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Enhanced Biological Activity of Antisense Oligonucleotides Complexed with Glycosylated Poly-L-lysine

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SUMMARY

We sought to exploit glycosylated poly-L-lysine (pLK) to increase the uptake and biological antisense activity of a phosphorothioate oligonucleotide (pt-odn) [pt-odn complementary to the 3′ noncoding region of intercellular adhesion molecule-1 (ICAM-1) (odn_{ICAM-1})] complementary to the 3′-noncoding region of ICAM-1 in A549 cells. Dose-dependent inhibition of ICAM-1 expression was obtained (IC_{50} = 500 nM) through treatment of cells with odn_{ICAM-1} complexed with pLK carrying fucose residues in the presence of 100 μM chloroquine. Alteration in the charge ratio between fucosylated pLK and pt-odn had a significant effect on the efficacy of inhibition (optimal conditions, charge ratio = 1.1). This effect was also dependent on the number of fucose moieties per pLK. Free pt-odn or pt-odn complexed with nonglycosylated pLK gave no inhibition at concentrations of ≤2 μM. Two control pt-odn (one was targeted against an unrelated gene not present in these cells, gag_{HIV}, and the other had a randomized sequence) gave no inhibition of ICAM-1 expression in the presence or absence of pLK carrying fucose residues at concentrations of ≤2 μM. When complexed with pLK carrying 100 fucose residues, the amount of cell-associated pt-odn was increased by 15-fold compared with the free pt-odn. Nongycosylated pLK also increased the amount of cell-associated pt-odn by >10 fold but did not alter the biological activity. These results demonstrate clearly the potential of glycosylated pLK as a pt-odn transporter.

Antisense odn have been used extensively in cultured cells to inhibit gene expression in a sequence-specific manner (for a review, see Ref. 1). Their use, however, is severely limited by their poor cellular uptake because they do not cross lipid bilayers or cell membranes (2, 3). Cellular association of odn can be enhanced through coupling the odn to hydrophobic moieties such as cholesterol (4) or through association with cationic lipids (5) or liposomes (6). An alternative approach is to associate the odn with a macromolecular carrier bearing a ligand that is recognized by a cell surface receptor, resulting in very efficient cell uptake of the odn (7–10). Inhibition of gene expression in a sequence-specific manner has also been shown using odn associated with such carriers (11–14).

Cell surface sugar-binding receptors named membrane lectins are potentially good targets for drug delivery using glycosylated carriers. Membrane lectins with different sugar specificities are expressed at the cell surface of normal and tumor cells (for reviews, see Refs. 15 and 16). Several membrane lectins are well characterized and have been used as targets for the delivery of cytotoxic drugs (for a review, see Ref. 17), immunomodulators, and, most recently, genes and odn (for reviews, see Refs. 18 and 19). In our laboratory, we have chosen to use small sugar units as recognition signals instead of glycoproteins. The preparation of such glycosylated carriers is much simpler and allows access to a large panel of specificities. Neoglycoproteins [i.e., serum albumin substituted with ~20 sugar residues (20)] can be used to screen for the presence of membrane lectins and to study the capacity of the cell to take up their ligands. We have also used such neoglycoproteins to increase the uptake of odn in cells expressing membrane lectins (7), and we have shown that an increase in uptake may induce an increase in the biological effect of an odn (14).

In this report, we describe the use of pLK-Fuc as an efficient carrier for antisense pt-odn by forming electrostatic complexes

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ABBREVIATIONS: odn, oligonucleotide(s); pt-odn, phosphorothioate oligonucleotide(s); pLK, poly-L-lysine; F-pt-odn, fluoresceinylated phosphorothioate oligonucleotide(s); odn_{ICAM-1}, phosphorothioate oligonucleotide(s) complementary to the 3′ noncoding region of intercellular adhesion molecule-1; odn_{gag}, phosphorothioate oligonucleotide(s) complementary to the initiation codon of the human immunodeficiency virus *gag* gene; odn_{random}, phosphorothioate oligonucleotide(s) with a randomized sequence; CR, charge ratio; BSA, bovine serum albumin; PBS, phosphate-buffered saline; FBS, fetal bovine serum; ICAM-1, intercellular adhesion molecule-1; TNF-α, tumor necrosis factor-α; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

between the polyanionic pt-odn. After the complex is formed, serum can be added. This type of complex avoids the need for further chemical modification of pt-odn or glycosylated pLK.

To demonstrate the use of glycosylated pLK, a pt-odn targeted to the 3′ noncoding region of ICAM-1 mRNA (pt-odn$_{ICAM-1}$) was chosen. The ability of this pt-odn to inhibit TNF-α-induced expression of ICAM-1 in A549 lung carcinoma cells has been described previously (pt-odn 1939; Ref. 21). This model is appropriate for testing pt-odn carriers because it was shown by Chiang *et al.* (21) that this pt-odn was inactive unless used in combination with cationic lipids. To achieve pt-odn delivery, fucose was selected as one of the best recognition signals, and fucosylated pLK was used to form electrostatic complexes with the pt-odn. We observed 50% inhibition of ICAM-1 expression at ~0.5 μM pt-odn$_{ICAM-1}$ complexed with pLK-Fuc, whereas the two control pt-odn that were used had no effect. The pt-odn carried by glycosylated pLK was efficient when it was used in conjunction with chloroquine, as occurs with delivery of genes from plasmid/glycosylated pLK complexes (22).The same antisense pt-odn, free or complexed with nonglycosylated pLK, did not inhibit ICAM-1 expression at concentrations of ≤2 μM. Our results demonstrate the use of glycosylated pLK as a pt-odn transporter.

Materials and Methods

The pt-odn. The pt-odn$_{ICAM-1}$ (20 mer: CCCCCACCACTTCCCCTCTC; ISIS1939) (21) was synthesized by Eurogentec (Seraing, Belgium). The pt-odn$_{gag}$ (25 mer: CTCTCGCACCCATCTCTCTCCTTCT; GEM91) and the 25-mer random-sequence pt-odn$_{random}$ (all four bases in equal proportions at each position) were kindly provided by S. Agrawal (Hybridon, Worcester, MA). SdC28 was synthesized at the Pasteur Institute (Paris, France).

