Evaluation of Adjuvants that Enhance the Effectiveness of Antisense Oligodeoxynucleotides

J. A. Hughes,[1,3] A. I. Aronsohn,[1] A. V. Avrutskaya,[2] and R. L. Juliano[2]

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Purpose. A factor limiting the effectiveness of antisense (AS) deoxyoligonucleotides (ODNs) is inefficient transport to their sites of action in the cytoplasm and in the nucleus. The extent of ODN transfer from endosomes to cytosol seems to be an important determinant of ODN effects. Consequently, the development of compounds (adjuvants) that enhance endosome to cytosol transfer may be vital in AS ODN therapeutics.

Methods. In this report, we evaluated compounds for their potential to enhance the effects of phosphorothioate ODNs. The test system used a CHO cell line expressing the enzyme chloramphenicol acetyltransferase (CAT) under the control of an inducible promoter. Several potential endosomal disrupting adjuvants were screened, including: (a) fusogenic peptides; (b) a pH sensitive polymer; (c) polymeric dendrimers, (d) cationic liposomes and (e) a pH sensitive surfactant N-dodecyl 2-imidazole-propionate (DIP). ODN effects were evaluated at the protein level by quantitating levels of CAT.

Results. The use of AS ODN in co-incubation with the GALA peptide, cationic liposomes or 5th generation dendrimers resulted in a 35–40% reduction in CAT expression. The mis-matched ODN had no effect on CAT expression. Only modest effects were observed with the other adjuvants. DIP did not increase ODN activity by itself; however, when the liposomal form was used a significant reduction (48%) in CAT activity was seen.

Conclusions. We found the fusogenic peptide GALA, dendrimers, as well as the liposomal form of DIP, could significantly enhance the effects of ODNs.

KEY WORDS: oligonucleotide; endocytosis; delivery; surfactant.

INTRODUCTION

An important limiting factor in the potential use of ODNs in therapeutics is the relatively inefficient uptake of these agents into sites of action in the nucleus and in the cytoplasm. ODNs are usually brought within cells by endocytosis (1) and accumulate within endosomes, an intracellular vesicular compartment having an acidic pH (2). While some observations suggest that certain types of ODNs may by-pass acidic compartments (3), most evidence suggests otherwise (4). From endosomes, the ODNs may traffic to lysosomes, to other intra-cellular membrane compartments, or they may be released into the cytosol by an, as yet, undefined mechanism (1). Once in the cytosol, ODNs rapidly enter the nucleus by a diffusional process (5,6). The rate and extent of transfer of ODNs from vesicular compartments, such as endosomes to the cytosol may, thus, be an important determinant of AS ODN efficacy.

A number of strategies, similar to DNA transfection methods, have been employed to facilitate the entry of ODNs into the cytoplasm. These strategies have two goals: the first is to increase the amount of ODN or DNA that associates with the cell; the second is to bring the nucleic acid into the cytoplasm by transiently disrupting the endosomal membrane or plasma membrane. A widely used approach involves complexation of the ODNs (or DNA) with cationic lipids (7,8). Another approach is the complexation of ODNs or DNA with cationic polymers (e.g, polylysine) (9). A third approach, used mainly thus far for transfection studies, involves mimicking mechanisms of delivery of viral nucleic acids by use of fusogenic peptides (10). In some studies, these approaches have been used conjunctively (11).

In the current investigation we had two goals. The first was to establish a simple screening system for testing effects of compounds that might enhance the action of ODNs. For this purpose we used CHO cells transfected with a reporter gene construct, with the reporter itself being the target for the ODN. Our second goal was to evaluate which compounds might enhance the effects of ODNs; henceforth, we term such compounds "adjuvants." We evaluated fusogenic peptides, dendrimers, a pH responsive polymer, and a newly synthesized pH sensitive surfactant, DIP. We found that certain fusion peptides, dendrimers and the liposomal form of DIP produced a significant enhancement of ODN effects in the model system.

