REVIEW

Department of Pharmaceutics, College of Pharmacy, University of Florida, U.S.A.

Oligonucleotide delivery: a cellular prospective

E. LIANG, P. S. AJMANI and J. A. HUGHES

The use of oligonucleotides (ONs) for gene therapy of certain diseases has been discussed since the late 1970s. ONs are single stranded chains of nucleic acids that can hybridize with target nucleic acid sequences to inhibit specific proteins, and therefore allow selective treatment of various diseases. The use of ONs is limited due to their instability in biological tissues and difficulty in delivery to the intracellular compartments of the cell. Chemical analog approaches have been used to address the instability issue and delivery systems have been developed to increase cellular uptake of ONs. It is generally thought that ONs with or without a delivery system are transported into cells by endocytosis, and then accumulate within endosomes where they are significantly inactivated. The rate and extent of movement of ON from endosomes appears to be important in determining ON effects. Consequently, developing accessory compounds or delivery methods that enhance endosome to cytoplasm transfer may be vital to ON therapy. This review focuses on investigating mechanisms of various delivery approaches at the cellular/intracellular level that have demonstrated utility in increasing ON activity or cellular accumulation. The future prospects of ON delivery are also addressed.

1. Introduction

The ability of short synthetic single-stranded nucleic acids to interfere with individual gene expression in a sequence-specific manner is the foundation for ON-based therapy. The first clear exploration of ONs was reported by Zamecnik and Stephenson [1]. However, due to a number of impediments (e.g., understanding the sequence and topology of the nucleic acid target, synthesis of adequate quantities of ONs, and modification of ONs to produce stable analogs), research in using ONs for biological studies was limited until the late 1980s. Following advances in ON chemistry and initial biological studies [2–4], interest in ON therapeutics began to increase.

ONs are short (12 to 40 mer) nucleotide polymers of a synthetic single-stranded nucleic acid and are designed to bind to a specific gene [5, 6], mRNA [7], or protein [8, 9]. The binding follows Langmuir isothermal binding similar to most drugs but with a greater affinity [10]. After interacting with their target in cells, ONs can prevent the production of a specific protein (Fig. 1). Detailed mechanisms of action are explained in excellent review articles [11–15]. In brief, a triplex forming ON (antigene ON) is capable of binding in the major groove of double-stranded DNA via Hoogsteen base interactions, thereby creating a triple helical structure resulting in sequence-specific inhibition of transcription. On the contrary, an antisense ON is complementary to a specific sequence of mRNA that can hybridize through Watson-Crick hydrogen bonds [16]. It can inhibit translation by several proposed mechanisms including activation of RNase H, inhibition of RNA processing, and blockade of ribosomal reading. RNase H is an endogenous cellular enzyme recognizing a hybrid duplex between DNA and RNA [17]. RNase H cleaves RNA and releases the DNA-ON. The freed DNA-ON is then able to hybridize to another RNA strand and repeat the RNase H dependent degradation, thus forming the basis for a catalytic effect.

ON therapy is most often directed at inhibiting production of disease causing proteins, and has the potential to be much more specific than conventional drug therapy [10], as it interacts specifically with target sequences.

2. Barriers to ON transfer and activity

2.1. In vitro/in vivo stability

Of all the possible obstacles, rapid degradation of unmodified DNA and RNA phosphorodiester ONs in the biological milieu is the first problem encountered [18, 19]. Enzymes (non-specific endo- and exo-nucleases) limit phosphorodiester ONs' physiological half-life to a few minutes [18]. This short biological half-life makes the therapeutic use of phosphorodiester ONs unlikely. Biologically stable ONs can be synthesized by chemically altering the phosphorodiester backbone or the nucleoside itself. To maximize the effect, the modified ONs should be stable in both serum and inside the cell, be able to reach their site of action, and form stable hydrogen bonds with target sequences (See Uhlman et al. for details [20]).

Based upon the above criteria, a number of structural analogues such as phosphorothioate [21, 22] and methylphosphonate [23, 24] ONs with nuclease resistance have been developed. Of these modified ONs, phosphorothioate ONs are possibly the most potent because they are highly resistant to nucleases, retain a net charge, are soluble in water, and can act as substrates for RNase H. Nonetheless, phosphorothioate ONs may also cause a variety of nonsequence dependent effects on cellular function, such as effects on fibroblast growth factor [25], Sp1 nuclear transcription factor [26], and NF-κB nuclear transcriptional regulatory factor [27].

2.2. Cellular transport

Another major encumbrance to the therapeutic use of ONs is the inefficient delivery of ONs to the cytoplasm or nucleus. There are two transport aspects, cellular uptake and entry into the cytoplasm/nucleus that need to be distinguished.

Cellular uptake refers to both ON membrane binding and a general internalization within the cell. Entry into the cytoplasm/nucleus concerns the amount of ONs that reach a pharmacologically active compartment. Internalization of ONs by cultured cells is inefficient [18, 28] and only a

small fraction of ONs can actually gain entry into cells. It is commonly assumed that most ONs are brought into cells through (receptor mediated, adsorptive, or fluid phase) endocytosis [29, 30].

After entry into cells, ONs must penetrate the endosomal membrane to exert their effects. Not all of the internalized ONs are necessarily available to interact with intended subcellular targets. Indeed, most of them are eliminated by lysosomes (Fig. 2). Unlike gene delivery, following cellular entry and escape from endosomal compartments, ONs are able to migrate to the nucleus without much difficulty [31–33] and nuclear pore size is not a barrier for ON delivery. An issue that needs to be addressed is that, like other drugs, ONs may bind to intracellular proteins that can cause side effects and limit free fraction; only free unbound ONs can interact with targets at the sites of action and demonstrate a biological effect [34].

By optimtizing ON transfer at each stage of the delivery process, the dose of ONs (with or without their carrier) to achieve the same biological effect can be minimized. Increasing the concentration of ON cellular uptake and/or escape of ONs from the endosomes may be of considerable value in improving the inhibition of certain protein expression. Hence, the cytotoxicity associated with a large amount of ONs and delivery systems can be minimized.

3. Toxic effects of ONs

High concentrations of phosphorothioate ONs have been reported to be harmful in animal studies and the toxicity