For uptake studies, pt-odn were fluoresceinylated on the phosphorothioate backbone using fluorescein-iodoacetamide (Molecular Probes, Leiden, The Netherlands) as described by Stewart *et al.* (23). Briefly, pt-odn in 0.1 M sodium phosphate, pH 8.0, were incubated with 5 equivalents of fluorescein-iodoacetamide for 18 hr at 50°. Excess fluorescein was removed through precipitation of the pt-odn in absolute ethanol before being passed over a Biogel P2 column (BioRad, Ivry-sur-Seine, France) equilibrated with 5% butanol in water. The peak of F-pt-odn eluted well in advance of the free fluorescein. After lyophilization, F-pt-odn was quantified by spectrophotometry and spectrofluorometry.

Iodination of F-pt-odn was achieved using Iodogen reagent (Pierce Chemical, Paris, France). Briefly, Iodogen-coated tubes were prepared by gentle evaporation of 400 μl of chloroform containing 500 μg/ml Iodogen. F-pt-odn in PBS (1× = 2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4) (400 μl of 0.5 mg/ml) was added to the Iodogen-coated tube followed by 5 μl of ^{125}I (Amersham, Paris, France; 581 MBq of ^{125}I/μg, 3.6 of GBq ^{125}I/ml). The reaction was stopped by transferring the reaction mixture to a tube containing 2 ml of absolute ethanol. The precipitated radiolabeled pt-odn was washed three times in ethanol. The pt-odn was then resuspended in 200 μl of PBS, and the incorporation of ^{125}I was measured using a γ counter (LKB, Paris, France). Specific activity was ~1.5 × 10^8 cpm/mol.

Cell culture. A549 human non-small cell lung carcinoma cells (CCL 185; American Type Culture Collection, Rockville, MD) were cultured in RPMI (Life Technologies, Renfrewshire, UK) containing 10% heat-inactivated FBS and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cells were grown at 37° in a humidified atmosphere containing 5% CO_2-95% air. Cells were harvested after treatment with PBS/trypsin/EDTA [PBS with 0.02% (w/v) EDTA and 2.5 μg/ml trypsin] at 37° for 5 min.

Endocytosis of neoglycoproteins. Fluoresceinylated neoglycoproteins, bearing ~23 sugar residues and 2.5 fluorescein molecules, were synthesized as described previously (20). A549 cells were incubated in normal growth medium in the presence of fluoresceinylated neoglycoproteins (50 μg/ml) for 4 hr at 37°. Cells were washed three times with PBS and harvested with PBS/trypsin/EDTA before being centrifuged (500 × *g* for 5 min) and resuspended in sheath fluid (134 mM NaCl, 3.75 mM KCl, 15.2 mM NaF, 1.9 mM KH_2PO_4, 16.5 mM $NaHPO_4$ buffer, pH 7.4, containing 2 mg/ml 2-phenoxyethanol). The cell fluorescence intensity was measured before and after a postincubation period, for 30 min at 4°, in the presence of 50 μM monensin (Calbiochem, La Jolla, CA). This ionophore neutralizes endocytic compartments, allowing the retrieval of the fluorescence quenched in an acidic environment (24), and provides information regarding the intracellular location of the endocytosed material (25). The cell fluorescence intensity was measured using an FACSort cytometer (Becton Dickinson, Le Pont de Claix, France). Data shown represent the mean values of 5000 cells obtained with the use of Cell Quest software (Becton Dickinson). The variations among experiments was ≤10%.

Glycosylated pLK. pLK hydrobromide (M_r, 95100; P-1274, Sigma Chemical, Poole, Dorset, UK) with a mean degree of polymerization of 455 was used for this study. After exchange of the bromide for a *p*-toluene-sulfonate counter-ion, the pLK was substituted with glycosidic moieties using 4-isothiocyanatophenyl-α-L-fucopyranoside in dimethylsulfoxide (26). The glycosylated pLK was purified through precipitation in isopropanol. The mean number of sugar residues bound per pLK molecule was determined using the resorcinol sulfuric acid micromethod (27).

pt-odn/pLK complex formation. pt-odn/pLK or pt-odn/pLK-Fuc electrostatic complexes were formed in serum-free RPMI by the addition of one volume of pt-odn (2× concentrated), with constant vortexing, to one volume of glycosylated or nonglycosylated pLK (2× concentrated). Complexes were allowed to form for 30 min before the addition of FBS (10% final concentration) and, where appropriate, chloroquine (100 μM final concentration). Complexes, in the presence of 10% serum, were then added to cell culture wells.

CRs for a given complex were defined as the molar ratio of positive charges (derived from the mean number of free lysines present on the pLK) to negative charges (derived from the number of negatively charged internucleotide bonds present in the pt-odn), with the assumption that all free ϵ-amino groups are protonated (NH_3^+) and that all phosphorothioate linkages are nonprotonated (—O—POS$^-$—O—). It is acknowledged that this is a theoretical measure because the exact number of charges per molecule is difficult to establish due to the heterogeneous nature of the pLK and due to the equilibria that exist between the different states of protonation. However, the CR is a useful approximation for study of the optimization of complex formation. pt-odn/pLK and pt-odn/pLK-Fuc complexes containing a CR of 1.1 and a final pt-odn concentration of 1 μM in 1 ml were formed by mixing 1 nmol of pt-odn (6.1 μg; an eicosamer containing 19 phosphorothioate linkages) with 0.042 nmol of pLK$_{455}$ (i.e, where 455 is the degree of polymerization) (5.8 μg; containing an estimated mean of 455 positive charges) or 0.054 mol of pLK$_{455}$Fuc$_{100}$ (8.2 μg; containing an estimated mean of 355 positive charges), respectively. Quantities were adjusted for the various concentrations and different CRs that were used.

