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Intracellular availability of unmodified, phosphorothioated and liposomally encapsulated oligodeoxynucleotides for antisense activity

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ABSTRACT

We have studied factors which may effect the intracellular availability of oligonucleotides to achieve antisense activity. 15–20 mer unmodified, phosphorothioate modified and liposomally encapsulated oligodeoxynucleotides have been tested in leukemia MOLT-3 cells. Phosphorothioate analogs penetrated and accumulated intact in cells in contrast to unmodified oligomers, which showed a high instability in cell culture medium. A slow decrease of intracellular concentration of undegraded phosphorothioate oligodeoxynucleotides was observed after cell treatment and could be predominantly explained by a significant efflux transport. Using laser-assisted confocal microscopy we have observed that fluorescein 5-end-labeled phosphorothioate derivatives predominantly distributed in intracytoplasmic endocytic vesicles following cell treatment. The end-capped version of phosphorothioate oligodeoxynucleotides exhibited greater cellular uptake than fully modified analogues while exhibiting similar biological stability. Liposome encapsulation made possible oligomer protection in serum-containing medium and substantially improved cellular accumulation. Furthermore, the efflux rate of oligomer initially introduced within liposomes is 2-fold lower than that observed in cells which have been incubated with free oligonucleotides. Liposomal preparations of oligodeoxynucleotides facilitate release from endocytic vesicles, and thus, cytoplasmic and nuclear localization are observed following cell treatment. Furthermore, intracellular distribution studies demonstrate that intracellular transport of unmodified oligomers is effectively achieved using the liposomal carrier.

INTRODUCTION

The use of antisense oligonucleotides as a therapeutic tool in modulating gene expression represents a newly established strategy for treating diseases (1). Such oligomers may be designed to complement a region of a particular gene or messenger RNA. Using this approach, oligonucleotides can serve as potential blockers of transcription or translation through sequence-specific hybridization with targeted genetic segments. Antisense inhibition of gene expression using oligodeoxynucleotides (oligo(dn)) has been demonstrated in a variety of *in vitro* models (1,2,3). However, there are two major drawbacks in using oligo(dn): 1) their large molecular weights result in a low and slow cellular uptake and 2) their high sensitivity to nucleases makes them very unstable in a biological environment. Investigations have been focused on designing more stable oligodeoxynucleotides derivatives such as methylphosphonates (4), phosphorothioates (5), phosphotriester (6), or α-oligo(dn) (7). Interestingly, chemical conjugation of oligo(dn) with molecules such as DNA intercalators, poly (L-lysine), peptide, fatty acid or cholesterol have been studied and proven to increase the stability and the cellular penetration of these compounds (1,2,3). Although all of these analogues exhibit interesting properties, literature on the subject shows that oligo(dn) phosphorothioates are being used predominantly. Their resistance to eukaryotic endo- and exonucleases makes possible the significant inhibition of the expression of genes such as oncogenes or antiviral genes (8,9).

We have developed a liposomal delivery system for antisense oligo(dn) (10,11). Encapsulation of highly concentrated oligo(dn) in lipid vesicles formed by the minimum volume entrapment (MVE) method has been shown to protect these oligomers from nuclease attack (11). Cellular penetration is greatly facilitated when oligo(dn) are introduced in MVE liposomes. The transport of functional oligo(dn) has been observed in various cell lines using liposomally encapsulated oligo(dn) (lip-oligo(dn)) (11). We have recently demonstrated the *in vitro* inhibition of the multidrug resistance (mdr-1) gene expression in human cancer cells with 5μM liposomally encapsulated phosphorothioates oligo(dn) (Thierry et al., submitted).

Other studies have focused on determining conditions conducive to better antisense activity of oligo(dn). The stability and specificity of these compounds are among the most researched experimental considerations. Drug therapy relies principally on the drug ability to access the biological target. This theoretical concern is critical in the successful application of antisense technology. However, this point is poorly documented; we therefore addressed the intracellular availability of different

oligo(dn) in this study. We have explored various features of '*in vitro*' cellular pharmacokinetics of unmodified and phosphorothioate oligo(dn) in the leukemia MOLT-3 cells, and have also described the potential of liposome encapsulation. Using fluorescein isothiocyanate (FITC) or ^{32}P-end-labeled oligo(dn), we assessed the cellular uptake, intracellular stability, efflux rate and intracellular localization for each type of oligo(dn) presentation.

MATERIALS AND METHODS

Oligodeoxynucleotides synthesis

Oligodeoxynucleotides were synthesized using cyanoethyl phosphoramidite chemistry on an Applied Biosystems model 381 A synthetizer (Applied Biosystems, Forter City, CA). Three types of oligo(dn) were used in this study: 15-mer unmodified oligo(dn) (PD); 20-mer oligo(dn) phosphorothioate analogue (PS) and a 15-mer oligo(dn) end-capped phosphorothioate (cap-PS). The latter partially modified oligo(d15) exhibited two phosphorothioate modifications at both ends on their nucleotides chain. Phosphorothioate linkages were prepared by using tetraethylthiuram disulfide in acetonitrile. Coupling, oxidation and/or sulfurization were performed according to manufacturers recommendations. After cleavage from the column and deblocking in ammonium hydroxide (55°C, 10−15 hrs) unmodified and modified oligo(dn) were purified on neusorb prep columns (NEN, Radiochemicals, Inc.).

