into the cytosol of CaSki cells (Lappalainen et al. 1997). Other common liposomal carriers include e.g. DC-Chol, 3β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol introduced by Gao and Huang (1991) for DNA delivery, DOGS, dioctadecylamidoglycylspermine, DMRIE, 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (Dassow et al. 2000, Takagi et al. 2000) and Cytofectin GS, Dimyristylamidoglycyl-N-isopropoxycarbonyl-arginine dihydrochloride in combination with DOPE (Lewis et al. 1996). After the introduction of these carriers, a large amount of novel transfection reagents have been introduced, but the data about their efficiency is mostly from DNA transfections and the structure of the commercial carrier often not available. The structures of some of the most used cationic lipids in ODN delivery are shown in Figure 4.

Dioleoylphosphatidylethanolamine: Most widely used transfection enhancing component (helper lipid) with cationic lipids is dioleoylphosphatidylethanolamine (DOPE, Fig. 4). The small and lowly hydrated headgroup gives DOPE its inverted cone-like structure and at physiological pH tends to form nonlamellar structures. Mui et al. (2000) found a 10- to 100-fold increase in DNA transfection activity in BHK cells with DODAC/DOPE complexes prepared in water compared to DODAC/DOPC complexes. In addition, about 10-fold enhancement (maximally about 80 %) in antisense activity against EGFR (epidermal growth factor receptor) was seen with a 15-mer PS-ODN.

They also concluded, that fusion (lipid mixing) was not the mechanism of the enhancing effect but more the ability to disrupt membrane integrity. Similar result, lack of correlation between lipid mixing and plasmid transfection activity, was established earlier (Stegmann and Legendre 1997). The particular cationic lipid affects the possible enhancing effect of DOPE and the cell lines respond differently to DOPE containing complexes (Wheeler et al. 1996), and this partly explains the discrepancies concerning the use of DOPE as an enhancer. As for transfection of PS-ODNs into immortalized avian embryonic cardiomyocytes, a tendency of enhancing activity with DOPE was established as opposed to plasmid DNA transfections (Conrad et al. 1998).

2.2.2. Cationic polymers

Polyethyleneimine: Polyethyleneimine (PEI) was introduced as a gene and oligonucleotide transfecting agent by Boussif et al. (1995). Presumably PEI has endosome buffering capacity, that prevents DNA from degradation and facilitates escape from the endosomes due to swelling and consequent endosomal membrane rupture (Boussif et al. 1995). They also showed uptake of rhodamine-labeled PO-ODN and its nuclear delivery into chicken embryonic neurons, although no antisense effect was established.

Dendrimers: Starburst polyamidoamino polymers (PAMAM) are prepared by stepwise polymerization of a core molecule and depending on the extent of polymerization different generations (increase in molecular weight) can be achieved. Ammonia (NH_3), ethylenediamide (EDA) are generally used as starting molecules and the dendrimers contain positively charged amino groups on their surface. PAMAM dendrimers have been shown to deliver plasmid DNA into the cells (Haensler and Szoka 1993, Kukowska-Latallo et al. 1996, Tang et al. 1996, Ruponen et al. 1999). Fractured (solvolyted) or dendrimers synthesized with defective branching are more active in plasmid transfections than intact dendrimers (Tang et al. 1996). Bielinska et al. (1996) used PAMAM dendrimers (generation 7 EDA) for PO-ODN transfection in D5 (mouse melanoma) cells expressing luciferase and observed 15-30% antisense inhibition effect. Interestingly, in a cell-free coupled transcription-translation system (reticulocyte lysate), inhibition of luciferase was similar for free PO-ODN and PO-ODN/dendrimer complexes incubated with the system.

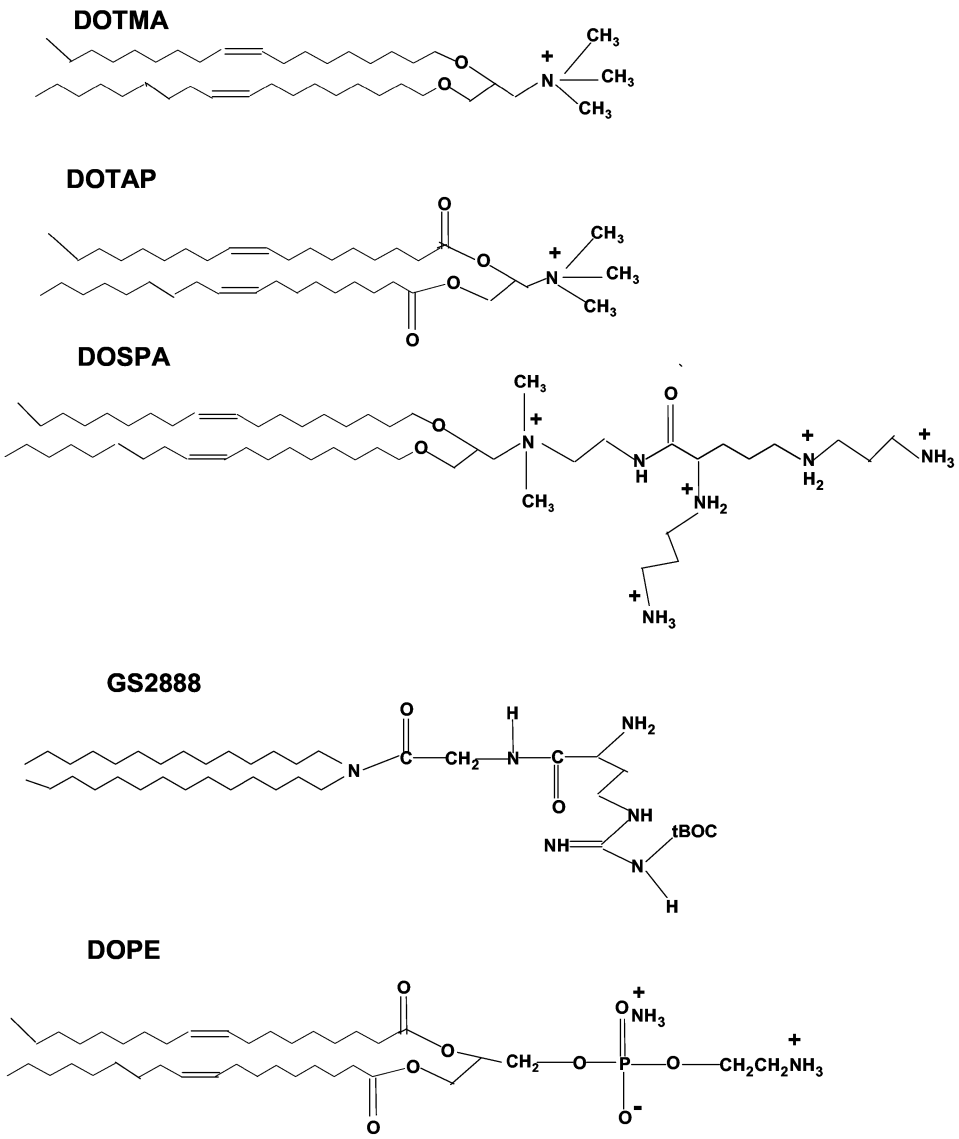


Figure 4. Structures of some commonly used liposomal ODN transfection agents and helper lipid DOPE. DOTMA = *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium (chloride); DOTAP = *N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium (propane); DOSPA = 2,3-dioleoyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate (= Lipofectamine in combination with DOPE; GS2888 = Dimyristylamidoglycyl-N^{w}-isopropoxycarbonyl-arginine dihydrochloride (= Cytofectin GS in combination with DOPE); DOPE = 1,2-Dioleoyl-3-phoshatidylethanolamine.

Polylysine: Polylysine (Poly-L-lysine, PLL) complexation enhances ODN uptake, but antisense activity is not seen without conjugation of ODNs to PLL, adding enhancing moieties to PLL (Clarenc et al. 1993, Rojanasakul 1996) or using > 100-fold ODN concentrations in sole PLL/ODN complexes (Clarenc et al. 1993). A more than 10-fold increase in cell-associated PS-ODN did not lead to any activity (≤ 2 μM ODN), but glycosylation of PLL led to antisense activity (Stewart et al. 1996). Conjugation of PLL (10-mer) with a signal import peptide derived from Kaposi fibroblast growth factor (K-FGF) led to enhanced cell uptake, but PLL alone did not have any effect on cell association (surface-bound PS-ODN was removed) even at 10 μM concentration, possibly due to low molecular weight PLL used (Dokka et al. 1997). Similarly PLL (20 kDa) was without activity in plasmid DNA transfections, while PLL 200 kDa showed low activity (Ruponen et al. 1999).

Other:

FuGENE6™: Reportedly non-liposomal transfection reagent FuGENE6™ (composition not available) has been used to inhibit human telomerase activity, although relatively high PS-ODN concentrations were needed (Tao et al. 1999, Tamura et al. 2000).

Nanoparticles: Nanoparticles are generally prepared from polymers and have been studied as ODN carriers (for reviews see Fattal et al. 2000, Lambert et al. 2001). Studies have mostly been done using poly(iso)hexylcyanoacrylate (PACA) polymer particles in combination with cationic copolymers or cationic hydrophobic detergents to enable ODN binding by ion pairing (Zimmer 1998). PO-ODN stablility against enzymatic degradation by phosphodiesterases, cellular uptake, as well as half-lives in cell growth medium have been shown to increase significantly when adsorbed to polyisohexylcyanoacrylate (PIHCA) nanoparticles (Chavany et al. 1994). Polymethylmethacrylate (PMMA) core shell nanospheres have been shown to increase the binding, cellular uptake, as well as antisense effect against *c-myb* (Tondelli et al. 1998). A similar inhibition of ecto-5′-nucleotidase for both PS-ODNs and PO-ODNs has been shown using monomethylaminoethylmethacrylate (MMAEMA) copolymer nanoparticles (Zobel et al. 2000). 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 (Nakada et al. 1996), and more recently these particles have been shown efficacy in inhibiting Ewing sarcoma-related tumor in mice (Lambert et al. 2000). Recently, poly(lactide-co-glycolide) nanoparticles have shown high encapsulation and slow release of ODN, when PEI/ODN –complexes are encapsulated in these microspheres (De Rosa et al. 2003).

Couvreur and his colleaques have also studied the use submicron cationic emulsions as carriers (Teixeira et al. 1999, 2000).

pH-sensitive liposomes: Lipids containing pH-sensitive groups include cholesteryl hemisuccinate (CHEMS), oleic acid (OA), palmitoyl homocysteine (PHC) (Yatvin et al. 1980) and succinyldioleylphosphatidylethanolamine (SOPE) (Schroit et al. 1986). These are charged at neutral pH but lose their charge at low (endosomal) pH and subsequently destabilize the membrane. Release of the contents depends also on auxiliary lipids, and especially DOPE is a usefull additional component, although other factors may contribute as well (Simões et al. 2001). Tumors, metastases, sites of inflammation and infection have usually lower than normal pH, and thus pH-sensitive liposomes may have advantages in targeting to these tissues (Yatvin et al. 1980).

Most studies have been done by using DOPE/CHEMS liposomes with greatly enhanced activity *in vitro* (Lubrich et al. 2000) and additionally containing cholesterol *in vivo* (Ponnappa et al. 2001). *In vivo,* steric stabilization with polyethylene glycol (PE-PEG) has been shown to increase the circulation time of these liposomes (Slepushkin et al. 1997).

2.2.3. Peptides

Peptides are recognized by receptors on the cell surface and amphiphatic peptides are able to destabilize cell membranes. Often derived from viruses, that use peptides for cell entry, they are potential targeting ligands and delivery enhancers for ODNs. The peptides can be utilized in different ways: complexed or conjugated with ODN or attached to liposomal or polymeric delivery system. Complexation is usually achieved by mere electrostatic interactions with ODNs and cationic peptides containing lysine and/or arginine residues (+ charged), while conjugation is achieved by chemical bonds between ODN and peptide.

Membrane active peptides: A DNA-peptide complex, consisting of a DNA condensing ($YKAK_nWK$) and a pH-sensitive endosomolytic peptide JTS-1 (GLFEALLELLESLWELLLEA) was introduced as an efficient in vitro transfection system. Lytic activity (hemolysis of erythrocytes) of JTS-1 as well as transfection activity of the complex was greater compared to an influenza fusion peptide INF-7 complex (Gottschalk et al. 1996). Later named GM225.1, the peptide expressed hemolytic activity and conformational transition in the presence of liposomes when the pH was lowered from 7.4 to 5 (Duguid et al. 1998). Branched cationic peptides (partly coupled to transferrin for receptor mediated transfection) were used for DNA compaction and transfection of K562 cells (Plank et al. 1999). A minimum of 6-8

cationic amino acid chain length for activity was found, in agreement with earlier findings (Gottschalk et al. 1996). Morris et al. (1997) introduced a MPG peptide (GALFLGFLGAAGSTMGAWSQPKSKRKV) for single and double stranded oligonucleotides and later for gene delivery (Morris et al. 1999). The peptide derives from the fusion sequence of HIV gp 41 and nuclear localization sequence of SV40 T-antigen. A rapid and efficient (a non-endocytotic pathway was proposed) nuclear delivery of fluorescein-labeled ODN was seen, although no antisense effect was reported.