Inhibition of ICAM-1 expression by pt-odn and pt-odn/pLK-Fuc complexes. The efficacy of antisense pt-odn carried by glycosylated pLK was assessed using the model previously described by Chiang *et al.* (21). A549 cells were plated onto 96-well microtiter plates (10^4 cells/well) and allowed to attach overnight. pt-odn/pLK or pt-odn/pLK-Fuc complexes were then formed as described above and added to the cells in the presence or absence of 100 μM chloroquine. It has been determined with the use of the MTT colorimetric assay (28) that the viability of A549 cells was not affected by chloroquine at concentrations of ≤100 μM for 4 hr. In control experiments, Lipofectin (5 μg/ml; Life Technologies) treatments were made using serum-

free OPTI-MEM (Life Technologies) at various pt-odn concentrations as described previously (21). Treatments lasted 4 hr, after which the medium was changed to a pt-odn-free complete medium containing 10 ng/ml TNF-α to induce the overexpression of ICAM-1 and 10% FBS. Control cells maintained in the absence of TNF-α enabled the measurement of basal ICAM-1 expression. After 18 hr, the cells were washed three times with PBS and fixed for 20 min at room temperature in paraformaldehyde (20 mg/ml in PBS). Cells were then washed three times with 200 μl of PBS and incubated in 100 μl of PBS containing 20 mg/ml BSA for 1 hr at 37°. An anti-ICAM-1 primary antibody (Becton Dickinson) diluted 1:20 in PBS containing 20 mg/ml BSA (100 μl) was incubated with the cells for 90 min at 37° before being washed three times with 200 μl of PBS. An anti-mouse horseradish peroxidase conjugate (diluted 1:2000 in 100 μl of PBS containing 20 mg/ml BSA; Becton Dickinson) was then added for 1 hr at 37°. After three washes with 200 μl of PBS, horseradish peroxidase activity was assessed using 100 μl of *o*-phenylenediamine dihydrochloride peroxidase substrate tablet set; Sigma P-9187), and the reaction was stopped after 15 min by the addition of 25 μl of 3 N H_2SO_4. The orange color that developed was read at 492 nm. All calculations were made relative to untreated controls (in the presence of chloroquine) in the presence or absence of TNF-α. The percentage of TNF-α-induced expression of ICAM-1 was calculated as follows:

$$[(A_{TNF\text{-}\alpha}{}^{pt\text{-}odn} - A_0)/(A_{TNF\text{-}\alpha} - A_0)] \times 100$$

where $A_{TNF\text{-}\alpha}{}^{pt\text{-}odn}$ is the ICAM-1 expression for pt-odn-treated, cytokine-induced cells, A_0 is the basal ICAM-1 expression, and $A_{TNF\text{-}\alpha}$ is the cytokine-induced ICAM-1 expression.

Where indicated, duplicate plates containing cells treated with pt-odn, pt-odn/pLK, or pt-odn/pLK-Fuc in the same way were examined for cell viability using the MTT colorimetric assay (28).

Cellular uptake and efflux of radiolabeled pt-odn. To study the influence of glycosylated pLK on the uptake of pt-odn, A549 cells in 24-well plates (2×10^5 cells/well) were incubated at 37° for the periods indicated in 0.5 ml of RPMI containing 10% FBS and 0.15 μM ^{125}I-labeled pt-odn, free or complexed with pLK or pLK-Fuc (and 100 μM chloroquine where appropriate). Cells were washed with PBS containing 10 mg/ml BSA, followed by a wash with 200 μl of PBS containing 1 μM SdC28 (a phosphorothioate homopolymer of cytidine that has been shown to remove pt-odn from the cell surface; Ref. 2) and then by an additional wash with PBS containing 10 mg/ml BSA. Cells were harvested by treatment with PBS/trypsin/EDTA and washed with PBS before lysis in 0.1 N NaOH. Radioactivity was measured using a γ counter (LKB), and protein content was measured according to the method of Lowry (29), which is compatible with the cell lysis in 0.1 N NaOH.

For efflux studies, cells were incubated at 37° for 4 hr with the radiolabeled pt-odn as described above and then washed twice with fresh medium. Cells were further incubated for the indicated periods in normal culture medium, in the absence of radiolabeled pt-odn, before harvest and measurement of radioactivity.

Cellular uptake of F-pt-odn: confocal microscopy analysis. For visualization of F-pt-odn uptake, A549 cells were seeded onto sterile coverslips in 20-mm wells (2×10^5/well) and allowed to adhere. Cells were incubated for 4 hr at 4° or 37° in 0.25 ml of RPMI containing 10% FBS and 0.15 μM F-pt-odn, free or complexed with pLK or pLK-Fuc. Cells were then washed twice with PBS (once with PBS containing 1 μM SdC28 and again with PBS) before being fixed for 30 min at ambient temperature in PBS containing 20 mg/ml paraformaldehyde. Cells were further washed before being mounted on slides in a PBS/glycerol mixture (1:1 v/v) containing 1% 1,4-diazodicyclo-(2,2,2)-octane to minimize photobleaching (30). Analysis was carried out using a BioRad MRC 600 laser-assisted confocal microscope equipped with an argon/krypton laser with optical sections of 1 μm.

Results

Endocytosis of neoglycoproteins by A549 cells. As a first step toward using glycosylated polylysine to deliver pt-odn into A549 cells, the presence of membrane lectins acting as glycoconjugate receptors, mediating endocytosis of their ligands, was examined using fluoresceinylated neoglycoproteins.

A549 cells were incubated for 4 hr in culture medium containing a 50 μg/ml concentration of a panel of fluoresceinylated neoglycoproteins (Fig. 1). Through examination of the cell fluorescence intensity before and after post-treatment with monensin, it was apparent that neoglycoproteins bearing fucose, mannose-6-phosphate, or mannose residues were efficiently endocytosed. The increase in the cell fluorescence intensity on post-treatment with monensin, a sodium/proton ionophore that neutralizes acidic vesicles (24), indicated that these neoglycoproteins were within acidic intracellular compartments. This is a good indicator of receptor-mediated endocytosis that directs vesicular contents to acidic endosomes. Other neoglycoproteins, such as lactosylated, β-glucosylated, and β-*N*-acetylglucosaminylated BSA, were internalized with a lower efficiency. The polymer pLK-M6P has poor solubility, making it unsuitable as a pt-odn carrier. Both pLK-Man and pLK-Fuc were prepared and used as pt-odn carriers of antisense pt-odn. However, to simplify the initial characterization of glycosylated pLK, it was decided to focus on one sole ligand, fucose, because preliminary experiments showed little difference between pLK-Man and pLK-Fuc.