Synthesis of FITC-labeled oligonucleotides was performed using the aminolink system (Applied Biosystems) on the 5′ end. After cleavage from the column and deprotection, FITC labeled oligo(dn) were incubated overnight in the dark with FITC in sodium bicarbonate buffer. Unincorporated FITC was removed by gel filtration on a sephadex G50 column and eluted with 20% ethanol. Samples were ethanol precipitated and resuspended in sterile water.

Oligo(dn) were 5′ end labeled using γ-^{32}P-ATP and T4 polynucleotide kinase by the exchange reaction method (specific activity = $50-250\times10^9$ cpm/μmole).

Oligo(dn) encapsulation by minimal volume entrapment (MVE) method

MVE liposome-encapsulated oligo(dn) were prepared by thin lipid hydration in highly concentrated oligo(dn) solution followed by sonication. Cardiolipin, phosphatidylcholine and cholesterol were mixed at a 0.5:10:7 molar ratio in a round-bottomed flask. A thin lipid film was formed by rotary evaporation under vacuum. Then the dry lipid film was first hydrated by adding 4μl/mg lipid of a 10 mg/ml oligo(dn) solution. After overnight incubation at 4°C corresponding to an absorption step, 4μl/mg lipid of oligo(dn) dilution were added to the hydrated lipid film and the mixture was vigorously vortexed. Then 8μl/mg lipid of PBS were added and the mixture was again vigorously vortexed. Following a 2 hr swelling incubation period at room temperature, the resulting suspension was sonicated for 3 min using a cup horn (Heat Systems, SW 370, Plainview, N.Y.). Free oligo(dn) were removed by washing the liposomes by centrifugation (3 times, at 70,000g for 30min) in PBS. Determination of encapsulation efficiency was ascertained by radioactivity counting of an aliquot of the preparation in which traces of ^{32}P-end labeled oligo(dn) were added to the initial oligo(dn). The entrapment rate was found to be 50−60% of the initial input dose. The final concentration of oligo(dn) in liposomes was 60−70 μg oligo(dn)/mg lipid. This result could be reliably reproduced from sample to sample. Lip-oligo(dn) were kept at 4°C and used within 3 weeks of preparation.

Study of oligo(dn) stability in culture medium

^{32}P-end labeled PD, PS, cap-PS in free or liposomal form were incubated at a concentration of 1μM (100,000 cpm/ml) at 37°C in D-MEM culture medium (Gibco, Long Island), containing 10% heat-inactivated fetal calf serum (Gibco). Following incubation, aliquots were quickly frozen and then electrophoresed in a denaturing 20% polyacrylamide gel and then submitted to autoradiography (6). Electrophoretic bands corresponding to the undegraded oligo(dn) were analyzed by a GS 200 scanning densitometer using a GS 370 densitometry Software (Hoefer, San Francisco). Gel electrophoresis coupled with autoradiography allows for the unequivocal detection of undegraded ^{32}P-end labeled oligo(dn). However, due to high phosphatase activity present in serum, oligo(dn) labeled at 5-phosphate lose radiolabel, making analysis of oligo(dn) by PAGE may be nonrepresentative of the total oligo(dn) pool (13). In order to normalize the phosphatase activity in cell culture medium, the same batch of serum was used in this study. Oligo(dn) stability was expressed as percent of initial input.

Detection of intracellular oligo(dn)

Leukemia MOLT-3 cells were grown in RPMI 1640 (Gibco) culture medium supplemented with 15% fetal calf serum and 2 mM glutamine. Cells were maintained at 37°C in a water-saturated atmosphere containing 5% CO_2. 2μM of ^{32}P-end labeled unmodified or modified oligo(dn) in free or liposomal form were added to logarithmically growing MOLT-3 cells ($2.5-5\times10^6$cpm/10^6 cells/ml). Following incubation, cells were centrifuged and washed twice in PBS. The pellet was suspended and centrifuged in 0.2M glycine (pH 2.8) and then washed again in PBS. Using this treatment membrane-bound oligo(dn) was strip off, and the remaining radioactivity was interpreted to represent intracellular ligands. Then the cell pellet was lysed in 10 mM Tris-HCl, 200 mM NaCl, 1% SDS, 200 μg/ml proteinase K, pH 7.4 for 2 hrs at 37°C. Samples were then extracted with phenol as described previously (6). Aqueous fractions were lyophilized. An aliquot was analyzed by scintillation counting in order to assess cell-associated radioactivity. Cellular oligo(dn) uptake was expressed as pmol/10^6 cells. For stability studies: treated cells were rinsed three times in PBS by centrifugation and resuspended in fresh drug-free culture medium. Following an incubation period, cells were washed and oligo(dn) were extracted as previously described. Lyophilized samples were redissolved in a 90% glycerol solution containing 0.01% bromophenol blue, and then submitted to electrophoresis in a denaturing 20% polyacrylamide gel. Subsequent gels were dried, and then submitted to autoradiography using X OMAT Kodak film. Stability of oligo(dn) was determined densitometrically. Intracellular oligo(dn) content was expressed as percent of undegraded oligo(dn) as compared to control.

