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Improved biological activity of antisense oligonucleotides conjugated to a fusogenic peptide

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ABSTRACT

Recently several groups reported a dramatic improvement of reporter gene transfection efficiency using a fusogenic peptide, derived from the Influenza hemagglutinin envelop protein. This peptide changes conformation at acidic pH and destabilizes the endosomal membranes thus resulting in an increased cytoplasmic gene delivery. We describe the use of a similar fusogenic peptide in order to improve the antiviral potency of antisense oligodeoxynucleotides (anti TAT) and oligophosphorothioates (S-dC$_{28}$) on *de novo* HIV infected CEM-SS lymphocytes in serum-free medium. We observed a 5 to 10 fold improvement of the anti HIV activities of the phosphodiester antisense oligonucleotides after chemical coupling to the peptide in a one to one ratio by a disulfide or thioether bond. No toxicities were observed at the effective doses (0.1 – 1 μM). No sequence specificity was obtained and the fusogenic peptide possessed some antiviral activities on its own (IC$_{50}$: 6 μM). A S-dC$_{28}$ – peptide disulfide linked conjugate and a streptavidin – peptide-biotinylated S-dC$_{28}$ adduct showed similar activities as the free S-dC$_{28}$ oligonucleotide (IC$_{50}$: 0.1 – 1 nM). As expected, all the compounds were less potent in the presence of serum but the relative contribution of peptide coupling was maintained.

INTRODUCTION

The use of antisense oligonucleotides as a therapeutic tool holds considerable promise and several clinical trials are currently under way (1). In order to improve their pharmacological properties, numerous chemical modifications of oligonucleotides have been described (1). As a result analogs with improved stability against enzymatic breakdown and/or better hybridization properties with complementary natural oligonucleotides in cell-free systems were obtained. Notwithstanding the large amount of research devoted to this subject, the intra-cellular delivery of oligonucleotides remains a major hurdle (2).

It is commonly assumed that all natural and chemically modified oligonucleotides are taken up by an endocytic pathway. Passive diffusion across the external cell-membranes has never been demonstrated conclusively for any oligonucleotide analogs including non polar derivatives (3). Oligonucleotides remain trapped inside endocytic vesicles and end up being degraded in the lysosomes. The observed biological activity is produced by a minute amount of compound that manages to escape the endosomal compartments. This escape has to occur at an early stage of the endocytosis before the enzymatic degradation becomes prevalent.

In order to improve the uptake and cytoplasmic transport of the oligonucleotides, delivery systems have been investigated such as the addition of polylysine (4), encapsulation into antibody targeted liposomes (5, 6) and nanoparticles (7, 8). Nearly all the delivery systems rely on an endocytic uptake mechanism. Only cationic lipids and liposomes (9–12) and Sendai virus derived liposomes (13, 14) seem to permit a direct passage of their oligonucleotide load through the external cell membrane.

Envelopped viruses possess efficient means to transfer their genome from the endosomes into the cytoplasm of their host cell. After endocytosis their envelop proteins are capable of destabilizing the membranes of the endosomes, as to permit the passage of the nucleocapsid into the cytoplasmic compartment.

Several researchers took advantage of this property for gene therapy purposes. They successfully transfected plasmids coding for reporter genes using inactivated viruses (14, 15–18), fusogenic peptides (19–21) or other membrane destabilizing peptides derived from viral envelop proteins (22).

Our strategy consisted in the direct coupling of an influenza derived fusogenic peptide to antisense phosphodiester or phosphorothioate oligonucleotides as a means for improved cytoplasmic delivery of the latter compounds at an early stage of their endosomal uptake. Several studies have indeed shown that this peptide alone is capable of disrupting lipid membranes after a conformational change induced by acidification (23–26). It is thought that several fusogenic peptides self-assemble at acidic pH to form a transmembrane channel.

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MATERIALS AND METHODS

Chemicals

Phosphatidylcholine type V-EA from Egg Yolk, calcein, 2,2′ dipyridyl disulfide (DTP), 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid 3-sulfo-N-hydroxysuccinimide ester (sulfo-SMCC) and streptavidin were purchased from Sigma, Triton X 100 from LKB-Bromma and 5-carboxymethylrhodamine N-hydroxysuccinimidyl ester from Molecular Probes. The 5′ biotinylated S-dC_{28} phosphorothioate oligonucleotide was synthesized with synthons provided by Cruachem (C-amidite), Milligen/Biosearch (cytidine immobilized on 500 A CPG) and Glen Research (Beaucage's sulfurizing reagent, BioTEG phosphoramidite biotinylation reagent) on a Cyclone DNA Synthesizer (Millipore) and the 5′ protected thiol S-dC_{28} was synthesized by Genset using the Thiol-Modifier C6 S-S from Glen Research. The TAT-N d(GGCTCCATTTCTTGCTCTC) and TAT-B d(GGTCTTACTCTCCGTCTCT) phosphodiester oligodeoxynucleotides were modified with a 2-propanol-3-amino group or a ribonucleotide at the 3′ end and a hexamethylene-bridged pyridyldisulfide or primary amine at the 5′ end. They were synthesized by Genosys and were kindly provided by the French AIDS Research Agency (ANRS).

FAB (pos) mass spectrometry was performed on a Jeol JMS-SX102A. Fluorescence was measured on a Kontron SFM 25 fluorimeter . Reversed phase HPLC was carried out on Hypersil C18−5μM and Zorbax Protein Plus (DuPont) analytical columns using a Beckman System Gold 126 AA solvent delivery module equipped with a System Gold 168 photodiode array detector.

Synthesis of the phosphorothioate S-dC_{28} oligonucleotide

The synthesis followed the phosphoramidite methodology using a standard program provided by Millipore. The sulfurisation was performed using Beaucage's reagent. The 5′ biotinylation and ammonia deprotection were performed manually. In order to maximize the yield no capping step was included and the progress of the reaction was followed by colorimetry of the trityl cations released after each coupling step (27). Global yields of 22−28% for the 5′ biotinylated S-dC_{28} and 57−60% of unmodified S-dC_{28} were obtained based on the released trityl groups. The lyophilized compounds were used without further purification. For quantification purposes a molar extinction coefficient of 204 000 $M^{-1}cm^{-1}$ at 260 nm and 262 000 $M^{-1}cm^{-1}$ at 280 nm were assumed (28). The same values were used for the 5′ thiol linker modified S-dC_{28} synthesized by Genset.

