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A two step model aimed at delivering antisense oligonucleotides in targeted cells

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Abstract

To be efficient in vivo antisense oligonucleotides must reach the targeted cells and then cross the cellular membrane. We propose a two step system where the oligonucleotide is first electrostatically bound to a peptide coupled to a ligand of a cellular receptor. A complex is formed which allows the oligonucleotide to be bound to the membrane of the targeted cells. These oligonucleotides are then delivered inside the cells by the subsequent use of a transfection agent. As a reductionist model of peptide coupled to a ligand we have used a lipopeptide and characterized by a filter elution assay the stoichiometry between the peptide and the oligonucleotide in the complexes. Using HeLa cultured cells we have shown that addition of these complexes to the cells triggers the oligonucleotide binding to the cell membrane. The subsequent addition of dendrimers allows these antisense oligonucleotides to inhibit a reporter gene inside the cells.

Keywords: Antisense oligonucleotide; Lipopeptide; Cell targeting; Delivery

The use of antisense oligonucleotides (ODNs) as therapeutic agents has been greatly developed in the past few years. Their efficiency is related to their ability to recognize their mRNA target into cells and then to block gene expression. The general mode of activity of antisense ODN is mainly dependent on RNase H hydrolysis of the RNA part of the duplex formed between the antisense ODN and the RNA target. However, one of the major limiting aspects of this strategy is the poor cell penetration for large anionic molecules such as antisense ODN. To enhance intracellular delivery of antisense ODN, different strategies using adsorption to cationic lipids [1] and polymers (dendrimer, poly(lysine), polyethyleneimine (PEI),) [1–3] or direct coupling to peptides to be able to go through the cellular membranes [4] or KDEL signal peptide [5] have been developed. Another approach with peptides has been obtained by the adsorption of the antisense ODN with a peptide which was designed to contain two domains, one in charge of the cell penetration and the other responsible for ODN binding [6].

These strategies constitute a major advance, however, one important drawback is the lack of cellular specificity. This type of delivery is not specially designed to recognize one particular type of cell in an organism. It has to be noticed that, for instance, in cancer most of the studied targets in the antisense field (with the exception of the fusion oncogenes) are also present in normal cells.

In this study, we propose a two step model for advancing towards a more specific cellular approach.

Our aim is to use in the first step a peptide to bind antisense ODN to bring them to the cellular membrane of the targeted cells. In the second step an agent able to pierce through the cellular membrane with no specificity is then used to deliver the antisense ODN into the cell. This can be done in the first step with specificity using a ligand of a cellular receptor. However to show the feasibility of such an approach we have used here in a reductionist approach a cationic peptide derived from SV40 T antigen nuclear signal peptide associated to a

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lipophilic palmitoyl part promoting the membrane interaction. We had then used dendrimers to deliver the membrane bound ODN inside the cells. We describe the synthesis, the ODN binding, and the biological properties of this lipopeptide (LP) as a vector for ODN delivery to the cell membrane and the consequences of its translocation into the cell using dendrimers.

Materials and methods

ODNs. The antisense ODNs used in this study were targeted towards the green fluorescent protein (GFP) mRNA. Their sequence is of the following one: d(GAGCTGCACGCTGCCGTC). It was provided by Eurogentech (Belgium) as a 18 mers phosphodiester chemistry with two phosphorothioate (PS) linkages at each one 3′ and 5′ extremities to improve its nuclease resistance. A control sequence with the same chemistry was used with the sequence: d(TGAACACGCCATGTCGATTCT).

LP synthesis. For the synthesis of palmitoylate peptides, we have used the same protocol as in [7]. The peptides were synthesized and then coupled to the carboxy end of the palmitoyl part to form the following molecules palmitoyl–PKSKRKVS-NH2 (LP) for the SV40 T antigen.