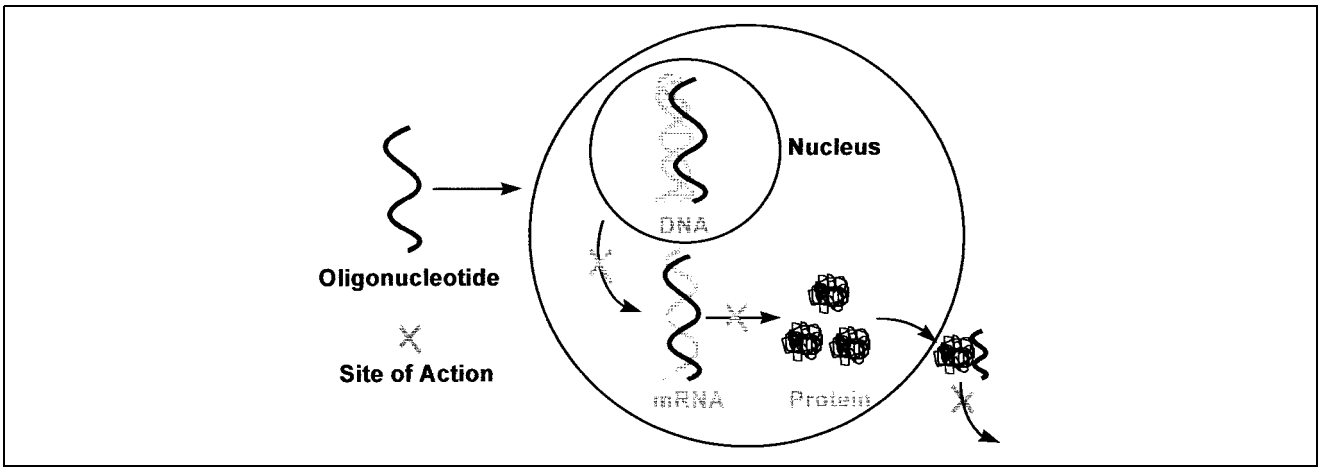

Fig. 1: Depiction of protein synthesis and possible sites of action for ONs. There are three potential sites where ONs can have actions. Firstly, ONs (antigenes) can be used to inhibit the transcription process from double stranded DNA to single stranded mRNA through Hoogsteen base pairing interactions. Secondly, complimentary ONs (antisense ONs) can be designed to bind with mRNA to restrain the translation process through Watson-Crick hydrogen bond interactions. Finally, ONs (aptamers) can interact with a synthesized protein to interfere with its activity via hydrogen bondings

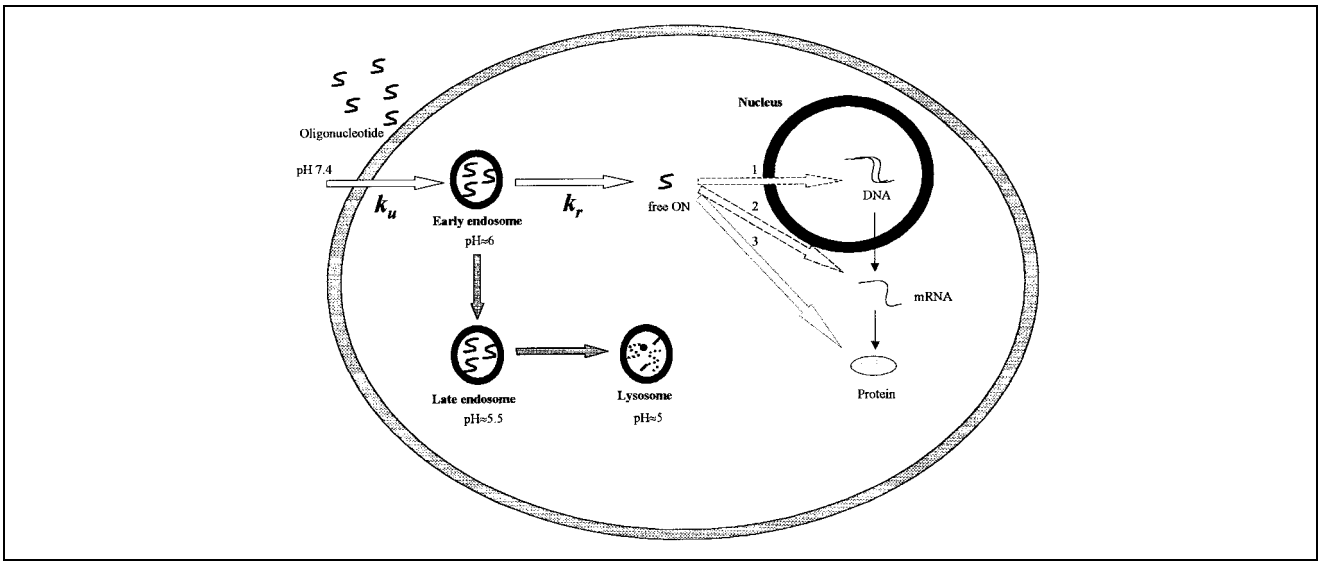

Fig. 2: Possible ON fates at the cellular level. It is suggested that most macromolecules like ONs are taken into cells through endocytosis. In order to reach their sites of action, ONs will face at least two rate-limited steps: cellular uptake (k_u) and release from endosome (k_r). If ONs can overcome both steps, they can act at their potential binding sites, 1, 2, and 3, as explained in Figure 1. If ONs are able to penetrate through the cellular membrane but not the endosomal one, they will be directed into the endocytosis process. Endocytosis is composed of continuous stages, characterized by various subcellular compartments (i.e., endosome and lysosome). Endosomes carry out several important processes associated with endocytosis, including fusion and sorting of internalized molecules, acidification, and maturation. The early sorting endosome is the organelle that clathrin-coated and pit-derived vesicles fuse with right after pinching off from the cell surface and the late endosome contains lysosomally destined molecules. Acidification caused by the presence of an ATP-dependent proton pump is one of the most significant properties during endocytosis, and has several important consequences. For example, many ligands that use the receptor-mediated endocytosis pathway dissociate from their receptors at acidic pH, some toxins undergo conformational changes at low pH, and hydrolytic enzymes generally have an acidic pH optima to develop their full activity. After endosomes mature into lysosomes, internalized materials (e.g., ONs) are completely broken down by lysosomal hydrolases

of ONs appears to be species dependent. Administration of ONs in mice and rats have resulted in significant toxicity including acute renal failure, liver and spleen damage, immune stimulation (e.g., spleen B-cell proliferation and immunoglobulin secretion), severe thrombocytopenia, and death [35, 36]. Intravenous (i.v.) bolus administration of ONs in monkeys produced a transient decrease in peripheral total white blood cell and neutrophil counts, prolonged activated partial-thromboplastin time, and resulted in hypotension and death [37]. Accumulation of ONs and their metabolites may be responsible for these toxicities due to the relatively long retention time in the reticuloendothelial system [38].

4. Strategies available to deliver ONs

A number of strategies have been pursued to facilitate entry of ONs into the cytoplasm. The strategies are used either alone or in combination with others to optimize their effect. Each system has its own advantages and drawbacks. According to the two mentioned ON transport aspects, these strategies can generally be classified into two groups (Table 1).

The first group includes delivery systems that increase the amount of ON association with target cells by either protecting them from degradation or by increasing their cellular interactions. These systems increase the probability of ONs escaping endocytotic degradation and reaching the cytoplasm. They include conjugation of molecules to ONs (i.e., conjugating agents), complexation of ONs with cationic molecules (i.e., complexing agents), encapsulation of ONs into vesicles (i.e., encapsulating agents), and labeling targets to either ONs or their delivery carriers (i.e., targeting agents).

To further optimize ON delivery, endosome destabilizing (escaping) systems have been developed. This group applies devices (i.e., ON cytoplasmic transfer techniques) or offers delivery systems (i.e., membrane destabilizing agents) to improve ON efflux to the cytoplasm.

4.1. Conjugating agents

Emphasis on the ability of ONs to penetrate biological membranes is one of the major elements in making ON therapy possible. One strategy is to conjugate hydrophobic anchor groups at either end of the ON through chemical reactions to extend their hydrophobicity and/or exo-nuclease resistance, thereby increasing the interaction with target cells (Table 2).

