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Preclinical report

Inhibition of Ras p21 synthesis by antisense undecamers with uniform and specifically arranged phosphorothioate linkages

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The design of chimeric oligodeoxynucleotides (ODNs) in which certain phosphodiester linkages are replaced by phosphorothioate (PS) aims to decrease non-sequence-specific effects of uniform PS ODNs and to preserve the PS-provided protection against exo- and endonucleases. This study has, for the fist time, directly compared the differences in nuclease resistance, cellular uptake, antisense potency and sequence specificity of PS and end-capped, pyrimidine-protected (PPS) undecamer ODNs, that are complementary to the initiation codon region of human Ha-*ras* mRNA. At concentrations above 5 μM, both PS and PPS undecamers were moderately and equally stable for over 48 h in complete medium with RS485 cells overexpressing Ha-*ras*. They were completely stable at 0.4 μM when complexed with Lipofectin reagent that enhanced cellular uptake up to 9-fold. Both the antisense PPS and PS undecamers produced well-defined inhibition of Ras p21 synthesis in both cell-free and cell-based assays. However, non-sequence-specific effects of the uniform phosphorothioates were still significant. In contrast, the antisense PPS undecamer, when delivered to RS485 cells with Lipofectin reagent, inhibits human Ras p21 synthesis by more than 90% at a concentration of 3.2 μM, while the effect of controls with inverted, mismatched or scrambled sequence was minimal (5% or less) on p21 synthesis and RS485 cell growth. [© 2000 Lippincott Williams & Wilkins.]

Key words: Chimeric antisense oligonucleotides, phosphorothioate, Ras p21 inhibition, sequence specificity.

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Introduction

Antisense oligodeoxynucleoside phosphorothioates (PS ODNs) are the first nucleic acid (NA) drugs that have currently advanced to human clinical trials.[1,2] The main therapeutic advantages of phosphorothioates over hundreds of other NA analogs and modifications are due to PS ODNs resistance to nuclease digestion, both in biological fluids and inside live cells, and through their unique capability of eliciting RNase H hydrolysis of mRNA in complementary PS ODN:mRNA heteroduplexes.[3,4] On the other hand, substitution of a sulfur for one of the non-bridging oxygens in natural phosphodiester linkages produces diminished affinity of phosphorothioates to complementary NA and, in parallel, enchances affinity for proteins which generate numerous non-antisense and non-sequence-specific biological effects.[1,5] In addition, the products of PS ODNs degradation may be toxic.

These negative effects can be significantly decreased by appropriately combining natural and phosphorothioate internucleoside linkages within an antisense ODN structure. As previously shown,[6,7] two or three phosphorothioate linkages in a row at both the 3′ and 5′ ends can protect the ODN against exonucleolytic hydrolysis, especially in serum. To prolong the lifetime of antisense ODN in cells, the end capping should also be supplemented by protection of internal pyrimidine residues which are the major sites of degradation by endonucleases.[8,9] Obviously, larger ODNs will require more phosphorothioate linkages to provide nuclease resistance. Theoretically, a 17 meric (or larger) ODN might have a unique nucleotide sequence and only one complementary target per genome.[10] However, such a long ODN can form complexes of partial (incomplete) complementarity and may induce RNase H hydrolysis of many different mRNAs.[11] The antisense

potency of comparatively short ODNs has still not been fully explored. Early experimentation with inhibiting BALB-*ras* and Ha-*ras* expression by undecamers[12–15] or by an end-derivatized nonamer[16] remains the rare exception to the rule.

