

Phosphorothioate oligodeoxyribonucleotides dissociate from cationic lipids before entering the nucleus

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ABSTRACT

Antisense oligonucleotides complementary to specific mRNA sequences are widely used inhibitors of gene expression *in vitro* and *in vivo*. *In vitro* cationic lipids have been demonstrated to increase the pharmacological activity of antisense oligonucleotides by increasing cellular uptake and facilitating nuclear accumulation. We have investigated the intracellular fate of oligonucleotide/cationic lipid complexes using fluorescently labeled lipids and oligonucleotides targeted to protein kinase C- α . After addition to cells the lipids initially co-localized with the oligonucleotide on the cell surface and in fine punctate structures within the cytoplasm. At later times the oligonucleotide began to accumulate in the nucleus as well as the cytoplasm. In contrast, the cationic lipid remained localized to the cell surface and the cytoplasm and was never found in the nucleus. Expression of protein kinase C- α mRNA did not begin to decline until after oligonucleotide was seen in the nucleus. This was also coincident with the transient appearance of a smaller mRNA transcript believed to result from RNase H cleavage of protein kinase C- α mRNA. These data suggest that although cationic lipids facilitate uptake of oligonucleotides, the complex must disassociate before the oligonucleotide can gain access to the nucleus and induce degradation of targeted mRNA.

INTRODUCTION

The idea of using oligonucleotides to modulate gene expression was first proposed by Zamecnik in 1978 (1). Since then, a large volume of data has demonstrated that oligonucleotides with sequences complementary to specific mRNAs (antisense oligonucleotides) can be used to decrease expression of the proteins encoded by these mRNAs both *in vivo* and *in vitro* (2–5). In animals, oligonucleotides have been successfully used to modulate gene expression in some cell types after systemic administration (6,7). However, not all organs or cell types internalize oligonucleotides to the same extent (8–11). The mechanisms by which oligonucleotides are functionally internalized *in vivo* are poorly understood, however, they are likely to differ from the processes of internalization *in vitro*. In tissue culture relatively high oligonucleotide concentrations (>5 μ M) are generally required in

order to demonstrate pharmacological activity. Oligonucleotide uptake is thought to proceed initially by interaction with cell surface proteins (12–16), followed by internalization through an endocytic mechanism (17,18). These events lead to a punctate distribution of the oligonucleotides in intracellular membrane-bound structures which are thought to represent endosomes and lysosomes (13,19–21). This sequestration of oligonucleotides into endosomal compartments may prevent their interaction with their target mRNA and hence decrease their activity (19,20).

The use of various enhancers to increase oligonucleotide intracellular accumulation has largely solved this problem and has greatly facilitated the application of oligonucleotides as research tools *in vitro*. These enhancers have allowed the use of greatly reduced oligonucleotide concentrations (typically nM). There have been many reported uptake enhancers, including: (i) cationic lipids (19,22), (ii) liposomes (23,24), (iii) peptides (25,26), (iv) dendrimers (27,28), (v) polycations (29,30), (vi) conjugation with cholesterol (31,32), (vii) aggregation with cell surface ligands (33,34) and (viii) electroporation (35,36).

One of the most commonly used enhancers is a mixture of a neutral lipid with a cationic lipid. The cationic lipid is the more crucial part of this mixture, as cationic lipids alone enhance antisense oligonucleotide activity, whereas neutral lipids cannot. The mechanism by which cationic lipids increase the activity of antisense oligonucleotides is poorly understood. It was recently demonstrated that oligonucleotide–lipid complexes are taken into the cell via an endocytic mechanism and do not simply fuse with the plasma membrane (37). It is also known that cationic lipids not only enhance cellular accumulation of the oligonucleotide, but also alter the intracellular distribution of the oligonucleotide by increasing the amount that escapes from the endosomal pathway and thus has an opportunity to interact with its target mRNA (19). The mechanism by which the oligonucleotide is released from the endocytic compartments in order to gain access to its RNA target is not yet fully understood, however, models have been proposed (38,39). Fluorescence studies by Szoka and colleagues have recently shown that when transfected with a cationic/neutral lipid mixture oligonucleotides reached the nucleus while the neutral lipid stayed in punctate structures within the cytoplasm (38). These punctate structures were presumed to be endosomes. However, the final destination of the cationic lipid was not examined in this study and whether the cationic lipid traffics together with the oligonucleotide to the nucleus has not yet been determined.

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To address this question we have synthesized fluorescinated versions of both an oligodeoxynucleotide and a cationic lipid. The oligodeoxynucleotide (ISIS 3521R) is targeted to protein kinase C- α (PKC- α). This has allowed us to concurrently evaluate the cellular pharmacokinetics and pharmacological activity of the oligonucleotide. We have previously characterized inhibition of PKC- α mRNA and protein expression by an unfluorescinated oligodeoxynucleotide with this sequence and have demonstrated a requirement for cationic lipids for activity in tissue culture (40). In the studies described here the cationic lipid-oligodeoxynucleotide complex initially appears to bind to the cell surface and then undergoes rapid (within 60 min) internalization into an endosomal compartment. At this time the complex disassociates, with the lipid remaining in the endosomal compartment while the oligodeoxynucleotide leaves the endosome and accumulates in the nucleus. We subsequently showed that the kinetics of reduction in the target mRNA matched the kinetics of oligonucleotide accumulation in the nucleus and that this was coincident with the rapid and transient appearance of a mRNA degradation product. These data demonstrate for the first time that the cationic lipid and oligodeoxynucleotide disassociate relatively rapidly upon internalization and accumulate in distinct intracellular compartments. These data also suggest that the nucleus is likely the site at which the oligonucleotide exerts its activity when utilizing RNase H to degrade its targeted mRNA.