ODN–LP titration. To determine the interaction between the LP and the ODN, we have used a membrane filtration assay using a multiscreen filtration system (Millipore, Bedford) with a mixed ester of cellulose or nitrocellulose membrane of 0.45 μm pore. Free LP or free ODN was passed through these membranes and was then collected in the filtrate. The complexes were carried on the filter after formation of the particles. We have used two techniques to determine the formation of complexes.

(i) Peptide quantification of the solution before filtration and in the filtrate by the colorimetric BCA protein quantification (Perkin).

(ii) Determination of the ODN concentration after [5′-^{32}P] labeling by T4 DNA polynucleotide kinase followed by purification on Sephadex G10 (Amercham Pharmacia Biotech).

The ODN radioactivity was measured by the Cherenkov effect using a liquid scintillation compter (1900TR Packard). Complexes were prepared in 150 μl final volume of Tris–HCl 10 mM, pH 7.5, NaCl 100 mM.

Biodegradation of radio-labeled ODNs. ODNs were labeled in 5′ by T4 polynucleotide kinase (Biolabs) as indicated by the shipper in the presence of [γ-^{32}P] ATP 3000 Ci/mmol (ICN). 10 μM-labeled ODNs were incubated in 100 μl of fetal calf serum. At different times, 5 μl aliquots were extracted by phenol:chloroform (29:1) and then precipitated with 100 μl of 2% $LiClO_4$ in acetone. The separation of digested ODN was performed on a 20% PAGE containing 7 M urea in TBE buffer. The gel was then analyzed with a phosphorimager (Storm 840, Molecular dynamic).

Cell culture and delivery at the membrane of ODN using LPs. HeLa cells (human epithelioid cervical carcinoma) were grown in DMEM medium containing 10% heat inactivated fetal bovine serum, penicillin, and streptomycin at 37 °C under 5% CO_2 in a moist atmosphere. Transient GFP expression was performed thanks to a double transfection of 0.8 μg of pEGFP-N1 (Clonetech) and 0.4 μg of pCMVβ-gal (Clonetech) used as a transfection control. Plasmids were first incubated in 150 μl final volume with 6 μl of Superfect (Qiagen) as indicated by the shipper and then added to the cells. The cells had been seeded one day before at 4.5×10^5 cell per well in a 24 well plate.

The membrane delivery of ODNs vectorized by the LP agent was performed as follows: we first prepared complexes between LP and ODN at a 10:1 ratio (LP:ODN) in 10 mM Tris–HCl, pH 7.5, 100 mM NaCl, and after a 20 min incubation at room temperature, the complexes were added to the culture at the final concentration indicated for each experiment.

After a 16 h incubation, the cells were washed twice in PBS and then lysis was performed in 400 μl of Reporter Lysis Buffer (Promega) for 10 min at room temperature. After scraping, the lysate was transferred into a tube, vortexed, and then centrifuged for 15 minutes at 14,000 rpm. The GFP fluorescence (emission 505 nm, excitation 473 nm) was recorded on an SFM23 spectrofluorometer (Kontron) using a 300 μl aliquot. The β-galactosidase activity was measured on 15 μl of lysate in 100 μl of reaction buffer (80 mM phosphate buffer, pH 7.4, 0.7% β-mercaptoethanol, 9 mM $MgCl_2$, 4.6 mg/ml chlorophenol red-β-D-galactopyranoside) in a 96 well plate and incubated for 10 min at 37 °C. The β-galactosidase activity was then detected by reading the absorbance at 570 nm using an automatic reader spectrophotometer (Dynatech Laboratories). All experiments were done in triplicate. The GFP expression was determined as the ratio of the GFP fluorescence to the β-galactosidase activity and expressed in % of the untreated transfected cells.