Peptide-oligonucleotide complexes: Hydrophobic and hydrophilic domains containing peptide (GALFLGFLGAAGSTMGAWSQPKSKRKV) termed MPG was introduced by Morris et al. (1997) for delivery of PO-ODNs. Complexes with ODN showed greatly enhanced uptake and nuclear delivery, even in the presence of serum, with proposed non-endosomal pathway of internalization. Although no antisense activity was established, they later showed efficient delivery of a plasmid containing antisense cDNA (Morris et al. 1999).

Peptide-oligonucleotide conjugates: To improve the cell delivery of ODNs, fusogenic peptides have been conjugated with ODNs (reviewed by Tung ans Stein 2000). Bongartz et al. (1994) used a peptide derived from influenza hemagglutinin envelop protein (GLFEAIAGFIENGWEGMIDGGGYC) to enhance antiviral activity of AS-ODN (anti TAT) on *de novo* HIV infected lymphocytes. A 5 to 10 fold increase was observed with conjugation, although no sequence specificity was observed. Pichon et al. (1997) used a KDEL motif containing peptide/PO-ODN conjugate against $gag_{HIV\text{-}1}$ in HepG2 cells. The result was a 5-fold increase in activity, although the internalization of the conjugate was less than that of the free ODN. Recently (Astriab-Fisher et al. 2000), inhibition of P-glycoprotein expression was achieved with 20-mer PS-ODN conjugated with either 35 amino acid sequence from HIV TAT protein (TAT) or 16 amino acid sequence from *Drosophila* Antennapedia protein (ANT).

2.2.4. Carrier-oligonucleotide complexes

2.2.5. Cellular uptake

Most studies about the uptake mechanism of ODN complexes have been performed with plasmid/carrier complexes. The first proposed mechanism of the cellular delivery (Felgner and Ringold 1989) was the fusion of complexes (Lipofectin) with negatively charged cell membranes enabling direct cytoplasmic delivery through the cell plasma membrane. Similar mechanism was proposed for ODN in early studies

(Capaccioli et al. 1993). Interestingly it was found, that although theoretically a slight positive charge excess in the complexes would be needed, about 2-fold excess of negative charges was found to be optimal (Felgner and Ringold 1989).

An endocytotic pathway was first proposed by Leventis and Silvius (1990), and in addition to direct plasma membrane fusion by Legendre and Szoka (1992). They showed in a study with several cell lines, that treatment of cells with chloroquine, ammonium chloride or monensin enhanced transfection activity of Lipofectin/plasmid complexes in some cases (or did not have any effect). Contrary to the hypothesis of direct fusion with plasma membrane, Zhou and Huang (1994) showed, that interfering with endocytosis by various chemicals led to about 6-fold increase in transfection activity of lipopoly(L-lysine) (LPLL), especially when the complexes contained DOPE.

In a study with a variety cationic lipid molecules, addition of chloroquine (inhibits endosomal maturation and thus delivery to lysosomes and subsequent hydrolysis) led to 4-fold increase in transfection activity for dioleoyl compound suggesting endosomal pathway of internalization. Although the activity of a dimyristoyl was decreased in similar conditions, the result was explained either by chloroquine toxicity or difference in the fusion ability (early vs. late endosome) (Felgner et al. 1994). Studies on HepG2 and CHO D^- cells using DOTAP or DC-Chol liposomes and lipid mixing studies indicated that binding to the cell surface was insufficient for liposome-cell fusion and endocytotic uptake is required for fusion to occur (Wrobel and Collins 1995). Similar results for DC-Chol were obtained by Farhood et al. (1995) with A431 cells suggesting destabilization of endosomal membrane and subsequent release of DNA into the cytosol. DOTMA/DOPE liposomes complexed with colloidal gold particles alone or with DNA, were found in coated pits in endosomes and lysosomes with gold particles dispersed throughout the cytoplasm (Friend et al. 1996). They also suggested occasional fusion with the nuclear envelope, indicated by formation of intranuclear membranes.

Cell surface proteoglycans, sulfated glycosaminoglycans bound to a core protein, have been shown to mediate the cellular uptake of of DNA/cationic liposome as well as DNA/cationic polymer complexes *in vitro* (Mislick and Baldeschwieler 1996) and also *in vivo* (Mounkes et al. 1998). Complexes bind to the highly negatively charged membrane-associated proteoglycans and the transfection activity diminishes dramatically e.g. after enzymatic removal of proteoglycans or inhibiting their sulfation. Additionally, transfection of mutant, proteoglycan deficient cell line led to 50-fold decrease in transfection activity (Mislick and Baldeschwieler 1996). On the other hand, proteoglycans secreted by target cells have been shown to decrease dramatically both the uptake of the complexes as well as transfection activity by competing with DNA binding to the cationic lipid (Belting and Peterson 1999). Likewise, glycosaminoglycans

inhibit transfection when they contact the complexes extracellularly (Ruponen et al. 1999, 2001). The role of glycosaminoglycans in ODN delivery is unknown.

A 6 – 15-fold increase in cell association and at least 1000-fold increase in activity was shown for PS-ODNs used with DOTMA compared to ODNs alone. The activity enhancement was attributed (as above with plasmid DNA, Legendre and Szoka 1992), in addition to increased cell association, to the altering of intracellular distribution (Bennett et al. 1992). Interestingly, this study, like a half of dozen other early studies, were done by adding the liposomes first and ODNs 15-30 mins later. This indicates, that *in vitro*, prior complexation may not be needed. DOTAP has been shown to increase uptake of ODN over 25-fold with slightly lower uptake for DOTMA and DOGS compared to free ODN (Capaccioli et al. 1993). Using ^{32}P-labeled PO-ODNs, the cell associated radioactivity was increased 2 – 4.5 –fold and nuclear accumulation 10-20 –fold, depending on the carrier (DOTAP or DDAB/DOPE), compared to ODNs alone (Lappalainen et al. 1994).

Using a fluorescently labeled ODN and DOTAP, Zelphati and Szoka (1996b) showed that ODN redistributes from punctate cytoplasmic regions into the nucleus. Inhibitors of actin microfilaments, energy metabolism and proteins involved in fusion of endosomes decreased nuclear accumulation independently from acidification of the endosomal vesicles. Thus, release of ODN from DOTAP and endosomal compartments at an early stage of endocytotic pathway was proposed. As has been shown in several studies, ODNs diffuse readily from cytoplasm into the nucleus (Chin et al. 1990, Leonetti et al. 1991, Fisher et al. 1993) and they have been shown to dissociate from cationic lipids (DOTAP, DOTAP/DOPE) before entering the nucleus (Zelphati and Szoka 1996b, Marcusson et al. 1998). A mechanism for ODN release from cationic liposomes (DOTAP) was proposed by Zelphati and Szoka (1996a). Flip-flop of anionic lipids from cytoplasmic facing monolayer occurs and anionic lipids mix into the complex leading to ODN-anionic lipid exchange in the complex and ODN release from the complex into the cytoplasm. Similar exchange mechanism was proposed for plasmid DNA (Xu and Szoka 1996), but this has not been shown at cellular level. These results got support later, as Bhattacharya and Mandal (1998) showed, that various anionic vesicular structures led to DNA release from the complexes if the negative charges in these structures were equal or in excess to negative charges in DNA. They also showed, that negatively charged micellar or vesicular aggregates (negatively charged compounds alone are not usually efficient) are needed for DNA release and the release requires both electrostatic and hydrophobic interactions.

Recently, different types of mechanisms for plasmid DNA delivery with polylysine (Zauner et al. 1999) and polyethyleneimine (Pollard et al. 1998) have been

proposed. For both compounds direct injection of the complexes into the cytoplasm enhances nuclear delivery compared to the naked DNA, whereas for lipid based complexes this is not the case.

It should be noted, that cationic liposomes (at least liposomes like Lipofectin) can be used without prior complexation with ODNs. Some of the earliest experiments with Lipofectin were done by first adding the liposomes to the cells and ODNs were added later (Chiang et al. 1991, Bennett et al. 1992, Colige et al. 1993, Bennett et al. 1994). ODNs can be added up to 30 minutes after addition of cationic lipid (Bennett 1998).

2.2.6. Protection against nucleases

ODNs without carrier: Stability of PS-ODN, PO-ODN, methylphosphonate (MP-ODN) and alternating PO-MP –ODNs was studied by Akhtar et al. (1991) in HeLa cell nuclear extract, S100 cytoplasmic extract, human and calf serum. In all media, PO-ODN was the most sensitive derivative. For example, degradation in human serum was complete in 30 mins. Mixed PO-MP-ODN was most resistant. Degradation was most profound in serum, less in nuclear extract and lowest in cytoplasmic extract. In cell culture medium, containing 10 % FBS, degradation of PO-ODNs was evident in 10 min, PS-ODNs in 30 min but no degradation was observed for PO-MP-ODNs in 3 hrs. Hudson et al. (1996) reported $t_{1/2}$ values of 30-40 and 50 minutes for PO-ODNs and PS-ODNs, respectively, after incubation with isolated rat liver lysosomal enzymes. In a buffer at pH 5.4 both ODNs remained stable for at least 48 hours indicating that acid-catalysed hydrolysis does not account for rapid degradation.

ODN/carrier complexes: DOTAP was shown to increase the half-life of PO-ODN from 15 to 40 minutes in human serum. Degradation was fastest in serum, slightly slower in cell culture medium and much lower in cell lysate or cell extract (Capaccioli et al. 1993). Increase in stability in cell culture medium, cytoplasm and nucleus for PO-ODNs complexed with DOTAP or DDAB/DOPE was shown by Lappalainen et al. (1994). A small fraction of free ODN was intact in medium after 2 hrs, but none in cytoplasm or nucleus. Part of the ODNs were intact when incubated with cationic liposomes after 2 and 5 hrs and, in the case of DDAB/DOPE, even after 8 h incubation. Therefore, it appears that ODNs are protected from nucleases when they are complexed with cationic lipids.

2.2.7. Complexes *in vivo*

Litzinger et al. (1996) found that after *i.v.* injection to mice via the tail vein, DC-Chol/DOPE liposome 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 from the lungs 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. In contrast to this result, Uyechi et al. (2001) showed, by confocal microscopy, that fluorescently labeled ODNs can be found in the nuclei after *i.v.* (endothelial cells) or *i.t.* (intra-tracheal administration, epithelial cells) administration of lipoplexes. Bennett et al. (1996) showed a broad distribution to many tissues of a plain PS-ODN, the main locations being liver, kidney, skeletal muscle and skin. ODN complex with DMRIE/DOPE led to increased distribution to liver, lung and spleen indicating that the biodistribution can be altered by complexing with cationic lipids. Since the complexes reach the lungs shortly after *i.v.* administration, they accumulate, due to their tendency to form large aggregates that may block small capillaries. On the other hand, liver readily takes up particles, especially charged, in the range of the complex sizes. These phenomena lead to short half lives *in vivo*.

Novel liposomal formulations have been developed to enhance the circulation time of ODNs for systemic applications. In these systems the complexes are covered with neutral lipids and possibly polyethyleneglycol (PEG) chains. This reduces protein binding as well as aggregation of the complexes leading to smaller (reduces lung uptake) and neutral (reduces liver uptake) complexes. Semple et al. (2001) 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. Encapsulation efficiency of about 70 % and greatly enhanced circulation times were reported as well as delivery to model sites of inflammation and tumor (Semple et al. 2000). A similar system utilizing cationic lipid (DOTAP) ODN complexes coated with neutral lipids (Stuart and Allen 2000) to avoid aggregation and liver/lung accumulation showed over 10-fold increase in blood half-life compared to free ODN (Stuart et al. 2000). A simple encapsulation of ODN in liposomes consisting of egg PC and Chol has been shown to increase the accumulation of ODNs to the sites of inflammation (Klimuk et al. 2000).

2.3. Regulatable cell lines

Measuring changes in endogenous protein levels after antisense treatment has many advantages, because the gene expression system is natural, but for antisense delivery studies it is tedious and time consuming. Regulatable cells lines expressing stable transgene offer a relatively fast, sensitive and easily measured system for such tests. Additionally, the target mRNA levels can be controlled efficiently in the regulated system. This may not be possible with endogenous proteins. The most widely used systems in antisense research are based on cell lines cloned to produce firefly luciferase, that can be measured easily at high sensitivity with a luminometer.