Laser-assisted confocal microscopy

Growing MOLT-3 cells were treated by 2μM FITC end-labeled unmodified and cap-PS oligo(dn) in free or liposomal form. After incubation, cells were rinsed twice in PBS by centrifugation. Subsequent pellets were resuspended in 90% glycerol, 20% 40

mM-Tris buffer, 1 mg/ml phenylenediamine. Cell suspensions were mounted on slides under coverslips for confocal microscopy. Samples were analyzed with an MRC-600 (Bio Rad) laser scanning confocal system equipped with a Nikon inverted microscope. The images shown were obtained using a 40×Nikon objective with a variable numerical aperture set to NA 1.1. Optical section of 1μm was obtained for each treatment.

RESULTS

Three oligodeoxynucleotides were tested. 15-mer phosphodiester (PD) and end-capped phosphorothioates (cap PS) oligo(dn) exhibit the same sequence, complementary to the 5′ end of the coding region of the *mdr-1* (multidrug resistance) gene, (5′AAGATCCATCCCGAC3′). 20-mer fully modified phosphorothioates oligo(dn) (PS) is complementary to the splice acceptor site at nucleotides 5349–5368 in the HIV genome (5′ACACCCAATTCTGAAAATGG3′) (12).

Stability in cell culture medium

The stability in culture medium of various forms of oligo(dn) at a concentration of 1μM is shown in figure 1. 'Natural' or unmodified oligo(d15) (PD) exhibited a high degradation rate in the experimental conditions as only 13% of undegraded PD remained after 1 hour of incubation. No detectable intact molecules were present after 4 hr. Following encapsulation in MVE liposomes, the same oligomers were completely protected by the lipid vesicles for more than 5 days (Fig. 1). PS and cap PS modified oligo(dn) are not fully stable after a long incubation showing a significant lysis at 48 hrs (21% and 40% oligomer remaining intact, respectively). No undegraded modified oligo(dn) could be detected after a 5 day incubation period. The liposomal preparation of PD oligo(dn) showed complete protection from degradation at 1 hr, 48 hrs and 5 days.

Cellular uptake

The kinetics of cellular uptake in MOLT-3 cells incubated with 2μM PS and cap PS in free or liposomal form are shown in figure 2. The level of uptake of PD was below detection under the experimental conditions. Incorporation of PS was found to be very slow and leveled off at 1.5 pmol/10^6 cells at approximately 18 hr. Cellular accumulation of cap PS appeared to be noticeably faster, exhibiting a maximum value (4 pmol/10^6 cells) at approximately 6 hr. (Fig. 2). In separate experiments, it has been demonstrated that cellular uptake of 15-mer and 20-mer capPS oligomers do not differ significantly (data not shown). In addition, no significant difference in cellular uptake has been found between 15 mer and 20 mer phosphorothioates in MCF-7 (14) and in MOLT-3 cell lines (data not shown). Consequently, we may compare cellular uptake of PS (20 mer) and capPS (15 mer). Internalization of PD, cap PS and PS encapsulated in liposomes are comparable. Oligo(dn) delivery reached a plateau around 2 hr at a concentration of 28 pmol/10^6 cells (Fig. 2). In terms of the percentage of the initial concentration of oligo(dn), approximately 0.3, 0.9 and 6.3% were incorporated in cells ($4-5\times10^6$ cells/assay) after PS, capPS and Lip-oligo(dn) exposure, respectively. Presentation in liposomes leads to an 18-fold and a 7-fold increase of cellular uptake as compared to PS and capPS, respectively. Using cell sorting analysis, we observed that FITC-end labeled cap PS had comparable cellular accumulation kinetics and the same free/liposomal cellular uptake ratio as ^{32}P-end labeled cap PS (data not shown).

Intracellular oligo(dn) content

In order to estimate the intracellular concentration of oligo(dn) in treated MOLT-3 cells, two different parameters have been taken in consideration: (1) the level of intracellular undegraded

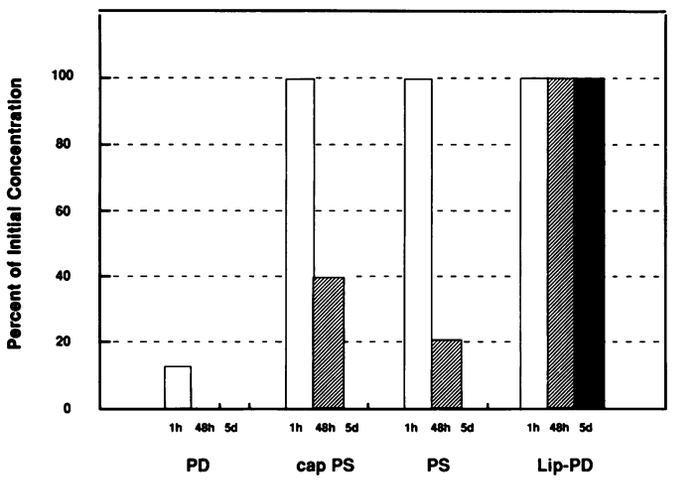

Figure 1. Stability of various form of oligodeoxynucleotides in cell culture medium. 1μM ^{32}P-end labeled oligomers were incubated in serum containing 10% heat-inactivated serum. Undegraded oligomers were detected following electrophoretic analysis and densitometry of subsequent autoradiogram (see 'materials and methods'). Results are the mean of two or more experiments. SE ± 8%.