Toxicity of LPs in cell culture. 100 μl of HeLa cells was seeded at 10^4 cells/ml of DMEM complete medium in a 96 well plate. After 24 h the LP–ODN complexes were then added and incubated for 16 h at 37 °C. The viability of the cells was then determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) method. 10 μl of 5 mg/ml MTT solution (Sigma) was added to the cells and then incubation was performed for 1 h at 37 °C. The cells were then lysed overnight at 37 °C with 100 μl 10% SDS, 10 mM HCl. The blue color was measured at 570 nm using an automatic reader spectrophotometer (Dynatech Laboratories). The relation between absorbance and cell number was determined using various dilutions of a calibrated cell solution.

Detection of GFP mRNA by Northern blot. After treatment, HeLa cells were washed with PBS buffer and then lysed in 400 μl of 4 M guanidium thiocyanate, 25 mM Na citrate, pH 7, 0.5% sarcosyl, and 0.1 M β-mercaptoethanol at 0 °C. After homogenization, RNA was phenol extracted as follows. We added 40 μl of 2 M Na acetate, pH 4, 400 μl of water saturated phenol, and 120 μl of chlorophorm:isoamyl alcohol (49:1). The mix was centrifuged at 13,000 rpm for 15 min and 300 μl of the aqueous phase was precipitated with 300 μl of isopropanol at −20 °C. The pellet was 70% ethanol washed, dried, and solubilized in 10 μl of water. Electrophoresis of 2 μg of total RNA was performed on a 1% agarose gel in MOPS buffer containing 6.3% of formalin. The RNAs are then blotted on a nitrocellulose membrane (BA-S85, Schleicher and Shuell) in [10X] SSC buffer. GFP mRNA was detected by the Northern technique [8] with a 600 bp fragment of GFP cDNA labeled with [α-^{32}P] dCTP 300 Ci/mM (ICN, France), and nonaprime II random priming kit (Appligen, France). After washing, the nitrocellulose was analyzed with a Storm 840 phosphorimager (Molecular Dynamic).

Results

ODN–LP titration

The determination of the LP binding capacity was done by a filtration assay. Both the free LP and ODN were passed through the filter and the complexes were retained by the membrane. The dosage of either free ODN or free LP in the filtrate indicates the capacity of these agents to form nonfilterable complexes. The free LP was measured by a colorimetric protein assay (Fig. 1A) and the free ODN was measured thanks to the use of [^{32}P]-labeled ODNs (Fig. 1B). We obtained a maximum of ODN association for a five time excess of peptide (Fig. 1A). These results, obtained by an original filtration technique, are in accordance with the results