In addition to variations in antisense molecule length, content and position of phosphorothioate linkages, the use of cellular uptake enhancers can also help to minimize side effects. For example, ODNs may be made inaccessable for cellular surface proteins and extracellular nucleases via inclusion of partially or all-phosphorothioated (PPS and PS, respectively) ODNs into liposomes, or via capturing the oligomers negative charge by carrier polycations or by lypophilic cations.[17–20]

Taking the above factors into consideration, we compared the ability of the PS and minimally protected PPS undecamers, either free or Lipofectin-bound, to inhibit Ras p21 synthesis in RS485 cells overexpressing this protein. The initiation codon region ...5′ AUG ACG GAA UA 3′... of human Ha-*ras* mRNA was targeted in the study for a number of reasons. This region was predicted to occur in a large bulge of the mRNA secondary structure,[21] a factor that may significantly increase specificity and efficacy for short antisense ODNs.[22] In a cell culture system, we have already shown that the complementing methylphosphonate undecamer inhibited Ha-*ras* p21 expression by 90% at a concentration of 50 μM.[15] Using a cell-free translation assay and a set of backbone-modified undecamers directed against the same region in BALB-*ras* mRNA, we found that at relatively low (12.5–25 μM) concentrations, the phosphorothioate ODN was a more potent inhibitor of p21 protein synthesis than the methylphosphonate ODN. However, at higher concentrations, a non-sequence-specific effect for the phosphorothioate was dominant.[12] Therefore, Ha-*ras* mRNA translation seemed to be an appropriate and sensitive experimental model for the comparison of antisense specificity and potency of the short PS and PPS ODNs.

Using both cell-free and cell-based translation assays and a complete set of control ODNs, we demonstrated in this paper that sequence specificity is highest for the antisense undecamer modified as an end-capped, pyrimidine-protected partial phosphorothioate. When antisense PPS undecamer is delivered to RS485 cells with Lipofectin, Ras p21 synthesis is inhibited by more than 90% at a concentration of 3.2 μM.

Table 1. Oligonucleotides used in this study

Sequence	Description abbreviation
All-phosphorothioated ODNs	PS
5′-TsAsTsTsCsCsGsTsCsAsT-3′	antisense, AS PS
AsTsGsAsCsGsGsGsAsAsTsA	sense, S PS
Ts**T**s**A**sTs**A**sCsGsTsCs**C**sT	mismatch, M PS
AsTsCsTsTsAsCsGsTsTsC	scrambled, SC PS
TsAsCsTsGsCsCsTsTsAsT	inverted, I PS
Partially phosphorothioated ODNs	PPS
5′-TsAsToToCsCoGoTsCsAsT-3′	antisense, AS PPS
AsTsGoAoCsGoGoAsAsTsA	sense, S PPS
Ts**T**s**A**oTo**A**sCoGoTsCs**C**sT	mismatch, M PPS
AsTsCoToTsAoCoGsTsTsC	scrambled, SC PPS
TsAsCoToGsCoCoTsTsAsT	inverted, I PPS

s = phosphorothioate internucleoside linkage.
o = phosphodiester internucleoside linkage.
A bold letter denotes a mispaired base, relative to the *ras* p21 mRNA sequence.

Methods

Oligonucleotide synthesis

The ODNs, listed in Table 1, were synthesized by standard phosphoramidite chemistry on an Applied Biosystems 394 DNA synthesizer (Applied Biosystems, Foster City, CA) After coupling, phosphorothioate linkages were introduced by sulfurization using the Beaucage reagent[23] followed by capping with acetic anhydride and *N*-methylimidazole. After cleavage from the solid support and final deprotection by treatment with concentrated ammonia, all ODNs were purified by FPLC on a Mono Q high-performance column (10/10; Pharmacia Biotech, Uppsala, Sweden) using a Pharmacia Biopilot system. ODNs were eluted with the 90 min, 0.3–1.5 M NaCl gradient in 10 mM NaOH, pH 12, and desalted via a C18 column (Millipore, Bedford, MA). The ODNs homogeneity was confirmed by analytical HPLC on a Gen-Pak Fax column (Millipore-Waters, Bedford, MA). The calculated masses in all cases were confirmed by negative ion electrospray mass spectroscopy (Fisons Bio-Q, UK).

5′ End-phosphorylation of ODNs

5′ Phosphorylation of the ODNs was done using ATP or [γ-^{32}P]ATP, 6000 Ci/mmol (NEN, Boston, MA) and T4 polynucleotide kinase in a reaction buffer provided by New England Biolabs (Beverly, MA) as recommended.[24] Before phosphorylation, the ODN water solutions were heated at 95°C for 5 min and then immediately cooled on ice. After phosphorylation, the ODNs were purified by electrophoresis on a 19% polyacrylamide/urea gel and desalted on NAP-5 columns (Pharmacia Biotech). The [5′-^{32}P]ODNs, diluted appropriately by the corresponding 5′ phosphorylated ODNs, were used in the uptake study (10^7 c.p.m./well).