Inhibition of ICAM-1 expression by pt-odn/pLK-Fuc complexes. As mentioned in detail, only a limited number of studies have demonstrated efficient inhibition of gene

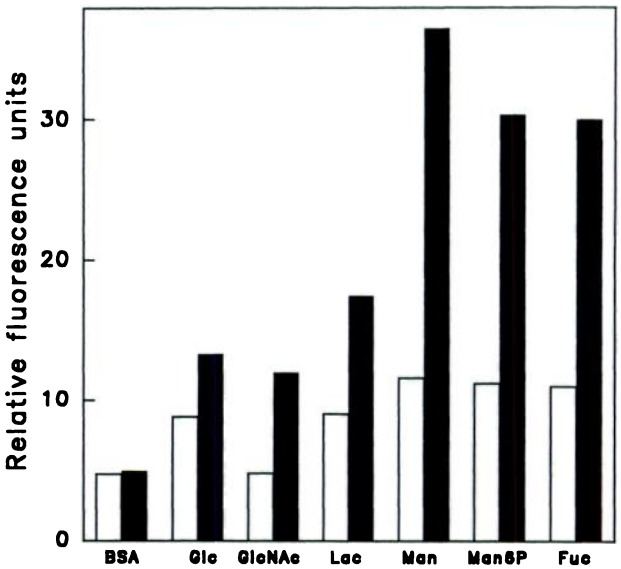

Fig. 1. Uptake of fluoresceinylated neoglycoproteins by A549 cells. Cells (2×10^5 cells/ml) were incubated at 37° for 4 hr in 0.5 ml of RPMI medium/10% FBS containing 50 μg/ml fluoresceinylated neoglycoproteins. Cell fluorescence intensities were measured by flow cytometry before (*open bars*) and after (*filled bars*) a postincubation at 4° for 30 min with 50 μM monensin, as detailed in Materials and Methods. Neoglycoproteins contained ~2.5 molecules of fluorescein and 23 residues of β-glucose (*Glc*), β-*N*-acetyl glucosamine (*GlcNAc*), lactose (*Lac*), mannose (*Man*), mannose-6-phosphate (*Man6P*), or L-fucose (*Fuc*).

expression using odn/pLK complexes. The ability of pt-odn$_{ICAM-1}$/pLK-Fuc complexes to inhibit ICAM-1 expression was therefore examined in A549 cells. Typically, in unstimulated A549, A_0 was ~0.3, and in TNF-α-stimulated cells, $A_{TNF-\alpha}$ was ~1.0; the level of control was ~0.2 for both the unstimulated cells and the TNF-α-stimulated cells but omitting the primary antibody.

In the first instance, it was decided to optimize complex formation between pt-odn$_{ICAM-1}$ and pLK-Fuc by varying the CR and directly measuring their ability to inhibit overexpression of the ICAM-1 gene. In the presence of 100 μM chloroquine, using a constant pt-odn concentration (1 μM) and an increasing concentration of pLK-Fuc, an optimal ratio was reached in which ICAM-1 expression was strongly inhibited (Fig. 2A). Inhibition was found to be maximal (~100% inhibition) when there was a small excess of positive charges (CR = 1.1). Inhibition of gene expression was significantly less when complexes were either globally negative (CR = ≤1.0) or more positive (CR = ≥1.5). The odn$_{random}$ had no effect on gene expression in the presence of nonglycosylated pLK or pLK-Fuc. It is important to note that pt-odn$_{ICAM-1}$ delivered with a nonglycosylated pLK had no significant effect on ICAM-1 gene expression at any CR tested, thus demonstrating that the efficiency was dependent on the pt-odn sequence and on the use of a pLK bearing the appropriate sugar recognition signal. In addition, there was an absolute requirement for chloroquine during the pt-odn treatment period because no inhibition was observed in its absence (data not shown).

It has been observed previously that free polycationic pLK can be toxic toward certain cells (31). However, by using pt-odn/pLK electrostatic complexes in which only a small residual positive charge remained (CR = 1.0–2.0), only minor cytotoxic effects were observed (Fig. 2B). The small decrease in cell viability (15% under optimal conditions) did not seem to depend on the type of complex used but was principally due to the presence of chloroquine because incubations in the absence of chloroquine showed no toxicity at all (data not shown). All further studies were carried out using the CR of 1.1, which was optimal with regard to the activity of pt-odn$_{ICAM-1}$/pLK-Fuc complexes.

Subsequent to the demonstration that nonglycosylated pLK was inactive, it was observed that the inhibition of ICAM-1 expression was highly dependent on the number of fucose residues present on the pLK (Fig. 3). There was little