Synthesis of the fusogenic peptide

The peptide with sequence GLFEAIAGFIENGWEGMIDGGG-YC was synthesized by the Fmoc procedure on a Milligen 9050 Pepsynthesizer (Millipore UK). A PEG-polystyrene graft polymer with a 4-hydroxymethylphenoxyacetic acid (HMP) linker (Millipore) was utilized as the matrix. The peptide was cleaved from the support with TFA/thioanisole/anisole/ethanedithiol (9:0.5:0.2:0.3 v/v) at room temperature for 4 hours. Following ether precipitation and washing at 4°C, the peptide was lyophilized and purified by HPLC on a Zorbax Protein Plus reversed phase column. The peptide was dissolved in 20 mM aqueous ammoniumbicarbonate containing 300 μl of 30 % ammonia per liter (buffer A) and treated with an excess of 1 M DTT before chromatographic purification. A linear gradient of 0 to 100 % methanol (solvent B) in buffer A eluted the peptide at about 56% methanol in buffer A. The purified peptide monomer was lyophilized immediately to prevent subsequent dimerization via intermolecular disulfide bond formation. The peptide was characterized by amino acid analysis and FAB(pos) mass spectrometry (M + H^+: 2506). For peptide concentration determinations we used a molar extinction coefficient of 6800 $M^{-1}cm^{-1}$ at 280 nm.

Synthesis of the TAT−peptide conjugate

6 OD (29 nmoles, estimated ϵ^{260}: 206000 $M^{-1}cm^{-1}$) of the 5′ thiol and 3′ aminemodified TAT oligonucleotide was mixed with a fourfold excess of peptide in 500 μl Tris buffer (10 mM Tris/1 mM EDTA, pH 7.2). The solution was submitted to repeated cycles (5−10) of quick freezing (−20°C) and slow thawing at room temperature. The purification and progress of the reaction was monitored by HPLC on a Hypersil C18−5μm or Zorbax analytical column using a linear gradient of 0−15 % acetonitrile in 10 mM triethylammoniumacetate pH 7.0. The coupling yield was about 60% conjugate based on the UV absorbance of the species. The identity of the conjugate was confirmed by HPLC after cleavage of the purified conjugate with DTT, which regenerated the free oligonucleotide and peptide as sole products. For quantification purposes, we used the same extinction coefficient for the conjugate as for the free oligonucleotide.

Fluorescent labelling of the TAT oligonucleotide

The oligonucleotide (100 nmoles) was dissolved in 700 μL 0.25 M $NaHCO_3$ pH 8.0 and reacted with a large excess of 5-carboxytetramethylrhodamine N-hydroxy succinimidyl ester (1700 nmoles in 20 μL DMSO) for 2 hours at room temperature in the absence of light. This was followed by the addition of 50 μL 1 M Tris pH 6.8 to quench the reaction. and gel filtration on Sephadex G25 previously equilibrated with 10 mM phosphate/1 mM EDTA pH 7.2 buffer containing 1 mg/mL bovine serum albumine (BSA). Elution was performed with the same buffer without BSA. The rhodamine labelled oligonucleotide was separated from the unlabelled compound by HPLC on a Zorbax Protein Plus reversed phase column with a linear gradient of 0 to 15% acetonitrile in 10 mM triethylammoniumacetate pH 7.0 using simultaneous detection at 260 and 560 nm. The labelled TAT was coupled to the fusogenic peptide using the same procedure as described above. The unreacted rhodamine dye recovered from the gel filtration was concentrated and used as a control for the fluorescence microscopy experiments (estimated ϵ^{560} : 60000 $M^{-1}cm^{-1}$).

Synthesis of the TAT-B-SMCC−peptide conjugate

60 nmoles (estimated ϵ^{260} : 206000 $M^{-1}cm^{-1}$) of 5′ aminolink and 3′ ribose modified TAT-B oligonucleotide in 400 μL phosphate buffer (0.25 M, pH 7.2) was mixed with 40 μL sulfoSMCC (3600 nmoles) in DMF and incubated at room temperature for 2.5 hours before separating the excess sulfoSMCC from crude TAT-SMCC by Sephadex G 25 gel filtration using 20 mM Tris/1 mM EDTA pH 7.0 as eluting buffer. The recovered oligonucleotide fraction (2mL) was immediately reacted with a fourfold excess of fusogenic peptide for one hour at room temperature before being stored at −20°C. The TAT-B-SMCC−peptide conjugate was isolated by HPLC on a Zorbax Protein Plus reversed phase column using a linear gradient of 0 to 100 % acetonitrile (solvent B) in 10 mM triethylammoniumacetate (solvent A). The coupling yield was about 50 % based on the UV absorbance of the total amount of

oligonucleotide used. For quantification purposes, we used the same extinction coefficient for the conjugate as for the free oligonucleotide.

Synthesis of the Streptavidin−peptide-S-dC$_{28}$ conjugate

5 mg Streptavidin (86 nmoles, estimated ϵ^{280} (29): 136 000 $M^{-1}cm^{-1}$) were dissolved in 800 µL phosphate buffer (0.1 M, pH 7.5) was mixed with 160 µL of a 5.5 mM solution of sulfoSMCC in dry dimethylformamide (880 nmoles). The solution was stirred at room temperature for 30 minutes and the excess sulfoSMCC was removed by gel filtration on Sephadex G50 SF using 10 mM Tris/1mM EDTA/300 mM NaCl pH 7.3 as eluting buffer. In the meantime 1.6 mg of fusogenic peptide was dissolved in 900 µL water/methanol (2:1 v/v) using a little NaOH to improve the solubility. Residual cystine bridged peptide homodimer was cleaved by the addition of 10 µL of a 10 mM tributylphosphine solution in isopropanol. The peptide solution was mixed with the pooled sulfoSMCC modified Streptavidin fractions (5 mL). The peptide−protein coupling proceeded for one hour at room temperature before mixing with 80 nmoles of biotinylated S-dC$_{28}$. The conjugate was lyophilized after a couple of hours of incubation. The residue was dissolved in 1 mL deionized water and purified on a Sephadex G50 SF gel filtration column using 10 mM Tris/1 mM EDTA pH 7.15 as eluent. The effluent was monitored at 280 and 260 nm. The first peak is the Streptavidin−peptide-S-dC$_{28}$ conjugate based on peptide labelling and spectroscopic measurements. Under the assumption of a fully biotinylated complex the recovered conjugate was estimated at 43 nmoles based on the absorbance at 280 nm (estimated ϵ^{280} : 1 200 000 $M^{-1}cm^{-1}$).