In the stability assay, ODNs incubated with RS485 cells were ^{32}P-post-labeled, and 10 μl of media was removed from cell culture, heat-denatured and labeled as above. The reaction mixtures were separated on 19% polyacrylamide/urea gels and bands visualized via autoradiograhpy using Biomax MS film (Kodak, Rochester, NY).

Preparation of ODN–Lipofectin complexes

ODN–Lipofectin complexes were prepared as recommended by the manufacturer using Lipofectin reagent (1 mg/ml; Life Technologies, Gaithersburg, MD), a 1:1 (w/w) mixture of *N*-[1-(2,3-dioleyloxy)propyl]-*n,n,n*-trymethylammonium chloride (DOTMA) and dioleyl phosphatidylethanolamine (DOPE). Briefly, 7 μl of Lipofectin was diluted into 100 μl DMEM and allowed to stand at room temperature for 30 min. The diluted Lipofectin was gently mixed with 100 μl of 4–32 μM ODN solution in DMEM and incubated for an additional 15 min. Finally, the mixture was combined with 800 μl of DMEM.

Cell culture

RS485 cells, an NIH 3T3 cell line containing multiple copies of a long terminal repeat-activated normal human Ha-*ras* gene,[25] were routinely maintained at 37°C, 5% CO_2, in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and 50 μg/ml each of penicillin, streptomycin and neomycin.

Oligonucleotide treatment

RS485 cells were subcultured in six-well plates (10^5 cells/well) and grown for 18–24 h. At 40–60% confluency, medium from each well was replaced by 1 ml of the fresh serum-supplemented DMEM containing the ODNs and the cells were cultured for 48 h.

In Lipofectin-mediated treatment, the subcultured RS485 cells were washed twice with serum and antibiotic-free DMEM, and then overlaid with 1 ml of DMEM containing an ODN–Lipofectin complex. After 6 h, 1 ml of DMEM supplemented with 4 mM L-glutamine and 20% FCS was added, and incubation was continued for an additional 42 h.

Preparation of cell lysates

Cells, washed twice or 5 times (in the uptake study) with phosphate-buffered saline (PBS), were overlaid with 0.5 ml of RIPA buffer and shook gently for 60 min at room temperature. RIPA buffer was composed of PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS. Total protein in the lysate was determined using the Pierce Micro BCA protein assay reagent (Pierce, Rockford, IL). In the uptake study, 100 μl of a lysate was mixed with a scintillation cocktail (Ultima Gold; Packard, Meriden, CT) and radioactivity was counted in an LS 7500 system (Beckman, Fullerton, CA).

In preparation for Western analysis, cells were washed with PBS, harvested with trypsin, collected after centrifugation at 1000 *g* for 7 min at 4°C and washed once more with cold PBS. The cells were resuspended in 50–100 μl of cold RIPA buffer, which contained 0.1 mg/ml PMSF, 30 μg/ml aprotinin and 1 mM sodium orthovanadate, and lysed for 20 min in an ice bath. Lysates were passed through a 21 gauge needle, incubated on ice for an additional 30 min and then centrifuged at 15 000 *g* for 20 min at 4°C. Protein concentrations of the supernatants were measured and the proteins stored at −80°C.

In vitro translation

Ha-*ras* mRNA used in the study was synthesized by *in vitro* run-off transcription of the expression vector pGEM4-Ha-*ras* and was purified via phenol–chloroform extraction followed by ethanol precipitation.[14] Prior to *in vitro* translation, mRNA and varying amounts of ODNs were annealed in a solution composed of 10 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$ and 25 mM KCl. *Escherichia coli* RNase H (Gibco/BRL, Gaithersburg, MD) was added, 20 U/ml, and the mixture incubated at 37°C for 1 h.