Cholesterol is a typical conjugating agent that has been used as a hydrophobic anchor group at either the 3′- or 5′-terminus of ONs [39–42]. Alkyl side chains are also commonly used as conjugating agents. Examples include hexadecyl moieties affixed to the 5′-end [43], dodecyl moieties to the 3′-end [44], hexanol to the 3′-end [45], aminohexyl to the 3′-end [45], and undecyl derivative to the 5′-end of ONs [46]. Poly(L-lysine) is another type of conjugating agent; by attaching ONs to poly(L-lysine) at the 3′-end [47–51], cellular uptake is increased possibly due to a better interaction with the negatively charged cellular membrane. In addition, the improved biological effect of poly(L-lysine) conjugates is perhaps due to better protective properties against nucleases.

As mentioned above, a major advantage of using conjugating agents is to increase the initial membrane interaction that leads to greater cellular accumulation of ONs. However, there are a number of disadvantages that could hinder the use of conjugating agents, for example, the che-

Table 1: Proposed routes to improve ON cellular transport

Aspect to enhance ON delivery	Strategy (delivery agent)	Selected Agent (reference)
Cellular uptake	Conjugation of compounds to ONs (conjugating agent)	Cholesterol [39–42]; Alkyl side chain [43–46]; Poly(L-lysine) [47–51]
	Complexation of ONs with cationic molecules (complexing agent)	Cationic polymers [53–62]; Cationic liposomes [59, 63–79]; Nanoparticles [80–82]
	Encapsulation of ONs into vesicles (encapsulating agent)	Liposomes [18, 91–96]; Cyclodextrins [97, 98]
	Labeling targets to either ONs or their delivery carriers (targeting agent	Intercalating agents [7, 99–112]; Receptor ligands [113–117]; Essential nutrients [53, 54, 74, 118–126]
Entry into the cytoplasm and/or nucleus	Use of a device to carry ONs to cytoplasm/nucleus (ON cytoplasmic transfer technique)	Electroporation [127–130]; Microinjection [131–135]
	Disruption of endosomal membrane (membrane destabilizing agent)	Viral peptides [59, 136, 137, 142–147]; Fusogenic and pH-sensitive lipids [123, 152–157]; BPS [59, 72, 163]

Table 2: Advantages of different systems to increase the cellular uptake of ONs

Delivery system	Major advantages	Major disadvantages
Conjugating agent	Increases initial ON membrane interaction	Time consuming and expensive chemical synthesis of the connector
Complexing agent	Increases initial ON membrane interaction Ease of production High capacity to retain ONs Prevent ONs from enzymatic degradation	High toxicity Immunological problems
Encapsulating agent	Increases initial ON membrane interaction Ease of production	Limited capacity to retain ONs
Targeting agent	Increases initial ON membrane interaction Enhances ON cellular uptake specifically	Time consuming and expensive linking process Receptor subtype dependency

mical synthesis of the connector. This process is time consuming and expensive. The addition of conjugating agents (e.g., poly(L-lysine)) could also account for additional nonspecific cytotoxic effects [52].

4.2. Complexing agents

Unlike conjugating agents, the basic principle behind the use of complexing agents is to bind ONs to a carrier in a strong but non-covalent manner, based upon an electrostatic attraction. This system carries more ONs into cells through endocytosis and hence increases their probability of reaching the cytoplasm (Table 2).

Cationic polymers such as poly(L-lysine) [53–55], polyethylenimine [56], polyamidoamine PAMAM starburst dendrimers [57–61], and avidin [62] have been used as complexing agents to enhance ON delivery. Cationic liposomes have also been investigated; cationic liposomes including *N*-[1-(2,3-dioleoloxy)-propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) [59, 62–66], *N*-[1-(2,3-dioleoloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP) [66–72], 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol) [73], spermi(di)ne-cholesterol [74], 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA) [75, 76], 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE) [64], and dimethyldioctadecylammonium bromide (DDAB) [77–79] as a partial list, have been shown to improve ON cellular delivery.

Adsorption of ONs on nanoparticles is achieved by forming ion pairs between the negatively charged phosphate groups of the nucleic acid chain and the hydrophobic cations. The cellular delivery of ONs was enhanced [80–82] due to ON protection and better cellular uptake. The approach behind the ON-nanoparticle interaction by using a third agent (hydrophobic cations such as tetraphenylphosphonium chloride or quaternary ammonium salts) is somewhat different from that of cationic polymers and liposomes. Still, this type of agent is loosely grouped as a complexing agent as it shares the same principles of electrostatic interactions used in cationic polymers and liposomes.

In contrast to conjugating agents, the biggest advantage of the complexing agents is their ease of production. No chemical linkage between ONs and complexing agents is required. In addition, they provide high capacity to retain ONs. Complexing agents may also prevent ONs from enzymatic degradation by forming a poor substrate [83]. However, a major concern in using complexing agents are their possible toxic effects. As the concentrations are increased [84–87], cationic polymers and cationic liposomes eventually become toxic to cells than their neutral counterparts. Also, the intrinsic properties of the carriers like liposomes or nanoparticles can lead to increased immunological problems with the ON complex [63, 88].

4.3. Encapsulating agents

Encapsulating and complexing agents are possibly the most popular systems used in ON delivery. Not only do both methods protect ONs from degradation [65, 89], but they also increase cellular uptake [90]. However, the principles behind the two methods are different. Complexing agents bind to ONs through an electrostatic attraction while encapsulating agents entrap ONs within vesicles (Table 2).

The most popular encapsulating approach currently being investigated is the use of liposomes. Liposomes are vesicles comprised of lipid bilayer(s) similar in structure to biological membranes. Utilizing their versatility (e.g., size, charge, and composition) and advantages (e.g., economical, ability to attach chemicals to their surface, and ease of production), liposomes can be used as vehicles for increasing ON delivery to the site of action. They can also be used to control or sustain ON release [22]. Numerous examples utilizing liposomes illustrate the improved effect of ONs [22, 91–96]. There are reports in the literature of using cyclodextrin analogs [97, 98] as possible carriers for delivering ONs.

Similar to complexing agents, the biggest advantage of encapsulating agents is their ease of production. Unlike complexing agents, encapsulating agents are believed to be less cytotoxic. However, compared to complexing agents, encapsulating agents have a limited capacity to bring ONs into cells, thereby reducing their efficiency.

4.4. Targeting agents

Targeting agents may be categorized into two groups. Members of the first group target the nucleic acid level and are known as intercalating agents. These agents are most often attached at the 3′- or 5′-end of ONs. The moieties linked to ONs interact strongly and nonspecifically with nucleic acids. After entering into cells and interacting with target nucleic acids, the hybrids are stabilized by the intercalation of agents in the RNA-DNA duplex. Hence, they increase the affinity of ONs to their targets (Table 2). Of all the intercalating agents, acridine is the most widely used and investigated [7, 99–106]. Other intercalators that have been studied include chlorambucil [107], benzopyridoquinoxaline [108, 109], benzopyridoindole [109, 110], benzophenanthridine [111], and phenazinium [112].

The second group of targeting agents utilizes moieties that can selectively and specifically transport ONs to a target cell population. The moieties can either be conjugated to ONs or attached to a carrier system (e.g., poly(L-lysine) or liposomes) linked to ONs. For cells that express the characteristics of specific receptor-mediated endocytosis, ligands are good candidates for initiating cellular uptake of ONs. Glycoproteins bearing an appropriate sugar residue specifically attach to sugar binding receptors [113]. By labeling ONs at the 3′-end to the neoglycoprotein (6-phosphomannosylated glycoprotein), an improved effect was observed in peritoneal macrophages and murine macrophage cells (J774) [114]. Similarly, asialoorosomucoid in NIH 3T3 [115] and human hepatoma (HepG2 2.2.15) cells [116] or mannosylated glycoprotein in alveolar macrophages [117] conjugated to poly(L-lysine) has been employed to target and enhance cellular uptake of ONs.