MATERIALS AND METHODS

Cell culture and chemicals

A549 (lung carcinoma) cells obtained from the ATCC were used throughout this study. Lipids were acquired from Avanti Polar lipids (Alabaster, AL), except for the *N*-[4,4-difluoro-5,7-diphenyl-4-boro-3a,4a-diaza-(5)-indacene-3-propionyl]-(*N*)-iodoacetylenedimimine (BODIPY)-labeled 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) derivative, which was synthesized as described below. All phosphorothioate oligodeoxynucleotides (ISIS 2105R, rhodamine-TTGCTTCCATCTTCCTCGTC; 3521, GTTCTCGCTGGTGAGTTTCA; 3521R, rhodamine-GTTCTCGCTGGTGAGTTTCA; 4559, GGTTTTACCATCGGTTCTGG) were synthesized as previously described (41,42). All cell culture medium was from Gibco/BRL (Bethesda, MD) and the FBS was from Irvine Scientific (Irvine, CA). 1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) was obtained from Molecular Probes (Eugene, OR). Four-chambered Permax plastic slides were obtained from Nunc (Nutly, NJ).

Synthesis of fluorescent cationic lipid

The fluorescent cationic lipid 1-*O*-oleoyl-2-[(BODIPY)-dodecanoyl]-*N,N,N*-trimethyl-3-aminopropane bromide was synthesized by the reaction of oleic acid with 3-bromo-1,2-propane diol, which when stirred together in anhydrous dichloromethane in the presence of EDC and DMAP gave the desired compound, 1-oleoyl-2-hydroxy-3-bromopropane. This compound was then reacted with anhydrous trimethylamine in a sealed tube in toluene as the solvent to give 1-*O*-oleoyl-2-hydroxy-*N,N,N*-trimethylaminopropane bromide as the desired product. The structure of this compound was confirmed by NMR and mass spectrometry (data not shown). The BODIPY-labeled fatty acid (Molecular Probes) was then reacted with the above derivatized secondary alcohol in the

presence of DMAP and EDC in anhydrous dichloromethane to give the final compound. It was purified, characterized and used for the current study. A detailed account of the synthesis of this fluorescent cationic lipid and its analogs will be published elsewhere (Balkrishen Bhat *et al.*, manuscript in preparation).

Preparation of lipid mixture for transfections

All lipids were dissolved in chloroform. The appropriate amounts of lipids were then added to glass vials and dried under argon. The vials were left under vacuum overnight to ensure that all traces of the chloroform were removed. The vials were capped and stored in the dark at -20°C . On the day of the experiment the vials were removed and the lipids rehydrated in water at a concentration of between 0.5 and 2 mg/ml. The lipids were sonicated in an inverted cup sonicator and then passed three times through an extruder with a 200 nm pore size filter in order to ensure uniformity of particle size.

Northern blot assays

A549 cells were grown in 75 cm² flasks in DME, 10% fetal bovine serum (FBS) until they reached ~75% confluency. The cells were then washed once in OptiMem (Gibco/BRL) and treated with 5 ml OptiMem containing the indicated concentrations of a 1:1 mixture of DOPE and DOTAP (5% of which was the BODIPY derivative of DOTAP shown in Fig. 1) and oligonucleotide. The cells were then incubated at 37°C for the indicated time before being washed once with cold phosphate-buffered saline (PBS) and harvested in 3.5 ml guanidinium isothiocyanate. The harvested solution was then passed twice through an 18 gauge needle before being layered on top of 1.5 ml 5.7 M CsCl and centrifuged at 35 000 r.p.m. in a SW55 rotor for 14–20 h. The pellet was resuspended and ethanol precipitated twice. The RNA was quantitated by spectrophotometer and 15 μg was resolved on a 1% agarose-formaldehyde gel. The gel contents were transferred to a nylon membrane and the blot prehybridized with QuickHyb (Stratagene, La Jolla, CA) before being probed with ³²P-labeled fragments that hybridize to the coding region of PKC- α or glyceraldehyde 3-phosphate dehydrogenase (G3PDH). The bands were quantitated on a phosphorimager and the PKC- α mRNA levels normalized to the G3PDH mRNA levels.