METHODS AND MATERIALS

ODNs used in this study had a phosphorothioate backbone a and were synthesized at the University of North Carolina (UNC) ODN Core Facility. Peptides were synthesized at the UNC Peptide Synthesis Core using literature reported sequences for GALA (12), and a peptide from the influenza virus (HA) (10) (Table 1). The pH-sensitive polyelectrolyte, poly (α-ethylacrylic acid):PEAA was a generous gift from David Tirrell, Carnegie-Mellon University (13). Starburst dendrimers (generation 5) polyamidoamine (PAMAM) were obtained from Polyscience Inc. (Warrington, PA) had an ethyldiamine core and 64 amino groups on the external surface.

Preparation of DIP

A mixture of dodecanol (0.02 mol), 2-bromopropionyl bromide (0.02 mol), and triethylamine (5 g) in 50 ml of dry chloroform was stirred at room temperature for 24 hours. The reaction was washed with water (20 ml) three times. The organic phase was dried over anhydrous magnesium sulfate and the solvent was removed (distillation) leaving the crude dodecyl-

Table I. Fusogenic Peptide Sequences

Name	Peptide Sequence
GALA	NH_2-WEAALAEALAEALAEHLAEALAEALEALAA-CO_2H
HA	NH_2-GLFEAIAGFIENGWEGMIDGWYG-CO_2H

[1] School of Pharmacy, Department of Pharmaceutics, University of Florida, Gainesville, Florida 32610.
[2] Department of Pharmacology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599.
[3] To whom correspondence should be addressed.

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2-bromo-propionate. The reaction product (4 g) was dissolved in 50 ml of dichloroethane to which triethylamine (500 mg), imidazole sodium (1.6 g), and a few crystals of potassium iodide were added. The resulting mixture was refluxed for 24 hours. The organic phase was washed with water (20 ml) three times, dried and solvent removed. The crude oil residue was purified on a silica gel (100–200 mesh) chromatography system using ethyl acetate as the solvent. The purified dodecyl 2-imidazole-propionate was subjected to NMR and elemental analysis. The ^1H NMR ($CDCl_3$) spectrum showed resonances of m 7.4 (1H C2), 5.72 (t C'2), 4.48 (br m, C4-H, C5-H), 1.9 (t, C'1, CH_3), 1.48 (br m, C'4–C'14 CH_2), .92 (t, C'15 CH_3). Elemental analysis was conducted by Quantitative Technologies Inc. (Whitehouse, NJ); experimental versus (theoretical) values are given with the results in percent C: 69.2 (70.12), H:9.9 (10.3), N:9.05 (9.09). A rough estimate of the apparent pKa was determined by Quantitative Technologies Inc. (Whitehouse, NJ) using a micro pH electrode.

Preparation of Liposomes

Lipid vesicles were prepared by hydration of a dried lipid film (14). The film was prepared by dissolving (w/w%) L-α-Lecithin (80%), and cholesterol (20%), or L-α-Lecithin (90%) and dodecyl 2-imidazole-propionate (10%) (25 mg total lipid amount), in 25 ml of anhydrous chloroform in a round bottomed flask. The cholorform was removed and one milliliter of phosphate buffered saline (PBS), pH 7.4, was added. Liposomes (L-α-Lecithin/cholesterol) containing an encapsulated fluorophore were prepared by adding 1 mg of fluorescein isothiocyanate (FITC) labeled dextran MW 3,000 (Molecular Probes) in 1 ml PBS during liposome formation. The liposomal mixtures were passed through a 0.2 μm polycarbonate filter (Nuclepore), for the DIP containing liposomes, or a 0.6 μm filter, for the FITC-dextran containing liposomes, 20 times using the Lipofast system (15). The resulting liposome dispersions were washed with 5 ml of PBS and the liposomes separated from the bulk fluid by centrifugation (3×). A similar procedure was used for ODN encapsulation with DIP (L-α-lecithin, cholesterol, and DIP) and control liposomes (L-α-lecithin/cholesterol) but 100 nmol AS or MM ODN was added directly to the PBS during the rehydration step. The liposomes were subjected to five freeze (dry ice/acetone) and thaw (37°C water bath) cycles. The liposomes were extruded through 0.2 μm double polycarbonate filters 10 times and non-encapsulated ODN removed by gel filtration (Sephadex G-25). ODN encapsulation was determined by removing an aliquot, lysing the liposomes with Triton X-100 and quantifying ODN concentration with the commercial DNA Dipstick Kit (Invitrogen; San Diego, CA).