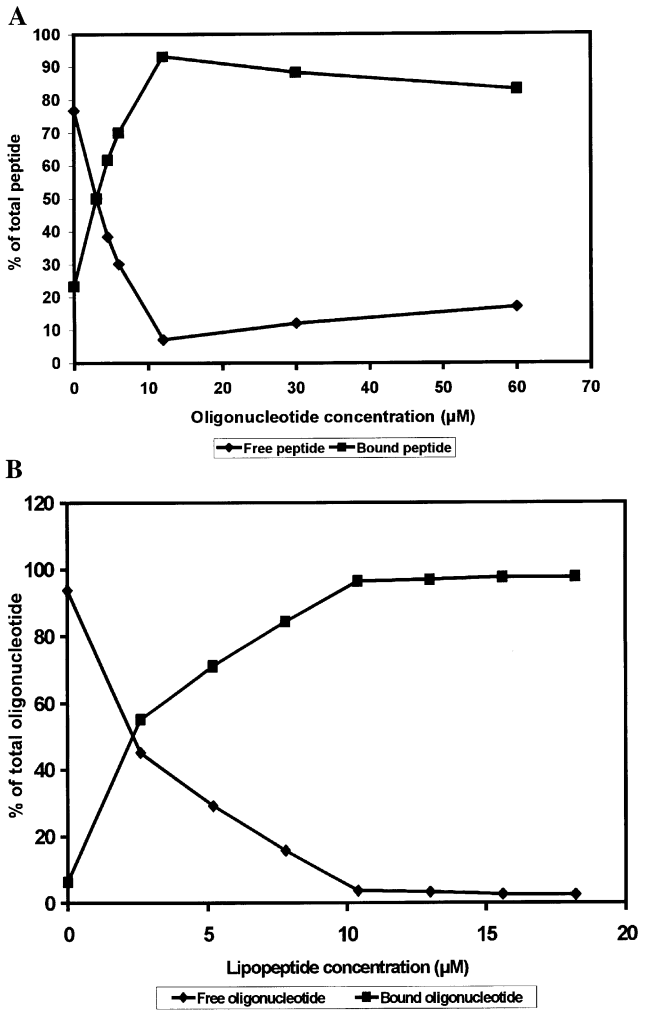

Fig. 1. Formation of the LP–ODN complex according to the ODN concentration or to the LP concentration. (A) A 60 μM solution of LP was incubated with ODN at various concentrations. (B) A 1.3 μM solution of [^{32}P]-labeled ODN was incubated with LPs at various concentrations. Bound and free ODN were measured after nitrocellulose filtration as described in Materials and methods.

obtained by Divita et al. [6] with a fusogenic peptide containing the same peptide fragment as a DNA binding domain. When studying the formation of complexes after ODN labeling a maximum of ODN association for a nine time excess of peptide (Fig. 1B) was obtained.

Stability of radio-labeled ODN

We have compared the degradation of either the PS modified ODN or the same ODN in the natural phosphodiester (PO) form in fetal calf serum at 37 °C (Fig. 2). We observed that PS ODNs, are more resistant than their counterparts, with the PO chemistry with a half-life of 40 and 10 min, respectively. When these ODNs were associated to the LP (Fig. 2), we observed a light protection of the ODN. At 30 and 120 min of incubation the intact ODN concentration was doubled when

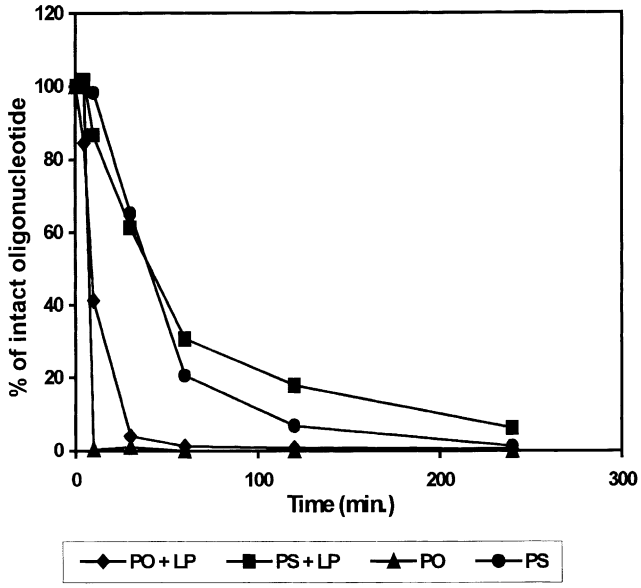

Fig. 2. Kinetics of degradation in fetal calf serum of either unmodified ODN (PO) or ODN with two phosphorothioate modifications at the 5′ extremity and two at the 3′ one (PS). Incubations have been performed with or without the LP. The full length ODN degradation was quantified with a phosporimager as described in Materials and methods.

either the PO or the PS ODNs (respectively) were associated to the LP.

Toxicity of the LP–ODN complex in cell culture

We have measured the cytotoxicity of LP–ODN complexes on growing HeLa cell by the MTT method (Fig. 3). No toxicity was observed due to the presence of the ODN–LP particles up to a concentration of 1 μM ODN (5 μM in LP).