2.3.1. Cloning of cell lines

Generation of cell lines that stably express a transgene requires a selection or screening of a cell population using a marker gene that can be either induced or suppressed. Selection is usually based on a drug resistance gene in the transfected gene construct. Therefore, the cells with drug resistance gene survive under the selection pressure. Generally used drugs in selection are e.g. hygromycin-B (HYG) and geneticin (G418). Reporter genes with easily analysed gene products are used to control the transfection. Commonly used reporter genes express chloramphenicol acetyltransferase (CAT), β-galactosidase (β-gal), luciferase (luc), secreted alkaline phosphatase (SEAP) and green fluorescent protein (GFP). After transfection, the cells are grown in the presence of selection drug, surviving cell colonies are selected, expanded and characterized in terms of reporter expression.

2.3.2. Controllable gene expression systems

Gene expression systems that can be reversibly and accurately controlled by extracellular signals allow detailed studies on gene function and physiological effects of a given cellular protein (e.g., regulation of toxicity). Ability to switch on or off genes enables also detailed kinetic analysis of mRNA and protein turnover. Additionally, these systems can be utilized to express foreign genes in transgenic animals and in the development of gene therapies.

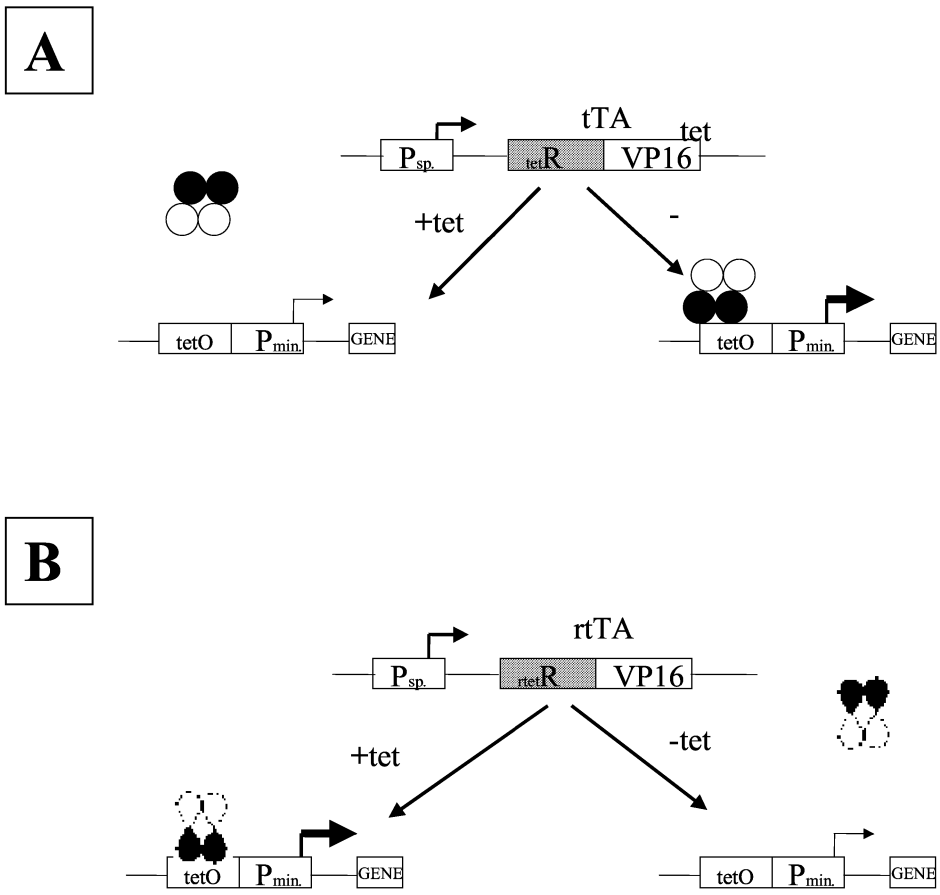
Early gene expression systems relying on mammalian heat shock-, heavy metal- or hormone-responsive components suffered from undesirable pleiotropic effects and leakiness of the inactive state (Yarranton 1992). Recently, however, Huang et al. (2000) have used heat shock response successfully to elevate 500-1000 fold the expression of a

gene with a heat shock protein 70 promoter. Additionally, up to about 1,4 x 10^4 –fold increase in cells was found for heat-inducible interleukin 12 (IL12) and up to 6.8 x 10^5 – fold for heat-inducible tumor necrosis factor (TNF-α). IL-12 also delayed tumor growth in a mouse melanoma tumor model (Huang et al. 2000). Earlier, ionising radiation was used by linking a radiation-inducible promoter to the gene encoding TNF-α and the results showed increased TNF-α production in human tumors in mice (Hallahan et al. 1995). These studies show, that cell-stress inducible gene promoters may have use in treatment of severe cases of cancer.

The problems associated with these early gene expression systems were mostly circumvented by the system based on the bacterial *tet* operon and its repressor (Gossen and Bujard 1992, Shockett and Schatz 1996). In the original bacterial system, a fusion protein (tTA = tetracycline-controlled transactivator) between the *tet* repressor and the activation domain of viral VP16 protein can bind to its cognate DNA element and activate transcription only in the absence of tetracycline (or doxycycline). In a reverse system (rtTA) the opposite happens (Fig. 5). Several versions of this system are now available (Blau and Rossi 1999). The VP16 moiety may be toxic and the accumulation of tetracycline in cells and tissues and its subsequent slow removal may hamper regulation. Moreover, cells do not necessarily respond gradually to changes in the tetracycline concentration (Shockett and Schatz 1996, Kelleher et al. 1990, Wu et al. 1995, Kistner et al. 1996, Hop et al. 1997).

Eukaryotic nuclear receptors are powerful ligand-dependent transcriptional activators (Mangelsdorf et al. 1995) that have been employed to control gene expression (Wang et al. 1994, No et al. 1996). These systems are restricted to a single or structurally similar ligands that may have undesired properties or side effects.

Commonly used systems are based on inducibility of antiprogestin mifepristone (RU486) or steroid hormone ecdysone. The improved mifepristone system is based on GLp65 transactivator (VP16 activation domain an earlier version is replaced by a region of human p65, a member of the NFκ B family). A mutated progesterone receptor combined with GAL4 DNA-binding protein and the activation domain binds, in presence of mifepristone, to the target gene containing GAL4 binding site leading to transactivation of the gene. Burcin et al. (1999) used human growth hormone as target gene and coupled the regulator to liver specific promoter. After adenoviral infection *in vitro* in HepG2 cells as well as *in vivo* in mice, high human growth hormone levels in the presence of mifepristone were achieved.



= fusion proteins between tetR and VP16 (A) or rtetR and VP16 (B)

Figure 5. Tetracycline system as an example of a controllable expression system. TetR = tet repressor, VP16 = activation domain of Herpes simplex virus protein-16, P_{sp} = specific promoter, tetO = multiple tet operator sequences, P_{min}= minimal promoter sequence. In the tTA system luciferase expression can be lowered with increasing TET concentrations. In the presence of tetracycline, repressor protein is cleaved from the operator. (**A).** In the reverse system rtTA there are mutations in the binding site leading to reverse phenotype to the repressor (rtetR) and the opposite happens (**B**).

Morphological changes induced by 20-OH ecdysone via the ecdysone receptor (EcR) in *Drosophila melanogaster*, has led to utilization of these receptors in mammalian cells. When modified, the system can be used to control transgene expression. The EcR domain is fused with VP16 activation domain, ultraspiracle gene (USP) is replaced by RXR for mammalian cells and the DNA binding site is mutated for higher inducibity. Major advantage of this system is that lipophilic ecdysone or its derivatives have better cell permeation ability as compared to more hydrophobic compounds. Additionally, the system has low toxicity and basal activity. No et al. (1996) achieved inductions up to 4 orders of magnitude with cells using either β-gal or luciferase as integrated markers. They also showed muristerone-dependent gene expression in transgenic mice.

Immunosuppressive chemicals, such as naturally occurring rapamycin or FK506 and cyclosporin bind to immunophilin proteins. Cyclosporin –FK506 dimer mediates interaction between the fusion proteins of a DNA-binding protein GAL4/FKBP12 and transcriptional activator VP16/cyclophilin. In the presence of the dimer the DNA-binding protein activates the transgene. The system has been improved to contain human components for the DNA-binding protein, a p65 activation domain and a rapamycin-binding domain from FRAP kinase instead of cyclophilin. This reduces the immunogenic responses and allows better inducibility and the use of naturally occuring dimerizator, rapamycin. Rapamycin derivatives are immunosuppressors and cause protein translation and cell cycle arrest. Nontoxic derivatives of rapamycin and site-directed mutagenesis of the binding domain may improve the situation (Liberles et al. 1997).

3. AIMS OF THE STUDY

The general objective of the study was to evaluate cationic liposomes as phosphorothioate oligonucleotide (PS-ODN) delivery agents, especially the physicochemical and morphological properties of the liposome/ODN complexes and their effect on cell delivery. The specific aims were:

1. To determine the roles of aggregation and fusion processes during formation of complexes between negatively charged ODNs and positively charged cationic liposomes.

2. To assess ODN-lipid complex morphology and external factors factors affecting complexation.

3. To model the complex interaction with endosomal membranes *in vitro*.

4. To assess the effects of complexing agents, membrane active compounds and medium on ODN transfection activity in cell culture.

5. To generate a novel regulatable cell line for antisense testing using luciferase reporter gene, as its expression can be induced or suppressed by various drugs and chemicals.

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Oligonucleotides (**I-IV**)

15-mer phosphorothioate ODNs (PS-ODNs) were synthesized with Applied Biosystems 381 A automatic DNA-synthesizer as antisense and random sequences used in studies against human c-myc (Heikkila et al. 1987) (**I, II**). In **III** and **IV** antisense sequences to the initiation to the initiation codon of firefly luciferase gene were synthesized on a PE Applied Biosystems 392 DNA synthesizer. The luciferase antisense sequences were 5′-TGG CGT CTT CCA TTT-3′ (in CV-1, D 407 and HEK293 31-4 clone cells) and 5′-TGG CGT CTT CCA TGG-3′ (in HEK293 M29 clone cells). Control sense sequence sense 5′-ACC GCA GAA GGT AAA-3′ and reversed ODN 5′-TTT ACC TTC TGC GGT-3′ were also used (**III, IV**).

The effects of the oligonucleotides on luciferase expression in a cell-free in vitro transcription-translation system was tested as described (Antopolsky et al. 1999) using TNT Coupled Wheat Germ Extract Systems kit (Promega).

4.1.2. Lipids (**I-IV**)

DDAB (dimethyldioctadecylammonium bromide), gangliosides (Gang, Type III from bovine brain), phosphatidylcholine (PC, egg hydrogenated), phosphatidylinositol (PI, bovine liver), phosphatidylethanolamine (PE, bovine liver), phosphatidylserine (PS, bovine brain), sphingomyelin (SM, egg yolk), and cholesterol (Chol) were from Sigma (St. Louis, MO, USA). DOPE (dioleoylphosphatidylethanolamine), *N*-Rh-PE [N-(lissamine rhodamine B sulfonyl)-PE], *N*-NBD-DOPE [N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-DOPE] were from Avanti Polar Lipids (Alabaster, AL, USA). DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) in aqueous solution was from Boehringer Mannheim GmbH (Germany) (**I**) or as powder from Avanti Polar Lipids (**II,III**). DOGS (dioctadecylamidoglycylspermine) was a gift from Dr. Jean-Serge Remy (CNRS, Strasbourg, France). Cytofectin GS (dimyristylamidoglycyl-N^w-isopropoxycarbonyl-arginine dihydrochloride) was from Glen Research (Sterling, VA, USA). DPPC and DPPG were from Orion Farmos Corp. (Turku, Finland). EPC was from Enzymatix Ltd - Lucas Meyer GmbH (Cambridge, UK).

4.1.3. Cationic polymers (**III**)

Polyethyleneimines (PEIs) with mean molecular weights of 25 and 800 kDa were from Aldrich and Fluka, respectively. Fractured sixth generation polyamidoamine (PAMAM) dendrimer was a kind gift from Dr. F.C. Szoka Jr. (University of California, San Francisco, CA, USA). Poly-(L-lysine) hydrobromides (PLLs) of mean molecular weights of 4000, 20 000 and 200 000 were purchased from Sigma.

4.1.4. JTS-1 peptide (**III**)

A membrane active, pH-sensitive peptide NH_2 -GLFEALLELLESLWELLLEA -COOH (JTS-1) (Gottschalk et al. 1996, Duguid et al. 1998) was synthesized and purified by Dr. Russ Henry (Protein Chemistry Laboratory at Department of Chemistry, University of North Carolina at Chapel Hill, NC, USA).