Transformation of Cell Lines

A parent Chinese Hamster Ovary cell line (AUXB1) was used to develop the test systems. From this parent line, a stable transfected line was produced using a Lipofection procedure (7). The inducible CAT producing line was made by a dual transfection procedure using a low background activity inducible CAT metalothionein promoter driven vector (16), a generous gift from Dr. Steve Haskill, University of North Carolina, along with the pSVNEO plasmid (Gibco BRL), allowing for selection.

Toxicity Screen

The adjuvant compounds were screened for cellular toxicity using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) based cytotoxcity assay (17). In these experiments CHO cells were plated at sub-confluent monolayers in 96 well plates. Adjuvants were added in 10 fold dilutions from 500 μg/ml to 0.5 ng/ml. The cells were maintained for 48 hours and then MTT (50 μg) was added to each well for 4 hours. After the incubation, the media was removed and 200 μl DMSO added. The plates were shaken for 2 minutes and the absorbance at 562 nm was obtained. The ID_{10} was defined as the concentration of adjuvant that reduced the absorbance by 10% as compared to untreated controls.

AS ODN Experiments

The CAT transfected cell line (5×10^5 cells) was plated in a 60 mm^2 (Falcon) plate containing 3 ml of Gibco α-minimum essential media (α-MEM) with deoxyoligoneucleotides +10% fetal calf serum (FCS) and 1 mg/ml G418 (Gibco). Cells were maintained at 37°C with 5% CO_2/95% air in a humidified incubator. An 18 mer phosphorothioate AS ODN sequence (5'-AGT GAT TTT TTT CTC CAT-3') or a 4 mis-matched sequence (5' AGT GTT TTA TCT CTT CAT-3') was used. The AS sequence was directed to the initation codon of CAT and has been reported to have biological effects (18). After pre-incubation (6 hr), the cells were rinsed with serum free media and incubated in 3 ml of serum free media along with the ODN and adjuvant. The plates were returned to the incubator for 4 hours and 300 μl fetal calf serum added followed by stimulation with $ZnCl_2$ (200 μM) for 18 hours. The cells were rinsed with PBS and lysed in 200 μl of lysis buffer (0.25M Tris, 0.5% Triton X-100, and 1 mM DTT) and stored for 15 min on ice. The lysate was centrifuged (10,000 x g) for 5 min at 4°C. The supernatant was transferred to a fresh tube and incubated for 10 min at 65°C. The cell lysate was centrifuged for 2 min (10,000 x g) at 4°C and transferred to a new tube. Fifty microliters of the supernatant lysate was evaluated using either a fluorescent CAT detection kit (Molecular Probes, Eugene OR) following manufacturer's instructions, or it was evaluated using a radioactive test system (16). Total protein concentration was determined using the BCA procedure (19). CAT activity was normalized for each experiment by assuming the positive control (stimulated with Zn^{+2}) as 100%, also a negative control (no Zn^{+2}) was included in all experiments serving as a background control. Significant differences between the various treatment regimens was analyzed by the Student's "t" test and one-way analysis of variance.

RESULTS

Synthesis and Characterization of DIP

DIP was designed to become a cationic detergent in its protonated form, while in its uncharged basic form it should display a lesser degree of surfactant activity. The apparent pKa of DIP is 6.8. Thus, at extracellular or cytoplasmic pH (7.3–7.4) approximately 80% of the compound is in the non-ionized form, while at early endosomal pH (6.2) 80% is in the charged form and can function as a cationic detergent; at lysosomal pH (5.2)