Fluorescence microscopy

A549 cells were grown on plastic 4-chambered slides until they reached ~75% confluency. The cells were then treated with 15 $\mu\text{g}/\text{ml}$ 1:1 mixture of DOPE and DOTAP (5% of which was the BODIPY-labeled derivative of DOTAP) and 300 nM ISIS 3521R. After the indicated times the slides were removed from the 37°C incubator and washed three times with DME, 10% FBS and twice with PBS. The cells were then covered and photographed live with a fluorescence microscope through either a 20 \times or 40 \times objective.

The endocytic compartments of A549 cells were stained by incubation with 12.5 $\mu\text{g}/\text{ml}$ TMA-DPH for either 3, 15 or 180 min. The cells were then washed with PBS and examined under the fluorescence microscope.

RESULTS

Dose-dependent reduction of PKC- α mRNA by ISIS 3521R

ISIS 3521, a 20 base phosphorothioate oligodeoxynucleotide, has been extensively studied, both *in vitro* and *in vivo*, and shown to

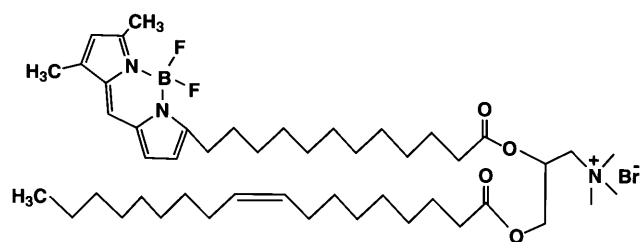


Figure 1. Structure of the fluorescent derivative of DOTAP. 1-*O*-Oleoyl-2-[(BODIPY)-dodecanoyl]-*N,N,N*-trimethyl-3-aminopropane bromide was synthesized by a method that will be described in detail subsequently (Balkrishen Bhat *et al.*, manuscript in preparation).

selectively reduce PKC- α mRNA and protein levels without affecting the mRNA or protein levels of other PKC isozymes (40,43,44). This oligodeoxynucleotide appears to require RNase H for activity, as a fully 2'-*O*-methyl-modified derivative of this sequence is unable to reduce PKC- α mRNA expression (40). As with most antisense oligonucleotides, the *in vitro* activity of this molecule is greatly increased by the use of cationic lipids to enhance cellular delivery and DOTAP is a commonly used cationic lipid for this purpose. In order to better understand the intracellular distribution and relationship of these molecules we conjugated rhodamine to ISIS 3521 (the labeled oligonucleotide is called ISIS 3521R) and synthesized DOTAP with a BODIPY label substituting for one of the acyl chains (see Materials and Methods and Fig. 1).

Experiments were first performed to determine whether PKC- α mRNA expression was reduced by ISIS 3521R and a lipid mixture, including the fluorescently labeled derivative of DOTAP, in a dose-dependent manner. A549 cells were incubated for 4 h at 37°C with the indicated amount of ISIS 3521R complexed with 15 μ g/ml 1:1 mixture of DOPE and DOTAP (5% of which was the BODIPY-labeled derivative). The levels of both the small and large transcripts of the PKC- α gene were determined by northern blot and normalized to the levels of G3PDH mRNA. The reduction in PKC- α mRNA levels by ISIS 3521R was found to be concentration dependent (Fig. 2). Maximum reductions in PKC- α mRNA expression were achieved at concentrations of 300–400 nM, while the oligodeoxynucleotide exhibited an IC₅₀ value of ~150 nM (Fig. 2B). The specificity of the oligodeoxynucleotide inhibitor was demonstrated by the fact that the mRNA level of another PKC isozyme (PKC- η) was not reduced by treatment with any of the concentrations of ISIS 3521R tested (Fig. 2A). This isozyme was chosen to determine the specificity of the oligonucleotide, as A549 cells do not express any other classical PKC isozymes. Treatment of cells with oligodeoxynucleotides that did not contain sequences complementary to PKC- α did not cause a significant reduction in PKC- α mRNA levels (Fig. 2A, ISIS 4559).

The IC₅₀ for ISIS 3521R with fluorescinated cationic lipids (~150 nM) was similar to the IC₅₀ for the unlabeled parent phosphorothioate oligonucleotide sequence (ISIS 3521) and an unlabeled cationic lipid (40). ISIS 3521R complexed with a 1:1 mixture of DOPE and pure BODIPY-labeled DOTAP derivative was just as effective at causing a reduction in PKC- α mRNA as ISIS 3521R complexed with DOPE and cationic lipids containing the mixture of labeled and unlabeled DOTAP (data not shown). These results demonstrated that a cationic lipid containing a BODIPY group on one of the fatty acyl chains of DOTAP was