Synthesis of the S-dC$_{28}$−peptide conjugate

14 OD (53 nmoles, estimated ϵ^{280} : 262000 $M^{-1}cm^{-1}$) of the 5′ thiol-modifier C6 S-S derivatized S-dC$_{28}$ was incubated in 100 µL 0.1 M DTT/0.1 M NaOH during 45 min in order to generate the free thiol group. The excess DTT and protecting group were extracted with ethylacetate (3×100 µL) and the remaining aqueous solution was treated with 100 µL 0.4 M 2, 2′- dipyridyl disulfide (DTP) for 60 min. All reactions were performed at room temperature. After removal of the excess DTP by Sephadex G 25 gel filtration in 10 mM Tris/1 mM EDTA pH 7.2, the recovered oligonucleotide (3 mL) was treated with a fourfold excess of fusogenic peptide and the solution was submitted to repeated cycles (5−10) of freezing (−20°C) and slow thawing at room temperature. The conjugate was recovered by HPLC on a Zorbax Protein Plus analytical column using a linear gradient of acetonitrile (0 to 48 %) in 10 mM triethylammoniumacetate pH 7.0. Based on the HPLC trace a 53% coupling yield of conjugate was obtained. For quantification purposes, we used the same extinction coefficient for the conjugate as for the free oligonucleotide.

Liposome leakage assay

The membrane destabilization induced by the fusogenic peptide or its conjugate was measured by the release of liposome encapsulated calcein in their presence at various pH (30). The liposomes were prepared by the method of dialysis using n-octyl glucoside as a detergent in a solution containing 10 µmoles egg yolk phosphatidylcholine dissolved in 50 mM calcein/20 mM Tris/1mM EDTA (31). The liposomes were freed from non-incorporated calcein by centrifugation on a 5 to 10 % (w/v) gradient of Nycodenz (32), followed by a gel filtration on Sepharose CL4-B (Pharmacia) using 20 mM Tris/1mM EDTA/110 mM NaCl pH 7.35 as eluting solvent. The mean size of the liposomes was 250 nm as determined with a Coulter NC4 counter. The calcein release measurements were performed after 20 minutes of incubation at room temperature on 1 mL solutions containing 990 µL buffer, 10 µL liposome stock solution and 2−6 µL aliquots of the destabilizing agent. The final concentration of the peptide, oligonucleotide and its conjugate was 0.2 µM. The fluorescence values (excitation at 490 nm, emission at 515 nm), which correspond to 100% leakage were obtained after addition of 10 µL of 10% (v/v) Triton X-100. At pH 6.3 to 7.5 we used a phosphate buffered saline (PBS) solution containing 1 mM EDTA. The pH 4.5 to 6.0 buffers contained 20 mM citrate/1mM EDTA/100 mM NaCl.

Cells and cell culture

CEM-SS lymphocytes were obtained through the AIDS Research and Reference Program, National Institutes of Health, from P.L. Nara (National Cancer Institute, Frederick, MD.). The cells were grown on RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and penicillin/streptomycin (100 units/mL each). The BHK 21 cells were grown on MEM supplemented with 10% fetal bovine serum.

Fluorescence microscopy

BHK 21 cells were plated on Lab-Tek (Nunc Inc) (500 µL capacity/well) coverslips (2×10^{4}cells/well) and cultured in MEM supplemented with 10 % FBS for 12−20 hours. Subsequently the culture medium was discarded and washed with MEM before incubating the cells 1 hour at 37°C with the rhodamine labelled compounds dissolved in 150 µL MEM supplemented with BSA at 1 mg/mL. The cells were washed with PBS at 4°C, fixed with 3.7 % p-formaldehyde in PBS for one hour at 4°C and mounted in a PBS/glycerol (2:1 v/v) mixture containing 15% Airvol (AIR Products Nederland BV) and 0.1% NaN_3. The cells were observed on a Zeiss Axiophot fluorescence microscope.

Biological assays

The biological assays were performed on microwell plates containing 10^4 cells per well in 200 µL serum-free medium (Gibco AIM) or RPMI supplemented with 10% FBS. Cells were infected with HIV-1 (LAI strain) at a TCID50 of 20. The antiviral agents were added after adsorption of the virus. The cells were grown 5 days before being screened for the reverse transcriptase (RT) activity (33, 34). The cytotoxicity was measured using the MTT assay (35).

Statistical treatments

The confidence limits of the data and the significance level of the difference between for example the peptide coupled and uncoupled oligonucleotide were assessed through analysis of variance (36). Because the RT activity of the controls (samples without drug) varied from one experiment to another, we normalized the data to the same basal level of RT counts. We verified that the variance among the data sets was homogeneous according to Bartlett's test (36). Orthogonal comparisons were performed by analysis of variance. When the difference between repeat experiments was not significant the data were pooled. The graphs are expressed as % of the RT activity. The error bars indicate the standard deviation computed from the analysis of variance.

RESULTS

Choice of the fusogenic peptide

The fusogenic peptide was derived from the influenza virus hemagglutinin envelop glycoprotein which is present at the N terminus of the HA-2 polypeptide subunit (26). The amino acid sequence of this peptide was nearly identical to the one utilized by Wagner *et al.* (19, 20). Its chemical coupling to polylysine strongly increased the transfection efficiency of a transferrin polylysine luciferase reporter gene complex in various cell types. An additional tyrosine residue has been included at the penultimate position of the C terminus of our peptide.

The C terminal position contains a cysteine group, which was used as a handle to couple the peptide to antisense oligonucleotides. Two coupling strategies were used; the formation of a disulfide bond, which should rapidly be cleaved in an intracellular reducing environment or the formation of a more stable thioether linkage (Fig. 1). Two types of oligonucleotides were used in this study as described below.

Conjugation of oligonucleotides to the fusogenic peptide

The TATN labelled compound is an oligomer with a regular phosphodiester backbone complementary to the AUG initiation site of the HIV TAT protein (37). This particular oligonucleotide promotes an efficient and sequence specific antiviral response when introduced in T lymphocytes cell lines through poly-L-lysine conjugation (38) or antibody targeted liposome encapsulation (6).

A scrambled sequence labelled TATB oligomer with the same base composition was synthesized as a control. In both cases the 5′ end was modified with an activated thiol containing spacer molecule, and the 3′ moiety was ended by an aminolink group.