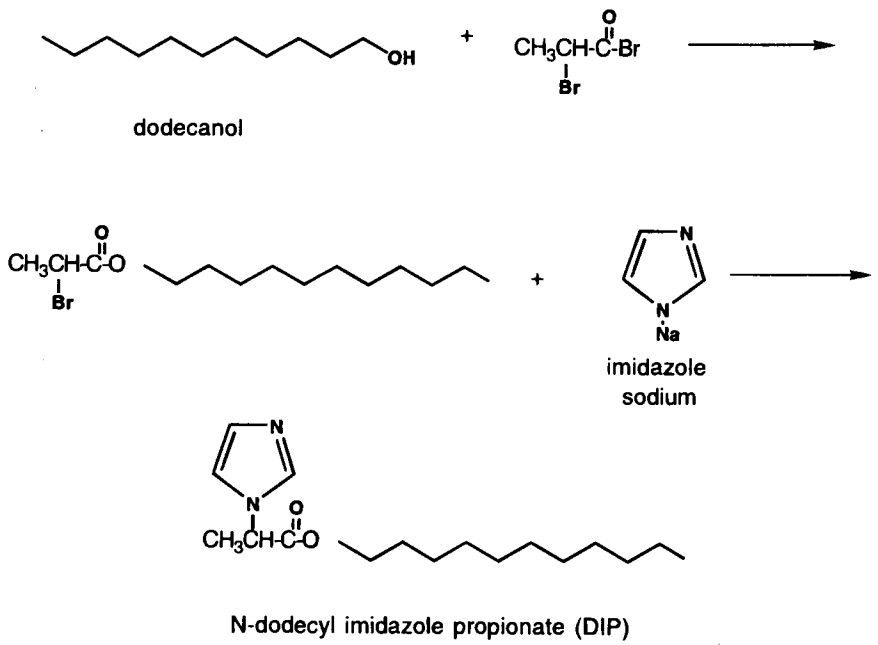

Fig. 1. Synthesis of N-Dodecyl 2-Imidazole-Propionate.

over 97% will be in charged form (20). After causing endosomal rupture the ester bond in dodecyl 2-imidazole should be readily cleaved in the intracellular environment (21), thus reducing the potential toxicity.

A scheme illustrating the synthesis of DIP is shown in Fig. 1. The surfactant activity of DIP is pH dependent as demonstrated in Fig 2, which shows the surfactant-induced release of a marker substance (FITC-dextran) from liposomes. Thus, at pH 4.5, 1.75 mM DIP caused the release of over 70% of an entrapped marker from liposomes within 10 min, while at pH 7.4 less than 10% of the marker was released.

Toxicity of Adjuvants

The toxicity of the potential adjuvant compounds was tested in CHO cells using a sensitive MTT assay. The concentrations required for 10% (ID_{10}) inhibition of cell growth are shown in Table 2. For studies of the adjuvants' effects on the action of ODNs, equitoxic concentrations of adjuvants were chosen corresponding to the ID_{10} for each compound. Thus all ODN studies were done under conditions where each adjuvant compound had equal effects on cell viability.

Table II. Adjuvant ID10 Values

Compound	ID10 (μg/ml)
DIP	25
GALA	250
HA	250
PEAA	10
Dendrimer (generation 5)	> 25
DIP-Liposome	> 1000

Effects of Adjuvants on AS ODN Inhibition of CAT Expression

CHO cells stably transfected with CAT were treated with AS or with MM ODNs. As an initial experiment the pharmacodynamic effects of a known adjuvant were evaluated. Lipofection has been demonstrated to increase the biological activity of ODNs (8). Figure 3 illustrates the inhibition effect of ODN complexed to cationic liposomes. As the amount of ODN is increased, the biological effect increases correspondingly; unfortunately at higher ODN concentrations sequence non-specific effects are seen (biological effects from the MM sequence). Based on this information we chose to use an ODN concentration of 2 μM. Cells receiving ODNs were also treated with equitoxic doses of the adjuvants, or were maintained as untreated controls. As seen in Fig 4a, use of AS ODN with GALA peptide resulted in an approximate 38% reduction in expression of CAT which is similar to the dendrimer and Lipofectin(R). The MM ODN had no effect on CAT expression. Only modest effects were observed with other adjuvants. DIP did not increase ODN activity; however, when the liposomal form of DIP was used, a significant reduction (48%) in CAT activity was seen (Fig 4B). This effect was potentiated when the ODNs were co-incorporated into the DIP-liposomes. Preliminary experiments with DIP-liposome encapsulated ODN demonstrated biological effects with both sequences (AS & MM) and marked cellular toxicity. In order to discriminate between the ODN sequences, a lower dose of DIP-liposome ODNs was used. Figure 4B demonstrates the effectiveness of the composite delivery system.