In vitro translation was performed in a rabbit reticulocyte lysate system from Promega (Madison, WI) according to the manufacturer's procedure. Each 10 μl of translation reaction mixture contained 0.06 μg of Ha-*ras* mRNA, ODN, 8 U RNasin, 0.8 mCi/ml [^{35}S]methionine, 20 μM amino acid mixture (minus methionine), 1 μM dithiothreitol and 3 μl nuclease-treated lysate. After 1 h incubation at 30°C, the mixture was treated with 10 μl RIPA buffer and placed in an ice bath for 20 min. Afterwards, 7 μl of 3× SDS sample buffer was added, the samples were heat-denatured, loaded (10 μl per lane) and separated by discontinuous electrophoresis on a 12.5% polyacrylamide–SDS gel.[24] The completed gel was fixed, dried and exposed to X-Omat AR film (Kodak) with an intensifying screen at −80°C. The relative amount of Ras p21 was estimated by densitometry of autoradiographs on a PDI (Huntington Station, NY) scanner,

model DNA 35, supported by PDI analysis software, Quantity One, version 2.0. The p21 level in the translation mixture without ODN was used as 100%.

Western blot analysis of Ras protein

Lysate samples, containing 40 μg of total protein, were denatured, separated on a 12.5% polyacrylamide–SDS gel and electroblotted onto Protran BA 85 nitrocellulose transfer membrane (Schleicher & Schuell, Keene, NH). To block non-specific binding, the membrane was incubated at room temperature for 1 h with 5% non-fat dry milk in 10 mM Tris–HCI buffer, pH 8.0, containing 150 mM NaCl and 0.05% Tween 20 (TBST). The blot was probed for 4 h with 0.1 μg/ml human Ras p21-directed primary antibody (C-20, rabbit polyclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA) and washed 3 times with TBST. Proteins were detected using 1:1000 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology), ECL Western blotting detection reagent and Hyperfilm ECL (Amersham, Piscataway, NJ). Films were scanned as described in the preceding section.

Results

Oligonucleotide design and modification

The sequences of PS and PPS ODNs used in this study are shown in Table 1. In the antisense PPS ODN, the phosphorothioate pattern was designed according to the 'minimal protection' strategy.[8] First, in order to render the ODN stable against 3′- and 5′-exonucleases it is capped by three and two phosphorothioate residues at the 3′- and 5′-ends, respectively. Secondly, an additional phosphorothioate linkage is placed between internal pyrimidine units since they are the major sites of degradation by endonucleases. Lastly, in order to avoid non-antisense effects, observed for all-phosphorothioates, no more than three phosphorothioate residues in a row were used. The positions of phosphorothioate linkages, as in antisense PPS undecamer, are also retained within the structures of several control PPS ODNs, having sense, mismatch, inverted and scrambled sequences (Table 1).

Inhibition of Ras p21 synthesis in an *in vitro* translation system

Results of the cell-free translation of mRNA in the presence of ODNs may be influenced by several factors. In particular, the antisense effect of ODNs containing phosphodiester, phosphorothioate linkages or both depends on the RNase H activity in the cell-free protein synthesizing.[26,27] In our study of methylphosphonates,[15] we have also noticed that the BALB-*ras* mRNA tertiary structure profoundly restricted interactions between the mRNA and the antisense ODNs. To avoid any uncertainty due to the above-mentioned factors, Ha-*ras* mRNA and ODNs were annealed in Mg^{2+}/K^{+}-containing buffer and treated by a limited amount of the *E. coli* RNase H1 before the translation assay.

The data, obtained in the rabbit reticulocyte system (Figure 1), show that both PS and PPS ODNs, that are complementary with the start codon and the downstream eight bases of Ha-*ras* mRNA, affect Ras p21 synthesis in a dose-dependent mode and provide complete inhibition of the synthesis at a concentration of 40 μM or below. However, the concentration dependences of the inhibitory effect appear different for these two ODNs. As shown in Figure 1, beginning at 18–20 μM the antisense PPS undecamer becomes more efficient than the PS counterpart. At the same concentration, the control sense PS ODN also begins to inhibit Ha-*ras* mRNA translation; at 40 μM, the inhibition reaches about 54% of p21 synthesis. In contrast, the control sense PPS ODN does not inhibit the synthesis at any of the concentrations tested (Figure 1).