Both modifications introduce functional groups, which can be used for coupling purposes to peptides or reporter groups such as fluorescent dyes. As an additionnal benefit they increase the stability of the oligomer against enzymatic hydrolysis by the exonucleases. These enzymes have been shown to contribute importantly to the oligonucleotide degradation *in vitro* (39, 40).

The disulfide bridged conjugate TAT−pep was obtained in good yield by simple mixing of the peptide and the oligonucleotide under optimized conditions and HPLC purification followed by lyophilisation. The same coupling conditions were used to produce the disulfide linked S-dC$_{28}$−peptide molecule.

The S-dC$_{28}$ phosphorothioate has been described as one of the most potent inhibitors of HIV reverse transcriptase (41, 42). Each internucleotide bridging phosphorus contains a thiol group, which we thought might form weak disulfide bridges with the cysteine group of the fusogenic peptide and result in the formation of an ill defined conjugate. This concern was unfounded as we learned later (Fritz Eckstein, personal communication).

Thus in order to circumvent our initial concerns, we made use of a streptavidin−biotin coupling strategy. The S-dC$_{28}$ phosphorothioate was modified at the 5′ end with a spacer molecule containing a biotin residue. The fusogenic peptide was coupled on streptavidin through a thioether bond using a heterobifunctional linker sulfoSMCC. Under these conditions, an average of four fusogenic peptides were bound to each streptavidin based on titration of the amount of sulfoSMCC covalently linked to streptavidin (43). The same modification with avidin resulted in the formation of an insoluble derivative. Mixing the biotinylated S-dC$_{28}$ with the streptavidin−peptide conjugate produced a stable complex because of the extremely strong binding affinity of streptavidin for biotin (29). Since streptavidin possesses four binding sites for biotin the complex should contain on the average one molecule of fusogenic peptide for each oligonucleotide. This is true under the unproven assumption that the introduction of sulfoSMCC did not inactivated some biotin binding sites and no nonspecific binding of peptide or oligonucleotide occurred.

At a later stage of this research we synthesized 5′ thiol linker modified S-dC$_{28}$, which could be coupled to the peptide via a disulfide bond.

Finally a fusogenic peptide was attached to a TAT oligonucleotide through a thioether bond (TATB-SMCC−peptide), which was obtained by introducing a maleimide functional group on a 5′ amino linker modified TATB molecule via sulfoSMCC.

Liposome leakage assay

In order to evaluate the membrane destabilization properties of the peptide and its oligonucleotide conjugate, we used a liposome leakage assay (30). It involved the measurement of the release of calcein (a fluorescent dye) from egg yolk phosphatidylcholine liposomes (Fig. 2).

As expected the fusogenic peptide caused leakage, which increased at acidic pH. The peptide-anti TAT oligonucleotide conjugate showed the same pH dependent profile but with a

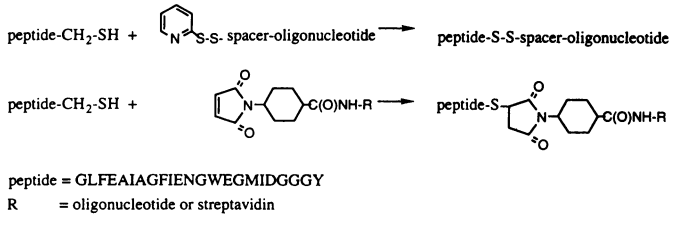

Figure 1. Reaction scheme for the chemical coupling of the fusogenic peptide to its target molecule.

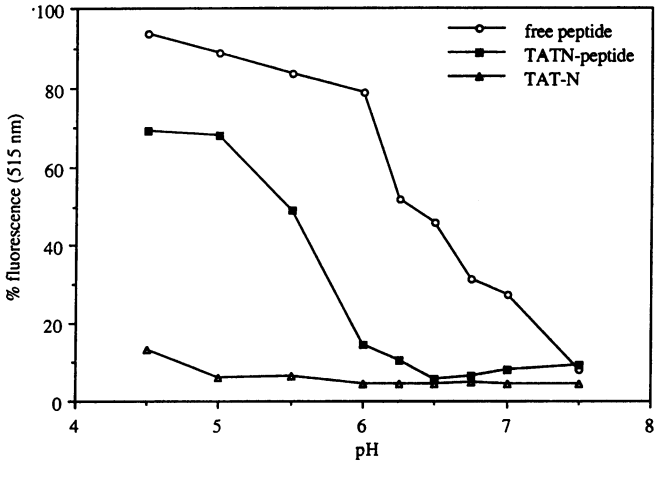

Figure 2. Destabilisation of calcein-filled egg yolk phosphatidylcholine liposomes (mean size 250 nm) after addition of 0.2 μM fusogenic peptide, 0.2 μM TAT-N-fusogenic peptide conjugate or 0.2 μM TAT-N oligonucleotide and incubation at room temperature for 20 minutes (see methods).

reduced destabilization as compared to the free peptide. The free oligonucleotide did not destabilize the liposomes significantly. Interestingly the pH dependency was stronger for the conjugate than for the isolated peptide. Releasing the peptide by cleavage of the disulfide bond of the conjugate with a large excess of DTT did not improve liposome destabilization (data not shown).

Pretreatment of a partially dimerized peptide stock solution with an excess DTT was necessary to observe a pronounced pH dependency of calcein release. This suggested that peptide dimers (oxidized) were more destabilizing than the monomers (reduced) especially at neutral pH. This observation has recently been confirmed by Plank *et al.* in an identical liposome leakage assay as well as in an erythrocyte lysis experiment (20).

Calcein loaded liposomes of different sizes (100−800 nm) but of the same lipid composition showed similar pH dependent leakage curves although the extent of the leakage differred. As predicted, the smaller liposomes were more unstable in the presence of the fusogenic peptide than the larger ones (data not shown).