DISCUSSION

A major goal of the work described was to define a simple screening procedure that could identify agents capable of enhancing ODN effects. Assays based on reporter genes seem

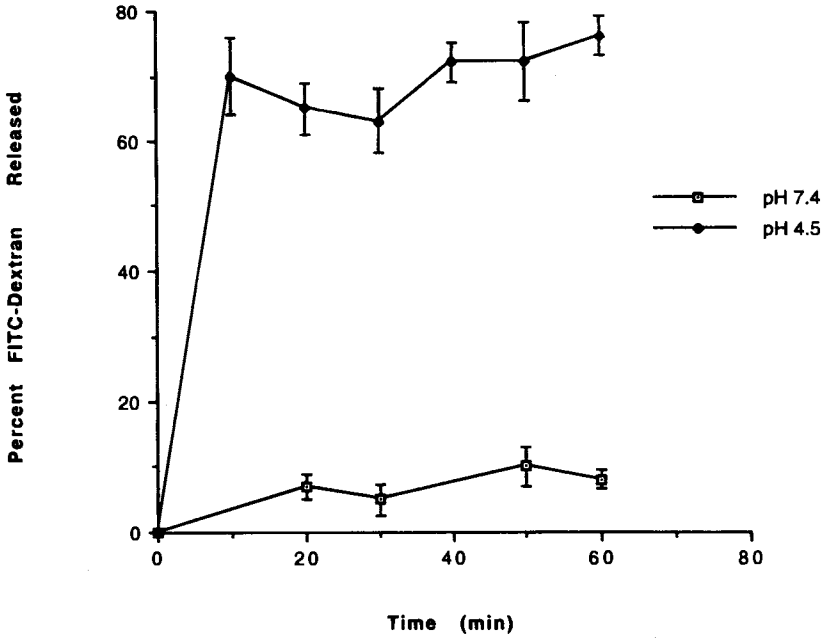

Fig. 2. pH dependent release of FITC-Dextran from liposomes. FITC-labeled-dextran containing liposomes (10 mg) were added either to 1 ml of PBS (pH 7.8) or 150 mM sodium acetate (pH 4.5). DIP (500 μg) was added to the liposomal suspension and 25 μl aliquots removed with time. The aliquot was added to 1 ml of PBS and centrifuged for 5 min at 10,000 × g. The supernatant (900 μl) was transferred to a flurometric cuvette and the amount of labeled dextran quantified (λ_{ex} = 495nm, and λ_{em} = 520nm). Total liposomal FITC-dextran amount was measured by removing an aliquot (25 μl) of the liposomal suspension and disrupting with Triton X-100, 10%. The graph is the mean ± s.d., n = 3.