Oligonucleotide stability in the presence of RS485 cells

In a cell culture system, the efficacy of antisense ODNs

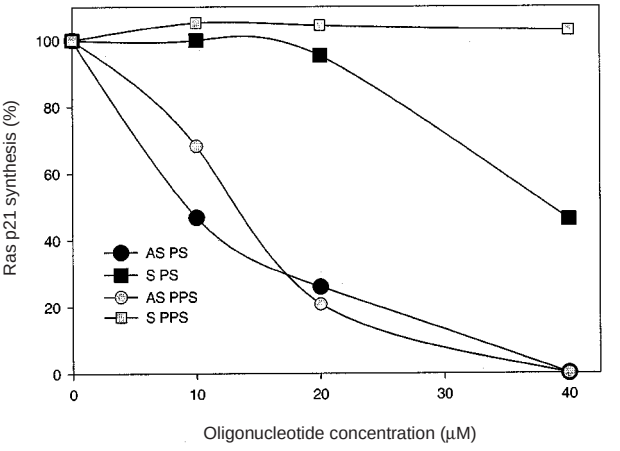

Figure 1. Effect of oligonucleotide concentrations on human Ras p21 expression in the rabbit reticulocyte lysate system. Before the translation assay, Ha-*ras* mRNA was annealed with antisense (AS) or sense (S) PS and PPS ODNs and then treated with the *E. coli* RNase H1.

correlates with their resistance to intracellular nucleases and to nucleases of the specifically used serum.[28–30] When exposed to 20% FCS alone, PS and end-capped, pyrimidine-protected PPS ODNs demonstrated similar stabilities: more than 60 and 50%, respectively, were undegraded in 48 h.[8] Since prolonged incubation of ODNs with RS485 cells in the complete medium could potentially change the extent of hydrolysis and hydrolysate patterns due to uptake and subsequent efflux of ODNs, we repeated the stability test using the postlabeling procedure as described in Methods.

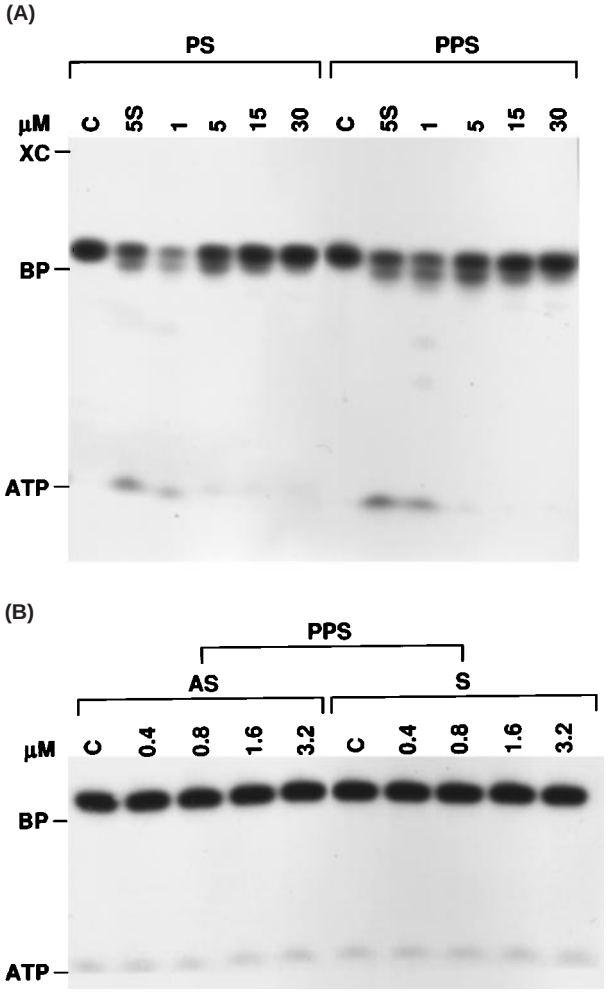

Figure 2. Autoradiographs of free (A) and Lipofectin-bound (B) ODNs incubated for 48 h at different concentrations in complete medium with RS485 cells. Antisense (AS) or sense (S) PS and PPS ODNs were ^{32}P-post-labeled and separated by electrophoresis in 19% polyacrylamide/urea gel. Lanes (C) and (5S) correspond respectively to control ODNs and to 5 μM ODNs incubated in complete medium without cells. Positions of xylene cyanole (XC), bromphenol blue (BP) and adenosine 5′-triphosphate (ATP) are also shown.