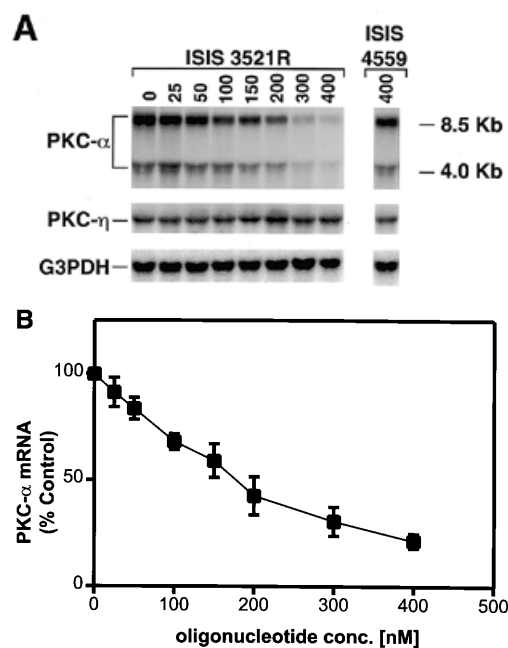


Figure 2. Dose–response characteristics of oligodeoxynucleotide inhibition of PKC- α mRNA expression. (A) A549 cells were treated with 15 μ g/ml DOPE/DOTAP (5% of which was labeled with a BODIPY group in one of the fatty acyl chains) and ISIS 3521R at the indicated concentration for 4 h at 37°C. The cells were then harvested and amounts of PKC- α , PKC- η and G3PDH were determined by northern blot. The 4 and 8.5 kb bands are two distinct transcripts of PKC- α . The far right-hand lane shows northern blots from cells treated with an oligodeoxynucleotide with the scrambled sequence of ISIS 3521R (ISIS 4559). (B) The levels of PKC- α mRNA from three independent samples treated with each of eight different concentrations of ISIS 3521R were determined by quantitation on a phosphorimager and normalized to the levels of G3PDH. The resulting values were plotted as a percentage of levels obtained from untreated samples. The error bars represent the SEM.

equally as effective as unlabeled DOTAP at enhancing uptake of oligodeoxynucleotides.

Time course of oligodeoxynucleotide–lipid complex uptake

The ability of the fluorophore-modified oligodeoxynucleotide and lipids to reduce PKC- α mRNA expression demonstrates that the fluorophores were not having a major effect on trafficking or activity of the oligodeoxynucleotide–lipid complex. Therefore, this allowed us to use the labeled molecules as tools to study the uptake properties of oligodeoxynucleotide–lipid complexes. A549 cells were seeded onto plastic chamber slides and incubated with 300 nM ISIS 3521R and 15 μ g/ml DOPE/DOTAP (5% of which was the BODIPY-labeled derivative). The slides were incubated at 37°C for times ranging from 0 to 240 min. At the indicated time the slides were removed from the incubator, washed thoroughly and examined under a fluorescence microscope. The cells were immediately observed while they were still alive to avoid any potential artifacts introduced by fixation procedures.

At time 0 no cellular fluorescence was detected, demonstrating a lack of background autofluorescence (data not shown). The cell surface was stained brightly at 30 min (Fig. 3) by the cationic lipid (green). ISIS 3521R (red) could also be seen, but in a more discrete punctate pattern. After 60 min incubation some of the cationic lipid could still be seen at the cell surface, but a portion had been internalized and could be found in very fine speckles with a pattern reminiscent of the endosomal system. The