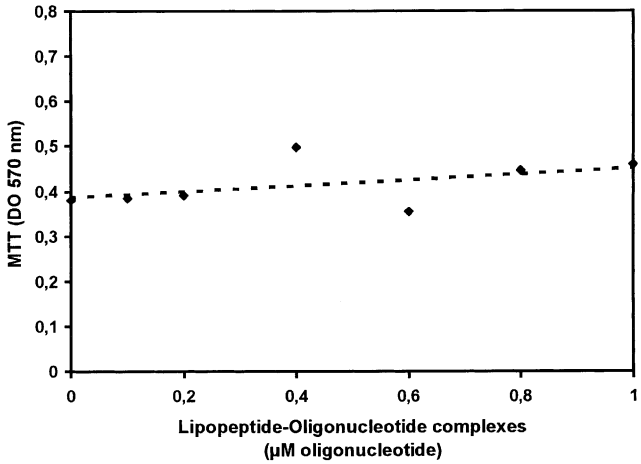

Fig. 3. Absence of cytotoxicity of the LP–ODN complexes on growing HeLa cells. The MTT test was performed as described in Materials and methods.

Binding of ODN at the cell membrane using the LP

Using an epifluorescence microscope we have observed the cells after either 4 or 24 h of incubation with FITC-labeled ODN. In these conditions free ODN do not bind on HeLa cells and no fluorescence is observed. When ODNs are associated to the LP, we observe a light fluorescence close to the cell membrane (results not shown).

Effect of peptide bound antisense ODNs on HeLa cells after treatment with a transfection agent

One way to evidence the uptake of antisense ODN associated to the LPs (first step) is to measure their antisense effect in the presence of Superfect (second step). To measure the inhibitory potential of GFP antisense ODNs associated to the LP particles, we have studied the effect of the LP–ODN complex at increasing concentrations on the transient expression of superfect transfected pEGFP-N1 plasmid. Inhibition occurs at concentrations in ODN higher than 0.6 μM (Fig. 4). At 1 μM ODN, a 45% inhibition of GFP using untreated cells as a control has been obtained. The control ODN triggers under the same conditions 115% GFP expression when compared to the untreated cells. The inhibition obtained in Fig. 4 might be explained according to two possibilities:

1. The peptide allows the delivery of a low amount of ODN in cells (not visible by fluorescence) which would nevertheless trigger gene inhibition.
2. The ODN has been allowed to leave the peptide at the membrane to bind Superfect used subsequently for plasmid transfection.

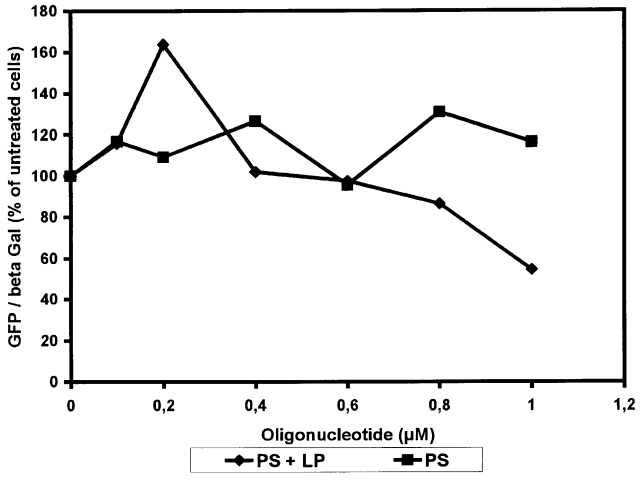

Fig. 4. Dose effect inhibition of GFP expression by either the LP vectorized antisense ODN or the LP vectorized control ODN. The GFP expression was determined after cotransfection of pEGFP-N1 and pCMVβ-Gal plasmids in HeLa cell and measurement after a 16 h incubation of the expression of GFP and β-Gal. The expression of the effect was normalized by the β-Gal expression on the same cells.