4.2. Methods

4.2.1. Liposome preparation (**I-IV**)

Cationic liposomes: DOTAP, DOTAP/DOPE (1:1 by mol), DOTAP/CHOL (1:1 by mol), DOTAP/DOPE/Chol (2:1:1 by mol) and DDAB/DOPE (8:15 by mol) liposomes were prepared in sterile water by the thin lipid film hydration method. Lipids were evaporated to dryness, water was added, and after 2 hours the samples were sonicated for about 10 minutes (50-60 Hz). For *N*-Rh-PE alone (1 mol %) or with *N*-NBD-PE (1 mol %) labeled liposomes, the labeled lipids were evaporated to dryness with cationic lipids and the samples were prepared as above, except in (**I**), where DOTAP as an aqueous solution was added to evaporated labeled lipids.
Cytofectin GS/DOPE (Cytofectin, dimyristylamidoglycyl-N^w-isopropoxycarbonyl-arginine dihydrochloride,) vesicles were prepared according to the manufacturer´s (Glen Research, Sterling, VA, USA) instructions. DOGS was used as described (Ruponen et al. 1999).

Other liposomes: Neutral and negatively charged liposomes with entrapped calcein at self-quenching (60 mM) concentration were prepared by the reversed phase evaporation (REV) method (Szoka and Papahadjopoulos 1978) in isotonic NaCl and extruded through two stacked 0.2 μm polycarbonate membranes. Unencapsulated calcein was separated on a Sephadex G-50 column (**II**).

Liposomes with lipid composition (PC:SM:PE:PS:PI:Gang:Chol, 5:1:1:1:1:1:3 by weight) mimicking that of endosomal membranes (Di Simone et al. 1994, 1995) were prepared similarly to cationic liposomes in 20 mM Hepes, 150 mM NaCl buffer (pH 7.4) at 60 ºC (**III**).

4.2.2. Liposome-oligonucleotide complex preparation

Complexes were prepared with mild vortexing in polystyrene tubes. ODNs in water were added into cationic lipid solution in Hepes-buffer (**I**), ODN/liposome complexes prepared in water into Hepes-buffer or DMEM (**II**) or complexes made by prior dilution of ODNs and liposomes in water, MES-Hepes or DMEM (**III**) Size determinations were performed after about 15 minutes. Charge ratios were calculated as 15 mer oligonucleotide having 14 negative charges and each cationic lipid one positive charge per molecule, except Cytofectin (**III, IV**) having two, and DOGS (**III**) having four positive charges.

4.2.3. Particle size determinations (**I-III**)

The sizes of ODN/lipid complexes and liposomes were determined by quasi-elastic light scattering using unimodal mode (Nicomp Submicron Particle Sizer, Model 370 (**I,II**) or Nicomp™ 380 ZLS Zeta Potential/Particle Sizer (**III**) (Particle Sizing Systems, Santa Barbara, CA, USA). Size determinations of the complexes were performed after about 15 minutes from complex preparation. Size distributions were established on the basis of vesicle number (**I-III**) and volume (**I**).

4.2.4. Calcein release (**I**)

The release of calcein was determined as the increase of fluorescence due to dilution (leakage into buffer and subsequent increase in fluorescence due to dilution of the dye) of self-quenching calcein (60 mM) from DPPC/DPPG (4:1 by mol), EPC/DOPE (1:1 by mol,), DPPC/DOPE (1:1 by mol) and EPC liposomes. The release was monitored in a cuvette with magnetic stirrer with ex 494 nm and em 515 nm for 5 minutes after adding ODN/DOTAP complexes to liposomes in 20 mM Hepes, 150 mM NaCl buffer (pH 7.4). Lipid concentration of 50 μM in 2 ml of buffer for both DOTAP and target liposome lipids was used. Triton X-100 at 0.2 % by volume was used to solubilize the liposomes and assess total liposomal calcein fluorescence.

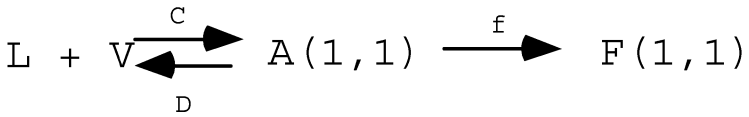
4.2.5. Resonance energy transfer (**I,II**)

Lipid mixing: Lipid mixing in cationic liposomes induced by oligonucleotides (**I**) or upon addition of complexes into different media (**II**) was monitored by resonance energy transfer (RET) at 460 nm (ex) and 530 nm (em). The method is based on having acceptor (*N*-Rh-PE) and donor (*N*-NBD-PE) probes in the same vesicle at high enough concentration to quench the emission of *N*-NBD-PE. Dilution of the probes during fusion with unlabeled vesicles will increase the donor fluorescence by increasing the distance between the labels. Fluorescence can be monitored continuously in a cuvette.

10 µl of *N*-Rh-PE and *N*-NBD-PE (1 mol % each) labeled and 40 µl of unlabeled liposomes in water were added in to 1.9 ml (37 °C) of Hepes-buffer. Subsequently 50 µl of ODNs in water were added (total volume 2 ml) and lipid mixing, as increase in *N*-NBD-PE fluorescence was followed for 3 minutes on a Perkin Elmer LS 50B Luminescence Spectrometer (Beaconsfield, Bucks, UK). Liposomes (50 µl) containing 0.2 mol % of both fluorescent probes were used to assess maximal (100 %) lipid mixing simulating the theoretical situation in which all labeled liposomes have fused with unlabeled liposomes (Düzgünes et al. 1989) (**I**).

In the case of the complexes, 20 µl labeled and 80 µl of unlabeled complexes were added simultaneously into HEPES-buffer or DMEM (1.9 ml) and lipid mixing was assessed as above. Triton X-100 at 0.2 % by volume was used to assess maximal (100%) fluorescence (**II**).

Kinetic analysis: (**I**) Kinetic analysis of fusion was based on principles of Nir (1991) and Bentz et al. (1983ab). Aggregation of the vesicles is assumed to obey second order kinetics, fractional rate of aggregation being dependent on aggregating vesicle concentrations. After aggregation, fusion takes place obeying the first order kinetics. During fusion labeled (L) and unlabeled (V) vesicles aggregate and fuse 1:1 according to following scheme:



C is the rate constant of aggregation and the aggregation rate is C x L x V (dissociation constant assumed to be negligible). Aggregate (A) fuses irreversibly at the rate f x A to form fused lipoidal structures (F). This scheme describes adequately the initial fusion resulting in fused particles of two liposomes.

The lipid concentrations were converted to approximate molar concentrations of liposomes as described (Bentz et al. 1983ab, Parente et al. 1988). Initial aggregation rate constants after addition of the ODNs were calculated assuming $I(t)=CV_0t$, where I(t) is the fraction of the maximal lipid mixing, C is the aggregation rate constant ($M^{-1}s^{-1}$), V_0 is the initial concentration of the unlabeled vesicles (M) and t is time (s). This approach is applicable at early times when $CL_0t<<1$ (Nir 1991). Slope of measured I(t) vs t was divided by V_0 to obtain C.

A kinetic model was constructed using STELLA 2.2 software (High Performance Systems, Hanover, NH). Initial vesicle concentrations in the cuvette (L_0,V_0) were used as the starting point. In the model, concentration dependent second-order aggregation of the vesicles (C x L x V) is followed by the first-order fusion step (f x A). Non-detectable fusion (e.g. among unlabeled vesicles) is distinguished from the fusion that produces the fluorescent signal by taking into account the initial fractions of labeled and unlabeled liposomes and the approximate number of fusing liposomes (size factor). The fraction of fluorescent signal of the total fusion taking place is estimated as $I = 2 \times [V/(L+V)][(n-1)/n]$ (Nir 1991), where n is the number of fusing particles estimated from the size distributions. At n=2 I is 0.4 and with increasing values of n it approaches 0.8.

Oligonucleotide release from the complexes (II): FITC-labeled PS-ODNs were complexed in sterile water with DOTAP or DOTAP/DOPE (1:1 by mol) liposomes containing 1 mol % of Rho-PE at different charge ratios. Release of ODN from the complexes was measured with Bio-Tek FL 500 fluorescence plate reader (Bio-Tek Instruments Inc., Vermont, USA) with 485 nm ex and 530 nm em after 15 min incubation at 37 ºC in Hepes buffer, DMEM or acetate-buffer (pH 5, 6 or 7.1). Effect of endosomal model membranes was evaluated by incubation of the complexes at 1.5 fold excess of negative charges from negatively charged phospholipids to those of ODN. Fluorescence dequenching was calculated by comparing with equal concentration of free FITC-oligonucleotide that was incubated simultaneously.

4.2.6. Freeze-fracture electron microscopy (**II**)

Freeze-fracture electron microscopy studies were performed by Dr. Brigitte Sternberg at the University of Jena, Germany.

Cationic liposomes and ODN/liposome complexes were quenched rapidly for freeze-fracture electron microscopy using the sandwich technique and liquid propane (cooling rate > 10^4 K/sec). The cryofixed specimens were fractured and shadowed in a Balzers BAF 400D freeze-fracture device at -120 ºC and 2×10^{-6} torr. The cleaned

replicas were examined in a transmission electron microscope (Jeol JEM 100B or Zeiss CEM 902 A) (Sternberg 1992).

4.2.7. PO-ODN stability

The effect of DOTAP and DOTAP/DOPE on PO-ODN (5′-CAT CTG TAT TGG ATC G -3′) stability was established by incubating the complexes at -/+ 0.5 and 2 charge ratios (ODN 5 μg/ml) in isotonic NaCl or DMEM, both containing 10 % FBS for 30 minutes at 37 °C. ODNs were extracted with phenol/chloroform/isoamyl alcohol. The aqueous phase containing ODN was washed with 10mM Tris, 1 mM EDTA-HCl – buffer (pH 8.0) and extracted with chloroform. The samples were directly injected from the aqueous phase into an uncoated CE capillary (67 cm x 50 μm) filled with a 12 % PEG 2000 solution in ammonium formate buffer (pH 4.5). The samples were injected in to the column by pressure and separated using reversed polarity (-30 kV at the injector end) with a Beckman P/ACE system 2100. Transient isotachophoresis (ITP) was used for focusing of the sample in the capillary before the CE separation. Up to 20 % of the capillary can be filled with the sample solution and focused to a narrow zone before the CE separation of the components. The background CE electrolyte (ammonium formate, pH 4.5) was selected so that the formate ion acts also as the leading ion in the ITP step. MES was used as the terminating slow anion in the ITP preconcentration. After the ITP preconcentration the terminator buffer vial was changed to a background buffer vial and normal CE run was started. The amount of intact ODN was measured by UV-detection at 254 nm (Auriola et al. 1996).

4.2.8. Generation of cell lines expressing luciferase (**IV**)

Tetracycline-regulated LUC in D 407, CV1-P and HEK293 cells: The D407 (cloned by Davis et al. in 1995 from human retinal pigment epithelial cells), CV-1 (monkey kidney fibroblast) and HEK293 (human embryonic kidney) cell lines expressing luciferase were developed as described for CV-1 cells (Antopolsky et al. 1999). Briefly, the cells were transfected with pTet-Off plasmid (Clontech Inc. Palo Alto, CA, USA) to generate stably integrated tetracycline-sensitive regulator. The recombinant cells were selected in the presence of 0.4 mg/ml G418 (Calbiochem, La Jolla, CA, USA). Resulting colonies were screened for expression of the tetracycline-sensitive regulator by transient transfection of pTRE-luc and pCMV-beta-galactosidase plasmids, culturing the cells in the presence or absence of 2 mg/l tetracycline for 40 hours before LUC and β-gal assays. The positive cell lines were expanded and

transfected with pTRE-luc plus pTK-hyg plasmids (Clontech Inc. Palo Alto, CA, USA), selected in the presence of both 0.4 mg/ml G418 and 0.1 mg/ml hygromycin B (Calbiochem) and screened again for tetracycline-repressible luciferase activity without transfection.

Constitutively active receptor (CAR) (IV: Fig. 1)**:** Cytochrome P450 (CYP) enzymes metabolize a variety of xenochemicals including drugs as well as endogenous compounds (Nielsen and Moller 1999, Masahiko and Honkakoski 2000). *In vivo* CAR controls expression of cytochrome P450 *CYP2B* genes by binding to the phenobarbital-responsive enhancer (PBREM). CAR can be suppressed by some androstane metabolites, while suppressed CAR can be activated by some chemicals including clinically used drugs leading to activation of PBREM (Masahiko and Honkakoski 2000).