Fluorescence microscopy

Subsequently we verified by fluorescence microscopy if peptide conjugation would induce a destabilization of acidic compartments in intact cells. These experiments were performed in BHK 21 fibroblasts , which are easier to manipulate than T lymphocytes; they are adherent, more resistant and possess better observable cytoplasm and nucleus. In keeping with our observations on liposomes, the rhodamine labelled TATB oligonucleotide showed a more punctate distribution than its peptide conjugate (Figure 3). However the difference is not very striking and needed repeated observations for confirmation. The same experiment was repeated at a 1 μM concentration with identical results except for a lower fluorescence signal. Likewise increasing the incubation time from 1 to 3 hours at 37°C did not significantly alter the overall pattern. When maintaining the cells at 4°C very little intra-cellular uptake of the oligo−peptide conjugate was observed and none of the free oligonucleotide. At 37°C in the presence of 50 μM chloroquine large vacuoles were formed, containing a lot of fluorescence, which masked the differences

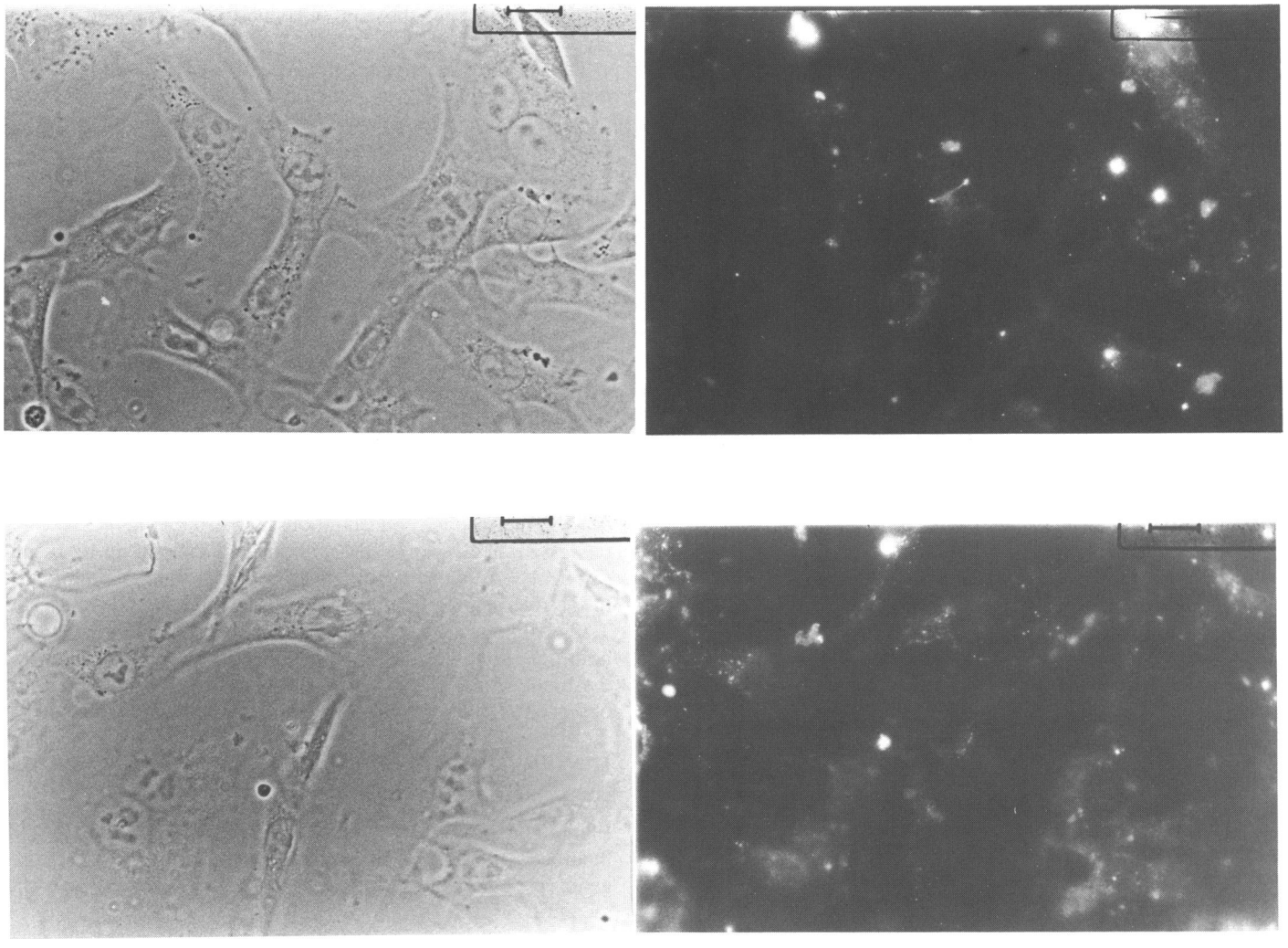

Figure 3. Distribution of 5-carboxymethylrhodamine labelled TATB and the TATB−peptide conjugate viewed after an incubation of BHK fibroblasts with 5 μM compounds at 37°C for one hour as described in methods. Images were taken at identical settings. On the left cells viewed under contrast-enhanced visible light, on the right fluorescence images. Top row TATB-rhodamine, bottom row TATB-rhodamine−peptide. Bar indicates 16 μm.

in distribution of the oligonucleotide and its peptide adduct. No free rhodamine dye was taken up by the cells in any of the experiments. Akhtar *et al.* reported similarly a punctate versus more diffuse intra-cellular distribution of methylphosphonate antisense oligonucleotides after addition of non-toxic doses of unconjugated Influenza derived fusogenic peptide (44).

Antiviral activity and cytotoxity of the anti TAT−peptide conjugates

The antiviral activity of the conjugates was evaluated in vitro on CEM-SS lymphocytes infected *de novo* by HIV-1 (strain LAI). The cells were preferably grown under serum-free conditions in the AIM medium (Gibco), which generates more reproducible results. Commercial fetal bovine serum possesses batch to batch variability in terms of protein content. Protease and nuclease activities in particular might be critical and cannot be reproducibly controlled even by heat treatment. For comparison purposes some experiments were run in parallel on cells incubated in RPMI medium supplemented with 10% (v/v) fetal bovine serum. The antiviral activities were determined by the RT activity measurement and the cellular toxicity on infected or non-infected cells was evaluated by the MTT test.