Since malignant cells are corpulent with an increased need for essential nutrients (e.g., folic acid and transferrin) than benign cells, these nutrients can be used as potential candidates to target ONs and inhibit cancerous cell growth. Further improvement of ON cellular uptake is seen in human promyelocytic leukaemia (HL-60) cells [118] and human melanoma (M-14) cells [54] when folic acid is linked to poly(L-lysine). Similarly, enhanced ON uptake is observed in adenocarcinoma A-549 cells [53] when epidermal growth factor is linked to poly(L-lysine), and in human promyelocytic leukaemia (HL-60) cells [119] when transferrin is linked to poly(L-lysine). Liposomes coated with maleylated bovine serum albumin, folic acid, or fer-

ric protoporphyrin IX, show increased cellular uptake of ONs in murine macrophages [120], KB cells [121], and 2.2.15 human hepatoma cells [74], respectively.
In order to increase ON uptake, liposomes could also be attached to antibodies for targeting to desired sites. Several monoclonal antibody-targeted liposomes, immunoliposomes, have been developed and used for mediatintg ONs to specific receptors on targeted cells (e.g., mouse L929 cells [122], human myeloid leukaemic (HL60, K562, and Meg-01) [123], CD2+ T-lymphoblastic leukaemic (Jurkatt and CEM) cells [123], H9 human T cells [124, 125], and T-lymphoblastoid cells [126]).
The major advantage of targeting agents is to specifically enhance ON cellular uptake. The targeting strategy can be incorporated with other systems to further increase the cellular biological activity of ONs. Similar to conjugating agents, a drawback that may hamper the development of targeting agents is the synthetic linking process. Furthermore, targeting particular routes of endocytosis depends upon receptor subtype, and therefore limits the use of some targeting agents.

4.5. ON cytoplasmic transfer techniques

Even if cellular uptake of ONs is increased by the use of a delivery system, escape from endosome must still be accomplished. One way to avoid this barrier is to transfer them directly into cytoplasm or nucleus (Table 3). This has been accomplished through electroporation [127–130] and microinjection [131–135].
Electroporation involves delivering a high-voltage pulse of a defined magnitude and length to the ON-cell system. The membrane structures of the cells are loosened and ONs can be introduced directly into the cell's cytoplasm. On the other hand, microinjection is performed by injecting ONs directly into the cytoplasm.
The above methods prevent lysosomal elimination without falling into the trap of the endocytosis pathway. However, since both methods are invasive, these techniques have limited use from the clincial therapy standpoint. It is therefore necessary to develop more practical delivery systems to improve ON therapy.

4.6. Membrane destabilizing agents

Membrane destabilizing agents function by disrupting endosomal or cellular membranes (Table 3). Some agents are directly conjugated to ONs, while others may be a part of the liposome composition to which ONs are either complexed or encapsulated.

4.6.1. Viral peptides

ONs incubated or coupled to viral peptides (derived from the haemagglutinin envelop protein of the Influenza virus [59, 136, 137]) provide a route for improving their cytoplasmic delivery. These peptides are able to form a transmembrane channel through a conformational change induced by the acidification following endocytosis [138–141]. Viral peptides can then help transfer ONs into the cytoplasmic compartment. When liposomes are decorated with viral peptides from the Sendai virus (hemagglutinating virus of Japan (HVJ)), ON delivery can also be improved [142–147]. Unlike the Influenza virus system, most HVJ-liposomes appear to employ a cell-membrane fusion mechanism [148], thus averting ONs from the endocytotic pathway and releasing them directly into the cytoplasm.
The advantage of fusogenic peptides is their ability to follow the natural virus entry pathway. However, fusogenic peptides are expensive to produce and could also pose immunogenicity problems on repeated administration.

4.6.2. Fusogenic and pH-sensitive lipids

Fusogenic and pH-sensitive lipids are used to form liposomes (i.e., encapsulating agents) and promote the efflux of ONs from the endosomal compartment [149–151]. Fusogenic lipids include phosphatidylethanolamine (PE) derivatives, and pH-sensitive lipids contain titratable carboxylic acids such as oleic acid [123, 152–155] and cholesteryl hemisuccinate [156, 157]. A fusogenic lipid is able to form a hexagonal II phase that influences membrane fusion and ON release. However, liposomes must maintain their integrity to encapsulate ONs before the endosomal membrane disruption occurs inside cells. A pH-sensitive lipid is therefore introduced into the liposomal matrix. With a chemical structure complementary to the hexagonal II phase (e.g., dioleoylphosphatidylethanolamine (DOPE)), the pH-sensitive lipid will assist in retaining the bilayer vesicle structure of the liposomes at an alkaline pH. When the pH decreases, as a result of the acidification of the endosome, the titratable head group of the pH-sensitive lipid is protonated causing liposome collapse. The pH-sensitive lipids then destabilize the bilayer structure and the fusogenic lipids promote membrane fusion [138]. Eventually, ONs are released out of the endosomes. Also, cationic liposomes (i.e., complexing agents) usually comprise a fusogenic lipid (e.g., DOPE) and a cationic lipid (e.g., DOSPA, DOTAP, DOTMA, DDAB, and DC-Chol) to improve ON delivery, [74, 76–79, 81, 158]. However, the mechanism is still not fully understood.

Table 3: Advantages and disadvantages of different systems to increase the transfer of ONs to cytoplasm and/or nucleus

Delivery system	Major advantages	Major disadvantages
Cytoplasmic transfer technique	Prevent lysosomal elimination of ONs	Limited use in clinical therapy
Membrane destabilizing agent	Viral peptides:	
	Minimize lysosomal elimination of ONs	Expensive generating process
	Follow the natural virus entry pathwy	Immunological problems
	Fusogenic and pH-sensitive lipids:	
	Minimize lysosomal elimination of ONs	Low capacity to entrap ONs
	Ease of production	
	BPS:	
	Disrupt the endosomal membrane to release ONs	Potentially Toxic
	High capacity to enclose ONs	
	Ease of production	

In addition to increasing cellular uptake of ONs, pH-sensitive liposomes also increase ON entry into the cytoplasm. However, since both pH-sensitive anionic lipids and nucleic acids have negative charges, they may have a low capacity to entrap ONs.

4.6.3. Biodegradable pH-sensitive surfactants (BPS)

The use of detergents to disrupt phospholipid bilayers (e.g., endosomal membranes) is efficient [159] and provides a rationale approach to enhance ON release from endosomes, but most detergents are indiscriminate of membrane type and attack the first cellular membrane they contact. In order to provide selectivity, a trigger is required to activate detergents in specific subcellular locations. A lysosomotropic amine (pKa 5–7) [160, 161] bearing a hydrophobic tail group is classified as a lysosomo-tropic detergent [162] and forms the basis of BPS [59, 72].

When incorporated into liposomes at an alkaline environment, BPS are predominated by its hydrophobic tail and reside within lipid bilayers due to limited surface-active properties [72]. After the whole system is taken into cells, BPS will be protonated as the pH decreases during endocytosis [163], and activate the membrane destabilization process using the surfactant-like characteristics from the ionized BPS. The biodegradable connector in the BPS would be digested into less toxic metabolites by the digestive enzymes in the cytoplasm following the ON release to their sites of action. The additional cytotoxic effects from the delivery system would thus be minimized [72].