CAR controlled LUC in HEK293 cells: HEK293 cells were transfected with the pCI-neo/CAR plasmid by the calcium phosphate method (Chen and Okayama, 1987) and selected with 0.4 mg/ml G418. Cells were screened by transfecting with pCMVβ plus pPBREMluc plasmids (100 ng each) and cultured for 40 hours in the presence of either 5 μM 5α-androst-16-en-3α-ol (ANDR) or 500 nM 1,4-*bis*[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) to repress or activate CAR-regulated LUC activity, respectively (Honkakoski et al. 1998a, Forman et al. 1998, Sueyoshi et al. 1999). Colonies yielding strong CAR-dependent responses were transfected with pTKhyg plus pPBREMluc (1:20 ratio), selected with 0.1 mg/ml HYG and G418- plus HYG- resistant colonies were tested for ANDR-repressed and TCPOBOP-activated LUC. Colonies exhibiting good CAR regulated LUC activity were expanded and characterized in further studies.

4.2.9. Cell cultures and oligonucleotide transfection (**III**,**IV**)

D 407, CV-1 (**III**) and HEK293 (clones M29 and 31-4, **IV**) cells were grown in DMEM (Gibco) with 5% (D 407) or 10% (CV1-P, HEK293) FBS and 1% penicillin-streptomycin (Gibco). One day before transfection the cells were plated into 24 well plates (1×10^5 cells/well). D 407 and CV-1 cells were treated with 360 nM ODN complexed with different carriers for the screening of different carriers and optimizing the +/- charge ratio (D 407 cells), and with 0-720 nM and 0-360 nM ODN for D 407 and CV-1 cells, respectively, for assessing the dose response of the antisense effect. DOTAP/ODN complexes were formed at charge ratio of +/- 4 and subsequently neutralized with JTS-1. Cells were incubated with the complexes for 4 hours in 400 μl of serum-free medium. Thereafter, the medium was replaced with normal growth

medium and the cells were grown for further 20 hours (**III**). HEK293 (M29 and 31-4) cells were transfected for 4 hours in 400 µl of normal growth medium with 2.5 µg/ml Cytofectin and 37.5-300 nM of ODNs. Fresh medium (400 µl) was added and the cells were cultured for 24 hours with 5 µM ANDR or 500 nM TCPOBOP for M29 cells, and in the presence or absence of 2 µg/ml TET for 31-4 cells (**IV**).

4.2.10. Determination of luciferase activity (**III**,**IV**)

The cells were washed with PBS and lysed with lysis-buffer (25 mM Gly-Gly/ 15 mM $MgSO_4$/ 4 mM EGTA/ 1 % Triton X-100/ pH 7.8). Luciferase activity was measured on a BioOrbit 1251 Luminometer by adding 100 µl of luciferase reagent (20 mM Tricine/ 1.07 mM magnesiumhydroxycarbonate/ 2.67 mM $MgSO_4$/ 0.1 mM EDTA/ 33.3 mM DTT/ 0.53 mM MgATP/ 270 µM coenzyme A/ 470 µM luciferin) to 50 µl of cell lysate. Protein was measured with BCA reagent kit (Pierce, Rockford, Il, USA). The results are calculated as relative light units (RLU)/mg protein and expressed as % compared to cells without treatment (=100%).

5. RESULTS AND DISCUSSION

5.1. Fusion of cationic liposomes and their complexes (**I,II**)

5.1.1. Lipid mixing during lipid-ODN complex formation (**I**)

During complexation oligonucleotide is added to the cationic liposomes at different -/+ ratios. In fluorescence resonance energy measurements increasing fluorescence indicate lipid mixing. In the case of non-exhangeable probes in liposomes (i.e. not exhangeable via medium), increased lipid mixing indicates aggregation and subsequent membrane fusion between liposomes or complexes. This may lead to the loss of the original liposome structure leading to particles with very heterogenous size distribution and morphology.

With increasing -/+ ratios at 20 μM cationic lipid concentration of DOTAP or DDAB/DOPE liposomes, there was a substantial increase in the initial rate of fluorescence increase and maximal fluorescence reached also higher levels. The increase was much slower, and the maximal fluorescence was lower with 2 μM DDAB or 4 μM DOTAP concentrations (**I**: Figs. 2 and 3). The initial rate of lipid mixing (%/s) in 20 μM mixture of cationic lipids increased with added ODN concentration until a plateau was reached. The plateau was achieved at smaller -/+ ratios in the case of DDAB than with DOTAP (**I**: Fig. 4). In a study with plasmid DNA (Mok and Cullis 1997), using DOTMA/DOPE and DOTMA/DOPC liposomes in 20 mM Hepes, similar results (i.e. lipid mixing induced by DOPE) were obtained. At 250 μM cationic lipid concentration, plasmid DNA caused similar lipid mixing and maximum was achieved with lower -/+ charge ratios with DOTMA/DOPE liposomes. Citrate and sodium chloride gave similar results: both induced lipid mixing at much lower concentration with DOTMA/DOPE liposomes, sodium chloride causing minimal lipid mixing in DOTMA/DOPC liposomes even at 2 M concentration.

Fusion of cationic liposomes appears to be controlled by the aggregation rate (different fractional initial rates of fluorescence increase at different vesicle concentrations) and therefore aggregation rate constants were calculated (**I**: Fig. 5). From the -/+ ratio of zero the added ODN increased the aggregation rate constant in DOTAP suspension about 5 times and 300 times at cationic lipid concentrations 4 μM and 20 μM (**I**: Fig. 5a), respectively. In the case of DDAB/DOPE the corresponding changes were 3 and 80 fold at DDAB concentrations of 2 μM and 20 μM, respectively (**I**: Fig. 5b).

Aggregation of the liposomes can take place only when the energetic barrier at the liposomal surface is overcome (Nir 1991), and, lipid fusion can take place only after membrane mixing. Rate controlling step (aggregation or fusion) can be distinguished by carrying out the RET experiments at two different vesicle concentrations (Nir 1991, Bentz et al. 1983a). At low concentrations aggregation is rate limiting due to the long distances between the vesicles. Lipid mixing rate was highly dependent on the vesicle concentration; therefore the vesicle aggregation is the overall controlling step, allthough phenomena such as membrane contact, membrane dehydration and early lipid mixing may also be of importance. Eventually ODNs increase the fusion of the cationic liposomes by enabling their aggregation.

Cationic liposomes without ODNs fused at the same rate independent of the liposome concentrations in DOTAP and DDAB/DOPE experiments. This suggests a completely fusion controlled system. Assuming that fusion step controls the lipid mixing in cationic liposomes, we calculated the apparent fusion rate constant using following equation of Nir (1991) in order to clarify whether aggregation or fusion is controlling the system.

$$I(t)/A_0 = 1 - e^{-f\,t}$$

f = fusion rate constant
t = time
I = fraction of the fluorescent signal from the total

For DDAB/DOPE the best fit was obtained at f values 0.0007-0.0008 s^{-1} ($r > 0.98$) and for DOTAP at 0.00033-0.00035 s^{-1} ($r > 0.98$). Due to the size factor 40 % is the theoretical maximum of lipid fusion. This was taken into account by dividing the first order rate constants above by 0.4 to obtain true rate constants for the simulations with the model. The obtained values were 1.7-2.0 x 10^{-3} s^{-1} (DDAB/DOPE) and 8.3-8.9 x 10^{-4} s^{-1} (DOTAP). After finding these values for fusion the aggregation rate constant was varied in the simulations. The aggregation rate constants should be > 5 x 10^7 $M^{-1}s^{-1}$ for DDAB/DOPE and > 5 x 10^8 M^{-1} s^{-1} for DOTAP in order to give the observed fusion rates in fusion controlled system at all lipid concentrations of our study. Compared to the initial aggregation rate constants (**I:** Fig.5) these values are unrealistic (> 5 times too high) for binding of cationic liposomes without ODNs. Charge repulsion should make the aggregation of the liposomes improbable. Thus, the concentration independent slow lipid mixing among the cationic liposomes without ODNs is not mediated by aggregation.

Further evidence for a process not mediated by aggregation was seen in simulations, where aggregation rate constant (C) was kept constant at realistic level (DDAB/DOPE: 0.1 x 10^7 $M^{-1}s^{-1}$ and DOTAP: 0.5 x 10^7 $M^{-1}s^{-1}$) and f was varied from 0.001 s^{-1} to 1.0 s^{-1}. For 4 μM DOTAP the observed lipid mixing was achieved only at $f \geq 0.5$ s^{-1} while at 20 μM the experimental rate was seen in simulation at $0.002 < f < 0.003$ s^{-1}. However, in all cases the simulated lipid mixing rate (%/s) was several times higher at 20 μM than at 4 μM suggesting aggregation rate controlled system. In the case of DDAB/DOPE, simulated fusion rate was also substantially higher at 20 μM than at 2 μM. These simulations suggest that at -/+ ratio of zero an aggregation initiated fusion rate controlled system is not a valid explanation for the observed concentration independent mixing of the lipids. Rather, the fluorescent lipid dilution without ODNs may be due to exchange of the fluorescent lipids via the medium. The fluorophores (NBD-PE, Rh-PE) were found to be virtually non-exhangeable between phospholipid vesicles (Struck et al. 1981, Hoekstra 1982). Similar extent of spontaneous lipid mixing as reported here was found for DOPE containing liposomes (Collins et al. 1990, Hazemoto et al. 1990) and for cationic DOTMA/PE liposomes (Düzgünes et al. 1989) in buffer. This may be due to cationic liposomes being SUVs, from which fluorophores may detach easier due to the relative instability of the highly curved bilayer.

Our studies based on oligonucleotide-cationic lipid complex size determinations and RET indicate that the oligonucleotides induce aggregation and, subsequently, fusion of the cationic liposomes. Aggregation and fusion of didodecyldimethylammonium bromide is similarly induced by dianions of dipicolinic acid (Rupert et al. 1985, 1986). When the ODN concentration is increased relative to the cationic liposomes the probability for formation of larger aggregates increases, but -/+ charge > 1 the mean size decreases again. This is probably due to repulsion by the negative charges that prevents the formation of the larger aggregates. The increase of the complex size of DDAB/DOPE liposomes took place at lower charge ratios than in case of DOTAP. Due to the larger initial size, smaller cationic charge density, and smaller surface area of DDAB/DOPE liposomes, less ODN is needed to decrease the electrostatic repulsion.

Interestingly, at -/+ ratio of 5.6 with ODN/DOTAP (20 μM) the lipid mixing was initially fast, but stopped abruptly at lower fluorescence level (about 60 %) than at charge ratios 0.8 and 1.2. The same phenomenon is seen also at 4 μM lipid concentration. At high (>0.8) charge ratios there is a decrease in the rate of fusion and initial aggregation. Probably, at higher -/+ ratios and later incubation times the ODNs have more chances to reverse the liposomal surface charge before the liposome binding and fusion. Accordingly the initial rates of fusion and aggregation are higher at high

vesicle concentrations compared to the lower concentrations. At high vesicle concentrations ODN-induced binding and fusion take place rapidly before the negative electrostatic repulsion becomes predominant. At low vesicle concentrations ODNs have more time to bind on the liposomes and to reverse the charge before the liposomes contact each other. DDAB/DOPE liposomes had a lower lipid mixing threshold possibly because DOPE is prone to undergo phase transition and fusion (Litzinger and Huang 1992).

The kinetic model was tested also for simulation of the time courses of ODN induced lipid mixing at the experimental conditions. In most cases the model provided good predictions, but often the simulations deviated clearly from the data (not shown). This is not surprising because at later times larger aggregates and very complex mixtures of fused and aggregated particles are formed. In order to take into account all the stochiometric possibilities in the fusing ODN-liposome system the model should be exceedingly complicated.

5.1.2. Lipid mixing of preformed complexes in different media (**II**)

Lipid mixing was tested with DOTAP and DOTAP/DOPE (1:1 by mol) as they are widely used and readily available (as pure lipids or readymade liposomes). Experiments were made in water, buffer and cell culture medium as they are all commonly used media in transfection studies.

When cationic lipid-ODN complexes (-/+ charge ratios 0, 0.3, 0.6, 1.2 and 2.8) were prepared in water and then added into Hepes-buffer the lipid mixing in the case of sole DOTAP liposomes was about 10 - 15 of maximal at charge ratios of 0.3, 0.6 and 1.2, while no significant mixing was found at 2.8. Maximal lipid mixing was about 40 % for DOTAP/DOPE (1:1 by mol) at ratios 1.2 and 2.8, and similar to that of DOTAP at ratios of 0.3 and 0.6 and about 15 % for DOTAP liposomes. For DOTAP/DOPE (1:2 by mol) the results were similar to DOTAP/DOPE (1:1 by mol), except that maximal lipid mixing was also achieved at a lower -/+ charge ratio of 0.6 (**II**: Fig. 2).

In DMEM lipid mixing of DOTAP-ODN complexes was only seen at around neutrality and to some extent with net negative complexes. With DOPE substantial lipid mixing was observed at all charge ratios, including zero (**II**: Fig. 3). Thus, DOPE induces, in addition to aggregation, also fusion at all charge ratios in DMEM. These data show that the complexes undergo more fusion after exposure to buffer or DMEM and the data is in agreement with results from the morphological studies (**II**: Figs. 4-6).