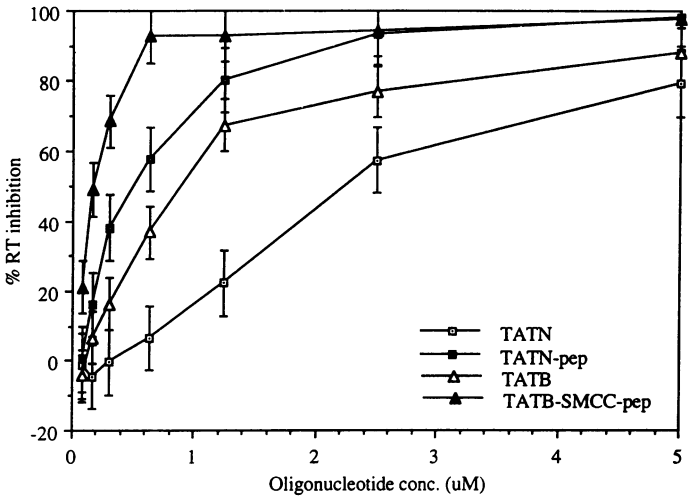

Figure 4. Measurement of the HIV inhibition produced by the anti TAT oligonucleotides and their fusogenic peptide conjugates on *de novo* infected CEM-SS lymphocytes. Error bars of ± one standard deviation are supplied. The cells were grown either in serum-free medium (Gibco AIM) (see methods).

Table 1. IC_{50} and CC_{50} values of the oligonucleotides and their peptide conjugates obtained on HIV infected CEM SS lymphocytes grown in AIM or RPMI + 10% FBS

Compound	IC_{50} (AIM) (μM)	IC_{50} (RPMI) (μM)	CC_{50} (AIM) (μM)	CC_{50} (RPMI) (μM)
TATN (3′ amino, 5′ thiol)	2.1	4.5	>10	>10
TATB (3′ amino, 5′ thiol)	2.9	10	>10	>10
TATN−pep	0.5	2.9	>10	>10
TATB−pep	0.2	1.5	>10	>10
TATB (3′ ribose, 5′ amino)	1.0	2.5	>10	>10
TATB-SMCC−pep	0.2	0.9	4.4	>5
S-dC_{28} (5′ biotinyl)	0.0015	0.016	>1	>1
Strep−pep-S-dC_{28}	0.0009	0.004	3.0	>1
S-dC_{28} (5′ thiol)	0.00008	0.002	>1	>1
S-dC_{28}−pep	0.00008	0.002	>1	>1

Figure 4 shows the dose-response curve of the TATN−pep and TATB-SMCC−pep conjugates versus the unmodified TATN and TATB oligonucleotides in AIM cell culture medium. In both cases the peptide coupled compound exhibited an increased antiviral activity as measured by the RT assay. In addition only the conjugate was able to inhibit HIV proliferation completely. Higher IC_{50} values were observed when cells were grown in the presence of serum but the relative improvement obtained by the conjugation of the fusogenic peptide remained similar (Table 1). The analysis of variance showed that the difference of antiviral activity between the coupled- and uncoupled oligonucleotide is highly significant in both culture media.

No sequence specific effects were observed because the random oligomer TATB and the coupled product TATB−peptide produced virtually identical biological activities as the antisense analogs when tested at the same time (Table 1). Analogous data have been published for both phosphorothioate (6) and phosphodiester (38) antisense oligonucleotides in acutely infected cells.

The MTT test revealed no toxicity of the peptide and its disulfide conjugate at concentration lower than 10 μM, on infected or uninfected cells grown either in AIM or in RPMI supplemented with 10% FBS (data not shown).

Repeated tests of TATB-SMCC−peptide revealed cytotoxic effects in AIM medium above 1 μM doses (CC_{50}: 4 μM) but none in serum supplemented RPMI. However the effective antiviral doses were lower (Table 1). Several hypotheses could explain this observation (modified cytoplasmic distribution, toxic metabolites) but we cannot support them by experimental data.

The importance of the covalent attachment of the peptide to the oligonucleotide is illustrated in Figure 5 as compared to Figure 4. By simply mixing the two molecules under serum-free conditions, at least a eightfold excess of the uncoupled peptide relative to the oligonucleotide had to be present in order to obtain the same antiviral activity as the conjugate at 1 μM. Moreover the antiviral effects of the peptide alone became predominant at those higher peptide concentrations. In AIM medium the peptide possessed an IC_{50} of 6 μM without any toxicity up to 10 μM (Table 1). A statistical comparison between the RT inhibition of the free peptide versus the mixture of free peptide and 1 μM TATN oligonucleotide showed no significant difference between both data sets. The contribution of the free oligonucleotide to the biological activity at 1 μM is therefore negligible and the observed HIV inhibition is entirely due to the peptide.

HIV inhibition and cytotoxity of the S-dC_{28}−peptide conjugates

Figure 6 shows the dose-response curve of the 5′ biotin or thiol modified S-dC_{28} phosphorothioate versus the streptavidin−fusogenic peptide-S-dC_{28} and S-dC_{28}−peptide adducts in AIM medium.

The 5′ thiol modified S-dC_{28} and its peptide conjugate were more potent than its biotinylated counterparts. This could be explained by a higher proportion of full length S-dC_{28} in the 5′ thiol modified S-dC_{28} produced by Genset as compared to the biotinylated compound synthesized in our laboratory. Studies by

Matsukura *et al.* have indeed shown a continuous increase of anti HIV potency of S-dC_n with n = 14, 18, 21 and 28 as n increases (41).

A statistically significant difference exists between the free biotinylated oligonucleotide and its streptavidin−peptide adduct in both AIM and RPMI + FBS but none between S-dC_{28}−peptide and 5′ thiol modified S-dC_{28}. However the concentration of the Strep−pep-S-dC_{28} complex was plotted as such and not its equivalent amount of S-dC_{28}. We calculated the absorbance of the Strep−pep-S-dC_{28} adduct on the assumption of a content of 4 molecules of phosphorothioate per conjugate. When normalizing the data to equivalent amounts of S-dC_{28}, the RT activity of the streptavidin conjugate compared to the free oligonucleotide would be severalfold lower and the difference would be cancelled. As observed on the phosphodiester compounds, the presence of serum reduced the potency of all the products several-fold.

We incubated 0.1 nM S-dC_{28} in the presence of increasing amounts of free peptide under serum-free conditions (Fig. 5). In the absence of the peptide less than 10% inhibition was observed. The inhibition was only doubled after the addition of 0.5 μM free peptide and about 2 μM was necessary to obtain 50% inhibition. At higher peptide concentrations the inherent HIV inhibitory properties of the free peptide prevented a reliable interpretation. Even at those higher concentrations no complete HIV inhibition was observed.

We observed some toxicities while using the Streptavidin−peptide-S-dC_{28} adduct in AIM medium (CC_{50}: 3 μM), but those concentrations were far above the HIV inhibitory concentrations (nanomolar range).

DISCUSSION

These studies showed that fusogenic peptides could be used as means for improving cellular delivery of oligonucleotides.