Like fusogenic and pH-sensitive liposomes, not only do BPS containing liposomes increase the amount of ONs taken into cells, but are also capable of increasing the ON release from endosomes into the cytoplasm. Unlike pH-sensitive lipids, BPS do not carry charges at an alkaline pH environment and can have an increased capacity to enclose nucleic acids. Nonetheless, if BPS were not completely degraded before endosomes mature into lysosomes or are unprotonated until the lysosome stage, the ionized BPS would end up disrupting the lysosomes and cause digestive enzyme release that can potentially kill all cells. Even though there is no significant toxicity observed in the current study, further investigations are necessary to address this issue.

5. Summary

An ideal ON should be stable in serum before exerting its therapeutic effect. *In vitro/in vivo* stability of ONs must therefore be considered. Chemical modification of ONs can be employed to increase their stability. However, modified ONs should still be able to form a stable complex with their target sequence. After stabilizing ONs in serum, the second barrier, cellular uptake, must be overcome (Table 1 and 2). Indeed, only a small portion of free unmodified ONs are able to enter cells. To increase cellular uptake, ONs and/or their delivery system can be modified in a number of ways. Most ONs with or without a delivery system are taken into cells through endocytosis and eliminated at the lysosomal stage. Hence, the endosomal membrane represents the third potential rate-limiting step. To increase ON transfer to the cytoplasm, ONs can be further modified to bypass endocytosis and/or the delivery system can be designed to deal with membrane destabilization (Table 1 and 3).

By optimizing ON transfer at each step during the delivery process, the amount of ONs that reach the cytoplasm can be maximized. Consequently, toxic effects associated with a large amount of ONs and/or the delivery system would be decreased.

Acknowledgements: The authors wish to express their gratitude to Dr. Guenther Hochhaus for his assistance in preparing the mannuscript. Funding from the National Institutes of Health (P01-AG10485 and R29 HL55770) is also gratefully acknowledged.

References

1 Zamecnik, P. C.; Stephenson, M. L.: Proc. Natl. Acad. Sci. USA **75**, 280 (1978)
2 Agrawal, S.; Goodchild, J.; Civeira, M. P.; Thornton, A. H.; Sarin, P. S.; Zamecnik, P. C.: Proc. Natl. Acad. Sci USA **85**, 7079 (1988)
3 Gao, W. Y.; Stein, C. A.; Cohen, J. S.; Dutschman, G. E.; Cheng, Y. C.: J. Biol. Chem. **264**, 11521 (1989)
4 Smith, C. C.; Aurelian, L.; Reddy, M. P.; Miller, P. S.; Ts'o, P. O.: Proc. Natl. Acad. Sci. USA **83**, 2787 (1986)
5 Cooney, M.; Czernuszewicz, G.; Postel, E. H.; Flint, S. J.; Hogan, M. E.: Science **241**, 456 (1988)
6 Moser, H. E.; Dervan, P. B.: Science **238**, 645 (1987)
7 Stein, C. A.; Cohen, J. S.: Cancer Res. **48**, 2659 (1988)
8 Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermaas, E. H.; Toole, J. J.: Nature **355**, 564 (1992)
9 Ellington, A. D.: Curr. Biol. **4**, 427 (1994)
10 Crooke, S. T.: Annu. Rev. Pharmacol. Toxicol. **32**, 329 (1992)
11 Crooke, S. T.: Faseb J. **7**, 533 (1993)
12 Scanlon, K: J.; Ohta, Y.; Ishida, H.; Kijima, H.; Ohkawa, T.; Kaminski, A.; Tsai, J.; Horng, G.; Kashani Sabet, M.: FASEB J. **9**, 1288 (1995)
13 Sharma, H. W.; Narayanan, R.: Bioessays **17**, 1055 (1995)
14 Helene, C.; Toulme, J. J.: Biochim. Biophys. Acta **1049**, 99 (1990)
15 Uhlman, E.; Peyman, A.: Chem. Rev. **90**, 543 (1990)
16 Zamecnik, P. C.; in: Agrawal, S. (Ed.): Antisense Therapeutics, p. 1, Humana Press, Totowa 1996
17 Ghosh, M. K.; Ghosh, K.; Dahl, O.; Cohen, J. S.: Nucleic Acids Res. **21**, 5761 (1993)
18 Akhtar, S.; Kole, R.; Juliano, R. L.: Life Sci. **49**, 1793 (1991)
19 Shaw, J. P.; Kent, K.; Bird, J.; Fishback, J.; Froehler, B.: Nucleic Acids Res. **19**, 747 (1991)
20 Uhlman, E.; Peyman, A.; in: Bertino, J. R. (Ed.): Encyclopedia of Cancer, p. 64, Academic Press, San Diego 1997
21 Connolly, B. A.; Potter, B: V.; Eckstein, F.; Pingoud, A.; Grotjahn, L.: Biochemistry **23**, 3443 (1984)
22 Cowsert, L. M.; Fox, M. C.; Zon, G.; Mirabelli, C. K.: Antimicrob. Agents Chemother. **37**, 171 (1993)
23 Blake, K. R.; Murakami, A.; Miller, P: S.: Biochemistry **24**, 6132 (1985)
24 Murakami, A.; Blake, K. R.; Miller, P. S.: Biochemistry **24**, 4041 (1985)
25 Guvakova, M. A.; Yakubov, L: A.; Vlodavsky, I.; Tonkinson, J. L.; Stein, C. A.: J. Biol. Chem. **270**, 2620 (1995)
26 Perez, J. R.; Li, Y.; Stein, C. A.; Majumder, S.; van Oorschot, A.; Narayanan, R.: Proc. Natl. Acad. Sci. USA **91**, 5957 (1994)
27 Khaled, Z.; Benimetskaya, L.; Zeltser, R.; Khan, T.; Sharma, H. W.; Narayanan, R.; Stein, C. A.: Nucleic Acids Res. **24**, 737 (1996)
28 Stein, C. A.; Cheng, Y. C.: Science **261**, 1004 (1993)
29 Akhtar, S.; Juliano, R. L.: Trends Cell. Biol. **2**, 139 (1992)
30 Loke, S. L.; Stein, C. A.; Zhang, X. H.; Mori, K.; Nakanishi, M.; Subasinghe, C.; Cohen, J. S.; Neckers, L. M.: Proc. Natl. Acad. Sci. USA **86**, 3474 (1989)
31 Aronsohn, A. I.; Hughes, J. A.: J. Drug Targeting **5**, 163 (1997)
32 Chin, D. J.; Green, G. A.; Zon, G.; Szoka, F. C., Jr.; Straubinger, R. M.: New Biol. **2**, 1091 (1990)
33 Leonetti, J. P.; Mechti, N.; Degols, G.; Gagnor, C.; Lebleu, B.: Proc. Natl. Acad. Sci. USA **88**, 2702 (1991)
34 Holford, N. H.; Benet, L. Z.; in: Katzung, B. G. (Ed.): Basic & Clinical Pharmacology, p. 34, Appleton & Lange, Stamford 1998
35 Krieg, A. M.; Yi, A. K.; Matson, S.; Waldschmidt, T. J.; Bishop, G. A.; Teasdale, R.; Koretzky, G. A.; Klinman, D. M.: Nature **374**, 546 (1995)
36 Sarmiento, U. M.; Perez, J. R.; Becker, J. M.; Narayanan, R.: Antisense Res. Dev. **4**, 99 (1994)
37 Galbraith, W. M.; Hobson, W. C.; Giclas, P: C.; Schechter, P. J.; Agrawal, S.: Antisense Res. Dev. **4**, 201 (1994)
38 Zhang, R.; Lu, Z.; Zhao, H.; Zhang, X.; Diasio, R. B.; Habus, I.; Jiang, Z.; Iyer, R. P.; Yu, D.; Agrawal, S.: Biochem. Pharmacol. **50**, 545 (1995)
39 Alahari, S. K.; Dean, N. M.; Fisher, M. H.; Delong, R.; Manoharan, M.; Tivel, K. L.; Juliano, R. L.: Mol. Pharmacol. **50**, 808 (1996)