5.2. Size distributions of the complexes (**I-III**)

Mean sizes of ODN/DOTAP complexes in Hepes-buffer increased gradually up to the -/+ ratio of about 1. Increasing the ratio further led to a gradual decrease of the complex size down to about 30 nm. With DDAB/DOPE liposomes the increase in size started at lower charge ratios (-/+ 0.02) and at high charge ratios mean particle size remained larger (**I**: Fig. 1). At higher ratios, fast complexation, aggregation and fusion occurs and the complex surface is covered with negative charges that block further aggregation thereby yielding small particles.

To study the effect medium on the size distributions, the complexes prepared in water were diluted with different media (**II**). Mean complex size in water increased with increasing -/+ ratio. At -/+ ratio of 1.2, the complex sizes were 5-10 fold that of the original liposomes. Again at higher -/+ ratios the complex sizes decreased (**II**: Fig. 1A). In Hepes buffer, complex sizes were about 2 –fold (100-200 nm) compared to those in water up to -/+ charge ratio of about 0.6 - 0.8. Thereafter there was a size increase to μm range in case of DOTAP/DOPE complexes. With DOTAP similar sized complexes as in water were seen at higher (≥ 1.5) -/+ charge ratios (**II**: Fig. 1B). In DMEM, cell growth medium DOTAP complexes showed similar size distribution profile as in Hepes, whereas DOTAP/DOPE complex mean sizes at both molar (1:1 and 1:2) ratios were 700-1200 nm (sd up to 60 %) at all charge ratios (**II**: Fig. 1C). After 2 hours, none of DOPE containing complexes were measurable and visible particles were formed in many cases.

Ions in buffer or components in cell growth medium are important in fusion reactions. This may take place via dehydration, as headgroup ionization, which favors lipid L_α phase (normal bilayer, Fig. 8), is reduced by salts (Seddon et al. 1983) and dehydration is considered to be necessary for H_{II} phase formation (inverted hexagonal phase i.e. tubular structures with acyl chains pointing outward, Fig. 8) (Katsaras et al. 1993). In the case of DMEM, however, ODN content did not seem to have effect on the size distribution of DOTAP/DOPE complexes. The mean size of DOPE containing complexes exceeded clearly the size limit, about 200 nm, for liposome endocytosis in most cells (Mönkkönen et al. 1994). Complexes with plasmid DNA have been shown behave similarly in cell culture medium: rapid increase in size during about 20 minutes for DOTAP/DOPE/DNA complexes, whereas replacing DOPE with DOPC led to stable size distribution for over 2 hours. Additionally, although DOPE enhanced transfection greatly compared to DOTAP alone, DOPC was more effective than DOPE (Hui et al. 1996), in terms of protection against particle size increase. They suggested that bigger DOPE containing particles hindered the uptake of smaller endocytable particles by first

making the contact with the cell membrane, whereas with small sized DOPC containing particles this was not the case. Mui et al. (2000) showed similar lipid mixing with cell membranes for DODAC/DOPE and DODAC/DOPC. They concluded, that lipid mixing with endosomal membranes is essential for DNA transfer, but that the transfection potency correlates better with the ability of the lipid to disrupt membrane integrity. Unfornately, although antisense effect and release of ODN at endosomal level (both were greatly enhanced by DOPE) were also studied, lipid mixing was only done with DNA.

5.3. Complex interactions with model membranes (**I**, **II**)

5.3.1. Calcein release from model liposomes (**I**)

Calcein release from neutral and negatively charged liposomes was used to study the destabilizing effect of the complexes prepared with DOTAP on lipid bilayers. In Hepes-buffer, highly charged hydrophilic calcein did not leak from the liposomes and due to the extrusion method, the degree of calcein entrapment was quite similar in all liposomes tested. DPPC/DPPG and EPC/DOPE liposomes showed rapid calcein release up to -/+ charge ratio of about 0.3 and slower release up to a charge ratio 1. Unlike negative DPPC/DPPG liposomes, EPC/DOPE liposomes showed calcein release also at higher (>1.6) charge ratios. Pure EPC liposomes did not show any significant release of calcein (**I**: Fig. 6).

For DPPC/DPPG liposomes the mechanism of calcein release seems to be simply the electrostatic attraction of opposite charges. With excess negative charges of ODN/DOTAP mixtures, no calcein release was seen. This was probably due to electrostatic repulsions, fewer collisions of the larger aggregates and the lack of DOPE in the liposomes.

Similar calcein release profile up to a charge ratio of about 1 was seen in the case of EPC/DOPE liposomes. Again, positive net charge was essential for interaction. At ratios of about 0.6 - 1.7 there was very low calcein release possibly due to the increased particle sizes leading to decreased collision rate. At higher ratios, there was slow leakage at the charge ratios above 2. Additionally, the slight negative charge of DOPE may contribute to the results.

Interestingly DOTAP did not cause significant calcein release from EPC liposomes at any -/+ ratio. The result is different from the EPC/DOPE liposomes and demonstrates that PE may be essential for the capability of DOTAP to fuse with cell membranes. In the case of negatively charged membrane (DPPC/DPPG), PE was not

required. Likewise, studies on interactions of PE and/or PC liposomes with low molar DOTAP fractions have shown aggregation with negative liposomes at physiological or lower ionic strengths and the enhancement of aggregation with increased PE/PC ratio (Bentz et al. 1983b).

Since PE and negatively charged phospholipids are present in the cell membranes, DOTAP liposomes have the ability to fuse with them (e.g. endosome).

5.3.2. Release of oligonucleotide from the complexes (**II**)

ODNs must be released from the complexes during interaction with endosomal membranes or later in the cytoplasm or nucleus. In the case of endosomal release, free ODNs must be released into cytoplasm for activity. To test the possible interaction, model liposomes with membrane phospholipid composition mimicking that of endosomes were used. As environmental pH of the cells (i.e. plasma membrane) is near neutral and acidic inside endomes (pH about 5-6), the interactions were studied at different pH values.

There was a significant fluorescence dequenching (increase in FITC-ODN fluorescence) and relaxation of resonance energy transfer between FITC-ODN and Rh-PE when ODN/DOTAP/DOPE complexes were incubated with endosomal model membranes at pH 7.1 and 7.4, especially at excess positive charges (-/+ ratio < 1). With ODN/DOTAP complexes, there was no significant fluorescence increase. These results suggest that DOPE is needed for ODN release from the complexes in contact with the bilayer (**II**: Fig. 7). The results are in agreement with later results of Mui et al. (2000). Endosomal release of FITC-labeled PS-ODN in KB cells was about 4 times higher with DODAC/DOPE than DODAC/DOPC complexes. An interesting hypothesis how DOPE facilitates DNA release, has been proposed by Harvie et al. (1998). They suggest that the negative charge in DNA phosphate group partly binds to positive charge in PE instead of the cationic lipid and this binding is much weaker and facilitates DNA release in contact with negatively charged membranes such as endosomal wall after endocytosis.

Experiments without endosomal model membranes showed considerable dequenching when ODN-lipid complexes were placed at pH 5 and 6 (typical pH in endosomes). This was most pronounced at lowest -/+ charge ratio (0.1) and was higher for DOTAP than DOTAP/DOPE complexes (**II**: Fig. 8). Additional fluorescence dequenching was observed when DOTAP/DOPE complexes were incubated with model membranes at pH 5 or 6, suggesting increasing ODN release upon contact with model membranes (**II**: Fig. 9A). Surprisingly, fluorescence quenching was seen when

DOTAP/ODN complexes were incubated at pH 5 and 6 endosome mimicking liposomes at -/+ charge ratios of less than 1.5 (**II**: Fig. 9B). The fluorescent labels may come in closer proximity when the complexes bind to the endosomal membranes, after partial loosening of the complexes at acidic pH. Eventually, DOTAP may have lower membrane destabilizing activity at endosomal pH (Wattiaux et al. 1997) than at neutral pH. The results are in agreement with previous findings, where DOTAP/DOPE liposomes were shown to fuse with negatively charged DOPC/DOPG liposomes at pH range 4.5 - 7, while DOTAP/DOPC liposomes did not fuse. Similar results were obtained in lipid mixing experiments with cells, where both liposomes showed similar binding and uptake, but significant lipid mixing was seen only with DOPE containing liposomes (Wrobel and Collins, 1995). Using synchrotron small-angle x-ray scattering (SAXS) and microscopy, it has been shown, that interactions of DOTAP/DOPE (H_{II} phase) and DOTAP/DOPC (L_{α} phase) DNA -complexes with 10 % negatively charged DOPC/DOPG (9:1) liposomes are very different. DOPC containing complexes attached to the liposomes without fusion, while DOPE containing complexes showed fusion, loss of the complex structure and appearance of free lipid lamellae (Koltover et al. 1998).

Furthermore, ODNs have been shown to be displaced from DOTAP liposomes when incubated with negatively charged fluid state liposomes, but not with neutral, or negative liposomes containing a mixture of fluid and solid state lipids, demonstrating the importance of the physical state of the target bilayer on ODN release (Zelphati and Szoka 1996b). Our results are in agreement with the results obtained for solid state membranes with respect to DOTAP (Zelphati and Szoka 1996b), as endosomal model membranes used in this study are partially composed of solid state lipids at 37 °C. However, DOPE in liposomes caused significant release of ODN even in the case of our endosomal liposomes that contain relatively high amounts of cholesterol and sphingomyelin (Belcher et al. 1987, Lange et al. 1989, Warnock et al. 1993). High cholesterol/phospholipid ratio has been found in endosomal membranes (Lange et al. 1989, Warnock et al. 1993). In fact TMA-DPH fluorescence anisotropy values for endosomal membranes for some cell lines are more in agreement with solid state than fluid state bilayers (Illinger et al. 1995). The lipid composition of model liposomes in this study is in agreement with the lipid composition of endosomal membranes and the results show clear difference between endosomal interactions of DOTAP and DOTAP/DOPE complexes.

5.4. Morphology of the complexes (**II**)

Since our previous experiments showed that DOPE induces changes in the complexes (size, interaction with target bilayers), it was of interest to study the morphology of ODN complexes in different situations.

Freeze-fracture electron micrographs of plain DOTAP, DOTAP/DOPE (1:1) and DOTAP/DOPE (1:2) liposomes incubated in Hepes-buffer (**II**: Figs. 4A,C and D, respectively) showed mainly small (< 300 nm) vesicles probably due to inhibition of aggregation by repulsive forces of the liposomes (Sternberg et al. 1994). DOTAP liposomes were similar in DMEM (**II**: Fig. 5A) and in buffer, but DOTAP/DOPE liposomes transformed to partial or full hexagonal-tubular (H_{II}) structures in DMEM (**II**: Fig. 5C,E). Based on its wedge-shaped structure, DOPE can adopt highly curved structures, such as H_{II} tubules. They are usually observed at high lipid concentrations, high temperatures (Cullis and Kruijff 1979), and/or high salt concentrations (Allen et al. 1990).

The ODN/DOTAP complexes in Hepes (**II**: Fig. 6A,6A′) and in DMEM (**II**: Fig. 5B) showed no apparent hexagonal structures, whereas DOTAP/DOPE complexes, especially at 1:2 molar ratios and -/+ ratio of 1.2, were extensively transformed to H_{II} tubular structures in Hepes (**II**: Fig. 4F) and in DMEM (**II**: Fig. 5F). Here, fusion of liposomes and hexagonal phase formation is possibly induced by cations (Allen et al. 1990). Morphological features of liposomes made of DOTAP/DOPE at 1:1 molar ratio (**II**: Figs. 4D, 5D, 6B, 6B′) were found to be intermediate between DOTAP and DOTAP/DOPE (1:2). Clearly increasing the concentration of DOPE and ions (DMEM > Hepes) favors lipid fusion and H_{II} tubule formation. This is seen also at -/+ ratios of 0.3 and 2.8.

Using SAXS, similar structural differences were found between DOTAP/DOPC and DOTAP/DOPE liposomes when complexed with DNA (Koltover et al. 1998). In this study, as well as in SAXS studies in general, a radical difference is that the complexes have to be made using high concentrations of both the carrier and DNA for sensitivity. Additionally, the complexes can be made in deionized water. This is based on very high concentrations of both DNA and liposomes complexed in a very small volume (tip of a closed capillary) leading to release of DNA counterions Na^+ and Cl^- during complexation and subsequent formation of different structures depending on the liposome composition (Rädler et al. 1997, Koltover et al. 1998).

It seems that the functional differences of ODN-cationic liposome complexes with and without DOPE are associated with clear morphological differences.