The liposome leakage measurements confirmed the pH dependence of the membrane destabilizing properties of the fusogenic peptide. The fluorescence microscopy observations on BHK 21 fibroblasts suggest an enhanced endosomal efflux of an oligo−peptide conjugate resulting in a more diffuse intra-cellular distribution of the rhodamine labelled antisense oligonucleotide in keeping with the cell-free observation.

The covalent attachment of the peptide to the oligonucleotide could enhance its resistance to enzymatic degradation, which would in turn result in an improved antiviral effect. Although we cannot rule out this possibility we assume that this should only be a minor contribution. Studies have indeed shown that the phosphodiester type oligodeoxynucleotides are mainly degraded through 3′ exonuclease activities present in the serum. The fusogenic peptide was coupled at the 5′ end of the oligonucleotide and therefore cannot protect the conjugate against 3′ exonucleases. Furthermore the presence of serum compared to serum-free conditions, did not alter significantly the relative contribution of the peptide coupling to the biological activity of the oligonucleotide.

The chemical coupling of the peptide to the antisense oligonucleotides in a one to one ratio, improved the potency of the phosphodiester oligomer 3 to tenfold as compared to the unmodified nucleotide depending on the batches of biological assays. However at these micromolar concentrations no specific antiviral effects were observed as expected from previous studies.

We and others have shown that only very efficient delivery systems such as the conjugation of antisense oligonucleotides to polylysine (38) or encapsulation in antibody-targeted liposomes (5, 6) produced sequence specificities in *de novo* HIV infected cells.

The S-dC_{28} phosphorothioate conjugates have a similar activity as an equivalent amount of free oligonucleotide. The phosphorothioate oligonucleotides were already extremely active (nanomolar range) in our assay. As a consequence the dose of the peptide might be too low to destabilize an endosomal membrane.

In order to maximize endosomal destabilization, a minimal concentration of the fusogenic peptide had to be present in the endosomal compartment. When the oligonucleotide was simply

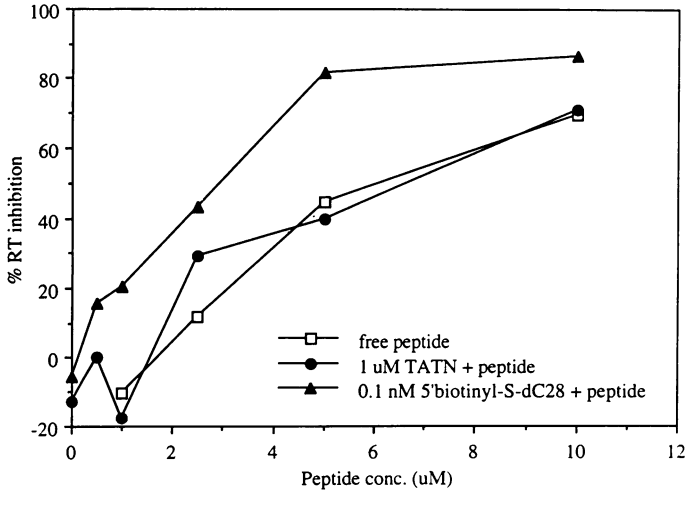

Figure 5. Measurement of the HIV inhibition produced by the fusogenic peptide added to a fixed amount of oligonucleotide as determined by the reverse transcriptase assay in *de novo* infected CEM-SS lymphocytes. The cells were grown under serum-free conditions (Gibco AIM medium).

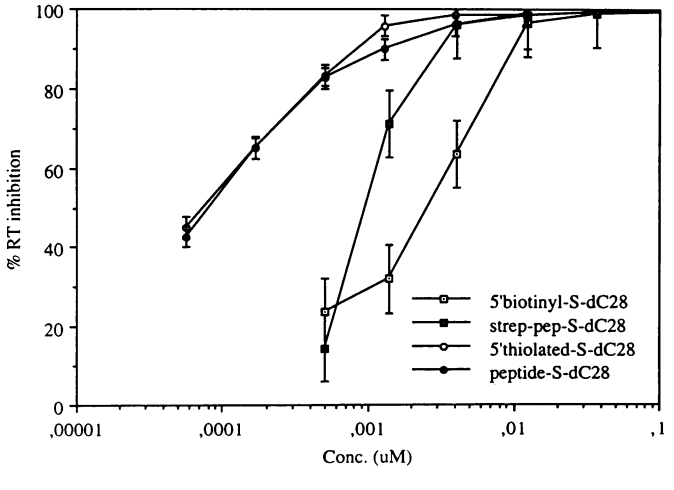

Figure 6. Measurement of the HIV inhibition produced by the S-dC_{28} oligophosphorothioates and their peptide adduct on *de novo* infected CEM-SS lymphocytes grown under serum-free conditions (Gibco AIM). The error bars represent ± one standard deviation (see methods).

mixed with the peptide in the cell culture, the minimal peptide concentration for a beneficial biological effect was around 1 μM. This was valid for both the phosphodiester and phosphorothioate oligonucleotides, which inherently possess quite different antiviral potencies. Since in our system the free peptide possessed inhibitory properties against HIV *de novo* infected cells (IC_{50}: 6 μM), the results are difficult to interpret at concentrations of several μM.

Enhancing the stability of the covalent linkage between the peptide and oligonucleotide moiety did not improve the biological results. Indeed coupling of the peptide to a 5′ aminolink, 3′ ribose derived anti TAT oligomer via a thioether bond provided by the heterobifunctional linker sulfoSMCC gave similar RT inhibition values as the disulfide bridged conjugate. In addition the sulfoSMCC linked conjugate induced some cytotoxicity above 1 μM under serum free conditions for some unknown reasons. However the fusogenic peptide, the TAT oligonucleotides and their disulfide bridged conjugate showed no toxicity on the CEM-SS cells grown under serum-free conditions up to 10 μM.

We are currently investigating several strategies to increase the effective concentration of the peptide relative to the oligonucleotide in order to boost further the cytoplasmic delivery of antisense compounds. Other membrane destabilizing peptides are also evaluated.