40 Boutorin, A. S.; Gus'kova, L. V.; Ivanova, E. M.; Koetz, N. D.; Zarytova, V. F.; Ryte, A. S.; Yurchenko, L. V.; Vlassov, V. V.: FEBS Lett. **254**, 129 (1989)
41 Godard, G.; Boutorine, A. S.; Saison, Behmoaras, E.; Helene, C.: Eur. J. Biochem. **232**, 404 (1995)
42 Letsinger, R: L.; Zhang, G. R.; Sun, D. K.; Ikeuchi, T.; Sarin, P. S.: Proc. Natl. Acad. Sci. USA **86**, 6553 (1989)
43 Shea, R. G.; Marsters, J. C.; Bischofberger, N.: Nucleic Acids Res. **18**, 3777 (1990)
44 Saison-Behmoaras, T.; Tocque, B.; Rey, I.; Chassignol, M.; Thuong, N. T.; Helene, C.: EMBO J. **10**, 1111 (1991)
45 Gamper, H. B.; Reed, M. W.; Cox, T.; Virosco, J. S.; Adams, A. D.; Gall, A. A.; Scholler, J. K.; Meyer, R. B., Jr.: Nucleic Acids Res. **21**, 145 (1993)
46 Kabanov, A. V.; Vinogradov, S. V.; Ovcharenko, A. V.; Krivonos, A. V.; Melik-Nubarov, N. S.; Kiselev, V. I.; Severin, E. S.: FEBS Lett. **259**, 327 (1990)
47 Degols, G.; Leonetti, J. P.; Mechti, N.; Lebleu, B.: Nucleic Acids Res. **19**, 945 (1991)
48 Degols, G.; Leonetti, J. P.; Gagnor, C.; Lemaitre, M.; Lebleu, B.: Nucleic Acids Res. **17**, 9341 (1989)
49 Lemaitre, M.; Bayard, B.; Lebleu, B.: Proc. Natl. Acad. Sci. USA **84**, 648 (1987)
50 Leonetti, J. P.; Rayner, B.; Lemaitre, M.; Gagnor, C.; Milhaud, P. G.; Imbach, J. L.; Lebleu, B.: Gene **72**, 323 (1988)
51 Stevenson, M.; Iversen, P. L.: J. Gen. Virol. **70**, 2673 (1989)
52 Brazeau, G. A.; Attia, S.; Poxon, S.; Hughes, J. A.: Pharm. Res. **15**, 680 (1998)
53 Deshpande, D.; Toledo Velasquez, D.; Thakkar, D.; Liang, W.; Rojanasakul, Y.: Pharm. Res. **13**, 57 (1996)
54 Ginobbi, P.; Geiser, T. A.; Ombres, D.; Citro, G.: Anticancer Res. **17**, 29 (1997)
55 Stewart, A. J.; Pichon, C.; Meunier, L.; Midoux, P.; Monsigny, M.; Roche, A. C.: Mol. Pharmacol. **50**, 1487 (1996)
56 Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P.: Proc. Natl. Acad. Sci. USA **92**, 7297 (1995)
57 Bielinska, A.; Kukowska Latallo, J. F.; Johnson, J.; Tomalia, D. A.; Baker, J. R., Jr.: Nucleic Acids Res. **24**, 2176 (1996)
58 Delong, R.; Stephenson, K.; Loftus, T.; Fisher, M.; Alahari, S.; Nolting, A.; Juliano, R. L.: J. Pharm. Sci. **86**, 762 (1997)
59 Hughes, J. A.; Aronsohn, A. I.; Avrutskaya, A. V.; Juliano, R. L.: Pharm. Res. **13**, 404 (1996)
60 Kukowska-Latallo, J. F.; Bielinska, A. U.; Johnson, J.; Spindler, R.; Tomalia, D. A.; Baker, J. R., Jr.: Proc. Natl. Acad. Sci. USA **93**, 4897 (1996)
61 Poxon, S. W.; Mitchell, P. M.; Liang, E.; Hughes, J. A.: Drug Del. **3**, 255 (1996)
62 Pardridge, W. M.; Boado, R. J.: FEBS Lett. **288**, 30 (1991)
63 Bennett, C. F.; Chiang, M. Y.; Chan, H.; Shoemaker, J. E.; Mirabelli, C. K.: Mol. Pharmacol. **41**, 1023 (1992)
64 Konopka, K.; Pretzer, E.; Felgner, P. L.;: Duzgunes, N.: Biochim. Biophys. Acta **1312**, 186 (1996)
65 Perlaky, L.; Saijo, Y.; Busch, R. K.; Bennett, C. F.; Mirabelli, C. K.; Crooke, S. T.; Busch, H.: Anticancer Drug Des. **8**, 3 (1993)
66 Saijo, Y.; Perlaky, L.; Valdez, B. C.; Wang, H.; Henning, D.; Busch, H.: Oncol. Res. **5**, 283 (1993)
67 Capaccioli, S.; Di Pasquale, G.; Mini, E.; Mazzei, T.; Quattrone, A.: Biochem. Biophys. Res. Commun. **197**, 818 (1993)
68 Lappalainen, K.; Urtti, A.; Soderling, E.; Jaaskelainen, I.; Syrjanen, K.; Syrjanen, S.: Biochim. Biophys. Acta **1196**, 201 (1994)
69 Liang, E.; Hughes, J.: Biochim. Biophys. Acta **1369**, 39 (1998)
70 Quattrone, A.; Papucci, L.; Morganti, M.; Coronnello, M.; Mini, E.; Mazzei, T.; Colonna, F. P.; Garbesi, A.; Capaccioli, S.: Oncol. Res. **6**, 311 (1994)
71 Takle, G. B.; Thierry, A. R.; Flynn, S. M.; Peng, B.; White, L.; Devonish, W.; Galbraith, R. A.; Goldberg, A. R.; George, S. T.: Antisense Nucleic Acid Drug Dev. **7**, 177 (1997)
72 Zelphati, O.; Szoka, F. C., Jr.: Pharm. Res. **13**, 1367 (1996)
73 Litzinger, D. C.; Brown, J. M.; Wala, I.; Kaufman, S. A.; Van, G. Y.; Farrell, C. L.; Collins, D.: Biochim. Biophys. Acta **1281**, 139 (1996)
74 Guy Caffey, J. K.; Bodepudi, V.; Bishop, J. S.; Jayaraman, K.; Chaudhary, N.: J. Biol. Chem. **270**, 31391 (1995)
75 Lappalainen, K.; Miettinen, R.; Kellokoski, J.; Jaaskelainen, I.; Syrjanen, S.: J. Histochem. Cytochem. **45**, 265 (1997)
76 Lappalainen, K.; Pirila, L.; Jaaskelainen, I.; Syrjanen, K.; Syrjanen, S.: Anticancer Res. **16**, 2485 (1996)
77 Jaaskelainen, I.; Monkkonen, J.; Urtti, A.: Biochim. Biophys. Acta **1195**, 115 (1994)
78 Ollikainen, H.; Lappalainen, K.; Jaaskelainen, I.; Syrjanen, S.; Pulkki, K.: Leuk. Lymphoma **24**, 165 (1996)
79 Rose, J. K.; Buonocore, L.; Whitt, M. A.: Biotechniques **10**, 520 (1991)
80 Chavany, C.; Saison Behmoaras, T.; Le Doan, T.; Puisieux, F.; Couvreur, P.; Helene, C.: Pharm. Res. **11**, 1370 (1994)