5.5. PO-ODN stability

Both DOTAP and DOTAP/DOPE complexation led to a marked decrease in degradation of ODNs in saline or DMEM containing 10 % FBS (Fig. 6). The degree of stabilization was dependent on the charge ratio. At -/+ 0.5 both liposomes considerably stabilized ODN in saline, but in DMEM only DOTAP complexation prevented degradation. With excess negative charges (-/+ 2), both liposomes showed similar stabilizing profiles but the effect was much weaker (Fig. 6). Positively charged complexes are small in saline and bind ODNs preventing degradation by nucleases, whereas in DMEM extensive lipid mixing and formation of hexagonal phases (**II**) probably leads to less tightly bound ODN in the case of DOTAP/DOPE explaining the higher degree of degradation. Perhaps, the different mechanism of ODN binding may explain the difference (Harvie et al. 1998). Excess of negative charges (-/+ 2) increased ODN degradation probably due to free ODN present and/or weaker binding.

The results show, that free or complexed PO-ODNs are quite rapidly degraded in serum containing cell culture medium, except in the case of positively charged (-/+ 0.5) ODN/DOTAP complexes. This may be of importance, since optimized complexes of DNA/DOTAP at the same charge ratio can transfect plasmid DNA equally effectively in the range of 0 – 10 % FBS in the cell culture medium (Hyvönen et al. 2002).

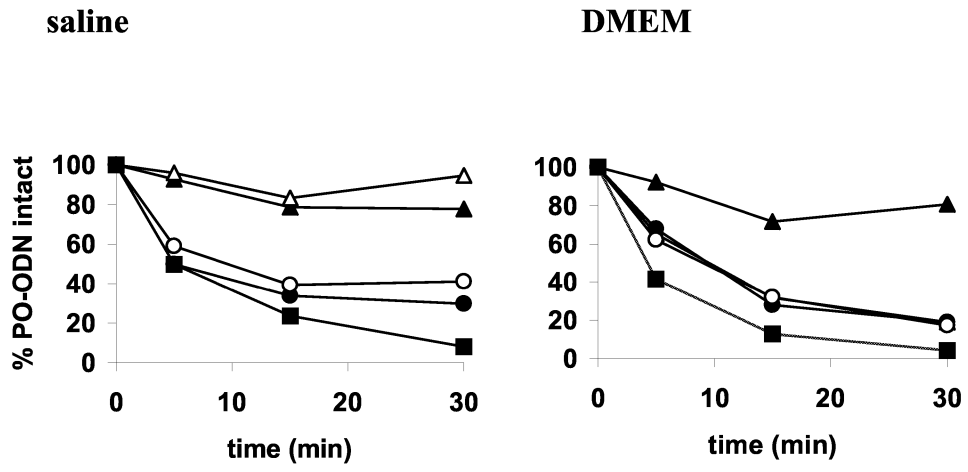


Figure 6. Stability of a 16-mer PO-ODN as a free ODN (■) or complexed with DOTAP (▲ = -/+ 0.5, △ = -/+ 2) or DOTAP/DOPE (● = -/+ 0.5, ○ = -/+ 2) in either saline or cell growth medium DMEM, both containing 10 % FBS.

5.6. Antisense effect using different carriers (**III**)

Antisense effect was tested using luciferase expressing cell lines and ODN against firefly luciferase.

5.6.1. D 407 cells

Retinal pigment epithelium is an interesting target for antisense ODN modifications. Retinal pigment epithelium may host viruses, it produces several growth factors, and it may proliferate in uncontrolled ways in proliferative vitreoretinopathy. Regulation of gene expression by ODNs may have therapeutic implications. Therefore, retinal pigment epithelial cell line, D 407, was used in the experiments.

The polymeric carrier/ODN complexes (PLL 4 000, PLL 20 000, PLL 200 000, PEI 25 kDa, PEI 800 kDa or 6^{th} generation dendrimer) did not have any detectable effect on luciferase activity. Neither did JTS-1 peptide in PLL 20 000 or PEI 25 complexes have any antisense effect, although it has been shown to improve delivery of plasmid DNA when it is used together with DNA condensing carrier peptide analogs (Gottschalk et al. 1996, Duguid et al. 1998).

Polylysines have been shown to deliver ODNs and DNA into cells. Contrary to DNA, antisense activity is only seen when PLL is conjugated with other peptides to increase cytosolic and nuclear uptake (Rojanasakul 1996, Dokka et al. 1997). In those studies, however, the PS-ODN concentrations were higher (1 μM or higher) and the PLL moiety was very small (10 mer) (Dokka et al. 1997). Importantly, antisense effect was not demonstrated in previous studies for unconjugated PLL, although the cell association of PS-ODN was increased by more than 10-fold with comparable ODN concentrations to our study (Stewart et al. 1996). Likewise, PLL delivers plasmid DNA well into the cells in culture, but transfection activity is very low (Ruponen et al. 2001).

Fractured PAMAM dendrimers have been shown to increase cytosolic and nuclear delivery of ODNs (Bielinska et al. 1996, DeLong et al. 1997) and to transfect plasmid DNA (Haensler and Szoka 1993, Tang et al. 1996, Kang et al. 1999, Ruponen et al. 1999). However, the antisense effects at the PO-ODN concentrations comparable to our study (≤ 360 nM) were only ≤ 20 % (Bielinska et al. 1996) and higher antisense effect was seen only at 6 fold higher PS-ODN (2 μM) (Hughes et al. 1996) or PO-ODN (1-2 μM) concentrations (Hélin at al. 1999). In D 407 cells, we did not see any antisense effect with PS-ODN delivered with 6th generation fractured dendrimer at PS-ODN concentration 360 nM and below. Kang et al. (1999) showed a great difference in the ability of Superfect™ (a commercially available fractured dendrimer) to transfect DNA

or methylated PS-ODN. DNA transfection was high compared to other carriers used, but antisense PS-ODN activity against splicing correction was about the lowest of the tested delivery systems.

PEI is known to deliver fluorescent PO-ODN into the cell nuclei (Boussif et al. 1995), but no antisense effect has been established. Recently, Dheur et al. (1999) showed, that although PS-ODNs are effectively delivered into the cells by PEI, the complexes with PS-ODN maybe too stable and ODN is not released from the complex to exert antisense activity. They used agarose gel electrophoresis, and showed that PS-ODN was not released from complexes with 10- or 20- fold excesses of positive charges, while PO-ODN was released to some extent. Similar results with 25 and 22 kDa PEI (and 54 kDa PLL) have been shown in mRNA transfection (target in cytoplasm). PEI 25 kDa and PLL 54 kDa complexes failed to express the mRNA in a cell-free translation and in the cytoplasm after microinjection. Decreasing the MW to 2 kDa (PEI) and 3.4 kDa (PLL) lead to efficient transfection, albeit chloroquine or melittin-conjugation was required for endosomolytic activity (Bettinger et al. 2001). Target cells may also be of importance, since the sole study showing PS-ODN antisense effect with PEI (50kDa) was done using peripheral and central neurons in primary culture (Lambert et al. 1996). Previously, PEI and dendrimers have been shown to be more effective than lipid based carriers in transfecting plasmid DNA into CV-1 (Ruponen et al. 1999) and retinal pigment epithelial cells (Urtti et al. 2000). In contrast, in our antisense study, PEI does not seem to deliver PS-ODN in effective form into the cells. These data suggest that there are important differences in the cellular delivery (cytosol or nuclei) of plasmid DNA and PS-ODNs with polymers.

ODN/lipid complexes without a fusogenic helper lipid (DOTAP, DOTAP/Chol or DOGS) did not cause inhibition of luciferase activity in D 407 cells. Significant antisense effect on D 407 cells was only seen with liposomal carriers DOTAP/DOPE/Chol (2/1/1), Cytofectin (**III**: Fig 1A) and DOTAP/JTS-1 (**III**: Fig 1B) at optimized +/- charge ratio of 1, 4, and 1 respectively. DOTAP/ODN complexes were formed at charge ratio of +/- 4 and subsequently neutralized with negatively charged JTS-1 peptide.

JTS-1 peptide, when added to DOTAP/ODN complexes, greatly enhances the antisense activity. JTS-1 seems also to be a more effective membrane active liposomal component than DOPE in increasing the ODN delivery. JTS-1 peptide has been shown to have lytic activity on PC liposomes and erythrocytes and the activity is more pronounced at pH 5 than pH 7 indicating high membrane activity on endosomal level (Gottschalk et al. 1996). JTS-1 peptide, together with DC-Chol and protamine, has been also been used for transfecting ribozymes against human proto-oncogene c-neu (Lui et

al. 2001). Additionally other peptides, such as HA (GLFFEAIAEFIEGGWEGLIEGC), derived from *Haemophilus influenza* hemagglutinin, show similar effects, at least with plasmid DNA transfections (Vaysse et al. 2000).

5.6.2. CV1-P cells

In studies with CV1-P cells with liposomal carriers, DOPE or JTS-1 peptide containing complexes had the highest activity, Cytofectin being the most effective formulation (**III**: Fig. 3). ODN complexes with DOTAP at +/- 4 or DOTAP/DOPE/Chol at neutral charge ratio were the least effective (about 20 % inhibition). Thus, the results in CV1-P cells are similar with D 407 cells, suggesting the importance of lipid fusion and ODN release in antisense delivery. GALA peptide has been shown to enhance plasmid DNA transfection when used with DOTAP/DOPE liposomes and the highest activity was found with neutral or negatively charged liposomes depending on the cell line (Simões et al. 1998). Liang et al. (2000) found that the use of 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. On the contrary, they did not see any effect on plasmid DNA distribution with similar complexes.

5.6.3. Medium effects

As endocytosis is at least the major pathway for the delivery of carrier/oligonucleotide complexes, the particle size of the complexes should be small, preferably well under 200 nm. Lipid-ODN complexes prepared in water are smaller compared to most complexes made in buffer or cell growth medium and, subsequently, they have the highest antisense activity. Although these complexes undergo aggregation and fusion after addition to cell growth medium, rapid binding of small particles to the cells may increase the endocytosis of ODN/carrier complexes and subsequently enhance antisense activity. On the other hand, larger particles, if endocytosed, can lead to higher intracellular concentration of ODNs as they have higher ODN cargo per complex.

Although DOTAP/ODN complexes are smallest in all media, they do not exert significant antisense activity in the cells studied. Lower fusogenic activity and poor release of ODN from DOTAP upon contact with lipid bilayers at endosomal level (**II** and Mui et al. 2000) may be responsible for poor activity. DOTAP destabilizes purified lysosomal membranes but the effect is more pronounced at pH 7.4 than pH 5 (Wattiaux et al. 1997). At higher cationic lipid concentrations (about 5-fold, charge ratio +/- 10),

DOTAP liposomes delivered ODN into CV-1 cells with similar antisense activity to our study, but the control ODN also inhibited the activity considerably (Zelphati and Szoka, 1996b). Although positive net charge in the complexes is considered to be necessary (Zelphati and Szoka 1996b), differences may rise due to experimental setup, since adequate amount of DOTAP in complexes seems to be efficient (Capaccioli et al. 1993, Rodríguez et al. 1999), albeit more ODN is naturally needed in negatively charged complexes for the effect. Contraversial results may be due to different way of thinking: is it the ODN or the carrier that is kept constant when doing charge ratio optimization, since the ability to transfer ODN from the endosomes needs high enough concentration of the carrier. Anyway, negative net charge may have impact *in vivo*, because the positive net charge renders the complexes reactive with various extracellular compounds (Ruponen et al. 1999).

Similarly to DOTAP, neutral DOTAP/DOPE/Chol complexes do not differ greatly in size in different media explaining the equal efficiencies of complexes made in water, buffer or DMEM. Interestingly, DOTAP/JTS-1 complexes delivered antisense effectively at neutral charge ratios, but again the complexes made in water were most effective.

5.6.4. Summary of *in vitro* transfection studies

ODNs, in contrast to plasmid DNA, can readily diffuse from cytoplasm into the nucleus (Chin et al. 1990, Leonetti et al. 1991, Fisher et al. 1993) because the molecular sizes of ODNs are smaller than the nuclear pores. Therefore, the release of ODN from the complexes in endosomes and release of free ODN into the cytoplasm are crucial for activity. Based on DSC (differential scanning calorimetry) studies, PO-ODNs interact with cationic lipids less efficiently than PS-ODNs (Bennett 1998). Too stable ODN binding to the carrier, as has been shown to be the case with PS-ODN complexed to PEI (Dheur et al. 1999) in cell culture studies, would be an obstacle for activity in two ways. Complexed ODN can not hybridize to mRNA in the cytoplasm and its entry to the nucleus is less than that of free ODN. Although cell association was 2-3 fold higher for PS-ODN compared to 3′-capped PO-ODN, only this protected PO-ODN showed antisense activity towards Ha-*ras* mRNA (Dheur et al. 1999). Plain PO-ODN did not have any activity, most probably due to degradation by nucleases. The antisense effect was established using similar (200-300 nM) ODN concentrations to our studies on T24 cells. Excess ($\geq$1) PS-ODN was able to release PO-ODN from the complex but PO-ODN, even at high (10 -fold) excess, could not release PS-ODN. In contrast, with Lipofectin, only PS-ODN showed activity in suppressing mRNA levels in the cells.