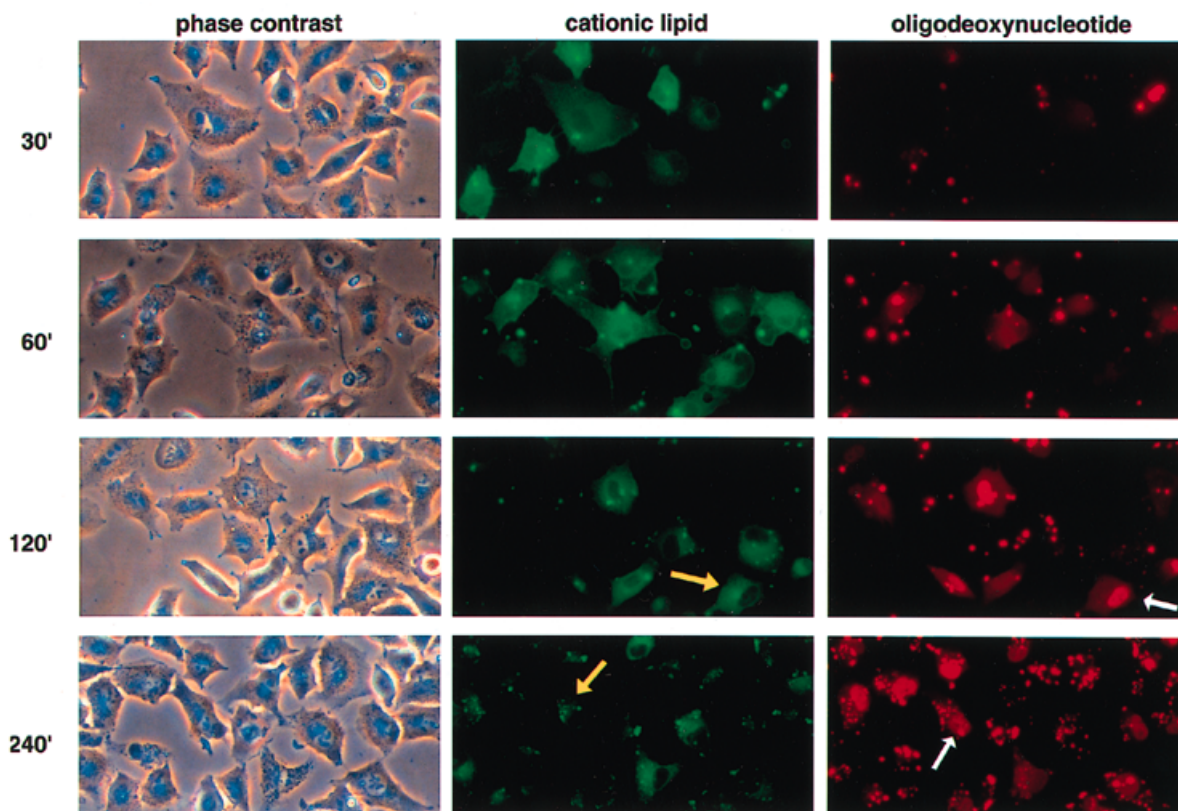


Figure 3. Time course of uptake of fluorescent oligodeoxynucleotide and cationic lipid. A549 cells were transfected for 30, 60, 120 or 240 min at 37°C with 15 µg/ml DOPE/DOTAP (5% of which was labeled with a BODIPY group in one of the fatty acyl chains) and 300 nM ISIS 3521R. The cells were then rinsed and examined live under a fluorescence microscope. The cationic lipid staining (green fluorescence) initially showed a cell surface pattern. Only a small amount of oligodeoxynucleotide staining (red fluorescence) could be seen at 30 min in large punctate structures. Over time the cationic lipid appeared to be gradually internalized and was found to reside in very fine speckles surrounding the nuclei (yellow arrows). The cationic lipid was never found to accumulate within the nuclei, causing the nuclei to appear as dark spots. The oligodeoxynucleotide was also internalized with time, but was found to accumulate within the nuclei of the cells (white arrows).

oligodeoxynucleotide could be seen in these same structures, however, in addition, a small percentage of nuclei also contained red fluorescence. By 120 min almost all of the cationic lipid could be seen in the very fine speckles within the cytoplasm, however, no nuclei stained green. In contrast, many more (~50%) of the nuclei were stained red with oligodeoxynucleotide at this time. This staining pattern did not change appreciably after an additional 2 h incubation, except that more nuclei (up to 80%) became positively stained with oligodeoxynucleotide. At this time nuclear staining was also much darker, indicating a greater accumulation of the oligodeoxynucleotide. At all times there were some larger punctate structures that appeared to contain both cationic lipid and oligodeoxynucleotide. These were possibly large aggregates of the oligodeoxynucleotide–lipid complex that adhered to the cell surface but were not internalized.

Double exposure photographs (Fig. 4A) showed that at both 120 and 240 min incubation a portion of the cationic lipid (green) and oligodeoxynucleotide (red) co-localized into cytoplasmic punctate structures (orange). However, a large percentage of the oligodeoxynucleotide (red) could be found in the nucleus. Higher magnification photographs (Fig. 4B) also showed the cationic lipid co-localized with a portion of the oligodeoxynucleotide in fine speckles and larger punctate cytoplasmic structures, while a large percentage of the oligonucleotide was found in the nucleus. At no time was the cationic lipid localized in the nucleus.

The final distribution of the cationic lipid appeared to be in very fine speckles scattered throughout the cytoplasm, which surrounded, but did not include, the nucleus of the cell. This pattern of staining would be anticipated if the cationic lipid remained in endosomal and lysosomal compartments. We have confirmed the identity of these structures by incubating A549 cells with TMA-DPH, a lipophilic fluorophore that has been used as a marker for endocytosis (45). At early times (3 min after addition to the cells) the staining pattern of TMA-DPH was consistent with a cell surface localization (Fig. 5) and was similar to the cationic lipid staining pattern at 30 min. As the incubation times were increased the TMA-DPH appeared to become internalized to a greater extent and the pattern of staining changed. Increasingly the dye stained fine perinuclear speckles reminiscent of the cationic lipid staining seen previously (Fig. 5). Unfortunately, the emission wavelengths of the light emitted by TMA-DPH and the BODIPY label of the cationic lipid were too close to allow for double staining with these compounds. However, the similarity of the staining patterns suggests that the final destination of the cationic lipid was within the endosomal system.

Time course of depletion of PKC- α mRNA

To determine the relationship between intracellular antisense oligodeoxynucleotide and the kinetics of target mRNA degradation, we examined PKC- α mRNA expression at different times after