A visual inspection of the autoradiograph presented in Figure 2(A) leads to several conclusions. First, a concentration dependence of the extent of hydrolysis is clearly seen with both antisense PS and PPS undecamers; a concentration of 30 μM results in the practically complete inhibition of digestion of these ODNs. Secondly, at concentrations above 5 μM, the only detected product of the PS or PPS undecamers hydrolysis is a decamer (band beneath undecamer) that might correspond only to the 3′-exonuclease action. Third, the partial degradation of the ODNs in medium without RS485 cells (see lanes 5S in Figure 2A) shows quite clearly that the standard heating of FCS was not sufficient to denature the serum exonuclease(s).

Recent tissue culture studies have shown that the pathway of ODN complexed with cationic lipids differs from that of free ODN.[31] Upon internalization, the complex dissociates, and ODN leaves the endosomes and accumulates in the nucleus while the lipid remains in the endosomal compartment. Modifying the ODN intracellular distribution, the DOTMA/DOPE formulation, at 7 μg/ml, also completely protects the complexed ODNs, both PS or PPS, against exonuclease hydrolysis in the medium with RS485 cells. As shown in Figure 2(B), after 48 h of incubation there is no evidence of any degradation of the ODNs at a concentration of 0.4–3.2 μM, where the free ODNs were most vulnerable to hydrolysis.

Uptake study

The uptake study was performed using 5′-^{32}P-end-labeled undecamers at a single concentration of 5 μM. Cell-associated radioactivity was measured at 6 h, a time that corresponds to the end of RS485 cell lipofection in serum-free medium. This time frame exceeds by 3- to 6-fold the time usually needed to reach a plateau in the kinetics of uptake without uptake enhancers.[28,32,33] In complete medium,

Table 2. Characteristics of ODNs uptake by RS485 cells

ODN	Uptake (pmol/10^5 cells)	
	Without Lipofectin	With Lipofectin
Antisense PS	1.75±0.20	16.0±1.5
Antisense PPS	2.10±0.25	13.7±1.2
Sence PS	2.60±0.25	11.0±0.9
Scrambled PPS	2.70±0.30	17.0±1.5

[^{32}P]orthophosphate was not detected after 6 and 24 h incubation of free undecamers with RS485 cells (data not shown). This finding supports the uptake data presented in Table 2. Only after 48 h was radioactivity (0.26%) found in the orthophosphate fraction as isolated by gel electrophoresis and evaluated by liquid scintillation counting.

Levels of the cell-associated radioactivity indicate that RS485 cells preferentially bind all of the partially phosphorothioted undecamers tested (1.4 times, on average) over the antisense undecamer which has uniform phosphorothioate linkages (see the first column in Table 2). Also, the cells take up more sense PPS ODN (73% purines) than antisense PPS ODN (27% purines) not only at a concentration of 5 μM (Table 2) but over the entire 1.6–20 μM range (data not shown). As expected, Lipofectin enhances internalization of all tested undecamers but the extent of enhancement differs: 4.2- to 6.5-fold for PPS undecamers and 9.1-fold for antisense PS undecamer (Table 2).

Inhibition of RS485 cell growth and intracellular p21 synthesis by free ODNs

Ras p21 has been shown to be involved in signaling from transmembrane receptors, activating downstream Ras effectors leading to either cellular proliferation or differentiation.[34,35] The correlations observed in this study between effects of ODNs on intracellular human Ras p21 synthesis and on RS485 cell growth are shown in Figure 3. After the cells were treated with antisense and control PS and PPS ODNs for 48 h, p21 was detected by Western blot analysis using human Ras p21-directed antibody (Figure 3A). Cell growth was monitored by determining the total protein in cellular lysates. As established, total cellular protein (*Pr*, in μg) varies directly with the number of RS485 cells (*N*, in thousands): $Pr=0.439N$.