Electrophoretically, PO-ODN but not PS-ODN, was shown to be readily released from the complex and the very low cell incorporation was explained by the release of PO-ODN already in cell culture medium (Dheur et al. 1999). Similar result with Lipofectin was obtained in a study by Cumin et al. (1993), where end-modified PO-ODNs did not show any activity against human prorenin mRNA, whereas PS-ODNs did inhibit the activity up to about 60 %. It has been shown that, although cell uptake of PS-ODNs is similar using cationic lipid with DOPE or DOPC, the cytosolic delivery and antisense activity are greatly enhanced with DOPE (Mui et al. 2000). In a study with 9 different cationic lipids, Bennett et al. (1997) found that DOPE was required for maximal activity for all lipids (including DOTMA), except a cationic lipid containing myristoyl (C14, fluid state lipid at RT) acyl chains, that are also present in highly active Cytofectin (Lewis et al. 1996, Flanagan et al. 1996). In the case of plasmid DNA transfections, C14 containing carriers (vectamidine and DMRIE-C) were more active than DOTMA/DOPE (El Ouahabi et al. 1997). Felgner et al. (1994) showed acyl chain length effect on DNA transfection activity (C14:0 > C18:1 > C16:0 > C18:0). This was confirmed by and Hyvönen et al. (2000) (C12:0 > C14:0 > C16:0 > C18:0) for fully saturated acyl chains. This can partly be explained by inefficient DNA interaction with less flexible solid state lipids (C16:0, C18:0) at room temperature and, subsequently, fluid state lipids are more efficient in transfections (Bhattacharya and Mandal 1998). Membrane active components facilitate membrane fusion, ODN release from the lipid complexes and antisense activity. Cationic polymers and lipids without fusogenic component were inactive at similar ODN concentrations, perhaps, due to their tighter structure and poor ODN release from complexes.

In contrast to free ODN, free plasmid DNA only poorly gains access from the cytoplasm to the nucleus, because of its large size (Zabner et al., 1995). With polymers like PEI (Pollard et al., 1998) and PLL (Zauner et al., 1999), but not with cationic liposomes, the carrier facilitates the nuclear entry of DNA from cytoplasm. It appears that different features of cationic carriers may be essential for antisense effect and DNA transfection in cells. Best activity with PS-ODN is seen with membrane active vehicles that may release ODN more efficiently upon contact with endosomal membrane.

5.6.5. Antisense effect in TET- and CAR regulated HEK293 cells (**IV**)

HEK293 (human embryonic kidney) cells were chosen to compare the antisense effect of often used tetracycline controlled and our novel CAR regulated system due to good performance of the CAR system in HEK293 cells (Honkakoski et al. 1998a, Masahiko and Honkakoski 2000).

LUC activity could be reduced 40-70% by antisense ODNs in both cell types (Fig. 7). The half-maximal values for inhibition were less than 100 nM and compare favorably with reported half-maximal values of 150-1000 nM and reasonably well with 60-80% maximal inhibition observed with cationic lipids and unmodified PS-ODNs (Cumin et al. 1993, Zelphati and Szoka 1996b, Bennett et al. 1998, Marcusson et al. 1998). This indicates the utility of these novel LUC-expressing cell lines in evaluating various ODN carriers.

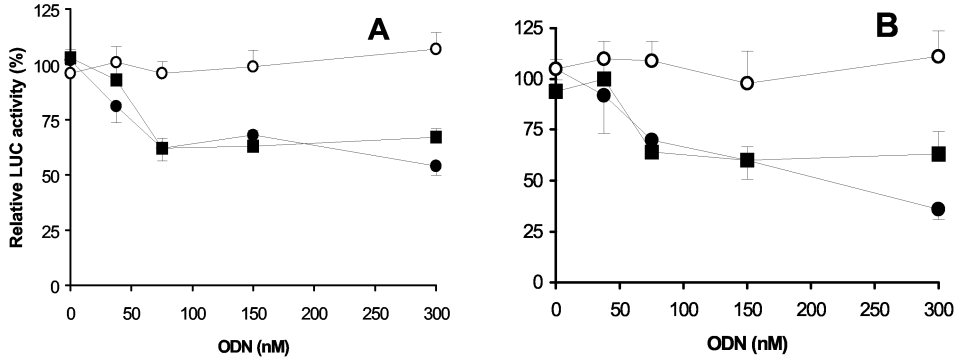


Figure 7. Antisense ODNs inhibit LUC expression. M29 cells (*panel A*) and 31-4 cells (*panel B*) were transfected with sense (*open symbols*) or antisense (*closed symbols*) ODNs and cultured in the presence of suppressing ANDR or TET (□, ■), or with TCPOBOP or in the absence of TET (○, ●). Regardless of pretreatment of the cells, sense ODNs did not inhibit LUC, and for clarity, results from one condition only are shown. LUC and protein were assayed 24 hours post-transfection. Data are expressed as relative to untransfected cells (= 100) and mean ± S.E.M. of 5-8 determinations.

5.7. Regulation of luciferase expression in new cell lines (**IV**)

The selection process yielded stable cell lines with similar growth characteristics to the parent HEK293 cells, similar number of cell colonies were obtained with both CAR or tTA (31-4 clone) expressing plasmids.

In M29 cells, the half-maximal concentrations for LUC activation and suppression were about 80 nM for TCPOBOP and 0.3 μM for ANDR (**IV**: Fig. 2A). In 31-4 cells,

the half-maximal concentration for repression was about 1 ng/ml tetracycline (**IV**: Fig. 2B). These values are in line with previous results with CAR (Forman et al. 1998, Sueyoshi et al. 1999, Tzameli et al. 2000) or tTA (Gossen and Bujard 1992, Wu et al. 1995, Hop et al. 1997). Suppression of LUC follows similar kinetics in M29 and 31-4 cells. LUC could be induced more than 12-fold by TCPOBOP in ANDR-pretreated M29 cells matching previous 8-15-fold regulation by CAR (Honkakoski et al. 1998b, Sueyoshi et al. 1999). The half-maximal increase occurred at 12 hours. In TET-pretreated 31-4 cells, derepression of LUC occurred with a similar or slightly delayed time scale. When TET concentrations higher than 10 ng/ml were used, a significant delay in derepression occurred. This indicates that, in some cases, kinetics of TET removal may hamper the tTA system.

CAR-dependent LUC can be suppressed by ANDR, its 16α-reduced, and 3-keto derivatives but not by some other steroids nor by TET in M29 cells (**IV**: Fig. 3A). In case of tTA, only TET but not ANDR-like steroids suppressed the activity. In addition to TCPOBOP, CAR-dependent LUC can be increased by structurally diverse, clinically used drugs but not by TET (**IV**: 3B). tTA-dependent LUC was not affected by the most potent CAR ligands. These data with stable cell lines and our previous work with transiently transfected genes (Honkakoski et al. 1998ab, Sueyoshi et al. 1999) indicate that CAR can be regulated by a wider range of structurally unrelated ligands than currently available in gene expression systems (Blau and Rossi 1999, Wang et al. 1994, No et al. 1996). The wide ligand specificity of CAR may eliminate the need to change the expression system if a chemical used to regulate CAR has undesirable properties or unintended effects.

The first-generation CAR system was regulated about 12-fold in stable cell lines and as an attempt to improve the response, we fused the ligand binding domain of CAR to the yeast DNA-binding protein GAL4 and replaced the PBREM enhancer with four copies of the 17-mer GAL4 binding site (Janowski et al. 1996). Original CAR and pPBREMluc plasmids (**IV**: Fig 4) were regulated in 16-fold overall by ANDR and TCPOBOP. When GAL4-CAR was used as the regulator, about 46-fold overall regulation was found (**IV**: Fig 4). The response to metyrapone improved from 30% increase to more than 2-fold activation by the GAL4-CAR fusion protein. This demonstrates that the ligand binding domain of CAR can be transferred to heterologous DNA binding proteins without changing its specificity.

6. CONCLUSIONS

The major conclusions of this study are described in Figure 8 showing different stages in cationic lipid mediated delivery of PS-ODNs into cells. In general DOPE affects significantly both complex formation and ODN delivery. Specific conclusions are as follows:

I: Upon addition of PS-ODNs to the DOTAP liposomes in buffer the particle size is increased significantly due to complexation and aggregation, especially in the range of -/+ 0.5-1.5. Morphology of individual liposomes is not significantly changed.

II: In the case of DOTAP/DOPE/ODN the size of the complexes increases in buffer, but at higher -/+ values (>1) there is no decline in the size, as opposed to DOTAP alone. Morphologically formation of tubular H_{II} – structures are seen.

III: In cell culture medium the size of DOTAP complexes and their morphology are not significantly changed. In the case of DOTAP/DOPE and its complexes with PS-ODN, however, extensive formation of tubular H_{II}–structures is seen culture medium.

IV: Detectable antisense effect in cells was seen with lipid based carriers with DOPE. Incubation of the complexes with endosome mimicking liposomes suggest that DOPE facilitates the release of ODN from the complexes and its escape from the endosomes.

V: Membrane destabilizing peptide JTS-1 enhanced the antisense delivery in DOTAP/ODN complexes even more than DOPE did.

VI: Complexation in water instead of buffer yielded smaller complexes that were more effective in the cells.

VII: Activity of the target gene under the control of CAR-receptor responsive PBREM element can be suppressed by various steroids or activated by many drugs. Stable transfectants with this regulated system cells can be used as a test method in antisense research.

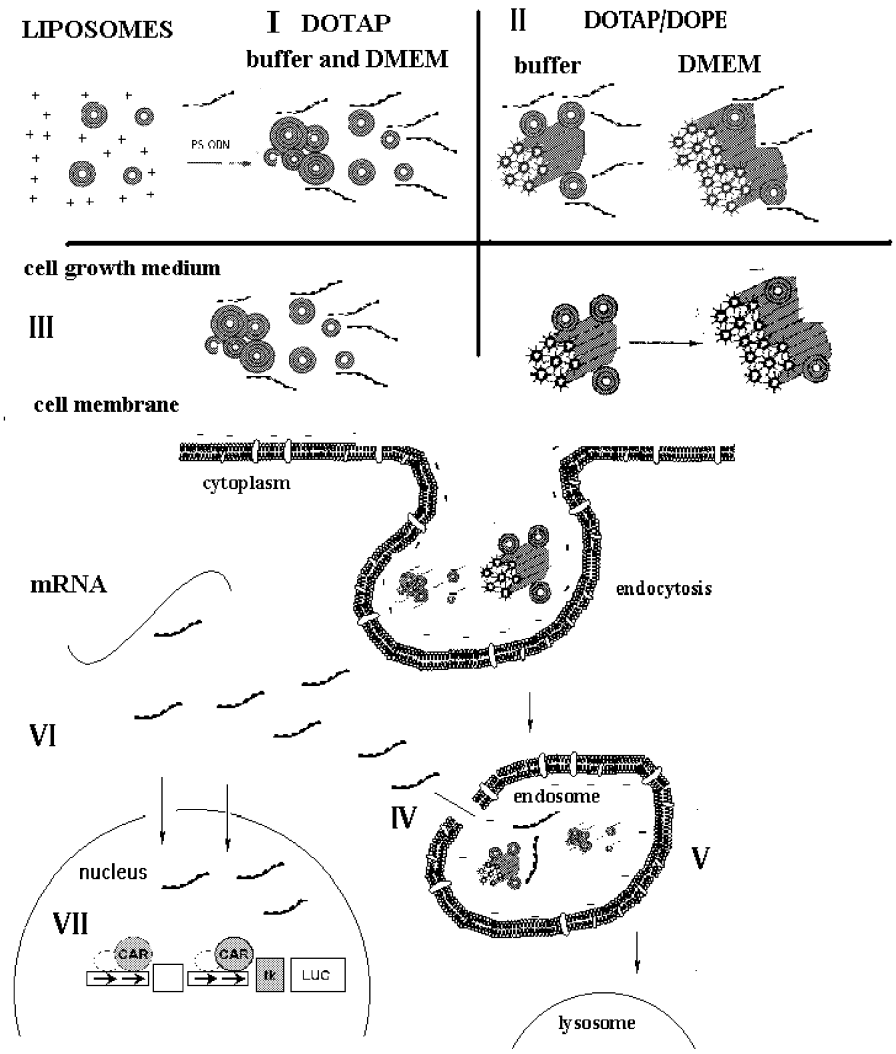


Figure 8. Overview of the conclusions. For explanation see ´CONCLUSIONS´.

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