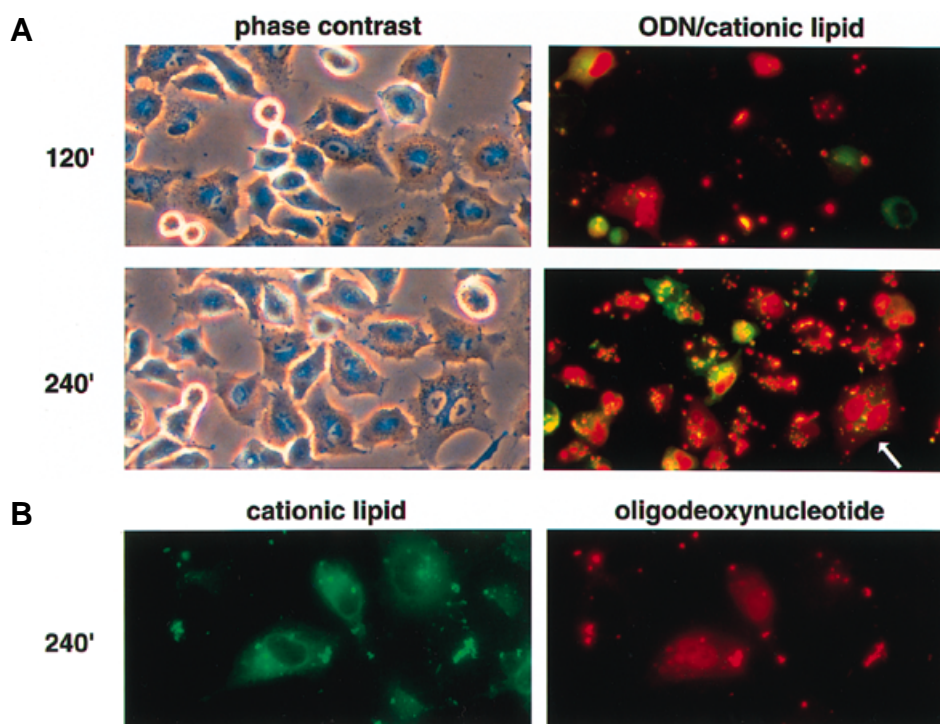


Figure 4. Localization of cationic lipid and oligodeoxynucleotide. (A) Double exposures of A549 cells treated with the lipid-oligodeoxynucleotide complex for 120 or 240 min at 37°C showed that there was some co-localization in punctate structures at these times (orange areas of the photographs). The arrow indicates a binucleate cell which shows both co-localization (orange) of cationic lipid and oligodeoxynucleotide (ODN) as well as cationic lipid staining (green) in punctate structures, while both of the nuclei have accumulated oligodeoxynucleotide (red). (B) Higher magnification photographs (through a 40× objective) of cells treated with the lipid-oligodeoxynucleotide complex for 4 h demonstrate the same distribution pattern.

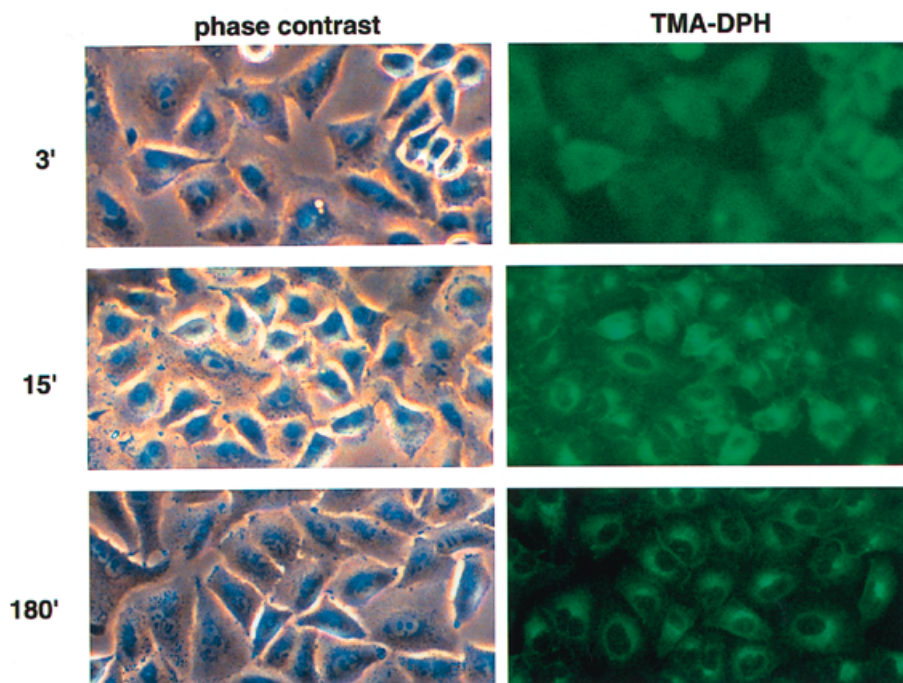


Figure 5. TMA-DPH staining of A549 cells. A549 cells were incubated with 12.5 µg/ml TMA-DPH at 37°C for 3, 15 or 180 min. The cells were then rinsed and examined live under a fluorescence microscope. The endocytic compartments stained by TMA-DPH appeared as very fine speckles.

addition of ISIS 3521R to the cells. A549 cells were treated with 20 $\mu\text{g}/\text{ml}$ DOPE/DOTAP (5% of which was the BODIPY-labeled derivative) and 400 nM ISIS 3521R for times from 0 to 240 min at 37°C. The cells were harvested and the RNA isolated as described in Materials and Methods.