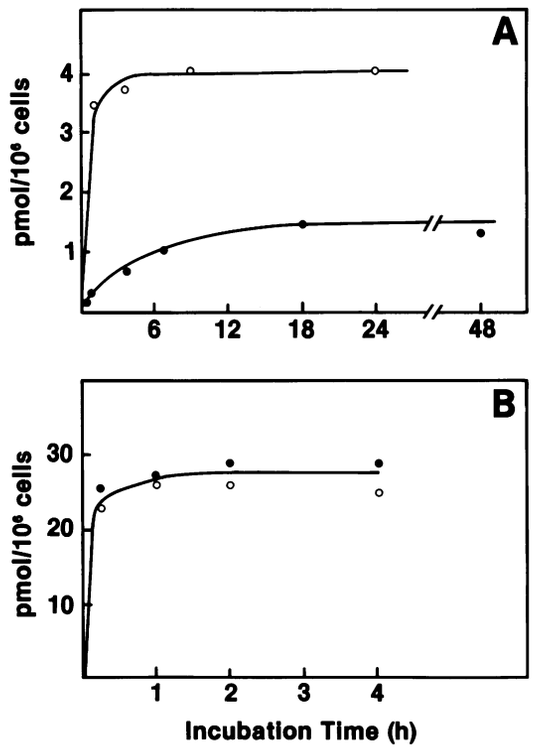

Figure 2. Cell uptake kinetics of oligo(dn) ^{32}P-end labeled by leukemia MOLT-3 cells. Cell were incubated with 2μM PS (●) or capPS (○) in free (A) or liposomal (B) form. Intracellular undegraded oligomers were detected as described in 'materials and methods'. Results are the mean of three or more experiments. SE ± 10%.

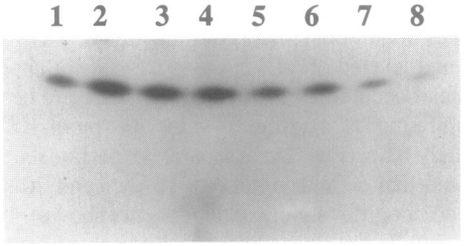

Figure 3. Intracellular content of PS (15-mer fully modified phosphorothioate oligo(dn)) in MOLT-3 cells during and after incubation of cells with 2μM liposomally encapsulated ^{32}P-end labeled PS. After treatment, cells were lysed, extracted with phenol, and extracts were analyzed by denaturing gel electrophoresis followed by autoradiography. Lane 1, control free PS, lane 2, 3 and 4, cells treated for 15 min, 1 hr and 4 hr with Lip-PS, respectively; lane 5, 6, 7, and 8, cells post-incubated in drug-free medium after 4 hr exposure to Lip-PS, for 1 hr, 4 hr, 24 hr and 48 hr, respectively.

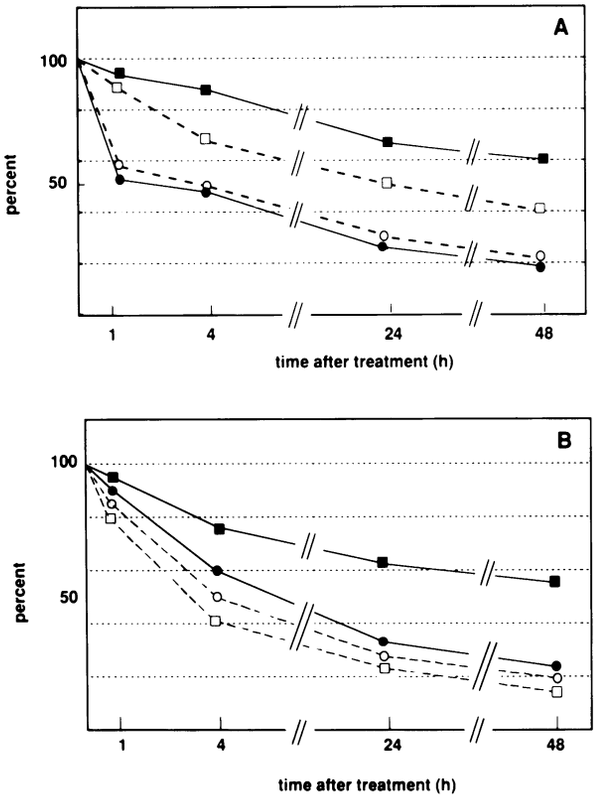

Figure 4. Reduction of intracellular oligo(dn) content in leukemia MOLT-3 cells following cell treatment. Cells were exposed to 2μM ^{32}P-end labeled PS (A) or cap PS (B) oligo(dn) in free (●,○) or liposomal (■, □) form. Intracellular undegraded oligomer content (full line) and cell associated radioactivity (dotted line) were estimated as described in 'materials and method' section. Results are expressed as percent of intact oligo(dn) initially incorporated after cell treatment. Results are the mean of two or more experiments. SE ± 10%.