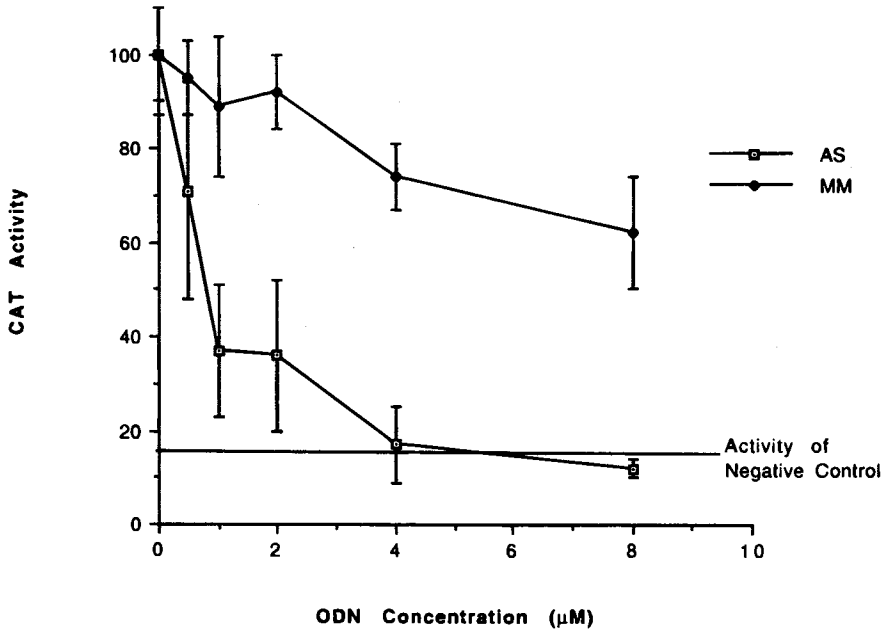

Fig. 3. Lipofection delivery of ODNs to a CAT producing cell line. ODNs (AS and MM) were incubated with Lipofectin (10 μg) for 15 minutes in 100 uL of serum-free D-MEM media. The complex was added to $ZnCl_2$ induced CHO cells as described in Methods. CAT activity was determined as described and corrected for by cellular protein. CAT activity was normalized to a non-treated sample. The horizontal line (~20%) represents a non-stimulated cell line control serving as a negative control. The graph is the mean ± s.d., n = 4.

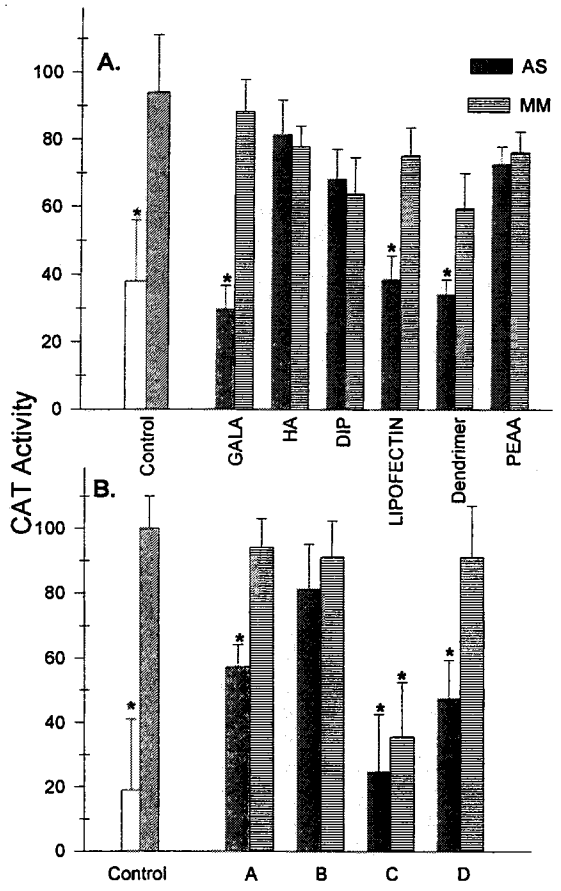

Fig. 4. A. Screening of the pH sensitive adjuvants. CAT cells were plated (n = 4) and treated as described. The ODN (2 μM) along with the adjuvants (GALA (250 μg/ml), PEAA (250 μg/ml), DIP (25 μg/ml), Dendrimer (25 μg/ml) and HA (250 μg/ml)) were incubated with the cells for 6 hours followed by stimulation with Zn^{+2}. After the incubation the cells were lysed and assayed for CAT and corrected for total protein. The controls (+) refers to stimulation with Zn while the negative control (−) is background CAT expression. The graph is the mean ± s.d., n = 5. A significant difference from the positive control is denoted by *. B. Effect of liposomal DIP and encapsulation on ODN activity. CAT producing cells were plated and treated as described. The liposomal form of DIP (200 μg lipid containing 20 μg of DIP) or liposomes (LIP) without DIP were used as controls. For the high-dose DIP-liposome and control liposome study the ODN concentration was 2 μM. The low dose DIP liposome treatment the oligonucleotide concentration was 0.05 μM. The graph is the mean ± s.d., n = 3. A significant difference from the positive control is denoted by *.

ODN (2 μM) + DIP/LIP	A
ODN-encapsulated in LIP (2 μM)	B
ODN encapsulated in DIP/LIP (2 μM)	C
ODN-encapsulated in DIP/LIP (0.05 μM)	D

ideal. They are rapid, convenient, and can be done with low numbers of cells. Although it is customary in ODN research to measure both the mRNA and protein levels of the gene that is being targeted, the approach utilized here, that of measuring reporter enzyme activity, should suffice for screening purposes. Confirmation that the observed inhibition of enzyme activity are due to an ODN effect can be gained by appropriate use of control ODNs such as sense, scrambled, or MM sequences. The possibility of aptomeric effects (22) can be excluded by testing direct effects of the ODNs on enzyme activity in broken cell preparations. A reporter assay cannot discriminate between effects due to translation arrest and those due to mRNA degradation. However, this distinction can easily be made for reporter gene systems such CAT by follow-up studies using commercially available cDNA probes and antibodies. The virtue of an enzymatic reporter assay is that one can deal with a large number of compounds, using modest amounts of reagents.