The kinetics of reduction of both the large and small mRNA transcripts of PKC- α was found to mirror the kinetics of nuclear accumulation of ISIS 3521R. No reduction in PKC- α mRNA expression was seen 30 min after oligonucleotide addition to the cells (Fig. 6), just as at this time there was no detectable nuclear accumulation of oligodeoxynucleotide (Fig. 3). After 60 min, when only ~5% of the nuclei contained oligodeoxynucleotide, there was a slight but not significant, reduction in PKC- α mRNA levels. After 120 min treatment the levels of PKC- α mRNA were reduced by >30% (Fig. 6). This was coincident with the great increase seen in nuclear accumulation of oligodeoxynucleotide (Fig. 3). By 240 min the reduction in PKC- α mRNA was even greater, approaching 85%, just as more nuclear accumulation of oligodeoxynucleotide was evident at this time.

At early times (30–120 min after oligodeoxynucleotide addition) a lower molecular weight transcript (~2 kb) that could hybridize to the PKC- α probe was sometimes evident in the blots (Fig. 6A). Levels of the transcript were generally relatively low (5% of the full-length transcript), but reached their highest between 60 and 120 min. This 2 kb transcript was undetectable at 240 min. This band possibly represents an oligonucleotide-mediated degradation product of the PKC- α mRNA. ISIS 3521R hybridizes to the PKC- α transcripts at the termination codon, which is 2 kb from the 5'-end of either transcript. Therefore, the size of the transient transcript was consistent with the expected 5' degradation product of PKC- α mRNA if the transcript is acted on by RNase H. This degradation product would be expected to be further degraded very rapidly, as the transcript would no longer be 3'-polyadenylated and would thus be susceptible to cellular RNases.

The specificity of the antisense oligodeoxynucleotide-mediated reduction in PKC- α mRNA expression was demonstrated by a lack of effect of two control oligodeoxynucleotides [ISIS 4559, a scrambled sequence of ISIS 3521R (Fig. 3), and ISIS 2105R, an unrelated sequence with a 5'-rhodamine label (data not shown)]. In addition, we confirmed that the control oligodeoxynucleotide ISIS 2105R also accumulated in the nucleus (data not shown).

DISCUSSION

The activity of antisense oligonucleotides *in vitro* was previously shown to be greatly enhanced by compounds that form complexes with the oligonucleotide and enhance intracellular accumulation and modify intracellular distribution by facilitating release of the oligonucleotide from an endosomal compartment. These uptake enhancers include cationic lipids (19,46), liposomes (23,47), dendrimers (27,28), polycations (48,49), peptides (26,50), and aggregation or conjugation with a ligand (51,52) or antibodies (53,54) to cell surface receptors. Similar effects can be obtained by using electroporation to aid uptake of oligonucleotides into cells (35,36). The most frequently used uptake enhancers have been a mixture of neutral lipid, such as DOPE, and a cationic lipid, like DOTAP. The mechanism by which cationic lipids facilitate oligonucleotide escape from the endosomal system is still unknown. Szoka and colleagues have proposed a model in which the cationic lipids in oligonucleotide-lipid complexes inside the endosome interact with anionic lipids in the outer

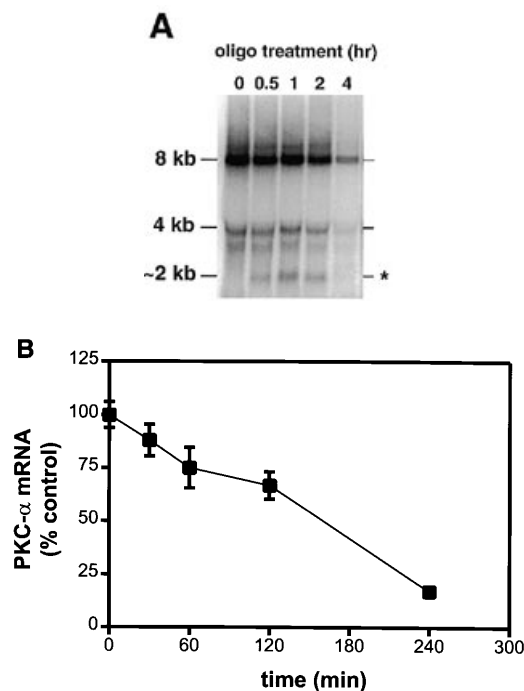


Figure 6. Time course of reduction of PKC- α mRNA. (A) A549 cells were treated with 20 $\mu\text{g}/\text{ml}$ DOPE/DOTAP (5% of which was labeled with a BODIPY group in one of the fatty acyl chains) and 400 nM ISIS 3521R for either 0, 30, 60, 120 or 240 min at 37°C. The cells were then harvested and the amount of PKC- α and G3PDH determined by northern blot. A 2 kb PKC- α related species (indicated by an *) appeared at 30, 60 and 120 min. (B) The level of PKC- α mRNA was quantitated on a phosphorimager and normalized to G3PDH mRNA level. The values expressed as a percent of the 0 min time point were plotted against time. The error bars represent the SEM.