oligo(dn) was determined in the cell extract following densitometric analysis of an autoradiogram made from an electrophoretic gel; and (2) cell associated radioactivity was assessed by scintillation counting in cell extract samples to be electrophoresed. Cell associated radioactivity mostly corresponded to either undegraded molecules or lysis fragments

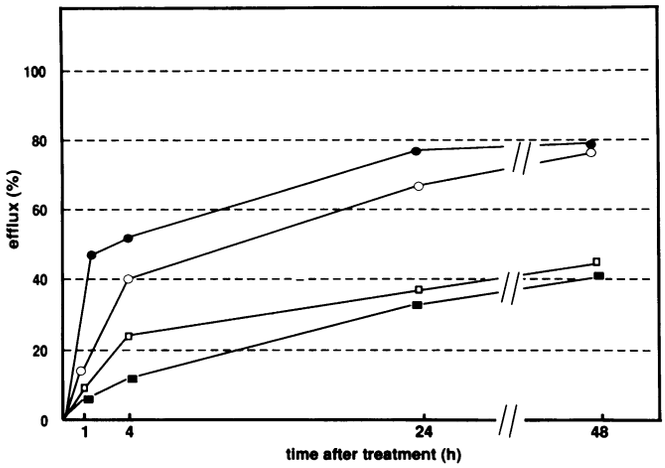

Figure 5. Relative efflux of oligo(dn) from treated MOLT-3 cells. Results are derived from cell associated radioactivity values presented in figure 4 and expressed as percent of oligomers initially incorporated in cells. Data are the mean of two or more experiments. SE ± 10%.

conserving the ^{32}P-end labeling, or to ^{32}P-incorporated cellular macromolecules. Fig. 3 illustrates the presence of intracellular undegraded PS delivered by the liposomal carrier to MOLT-3 cells during and following treatment.

Cells were exposed to 2μM for either 4 hr or 24 hr for liposomally encapsulated or free oligo(dn), respectively; and then were incubated in drug free-containing medium. Following free PS exposure, the undegraded PS cellular content quickly decreased leading to a loss of 50% within 4 hr (Fig. 4B). After a 4 hour incubation period, the reduction of the intracellular level of undegraded PS slowed resulting in approximately 20% of PS remaining intact in the cell after 48 hr. While cellular accumulation was much more important for encapsulated oligo(dn), the intracellular level of PS exhibited a comparable kinetic of decrease during post-treatment incubation (Fig. 4B). Interestingly, the cell associated radioactivity of PS treated cells diminished at a rate comparable to intracellular undegraded PS. In contrast, cell associated radioactivity corresponding to cells which have been exposed to Lip-PS, exhibited a smaller decrease during post-treatment incubation (75 and 55% at 4 and 48 hr, respectively).

After free cap PS exposure, cell associated radioactivity and undegraded cap PS concentration exhibited a comparable diminution during incubation (Fig. 4A). This pattern is characterized by a fast reduction within the first hour (53−55% remaining in cells) and then a slower phase over the next 48 hr. (21−23%). Following Lip-cap PS exposure, cell associated radioactivity decreases slowly for at least 48 hr. (60%, remaining). The undegraded cap PS concentration decreases at a higher rate (41%, remaining after 48 hr of post-treatment incubation). These data clearly demonstrate that the intracellular cap PS concentration was reduced more slowly when introduced into cells within liposomes.

In order to better analyze and compare the release of radioactive materials from MOLT-3 cells, fig. 5 presents the data of cell associated radioactivity from fig. 4 plotted in terms of cell efflux. It must be noted that little or no difference has been found between the reduction of cell associated radioactivity and undegraded oligo(dn) concentration after PS or cap PS treatment. The cell