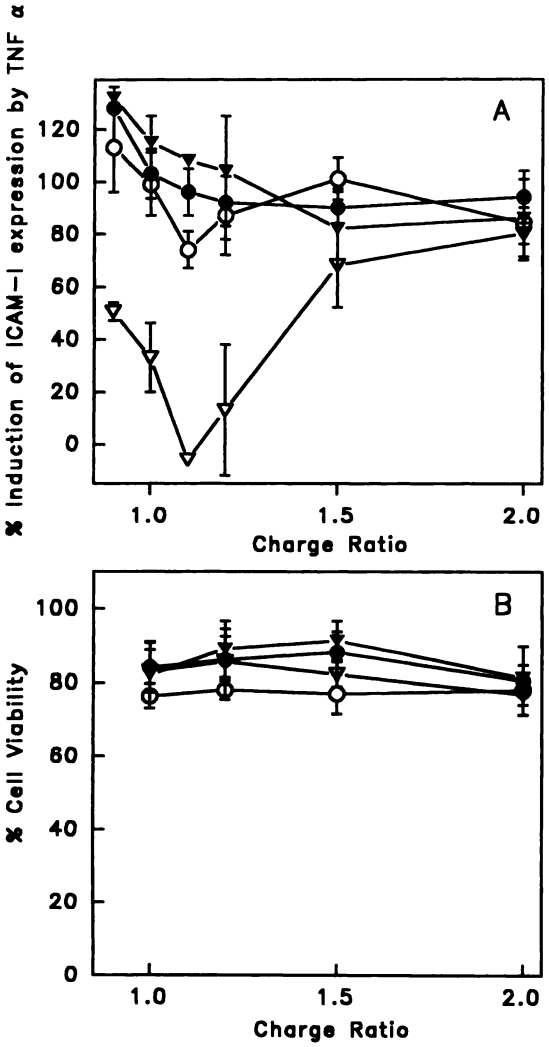

Fig. 2. The effect of pt-odn/pLK or pt-odn/pLK-Fuc$_{100}$ complexes containing different CRs on (A) TNF-α-induced ICAM-1 expression and (B) cell viability of A549 cells. Cells were seeded onto 96-well plates (10^4 cells/well) 24 hr before treatment for 4 hr at 37° in the presence of 100 μM chloroquine with (○) pt-odn$_{ICAM-1}$/pLK, (●) random pt-odn/pLK, (▽) pt-odn$_{ICAM-1}$/pLK-Fuc$_{100}$, and (▼) random pt-odn/pLK-Fuc$_{100}$ complexes containing the indicated CR; in each case, the pt-odn concentration was 1 μM. Medium was then replaced with normal growth medium containing 5 ng/ml TNF-α to stimulate ICAM-1 expression. After 18 hr, (A) ICAM-1 expression was measured by enzyme-linked immunosorbent assay, and (B) cell viability was measured by MTT assay as detailed in Materials and Methods. The results shown are typical of experiments carried out in triplicate and repeated at least twice. Data are expressed as the mean ± standard deviation of the percentage of control ICAM-1 expression induced by TNF-α.

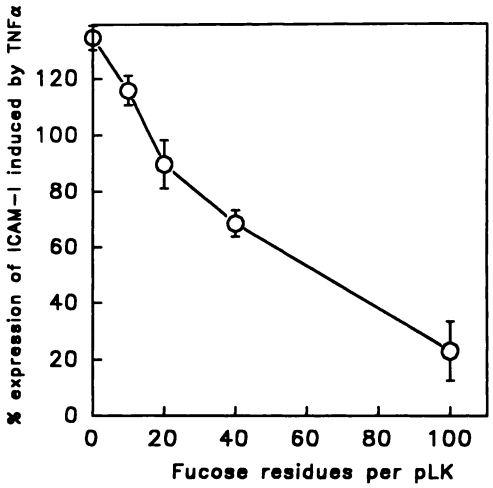

Fig. 3. The effect of pt-odn/pLK-Fuc complexes containing different levels of fucose substitution on the TNF-α-induced ICAM-1 expression in A549 cells. Cells were seeded onto 96-well plates (10^4 cells/well) 24 hr before treatment for 4 hr at 37° in the presence of 100 μM chloroquine with pt-odn$_{ICAM-1}$/pLK-Fuc (○) containing the indicated number of fucose residues per pLK molecule. Medium was then replaced with normal growth medium containing 5 ng/ml TNF-α to stimulate ICAM-1 expression. After 18 hr, ICAM-1 expression was measured by enzyme-linked immunosorbent assay as detailed in Materials and Methods. The results shown are typical of experiments carried out in triplicate and repeated at least twice. Data are expressed as the mean ± standard deviation of the percentage of control ICAM-1 expression induced by TNF-α.

inhibition using 1 μM pt-odn$_{ICAM-1}$ complexed with pLK carrying 10 or 20 residues, whereas pLK-Fuc$_{40}$ (where 40 is the number of fucose residues being carried by pLK) gave ~30% inhibition. Approximately 80% inhibition was observed using a pLK carrying 100 fucose residues. This result emphasizes the importance of the degree of sugar substitution on biological activity of pt-odn/pLK-Fuc in A549 cells. All further experiments were conducted with pLK-Fuc$_{100}$, which corresponds to a substitution close to 22% of the ε-amino groups of the pLK$_{455}$.

The inhibition of ICAM-1 expression, using pt-odn$_{ICAM-1}$ complexed with pLK-Fuc$_{100}$, was dose dependent (Fig. 4A). Inhibition was noticeable at 0.25 μM, and 50% inhibition was observed at 0.5 μM. At concentrations of >0.75 μM, ICAM-1 gene expression was inhibited by ~75-80%. This inhibition was sequence specific because two control pt-odn, pt-odn$_{random}$ and pt-odn$_{gag}$, had no significant effects over the same concentration range. The inhibition of ICAM-1 expression was again dependent on glycosylation of the pLK carrier because pt-odn$_{ICAM-1}$ complexed with the nonglycosylated pLK had no effect on ICAM-1 expression, even at concentrations of 1.5 μM (Fig. 4B). Similar experiments (data not shown) performed in serum-free medium with pt-odn$_{ICAM-1}$ in the presence of cationic lipids (Lipofectin) showed an IC$_{50}$ value of 0.5 μM, which was in agreement with previously reported values (21).