leaflet of the endosomal membrane and flip-flop into the outer leaflet (39). It is assumed that during this flip-flop event the oligonucleotides escape from the endosome into the cytoplasm while the cationic lipid remains in the outer leaflet of the endosomal membrane. No previous studies have, however, demonstrated the final destination of the cationic lipid in the cell. A previous study used a fluorescently labeled DOPE derivative with unlabeled DOTAP to investigate oligonucleotide uptake (38). The investigators found that this neutral lipid (DOPE) remained in the endosomal structures while the oligonucleotide was delivered to the nucleus. We have confirmed these studies with fluorescently labeled DOPE and obtained the same results (data not shown). However, several studies have shown that the cationic lipid component of the lipid complex is important for enhancement of antisense oligonucleotide uptake and increased activity (24,37). Therefore, the relationship between the cationic lipid and the oligonucleotide subsequent to internalization is still not clear.

To address these issues we have used both fluorescently labeled cationic lipids and oligodeoxynucleotides. We found that the oligonucleotide-lipid complexes initially associate with the cell surface. With time the complexes internalize and appear to reside within very fine speckles within the perinuclear region of the cytoplasm. These structures were identified as part of the endosomal/lysosomal system by the fact that they are also stained by the endocytic marker TMA-DPH. At later times (>60 min) a small percentage of nuclei began to stain positively for oligodeoxynucleotide. The percentage of nuclei stained with fluorescent oligodeoxynucleotide increased over time, reaching a peak

(~80% stained) at 4 h. In contrast, the cationic lipid remained in the endosomal compartment and was never seen to traffic into the nucleus. These results clearly demonstrate that the oligodeoxynucleotide–cationic lipid complex disassociates, with the oligodeoxynucleotide entering the nucleus and DOTAP remaining in the endosomal system.

Previous studies have shown that fluorescently labeled oligonucleotides microinjected directly into the cytoplasm quickly redistributed into the nucleus (55,56). Therefore, it was known that the cationic lipids are not necessary for delivery of oligonucleotides into the nucleus once they have reached the cytosol. This is in contrast to DNA plasmids, which have a difficult time passing from the cytosol to the nucleus (57). This difference may in part be due to the size discrepancy of the two types of molecules. It is believed that the smaller oligonucleotides move passively through nuclear pores, while plasmids are too large to diffuse through nuclear pores. An additional difference between the oligonucleotide studies we describe here and plasmid DNA intracellular trafficking is the observation that cationic lipids remain associated with the plasmids in the cytosol and may in fact retard their accumulation in the nucleus (57).

The kinetics of the antisense action of oligonucleotides has not previously been studied in detail, as most investigators wait 12–24 h after oligonucleotide addition to cells before measuring inhibition of mRNA or protein expression (40,41). We have therefore investigated the kinetics of degradation of PKC- α mRNA targeted by ISIS 3521R and have found a relatively rapid oligonucleotide-mediated effect on gene expression. This degradation of mRNA correlated very well with the appearance of the antisense oligonucleotide in the nucleus. For example, after 60 min only a small percentage of the nuclei were labeled with oligodeoxynucleotide and at this time a slight, but not significant, reduction in PKC- α mRNA was seen. Only after 2 h, when ~50% of the nuclei contained oligonucleotide, did a significant reduction in PKC- α mRNA become apparent. After 4 h the decrease in PKC- α mRNA continued as more oligonucleotide accumulated in the nuclei. We also observed oligodeoxynucleotide-dependent accumulation of an additional PKC- α mRNA transcript that was ~2 kb in size. The appearance of this transcript was rapid, appearing by the 30 min time point, and transient, disappearing by the 240 min time point. The size of this transient mRNA transcript is consistent with it being a 5' cleavage product of the PKC- α mRNA transcripts and would seem to indicate that some cleavage of PKC- α mRNA is occurring as early as 30 min after oligodeoxynucleotide addition (Fig. 6A). This cleavage product, however, would not contain a poly(A)-modified 3'-terminus and would therefore be highly susceptible to degradation by 3'-exonucleases. The probe, which hybridizes to the coding region, would not be able to hybridize to the expected 3' cleavage product, so we were unable to detect this fragment.

Phosphorothioate oligodeoxynucleotides are believed to induce degradation of mRNA by annealing to their targeted message and thereby acting as a substrate for RNase H. RNase H is an enzyme whose activity is found in both the cytoplasm and nucleus and that degrades RNA in a RNA–DNA duplex (58). The correlation between the kinetics of oligonucleotide appearance in the nucleus and degradation of PKC- α mRNA further supports the hypothesis that antisense oligodeoxynucleotides exert their effects by activating RNase H in the nucleus.

In conclusion, the studies reported here describe for the first time the relationship between an antisense oligonucleotide and a

cationic lipid used to enhance oligonucleotide activity. The oligodeoxynucleotide–lipid complex was found to disassociate after entry into the cell, with the lipid remaining in an endosomal compartment whilst the oligodeoxynucleotide traffics into the nucleus. The appearance of the oligodeoxynucleotide in the nucleus is coincident with the decrease in expression of target mRNA, suggesting that the nucleus is the major site of action of the oligodeoxynucleotide.

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