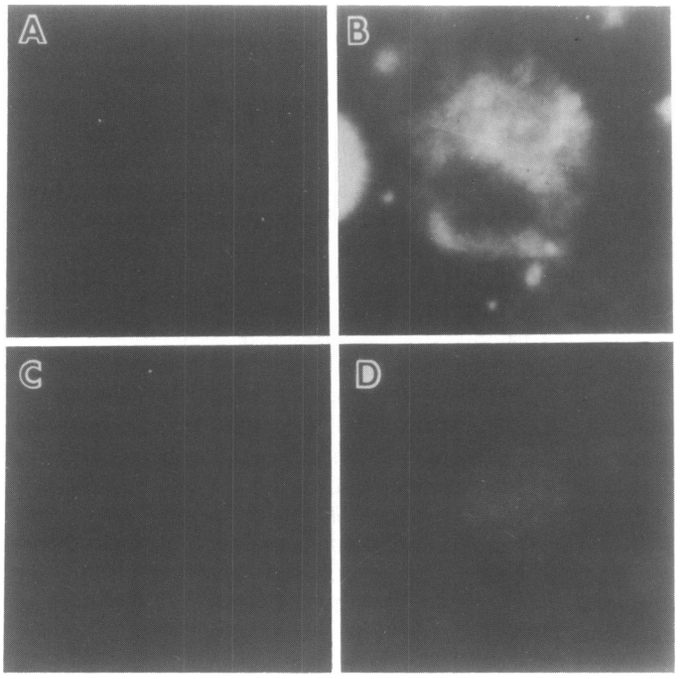

Figure 6. Intracellular localization of FITC-end labeled PD in leukemia MOLT-3 cells following PD (a,c) and Lip-PD (b,d) treatment. Cells were exposed to 2μM PD for 24 hr (a) and then post-incubated in drug-free medium for 24 hr (c). Cells were treated with 2μM Lip-PD for 4 hr (b) and then incubated in drug-free medium (d). Photographs represent computer-enhanced images from laser-assisted confocal microscopy. Magnification, ×320.

associated radioactivity could largely consist of intact ^{32}P-end labeled nucleotide chains. Thus, the efflux of free oligo(dn) observed in fig. 5 seems to correspond to the efflux of undegraded molecules. The efflux of radioactive materials was slightly faster in cap PS treated cells than in PS treated cells. However, the efflux appeared to end 48 hr after treatment in both cases (around 78% were extruded). Remarkably, the extrusion rate of radioactive materials from Lip-PS or lip-capPS treated cells appeared significantly slower, especially within 1 hr of post-treatment incubation. 40−45% of the radioactivity initially incorporated in cells was found to be extruded 48 hr after treatment. Using free oligo(dn) compared to Lip-oligo(dn) (Fig. 5), almost twice as much efflux was observed during this interval. In addition, the difference of efflux undegraded oligomers should be higher, considering that Lip-oligo(dn) treated cells showed a cell associated radioactivity value in which a significant part (25 to 50%) corresponded to degraded oligo(dn). It should be noted that there was a strong correlation between radioactivity counts of aliquots of culture medium where cells were incubated and cell associated radioactivity values (data not shown).

Intracellular localization

Intracellular localization was ascertained using FITC-end labeled oligo(dn) and laser-assisted confocal microscopy. The latter technique allows for the high resolution of optical sections of suspension cells preparations and can readily specify the intracellular distribution of a fluorescent compound. Fig. 6 presents images of cells treated with unmodified (PD) oligomers in free or liposomal form. Only a very weak cytoplasmic fluorescence has been observed in MOLT-3 cells treated for 24 hr with 2μM PD (Fig. 6A). No detectable fluorescence has been found in cells post-incubated for 24 hr. (Fig. 6C). In contrast, after a 4 hr. cell treatment with 2μM Lip-PD, unmodified oligomers were easily detectable and appeared to be distributed only in the cytoplasm in a nonhomogeneous pattern (Fig. 6B). After a 24 hr incubation, most of the fluorescence disappeared and only a weak intensity localized in the nucleus (Fig. 6D).