A second goal of this work was to identify adjuvants that might enhance the effectiveness of AS ODNs. The compounds tested here were intended to affect the transport of ODNs from endosomes to the cytosol. However, the assay used did not offer direct insights into mechanism. Commonly used adjuvants for AS ODN are cationic lipids; the prototype compound DOTMA (1,2-dioleoyl-3-trimethylammonium propane) (7) is now widely used for ODN (8) and transfection (23) studies, while a variety of related cationic lipids have been developed and tested more recently (24, 25). Another approach has been the use of peptides for enhancing transfer of nucleic acids into the cytoplasm. Initial DNA transfection studies used peptides based on influenza virus hemagglutinin (26) or even whole adenovirus particles (27). Recently, a number of fusogenic peptide derivatives based on influenza HA peptide or on the pH sensitive GALA peptide, designed by Szoka and colleagues (12), have been tested for their ability to enhance DNA transfection (10,11).

In the current study we examined the biological effects of potential adjuvants on ODNs activity. GALA and influenza fusion peptides were tested. In addition, we examined other novel compounds. The first was a pH sensitive polymer PEAA (poly(α-ethylacrylic acid)) (13). The second was a novel pH sensitive surfactant DIP. Finally, a unique nucleic acid complexing agent, Starburst dendrimers (11) were used. Dendrimers have been demonstrated to enhance plasmid DNA expression in tissue culture by condensing nucleic acids and serving as a endosomal pH buffer. All compounds were examined, at equitoxic concentrations, for the ability to enhance the effects of AS ODNs directed against CAT. There was a wide variation in the effectiveness of the adjuvant compounds with GALA peptide, with the dendrimer, and with dodecyl 2-imidazole liposomes being the most promising.

The pH dependent, alpha helical forming GALA peptide was able to facilitate ODN activity. The peptide most likely requires endocytosis for cellular entry, localizing within endosomes before eliciting its effects. The HA peptide also facilitates endosomal gene transfer (10), but in the current study minimal ODN activity enhancement was noted. The efficiency of endosome disruption by peptides is strongly dependent on a locally high concentration of the membrane active peptides. At the peptide concentrations used in our study the effective concentration may not have been reached. The GALA peptide sequence has been reported to elicit pH dependent leakage from liposomes which was not demonstrated with the HA peptide (10).

The approach of using pH sensitive fusogens or membrane destabilizing agents to assist in the delivery of DNA or ODNs from endosomes into the cytosol seems promising. However, there are a number of potential complications. Agents that become fusogenic or membrane active only at pHs comparable to those that exist in lysosomes (pH 5.2) will not be able to release ODNs or DNA from early endosomes; however, they