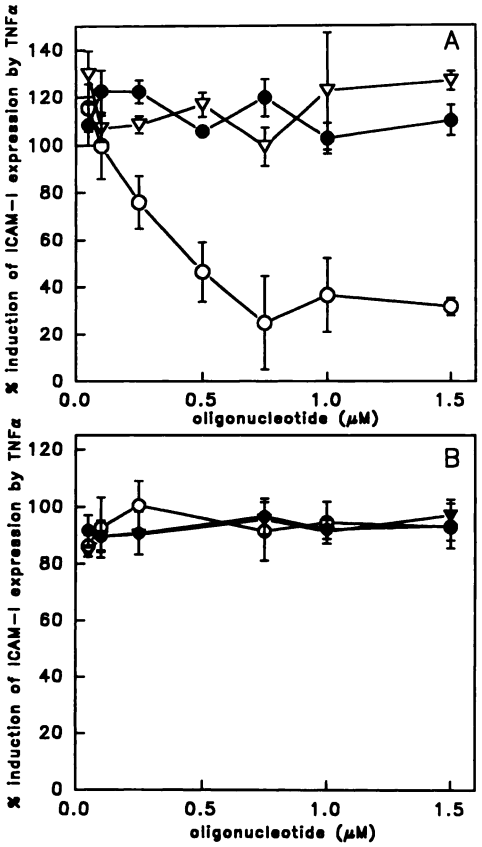

Fig. 4. The dose-dependent effects of pt-odn$_{ICAM-1}$ and two control pt-odn complexed with pLK-Fuc$_{100}$ or nonglycosylated pLK on the TNF-α-induced ICAM-1 expression in A549. Cells were seeded onto 96-well plates (10^4 cells/well) 24 hr before treatment for 4 hr at 37° in the presence of 100 μM chloroquine with (A) pLK-Fuc$_{100}$ or (B) nonglycosylated pLK complexed with (○) pt-odn$_{ICAM-1}$, (●) pt-odn$_{random}$, or (▽) pt-odn$_{gag}$ at the indicated concentrations. Medium was then replaced with normal growth medium containing 5 ng/ml TNF-α to stimulate ICAM-1 expression. After 18 hr, ICAM-1 expression was measured by enzyme-linked immunosorbent assay as detailed in Materials and Methods. The results shown are typical of experiments carried out in triplicate and repeated at least twice. Data are expressed as the mean ± standard deviation of the percentage of control ICAM-1 expression induced by TNF-α.

Endocytosis of pt-odn/pLK-Fuc complexes. When A549 cells were incubated with 0.15 μM radiolabeled pt-odn complexed with pLK-Fuc$_{100}$ the amount of cell-associated pt-odn increased rapidly over the first hour and reached a plateau by 4 hr (Fig. 5). After 4 hr, the amount of cell-associated pt-odn was increased by 15-fold using pt-odn/pLK-Fuc$_{100}$ complexes compared with free pt-odn. Surprisingly, nonglycosylated pLK also increased the amount of cell-associated pt-odn to a significant level, close to that observed with pLK-Fuc$_{100}$.

Confocal microscopy studies of A549 cells incubated at 37° with 0.15 μM carrier-free F-pt-odn showed very faint intracellular fluorescence (Fig. 6A). When F-pt-odn was complexed with pLK-Fuc$_{100}$, fluorescence was clearly visible and was shown to reside principally in vesicles within A549 cells (Fig. 6C). Similar images were obtained using nonglycosylated pLK (Fig. 6B). In both cases, fluorescence was clearly intracellular and not simply stuck to the cell surface.

Cells incubated under similar conditions but at 4° barely showed intracellular fluorescence in the presence of free F-pt-odn or complexed with pLK or pLK-Fuc$_{100}$, confirming that the uptake of the complexes occurred via an active process (Fig. 6, D–F).

The effects of chloroquine on pt-odn transfer. Because the inhibition of ICAM-1 expression by pt-odn$_{ICAM-1}$/pLK-Fuc$_{100}$ was observed only in the presence of chloroquine, the kinetics of uptake and efflux of radiolabeled pt-odn were investigated over a 4-hr incubation period at 37° in the absence and presence of 100 μM chloroquine and over a 20-hr chase period at 37° in the absence of both pt-odn and chloro-

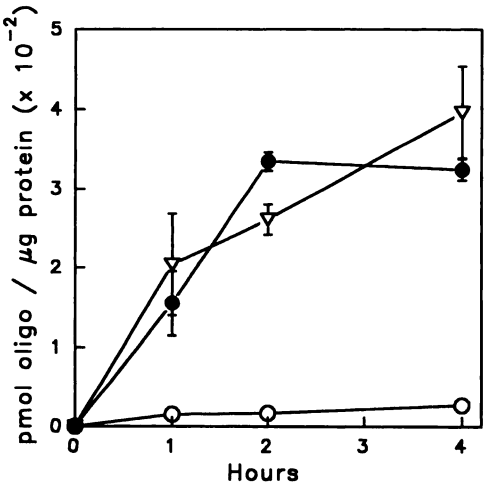

Fig. 5. The influence of glycosylated pLK on the uptake of pt-odn was studied in A549 cells. Cells in 24-well plates (2×10^5 cells/well) were incubated at 37° for the periods indicated in 0.5 ml of RPMI/10% FBS containing 0.15 μM ^{125}I-labeled pt-odn, (○) free or complexed with (●) nonglycosylated pLK or (▽) pLK-Fuc$_{100}$. Cells were washed and harvested as described in Materials and Methods. Radioactivity was measured using a γ counter, and protein content was measured according to the method of Lowry. The results shown are typical of experiments carried out in triplicate and repeated at least twice. Data are expressed as the mean ± standard deviation.