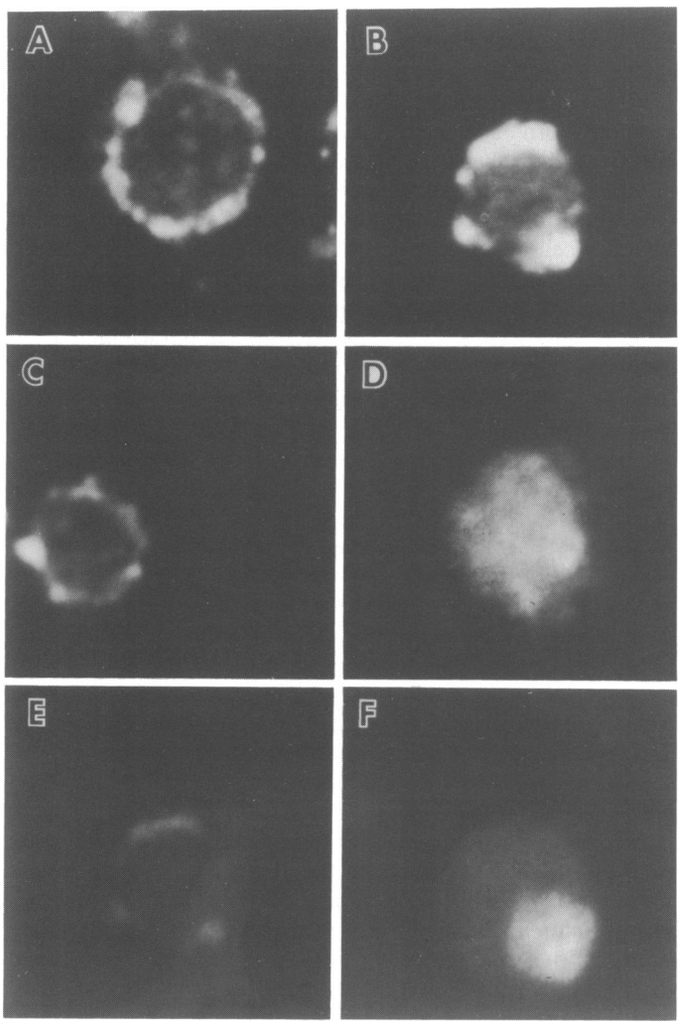

Figure 7. Intracellular distribution of FITC-end labeled cap PS in leukemia MOLT-3 cells following cap PS (a,c,e) and Lip-cap PS (b,d,f). Cells were exposed to 2μM cap PS for 24 hr (a) and then incubated in drug-free containing medium for 24 hr (c) or 48 hr (e). Cells were treated with 2μM Lip-cap PS for 4 hr (b) and then post-incubated in drug-free containing medium for 24 hr (d) or for 48 hr (f). Each photograph represents images from laser-assisted confocal microscopy. Magnification, ×320.

Images of cells treated with cap PS in free or liposomal form are presented in figure 7. Following a 24 hr treatment with 2μM free cap PS, fluorescence was mainly distributed in the cytoplasm, appearing granular or punctuate in nature (Fig. 7A). An apparent nuclear accumulation was observed, but it was estimated to be less than 10% of the total intracellular accumulation. 24 hr after capPS exposure, MOLT-3 cells showed the same pattern of distribution as previously described (Fig. 7C). Only a slight decrease in fluorescence intensity could be discerned. When cells were incubated for 48 hr. (Fig. 7E) or 72 hr (data not shown) the intracellular fluorescence intensity decreased but the

distribution pattern remained unchanged. Subcellular localization of PS incubated in the same conditions with MOLT-3 cells was equivalent to that observed using cap-PS (data not shown).

After a 4 hr treatment with 2μM Lip-capPS, we observed a similar pattern of fluorescence distribution in MOLT-3 cells as previously described using free cap PS (Fig. 7B). Only a higher fluorescence intensity distinguished Lip-cap PS treated cells from free cap PS treated cells. However, after a 24 hr after incubation MOLT-3 cells exhibited a completely different drug distribution. Fluorescence could be observed in the cytoplasm and predominantly in the nucleus, in a relatively homogenous and diffuse pattern (Fig. 7D). This shifting of intracellular drug distribution seems to be accentuated after 48 hr of incubation (Fig. 7F), with fluorescence being predominantly distributed in the nucleus.

DISCUSSION

As the first generation of antisense oligo(dn) compounds, natural phosphodiester (PD) sequences were studied for their therapeutic application. Significant inhibitory effects have been previously demonstrated in the 'pioneering' (6,15,16) and in recent studies (17−20). Much of this research underscores the low stability of PD in cell culture conditions. Some investigators (18−20) have used serum-free cell culture medium to circumvent this problem. To simulate the biological environment, we performed the stability studies in medium containing a 10% serum. As previously described, PD showed a high degradation pattern principally due to exonucleases present in serum (in the same experimental conditions but in the absence of serum, PD are stable for more than 4 days). A large variation in the degradation rate in culture medium of PD as well as modified oligo(dn) have been reported, suggesting the importance of the serum origin (6,21−23). No detectable cellular uptake has been observed, most probably, due to this high instability. It seems likely that a small fraction should enter the cells, but the extent of this incorporation may be below experimental detection. It must be noted that several investigators (6,16,24,25) have demonstrated cellular uptake but that a nearly complete intracellular degradation occurred within a few hours after incubation.

Increased antisense activity has been demonstrated using a second generation of oligo(dn) consisting of oligomers with internucleoside bridging phosphate modifications (such as phosphorothioates, or methylphosphonates). Phosphorothioates analogues have sulfur in place of an oxygen atom linked to the phosphorus atom of the nucleotide backbone (5). Due to this chemical modification, these oligomers are much less sensitive to nuclease attack (5,22,23,25). Our results reveal that PS exhibited a much higher stability than PD in the cell culture medium as previously described (8,13,22,23). Nevertheless, PS have proven to be sensitive to nucleases during long periods of incubation (>24 hr). The level of intracellular accumulation of PS in MOLT-3 cells was found to be in agreement with previous works (23,26). Determination of undegraded PS into MOLT-3 cells showed a quick reduction of the intracellular concentration of intact oligomers within 24 hr of incubation as observed in chick embryo cells using the same PS sequence (9). Our study demonstrates that this decrease corresponds to an efflux transport from the cells rather than an intracellular hydrolysis of the nucleotide chain. The same observation can be made for the phosphorothioates end-capped version (cap PS). Therefore, we suggest that an efficient efflux mechanism for oligo(dn) may be present in the cell. Accordingly, Crooke et al (23) noted that 80% of the radiolabel was lost from HeLa cells over a 4 hour-period while PS was stable within cells for up to 3 days. In general terms, as suggested by Akhtar et al (22), the major site of oligo(dn) degradation lies outside of the cells when PS are added exogenously to cells. In contrast, when microinjected in oocytes, PS appeared to be degraded with a half-life greater than 3 hr (25).