81 Chavany, C.; Le Doan, T.; Couvreur, P.; Puisieux, F.; Helene, C.: Pharm. Res. **9**, 441 (1992)
82 Schwab, G.; Chavany, C.; Duroux, I.; Goubin, G.; Lebeau, J.; Helene, C.; Saison Behmoaras, T.: Proc. Natl. Acad. Sci. USA **91**, 10460 (1994)
83 Gershon, H.; Ghirlando, R.; Guttman, S. B.; Minsky, A.: Biochemistry **32**, 7143 (1993)
84 Barry, E. L.; Gesek, F. A.; Friedman, P. A.: Biotechniques **15**, 1016 (1993)
85 Clarenc, J. P.; Degols, G.; Leonetti, J. P.; Milhaud, P.; Lebleu, B.: Anticancer Drug Des. **8**, 81 (1993)
86 Wagner, R. W.; Matteucci, M. D.; Lewis, J. G.; Gutierrez, A. J.; Moulds, C.; Froehler, B. C.: Science **260**, 1510 (1993)
87 Yeoman, L. C.; Danels, Y. J.; Lynch, M. J.: Antisense Res. Dev. **2**, 51 (1992)
88 Phillips, N. C.; Emili, A.: Immunol. Lett. **30**, 291 (1991)
89 Thierry, A. R.; Dritschilo, A.: Nucleic Acids Res. **20**, 5691 (1992)
90 Juliano, R. L.; Akhtar, S.: Antisense Res. Dev. **2**, 165 (1992)
91 Anazodo, M. I.; Wainberg, M. A.; Friesen, A. D.; Wright, J. A.: J. Virol. **69**, 1794 (1995)
92 Hatta, T.; Takai, K.; Nakada, S.; Yokota, T.; Takaku, H.: Biochem. Biophys. Res. Commun. **232**, 545 (1997)
93 Hatta, T.; Nakagawa, Y.; Takai, K.; Nakada, S.; Yokota, T.; Takaku, H.: Biochem. Biophys. Res. Commun. **223**, 341 (1996)
94 Marzo, A. L.; Fitzpatrick, D. R.; Robinson, B. W.; Scott, B.: Cancer Res. **57**, 3200 (1997)
95 Ogo, H.; Hirai, Y.; Miki, S.; Nishio, H.; Akiyama, M.; Nakata, Y.: Gen. Pharmacol. **25**, 1131 (1994)
96 Wielbo, D.; Simon, A.; Phillips, M. I.; Toffolo, S.: Hypertension **28**, 147 (1996)
97 Habus, I.; Zhao, Q.; Agrawal, S.: Bioconjug. Chem. **6**, 327 (1995)
98 Zhao, Q.; Temsamani, J.; Agrawal, S.: Antisense Res. Dev. **5**, 185 (1995)
99 Fukui, K.; Tanaka, K.: Nucleic Acids Res. **24**, 3962 (1996)
100 Grigoriev, M.; Praseuth, D.; Robin, P.; Hemar, A.; Saison Behmoaras, T.; Dautry Varsat, A.; Thuong, N. T.; Helene, C.; Harel Bellan, A.: J. Biol. Chem. **267**, 3389 (1992)
101 Klysik, J.; Kinsey, B. M.; Hua, P.; Glass, G. A.; Orson, F. M.: Bioconjug. Chem. **8**, 318 (1997)
102 Lacoste, J.; Francois, J. C.; Helene, C.: Nucleic Acids Res. **25**, 1991 (1997)
103 McConnaughie, A. W.; Jenkins, T. C.: J. Med. Chem. **38**, 3488 (1995)
104 Toulme, J. J.; Krisch, H. M.; Loreau, N.; Thuong, N. T.; Helene, C.: Proc. Natl. Acad. Sci. USA **83**, 1227 (1986)
105 Verspieren, P.; Cornelissen, A. W.; Thuong, N. T.; Helene, C.; Toulme, J. J.: Gene **61**, 307 (1987)
106 Walter, N. G.: J. Mol. Biol. **254**, 856 (1995)
107 Belousov, E. S.; Afonina, I. A.; Pudyminogin, M. A.; Gamper, H. B.; Reed, M. W.; Wydro, R. M.; Meyer, R. B.: Nucleic Acids Res. **25**, 3440 (1997)
108 Marchand, C.; Bailly, C.; Nguyen, C. H.; Bisagni, E.; Garestier, T.; Helene, C.; Waring, M. J.: Biochemistry **35**, 5022 (1996)
109 Silver, G. C.; Nguyen, C. H.; Boutorine, A. S.; Bisagni, E.; Garestier, T.; Helene, C.: Bioconjug. Chem. **8**, 15 (1997)
110 Giovannangeli, C.; Perrouault, L.; Escude, C.; Nguyen, T.; Helene, C.: Biochemistry **35**, 10539 (1996)
111 Chen, J. K.; Weith, H. L.; Grewal, R. S.; Wang, G.; Cushman, M.: Bioconjug. Chem. **6**, 473 (1995)
112 Levina, A. S.; Tabatadse, D. R.; Khalimskaya, L. M.; Prichodko, T. A.; Shishkin, G. V.; Alexandrova, L. A.; Zarytova, V. P.: Bioconjug. Chem. **4**, 319 (1993)
113 Sharon, N.; Lis, H.: Science **246**, 227 (1989)
114 Bonfils, E.; Depierreux, C.; Midoux, P.; Thuong, N. T.; Monsigny, M.; Roche, A. C.: Nucleic Acid Res. **20**, 4621 (1992)
115 Bunnell, B. A.; Askari, F. K.; Wilson, J. M.: Somat. Cell Mol. Genet. **18**, 559 (1992)
116 Wu, G. Y.; Wu, C. H.: J. Biol. Chem. **267**, 12436 (1992)
117 Liang, W. W.; Shi, X.; Deshpande, D.; Malanga, C. J.; Rojanasakul, Y.: Biochim. Biophys. Acta **1279**, 227 (1996)
118 Citro, G.; Szczylik, C.; Ginobbi, P.; Zupi, G.; Calabretta, B.: Br. J. Cancer **69**, 463 (1994)
119 Citro, G.; Perrotti, D.; Cucco, C.; I, D. A.; Sacchi, A.; Zupi, G.; Calabretta, B.: Proc. Natl. Acad. Sci. USA **89**, 7031 (1992)
120 Chaudhuri, G.: Biochem. Pharmacol. **53**, 385 (1997)
121 Wang, S.; Lee, R. J.; Cauchon, G.; Gorenstein, D. G.; Low, P. S.: Proc. Natl. Acad. Sci. USA **92**, 3318 (1995)
122 Leonetti, J. P.; Machy, P.; Degols, G.; Lebleu, B.; Leserman, L.: Proc. Natl. Acad. Sci. USA **87**, 2448 (1990)
123 Ma, D. D.; Wei, A. Q.: Leuk. Res. **20**, 925 (1996)
124 Renneisen, K.; Leserman, L.; Matthes, E.; Schroder, H. C.; Muller, W. E.: J. Biol. Chem. **265**, 16337 (1990)
125 Selvam, M. P.; Buck, S. M.; Blay, R. A.; Mayner, R. E.; Mied, P. A.; Epstein, J. S.: Antiviral Res. **33**, 11 (1996)