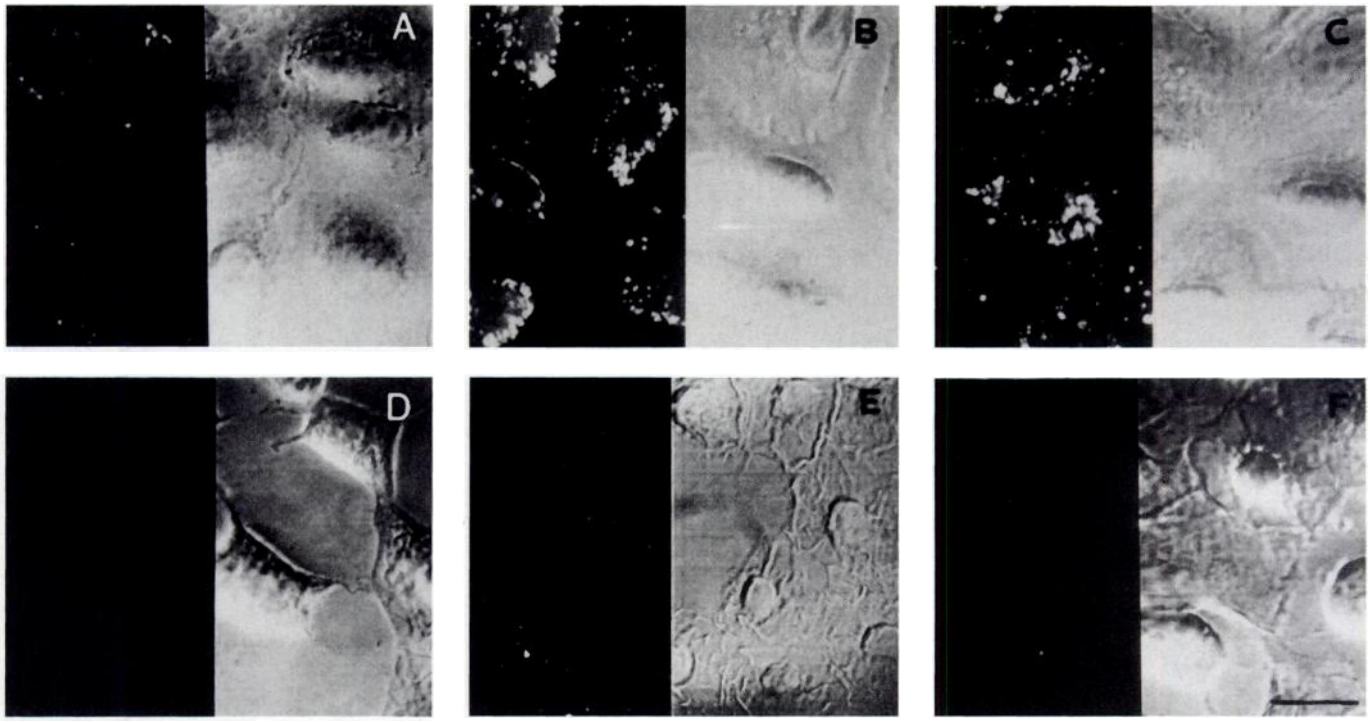

Fig. 6. The influence of glycosylated pLK on the uptake of pt-odn with the use of confocal microscopy. Cells (10^5 cells/ml) were incubated in 0.2 ml of RPMI/10% FBS at (A–C) 37° or (D–F) 4° for 4 hr in the presence of 0.15 μM F-pt-odn, free or complexed with pLK. Cells were washed and fixed as detailed in Materials and Methods. A and D, F-pt-odn; B and E, F-pt-odn/pLK; C and F, F-pt-odn/pLK-Fuc. *Scale bar*, 25 μm.

quine (Fig. 7). Uptake of pt-odn (see Fig. 5) was increased ~15-fold in the presence of pLK-Fuc_{100} and ~10-fold in the presence of nonglycosylated pLK compared with free pt-odn.

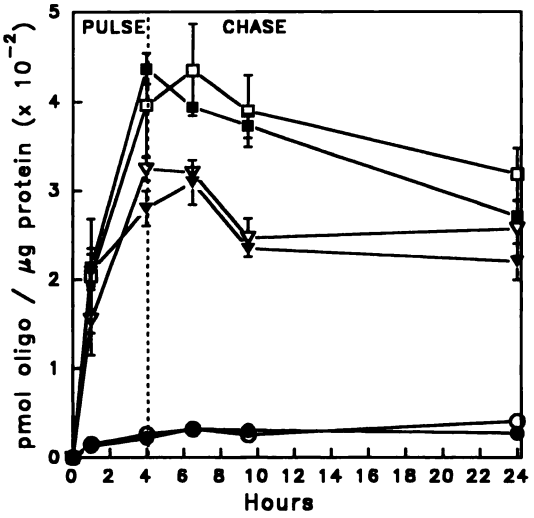

Fig. 7. The influence of chloroquine on the uptake and efflux of pt-odn complexed with nonglycosylated pLK or pLK-Fuc was studied in A549 cells. Cells in 24-well plates (2×10^5 cells/well) were incubated at 37° for the periods indicated in 0.5 ml of RPMI/10% FBS containing 0.15 μM ^{125}I-labeled pt-odn, (○ and ●) free or complexed with (▽ and ▼) nonglycosylated pLK or (■ and □) pLK-Fuc_{100} in the (*filled symbols*) presence or (*open symbols*) absence of 100 μM chloroquine. After 4 hr (*PULSE*), the medium was removed, and the cells were washed twice with fresh medium and then further incubated at 37° in fresh medium for the time periods indicated (*CHASE*). Cells were washed and harvested as described in Materials and Methods. Radioactivity was measured using a γ counter, and protein content was measured according to the method of Lowry. The results shown are typical of experiments carried out in triplicate and repeated at least twice. Data are expressed as the mean ± standard deviation.

The presence of chloroquine had no significant effect on the uptake of free or complexed ^{125}I-pt-odn (Fig. 7, *filled symbols*). During the 20-hr period after removal of pt-odn complexed with nonglycosylated pLK or pLK-Fuc_{100}, the cell-associated radioactivity was reduced to ~75% of the maximum. After a 20-hr chase, the amount of cell-associated pt-odn on delivery with pLK-Fuc was still 10 times higher than that observed in the presence of free pt-odn. Chloroquine had no significant effect on the amount of cell-associated pt-odn in the presence or absence of carrier over the incubation period used for measuring inhibition of ICAM-1 expression.

Discussion

Several groups have recognized the potential of pLK to increase the uptake of odn. With the use of odn covalently linked to polycationic pLK, Lemaitre *et al.* (32) and Degols *et al.* (33) obtained significant inhibition of gene expression. This approach, however, is quite different from the use of pLK carrying a ligand to induce receptor-mediated endocytosis of odn. Ligand-directed targeting has been studied almost exclusively using complexes in which the odn has been electrostatically bound to the carrier, thus requiring no chemical modifications. Several ligands have been used, including transferrin (12), epidermal growth factor (9), asialoglycoprotein (8, 11), and simple carbohydrate moieties (10), to target their respective receptors. In most cases, high levels of receptor-mediated uptake were demonstrated. However, data concerning sequence-specific inhibition of gene expression have been limited. Wu and Wu (11) showed inhibition of hepatitis B viral gene expression using an antisense pt-odn complexed with a pLK-asialoglycoprotein conjugate. Inhibition of gene expression was observed only at the exception-

ally high concentration of 50 μM pt-odn. Such a high concentration of pt-odn would normally be expected to induce a multitude of nonspecific effects (34), although they showed that a control pt-odn had no effect. In our experience, complexes with an pt-odn concentration of >2 μM tend to form large aggregates that were visible with the use of simple light microscopy.[2] The effect of such aggregates on gene expression and their mechanism of uptake is unknown.

Citro *et al.* used pLK-transferrin (12) and pLK-folate (13) conjugates to carry anti-*myc* and anti-*myb* phosphodiester odn, respectively, and obtained growth inhibition at ~4 μM. This is a significant advancement over free odn, which was not active in these studies. These results, however, may be due to a nonantisense effect because the odn sequence that was used has recently been shown to form G-quartet structures (35). Other recent articles have shown excellent efficacy in terms of cellular uptake but have not given any data concerning activity (i.e., inhibition of gene expression) (9, 10).