An end-capped version of phosphorothioate oligo(dn) exhibiting two modifications at both ends on their nucleotide chain has also been tested in this work. It has been demonstrated that in the reticulocyte lysate, an end-capped phosphorothioate oligo(d15) exhibited specific behavior close to that of PD in terms of DNAse resistance, activation of RNAse H and inhibition of translation (27). In this study, cap PS show a comparable DNAse resistance in culture medium when compared to PS. Interestingly end capped methylphosphonate preparations of the same sequence exhibited a similar stability pattern under the same conditions (data not shown). Our results showed a faster and a higher uptake of cap PS in MOLT-3 cells compared to PS. Similar observations were made using human ovarian cancer SKVLB cells (Thierry et al., submitted). Thus, cap oligo(dn), appear to be a good model since they exhibit an effective protection against nuclease attack while preserving an optimal capacity for antisense hybridization.

Study of intracellular distribution demonstrate that cap PS as PS (data not shown) was mainly localized to intracytoplasmic vesicles. As shown in previous studies (28) this localization pattern seems to confirm the involvement of an endocytic transport in oligo(dn) cellular internalization. However, the ultimate fate of this vesicular transport is not well understood. Our data indicate that this intracellular distribution pattern does

Table 1. Summary of the results

	Stability in cell culture medium	Cellular uptake	Intracellular concentration after cell treatment	Extravesicular localization after cell treatment
PD	+	ND	ND	ND
PS	++	+	++	+
capPS	++	++	++	+
Lip-capPS	+++	+++	+++	+++
Lip-PD	+++	+++	+	+++

Estimated relative capability has been expressed as low (+), medium (++), and high (+++). ND (not detectable). PD, phosphodiester oligo(dn); PS, phosphorothioates oligo(dn); capPS, end capped phosphorothioates oligo(dn); Lip-oligo(dn), liposome-encapsulated oligo(dn).

not change over a 2 day post-incubation period. The release of oligo(dn) from these vesicles seems to be minimal, but appears to be sufficient to lead to the observation of antisense activity as it has been shown in many works (2,3).

In this study we report on the potential of MVE liposomes to carry oligo(dn) into cells. MVE liposome preparations allow a high encapsulation efficiency of oligo(dn). The final concentration of oligomers per liposome content (60–70 μg oligo(dn)/mg lipid) appears to be 6-fold and 11-fold greater than that observed in the preparations of Leserman (29) and Juliano (30), respectively. Our data demonstrate that oligo(dn), even the highly degradable PD, are fully protected from environmental degradation. In previous investigations (10,11), we indicated that more than 90% of encapsulated oligo(dn) are inside the lipid vesicles. As recently demonstrated in various cell lines (11) liposomal delivery leads to an improved cellular uptake. We have previously shown using human lung carcinoma A549 cells, that Lip-oligo(dn) uptake is saturable and may be inhibited by increased concentration of empty liposomes (11). In addition, we have determined that the major route of cellular uptake to be endocytosis (10). As described before, for oligo (dn) uptake, liposome uptake is accompanied by an efflux mechanism, even though the efflux rate is lower. Furthermore, due to a higher cellular accumulation, Lip oligo(dn) exhibit a consistently higher intracellular concentration. It has been established that microinjected PD or PS do not distribute in intracytoplasmic vesicles and are rapidly transported to the nucleus (28,31). Observation of a nuclear localization following Lip-oligo(dn) treatment provided evidence that a significant release of oligo(dn) from endocytic vesicles in the cytoplasm took place after cell treatment. This leads to the suggestion that either the endocytic transport process is different for free oligo(dn) and Lip-oligo(dn), or that the liposome membrane may facilitate the release of liposome content from the endosomes into the cytosol.

Use of a liposomal carrier allows us to demonstrate the improved intracellular stability of cap PS compared to PD because a large part of the initially incorporated Lip-cap PS was transported through the cell. However, our results clearly show that effective delivery of unmodified oligo(dn) into cells is possible through the liposomal carrier. This confirms our previous investigation in which significant amounts of PD have been detected in human squamous carcinoma SQ 20B cells during Lip-PD exposure (11). Our study underscores the benefits of using liposomal packaging for biological stability, cell uptake and intracellular availability of oligo(dn). A summary of our observations is shown in Table 1. The '*in vitro*' antisense activity of oligo(dn) targeted to the multidrug resistance (mdr) gene was signigicantly enhanced using liposome encapsulation illustrating the usefulness of the liposomal delivery (11,32, Thierry et al., submitted).

In a clinical setting there will be a series of barriers in achieving a successful therapy employing antisense oligo(dn). Among the most important is that the antisense molecule must reach its cell targets at a satisfactory level. Although recent cytotoxicity studies in rodents look promising for the therapeutical use of methylphosphonates and phosphorothioates analogues (33–35), there are some apparent limitations regarding blood clearance, elimination and biodistribution. In particular, the plasma half-life of PS was found to be 10–20 min and this may restrict clinical use such as HIV therapy. Small unilamelar vesicles such as MVE liposomes have been shown to exhibit a plasma half-life ranging from 5 to 15 hr. Consequently, liposomal delivery may improve the bioavailability of antisense oligo(dn). Hence MVE liposomes seem to present a promising approach for the therapeutic utilization of this new class of compounds.

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