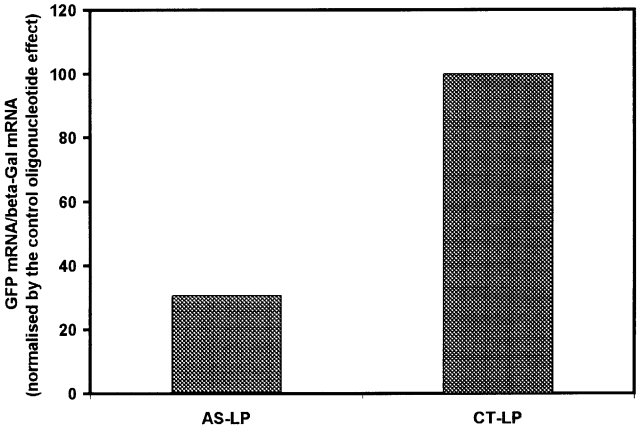

Fig. 5. Inhibition of GFP mRNA in HeLa cells by the LP vectorized antisense ODN (1 μM). The GFP mRNA was quantified after Northern blot and normalized by the β-Gal mRNA level. The result is expressed as the ratio between the effect of the antisense ODN over the effect of the control ODN.

The first hypothesis is unlikely because we had already shown in this cellular system that the gene inhibition is related to the ODN uptake as measured by intracellular fluorescence [9]. As described above we observed fluorescence only at the cell membrane.

GFP mRNA inhibition in HeLa cells

We have measured by Northern blot the GFP mRNA expression in HeLa cells after treatment by either antisense or control ODN vectorized with the LP (Fig. 5). When compared to the untreated cells GFP mRNA synthesis is strongly inhibited by the antisense ODN. This result confirms that the GFP targeted ODN is indeed acting by an antisense mechanism.

Discussion

For the purpose of demonstrating the feasibility of this two step approach to deliver antisense ODN in cells we have developed a new vector peptide. These molecules are made of two fragments. The first one is a lipid which is in charge of delivering the complexes at the cellular membranes, the second is a peptide moiety responsible for the ODN binding to the vector. We have observed a good association of the peptide and the ODN with a ratio of 1 ODN for nine peptides. This result is in accordance with the association of ODN with the same peptide as observed by Divita et al. [6]. Nevertheless, the complex formed presents a high affinity for the tube surface. The different types of tubes used, such as glass, polypropylene, siliconed tube, display between 30% and 60% of bound complexes. We had to take this

fact into account to determine the true amount of complex added to HeLa cells.

The SV40 T antigen fragment peptide was tested as a nuclease protecting agent in the range from 1 ODN for five peptides to 1 ODN for nine peptides. We observed a low protection of the ODN against degradation in the complexes (Fig. 2). This is not unexpected since the binding of the ODN to the peptide is reversible and the ODN is not hidden from the nucleases.

The capacity of these LPs to bind the ODN at the cell membrane was measured with an epifluorescence microscope using FITC-labeled ODN. A light fluorescence at the membrane was observed after cell treatment by such complexes.

The feasibility of the two step concept in HeLa cells is shown by the antisense effect (evidenced by the measurement of RNA and protein inhibition) of the ODN bound to the lipid peptide. We have indeed shown on one hand that the lipid peptide does not deliver the ODN inside the cells but only brings it to the membrane, and on the other hand that the ODN alone cannot be delivered inside the cells. It is therefore thanks to the addition of Superfect that the ODNs are able to penetrate into the cells. We propose that this can be achieved at the membrane only by dissociation of the ODN bound to the peptide lipid followed by a secondary binding to the cationic dendrimer (Superfect). According to this result the next step will be to use a ligand instead of a lipid and to proceed in a programmed two step system. The best would then be to use cells expressing permanently a reporter gene, then first to treat them by an ODN coupled to the ligand of a specific cell receptor, to wash the cells, and then second to add a cationic agent known to vectorize efficiently antisense ODN inside the cell. According to this protocol we expect that no antisense effect will be detected in the cells treated in the first step by ODN not coupled to the ligand.

Acknowledgments

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