In this respect, our results represent a considerable improvement because we reproducibly obtained 80% inhibition of gene expression after a single treatment with 1 μM pt-odn$_{ICAM-1}$ and observed 50% inhibition at ~400-500 nM pt-odn. This is particularly significant because only one of the several studies cited carried out experiments in the presence of serum and obtained significant inhibition of gene expression at ~3 μM in the presence of 50 μM chloroquine (36). Inhibition of ICAM-1 expression was observed at submicromolar concentrations of pt-odn$_{ICAM-1}$ when complexed with pLK-Fuc$_{100}$, but no effect was observed with the use of nonglycosylated pLK. Larger pLK molecules (average degree of polymerization, 633), glycosylated in the same way, were also effective in transporting antisense pt-odn and resulted in an efficient inhibition of gene expression with an IC_{50} similar to that reported in this study.[2]

The role of the fucose ligand is complex because the activity of pt-odn$_{ICAM-1}$ was observed only when carried by pLK-Fuc$_{100}$, whereas the amount of pt-odn associated with the cell was only slightly higher (1.2-fold) in the case of pLK-Fuc$_{100}$ than in the case of nonglycosylated pLK. In addition, both conjugates led to similar patterns of intracellular fluorescence as observed with confocal microscopy. The influence of the two carriers on the precise intracellular localization of pt-odn is currently under investigation because pt-odn could be routed to different intracellular compartments depending on the nature of the carrier (i.e., glycosylated or nonglycosylated). The mechanisms involved in the uptake of these complexes are unknown, and the putative lectin in A549 cells, which is responsible for the endocytosis and intracellular routing of fucose glycoconjugates, has not yet been isolated. However, we can postulate that the difference in activity may stem from differences that exist between the pinocytic and receptor-mediated uptake pathways (for a review, see Ref. 37). (Another possible explanation for the importance of fucose as a ligand is discussed later in relation to chloroquine.)

We also showed the importance of the CR between the anionic pt-odn and the cationic pLK. It is difficult to estimate the exact nature of the pt-odn/pLK complexes because the commercially available preparations of pLK are heterogeneous in size. For this reason, the ratios of positive to negative charges used in this article are purely theoretical, but they do represent a useful approximation for comparison of the different complexes. The majority of studies, with the exception of that of Liang *et al.* (CR = 1.5, Ref. 10), have used complexes containing a large excess of positive charges, which in our model is inhibitory. Reported methods for the optimization of complex formation have relied on the migratory characteristics of the complexes on gel electrophoresis (CR = 2.7, Ref. 11; CR = 8.4, Ref. 36) or on the cellular uptake of the complexes (CR = 16, Ref. 9). It may be noteworthy that these reports also used smaller pLK molecules. However, the effect this may have on the results is unknown.

The higher efficiency of the complexes used in this report compared with those of other reports is probably due to the use of complexes with a small excess of positive charge and the use of chloroquine, which dramatically increased the efficacy of pt-odn complexed with pLK-Fuc. The target of pt-odn action is either in the cytosol or in the nucleus, but endocytosis of the pt-odn/pLK-ligand complexes results in accumulation within vesicular structures. Adjuvants such as chloroquine (36) have been shown to increase the activity of antisense phosphodiester odn carried by pLK, and two recent reports provide evidence that fusogenic peptides are able to help phosphodiester odn to escape from vesicles and reach their target (9, 10). The requirement for adjuvants in gene transfer experiments using plasmid/polymer-ligand conjugates is well established (22, 26, 38, 39).

Chloroquine causes a number of effects that may influence the intracellular fate of the nucleic acid/pLK complexes (see Ref. 22 and references therein): it induces the neutralization and the swelling of acidic vesicles leading to membrane destabilization; by raising the pH of the lysosomes, it inhibits degradative enzymes, which require an acidic environment; and it inhibits vesicular transport, thus reducing delivery to the lysosomes. Chloroquine is also able to bind to DNA. Taking into account these properties, it is possible to envisage several mechanisms by which chloroquine could increase the efficacy of pt-odn in this system. Chloroquine could extend the effective intracellular half-life of the pt-odn by avoiding the lysosomal degradation and reducing excretion, destabilize the endocytic compartments, allowing the pt-odn to escape from them, or allow the release of pt-odn from the electrostatic complex. Surprisingly, unlike Reinis *et al.* (8), we found that chloroquine did not alter the endocytosis or efflux of pt-odn/pLK-Fuc$_{100}$ complexes in A549 cells. Differences in cell lines and the shorter incubation times in the presence of chloroquine used in our experiments may account for this discrepancy.

Erbacher *et al.* (22) showed that at high concentrations, chloroquine is able to dissociate plasmid DNA/glycosylated-pLK complexes and that such high concentrations (~10 mM) are encountered inside cell vesicles within a 4-hr incubation period. It was also observed that dissociation of plasmid/glycosylated-pLK complexes occurred at chloroquine concentrations that were lower than that required to dissociate plasmid/nonglycosylated pLK complexes. This suggests that the presence of the recognition signal results in a complex that is inherently less stable than when unmodified pLK is used. It is possible that these observations may be relevant for the results of the current study, in which pt-odn complexed with pLK or pLK-Fuc$_{100}$ led to a similar level of uptake, whereas only the latter complex inhibited gene ex-

[2] A. J. Stewart, C. Pichon, L. Meunier, P. Midoux, M. Monsigny, and A. C. Roche, unpublished observations.

pression. The pt-odn have an *in vivo* half-life of ~24 hr (1); chloroquine may partly increase the efficiency of pt-odn by protecting them against degradative processes that may occur in the endosome/lysosome compartments.

In summary, we have shown an effective uptake of pt-odn/pLK-Fuc_{100} complexes that, under optimal conditions in the presence of chloroquine, are able to specifically inhibit gene expression at submicromolar doses, even in the presence of 10% serum. These results demonstrate the use of glycosylated pLK in antisense pt-odn applications, particularly because of its simple preparation and high efficiency.

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