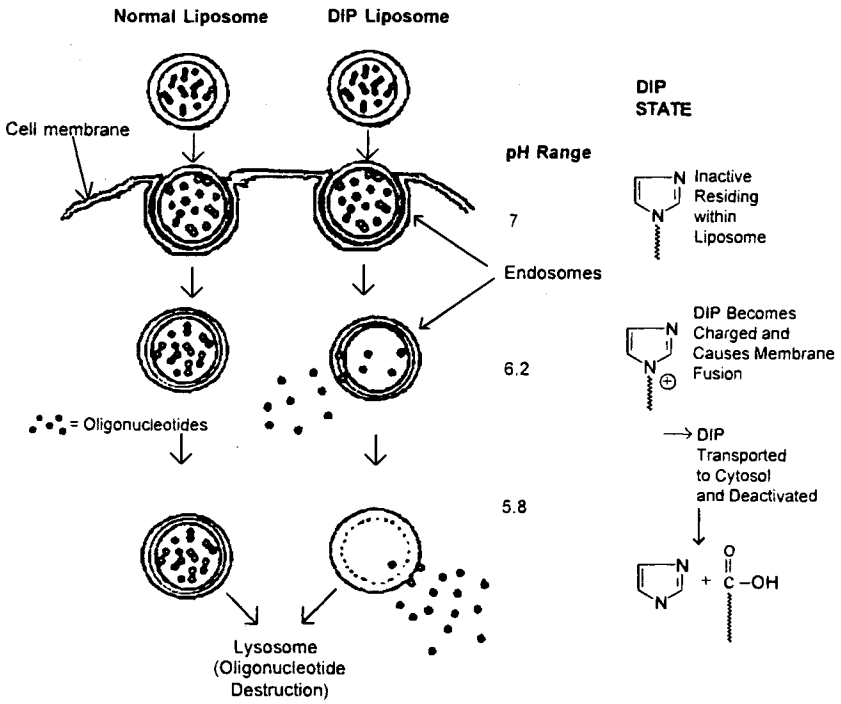

Fig. 5. A schematic representation of possible intracellular fates of DIP and of normal liposomes.

may cause rupture of lysosomes thus releasing proteases and other degradative enzymes. Agents that are designed to be inactive at extra cellular or intra cytoplasmic pH while becoming active/fusogenic at the pH prevalent in early endosomes seem more promising. Such agents may be able to release DNA or ODNs from endosomal compartments, prior to mingling with lysosomes. However, a practical difficulty with this approach is that the pH gradient between the extracellular compartment and early endosomes is rather shallow (1–1.2 pH units). Thus the conversion from inactive to active form may be incomplete as the pH sensitive adjuvant goes from extracellular to endosomal milieus. This is true of dodecyl 2-imidazole since at pH 7.4 (extracellular) approximately 80% of the compound is in the non-surfactant basic form, while at pH 6.2 (early endosomal) 80% is in the acidic surfactant form. The surfactant should be driven by a pH gradient to accumulate in acidic compartments within the cell (28,29).

Imidazole based surfactants, (e.g. dodecyl imidazole) have been evaluated as anticancer drugs but were found to be non-selective, causing lysomal rupture and eventually cell death (29). The use of a "soft surfactant" (SS), one which is easily deactivated by cellular enzymes, might have distinct advantages over other membrane destabilizing agents. A SS should be able to function (at the early endosome stage) and be deactivated before reaching the lysosome; this was the idea which drove the synthesis of DIP. The linkage unit between imidazole and dodecyl tail has previously been shown to be metabolically labile (21). Unfortunately, when this compound was tested at non-toxic doses, little or no ODN activity enhancement was observed. However, when DIP was incorporated into liposomes at 10 wt%, a dramatic difference was noted in adjuvant activity. The liposomal form demonstrated little toxcity at the highest dose tested (1000 μg/ml liposome; 100 μg/ml DIP). This discrepancy between free DIP and liposomal DIP could arise from 1) liposomal protection of surfactant from metabolic cleavage in the culture media before becoming activated in the acidic compartments, 2) increased local concentration of the surfactant within the endosomes due to liposomal delivery, or 3) local differences of pKa values of the surfactant caused by entrapment within liposomes (30). As a final test, the encapsulation of the ODN into both DIP liposomes and control liposomes was tested. Encapsulation efficiency was similar for each liposome (8–12%). When equal molar concentrations of the ODN DIP (AS and MM) liposomes were used, no discrimination between the two sequences could be seen even though biological activity was evident. An explanation could be found in non-specific effects of ODNs when high concentrations of phosphorothioate ODNs were used. In order to achieve discrimination a much lower dose of ODN was used (0.05 μM). Thus, a 40 fold reduction in dose could be obtained using this test system. A tentative model of the effect of the DIP liposomes is given in Figure 5.

A major incentive to study the endosome-disruptive properties of various adjutants has been their potential to enhance endosome to cytosol transfer. This important transport route has applications in nucleic acid (ODN and gene) therapy, the treatment of intracellular bacterial infections, and in the stimulation of MHC-1 immunity. Endosomal disrupting agents will likely require a local high concentration of adjuvant to reach a threshold membrane disrupting activity. One method to achieve this concentration is through the use of liposomes or other particulate systems.

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