126 Zelphati, O.; Imbach, J. L.; Signoret, N.; Zon, G.; Rayner, B.; Leserman, L.: Nucleic Acids Res, **22**, 4307 (1994)
127 Bergan, R.; Hakim, F.; Schwartz, G. N.; Kyle, E.; Cepada, R.; Szabo, J. M.; Fowler, D.; Gress, R.; Neckers, L.: Blood **88**, 731 (1996)
128 Flanagan, W. M.; Wagner, R. W.: Mol. Cell Biochem. **172**, 213 (1997)
129 Griffey, R. H.; Monia, B. P.; Cummins, L. L.; Freier, S.; Greig, M. J.; Guinosso, C. J.; Lesnik, E.; Manalili, S. M.; Mohan, V.; Owens, S.; Ross, B. R.; Sasmor, H.; Wancewicz, E.; Weiler, K.; Wheeler, P. D.; Cook, P. D.: J. Med. Chem. **39**, 5100 (1996)
130 Schaal, H.; Klein, M.; Gehrmann, P.; Adams, O.; Scheid, A.: J. Virol. **69**, 3308 (1995)
131 Blondel, D.; Harmison, G. G.; Schubert, M.: J. Virol. **64**, 1716 (1990)
132 Fenster, S. D.; Wagner, R. W.; Froehler, B. C.; Chin, D. J.: Biochemistry **38**, 8391 (1994)
133 Lamprecht, R.; Hazvi, S.; Dudai, Y.: J. Neurosci. **17**, 8443 (1997)
134 O'Keefe, R. T.; Mayeda, A.; Sadowski, C. L.; Krainer, A. R.; Spector, D. L.: J. Cell Biol. **124**, 249 (1994)
135 Sagata, N.; Oskarsson, M.; Copeland, T.; Brumbaugh, J.; Vande-Woude, G. F.: Nature **335**, 519 (1988)
136 Bongartz, J. P.; Aubertin, A: M.; Milhaud, P. G.; Lebleu, B.: Nucleic Acids Res. **22**, 4681 (1994)
137 Yu, Y. G.; King, D. S.; Shin, Y. K.: Science **266**, 274 (1994)
138 Carrasco, L.: FEBS Lett. **350**, 151 (1994)
139 Fattal, E.; Nir, S.; Parente, R. A.; Szoka, F. C., Jr.: Biochemistry **33**, 6721 (1994)
140 Marsh, M.: Biochem. J. **218**, 1 (1984)
141 Parente, R. A.; Nir, S.; Szoka, F. C., Jr.: Biochemistry **29**, 8720 (1990)
142 Aoki, M.; Morishita, R.; Higaki, J.; Moriguchi, A.; Kida, I.; Hayashi, S.; Matsushsita, H.; Kaneda, Y.; Ogihara, T.: Biochem. Biophys. Res. Commun. **231**, 540 (1997)
143 Dzau, V. J.; Mann, M. J.; Morishita, R.; Kaneda, Y.: Proc. Natl. Acad. Sci. USA **93**, 11421 (1996)
144 Kumar, M.; Hassan, M. Q.; Tyagi, S. K.; Sarkar, D. P.: J. Virol. **71**, 6398 (1997)
145 Mann, M. J.; Morishita, R.; Gibbons, G. H.; von der Leyen, H. E.; Dzau, V. J.: Mol. Cell Biochem. **172**, 3 (1997)
146 Morishita, R.; Gibbons, G. H.; Ellison, K. E.; Nakajima, M.; Zhang, L.; Kaneda, Y.; Ogihara, T.; Dzau, V. J.: Proc. Natl. Acad. Sci. USA **90**, 8474 (1993)
147 Yonemitsu, Y.; Kaneda, Y.; Muraishi, A.; Yoshizumi, T.; Sugimachi, K.; Sueishi, K.: Gene Ther. **4**, 631 (1997)
148 Okada, Y.; Koseke, I.; Kim, J.; Hashimoto, T.; Kanno, Y.; Matsui, Y.: Exp. Cell Res. **93**, 368 (1975)
149 Bentz, J.; Ellens, H.; Lai, M. Z.; Szoka, F. C., Jr.: Proc. Natl. Acad. Sci. USA **82**, 5742 (1985)
150 Connor, J.; Yatvin, M. B.; Huang, L.: Proc. Natl. Acad. Sci. USA **81**, 1715 (1984)
151 Duzgunes, N.; Straubinger, R. M.; Baldwin, P. A.; Friend, D. S.; Papahadjopoulos, D.: Biochemistry **24**, 3091 (1985)
152 De Oliveira, M. C.; Fattal, E.; Ropert, C.; Malvy, C.; Couvreur, P.: J. Cont. Rel. **48**, 179 (1997)
153 Ropert, C.; Mishal, Z., Jr.; Rodrigues, J. M.; Malvy, C.; Couvreur, P.: Biochim. Biophys. Acta **1310**, 53 (1996)
154 Ropert, C.; Malvy, C.; Couvreur, P.: Pharm. Res. **10**, 1427 (1993)
155 Ropert, C.; Lavignon, M.; Dubernet, C.; Couvreur, P.; Malvy, C.: Biochem. Biophys. Res. Commun. **183**, 879 (1992)
156 Chu, C. J.; Dijkstra, J.; Lai, M. Z.; Hong, K.; Szoka, F. C.: Pharm. Res. **7**, 824 (1990)
157 Slepushkin, V. A.; Simoes, S.; Dazin, P.; Newman, M. S.; Guo, L. S.; Pedroso de Lima, M. C.; Duzgunes, N.: J. Biol. Chem. **272**, 2382 (1997)
158 Zelphati, O.; Szoka, F. C., Jr.: Proc. Natl. Acad. Sci. USA **93**, 11493 (1996)
159 Pinnaduwage, P.; Schmitt, L.; Huang, L.: Biochim. Biophys. Acta **985**, 33 (1989)
160 De Duve, C.: Eur. J. Biochem. **137**, 391 (1983)
161 De Duve, C.; De Barsy, T.; Poole, B.; Trouet, A.; Tulkens, P.; Van Hoof, F.: Biochem. Pharmacol. **23**, 2495 (1974)
162 Firestone, R. A.; Pisano, J. M.; Bonney, R. J.: J. Med. Chem. **22**, 1130 (1979)
163 Liang, E.; Hughes, J. A.; J. Membr. Biol. **166**, 37 (1998)

Received November 19, 1998
Accepted March 22, 1999

Dr. J. A. Hughes
P.O. Box 100494, JHMHC
University of Florida
Gainesville, FL 32610-0494
U.S.A.
hughes@cop.ufl.edu