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REFERENCES

1. Crooke S.T. and Lebleu B. (1993) Antisense Research and Applications CRC Press, Boca Raton.
2. Clarenc J.P., Degols G., Leonetti J.P., Milhaud P. and Lebleu B. (1993) Anti-Cancer Drug Design, 8, 81–94.
3. Akhtar S., Basu S., Wickstrom E. and Juliano R.L. (1991) Nucleic Acids Res,. 19, 5551–5559.
4. Leonetti J.-P., Degols G. and Lebleu B. (1990) Bioconj. Chem., 1, 149–153.
5. Leonetti J.-P., Machy P., Degols G., Lebleu B. and Leserman L. (1990) Proc. Natl. Acad. Sci. U.S.A., 87, 2448–2451.
6. Zelphati O., Zon G. and Leserman L. (1993) Antisense Research and Development, 3, 323–338.
7. Rajaonarivony M., Vauthier C., Couarraze G., Puisieux F. and Couvreur P. (1993) J. of Pharmaceutical Sciences, 82, 912–917.
8. Haensler J. and Szoka F.C. (1993) Bioconj. Chem., 4, 372–379.
9. Felgner P.L., Gadek T.R., Holm M., Roman R., Chan H.W., Wenz M., Northrop J.P., Ringold G.M. and Danielsen M. (1987) Proc. Natl. Acad. Sci. U.S.A., 84, 7413–7417.
10. Capaccioli S., Di Pasquale G., Mini E., Mazzei T. and Quattrone A. (1993) Biochem. Biophys. Res. Commun., 197, 818–825
11. Boutorine A.S. and Kostina E.V. (1993) Biochimie, 75, 35–41.
12. Zhu N., Liggitt D., Liu Y. and Debs R. (1993) Science, 261, 209–211.
13. Compagnon B., Milhaud P. G., Bienvenüe A. and Philippot J. R. (1992) Experimental Cell Research, 200, 333–338.
14. Morishita R., Gibbons G. H., Ellison K. E., Nakajima M., Zhang L., Kaneda Y., Ogihara T. and Dzau V. J. (1993) Proc. Natl. Acad. Sci., 90, 8474–8478.
15. Curiel D.T., Agrawal S., Wagner E. and Cotten M. (1991) Proc. Natl. Acad. Sci. U.S.A., 88, 8850–8854.
16. Cotten M., Wagner E., Zatloukal K., Phillips S., Curiel D.T. and Birnstiel M.L. (1992) Proc. Natl. Acad. Sci. U.S.A., 89, 6094–6098.
17. Wagner E., Zatloukal K., Cotten M., Kirlappos H., Mechtler K., Curiel D.T. and Birnstiel M.L. (1992) Proc. Natl. Acad. Sci. U.S.A., 89, 6099–6103.
18. Cristiano R.J., Smith L.C. and Woo S.L.C. (1993) Proc. Natl. Acad. Sci. U.S.A., 90, 2122–2126.
19. Wagner E., Plank C., Zatloukal K., Cotten M. and Birnstiel M.L. (1992) Proc. Natl. Acad. Sci. U.S.A., 89, 7934–7938.
20. Plank C., Oberhauser B., Mechtler K., Koch C. and Wagner E. (1994) J. Biol. Chem., 269, 12918–12924.
21. Midoux P., Mendes C., Legrand A., Raimond J., Mayer R., Monsigny M. and Roche A.C. (1993) Nucleic Acids Res., 21, 871–878.
22. Kamata H., Yagisawa H., Takahashi S.and Hirata H. (1994) Nucleic Acids Res., 22, 536–537.
23. Murata M., Sugahara Y., Takahashi S. and Ohnishi S.I. (1987) J. Biochem., 102, 957–962.
24. Murata M., Takahashi S., Kagiwada S., Suzuki A. and Ohnishi S.I. (1992) Biochemistry, 31, 1986–1992.
25. Düzgünes N. and Shavnin S.A. (1992) J. Membrane Biol., 128, 71–80.
26. Bullough P.A., Hughson F.M., Shekel J.J., Wiley D.C. (1994) Nature, 371, 37–43.
27. Eckstein F. (1991) Oligonucleotides and Analogues, A Practical Approach, Oxford University Press, NY, pp. 13–14.
28. Sambrook J., Fritsch E.F. and Maniatis T. (1989) Molecular Cloning: A Laboratory Manual 2 nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 11.21.
29. Green N.M. (1990) Meth. Enzymology, 184, pp. 55–67.
30. Bondeson J., Wijkander J. and Sundler R.(1984) Biochim. Biophys. Acta, 777, 21–27.
31. Philippot J.R., Mutafschief S., Liautard J.P. (1983) Biochim. Biophys. Acta, 734, 137–143.
32. Rickwood D., Ford T. and Graham J. (1982) Anal. Biochem., 123, 23–31.
33. Gosselin G., Schinazi R.F., Sommasossi J.-P., Mathé C., Bergogne M.-C., Aubertin A.-M., Kirn A. and Imbach J.-L. (1994) Antimicrobial Agents and Chemotherapy, 38, 1292–1297.
34. Moog C., Wick A., Le Ber P., Kirn A. and Aubertin A.-M. (1994) Antiviral Research, 24, 275–288.
35. Mossman T.(1983) J. Immunol. Methods, 65, 55–63.
36. Snedecor G.W. and Cochran W.G. (1967) Statistical Methods 6th Ed., Iowa State University Press, Ames, Iowa, USA.
37. Goodchild J., Agrawal S., Civeira M.P., Sarin P.S., Sun D. and Zamecnik P.C. (1988) Proc. Natl. Acad. Sci. U.S.A., 85, 5507–5511.
38. Degols G., Leonetti J.P., Benkirane M., Devaux C. and Lebleu B. (1992) Antisense Research and Development, 2, 293–301.
39. Wickstrom E. (1986) J. Biochem. Biophys. Methods, 13, 97–102.
40. Akhtar S., Shoji Y. and Juliano R.L. (1992) In Erickson R.P. and Izant J.G. (eds.), Gene Regulation: Biology of Antisense RNA and DNA Raven Press, New York, pp. 133–145.
41. Matsukura M., Shinozuka K., Zon G., Mitsuya H., Reitz M. Cohen J.S. and Broder S. (1987) Proc. Natl. Acad. Sci. U.S.A., 84, 7706–7710.
42. Matsukura M., Zon G., Shinozuka K., Stein G.A., Mitsuya H., Cohen J.S. and Broder S. (1988) Gene, 72, 343–347.
43. Kitagawa T., Shimozono T., Aikawa T., Yoshida T. and Hishimura H. (1981) Chem. Pharm. Bull., 29, 1130–1135.
44. Akhtar S., Fisher M., Lentz B. and Juliano R.L. (1992) J. Pharm. Pharmacol., 44 (suppl.), 1046.