The data in Figure 3(B) provide evidence that antisense PS and PPS ODNs affect cell growth similarly at all tested concentrations. At 5 and 15 μM the decrease in total protein level is practically identical,

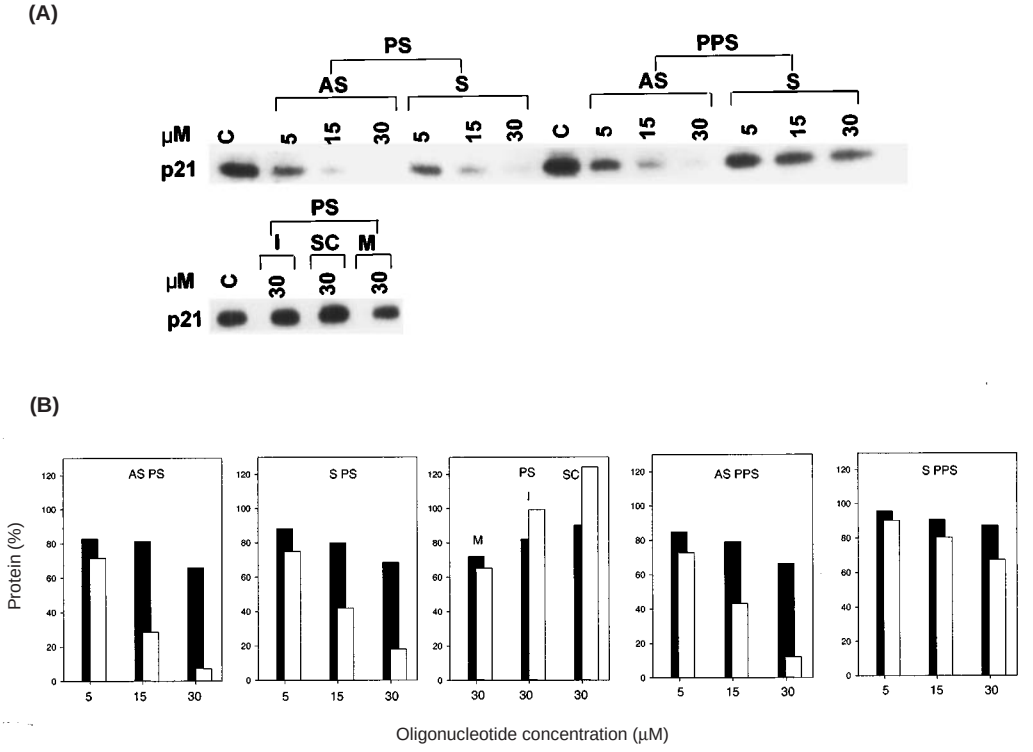

Figure 3. Effect of ODNs on intracellular Ras p21 synthesis (A and white bars in B) and on RS485 cell growth (black bars in B) as monitored by measurement of total protein. (A) represents results of Western blot analysis. The PS and PPS undecamers with antisense (AS), sense (S), mismatch (M), inverted (I) or scrambled (SC) sequence were incubated with RS485 cells for 48 h. At a fixed ODN concentration, three experiments were run in parallel in three separate wells. Before quantitation, lysates from the wells were combined to obtain an average value.

up to 82±3%, while p21 synthesis simultaniously diminishes from 72 to 28 and 43% with antisense PS and PPS ODNs, respectively. As the concentration of these ODNs increases to 30 μM, a 4-fold decrease of p21 synthesis is observed and cell growth is supressed up to 66%.

Like in the cell-free translation assay (Figure 1), sense PS ODN strongly inhibits the intracellular p21 synthesis (Figure 3B). The overall effect at 30 μM is 82% versus 93% specific for antisense PS ODN. In addition, the sense PPS ODN inhibitory effect (33% at 30 μM, Figure 3B), not detected by the cell-free translation assay, becomes detectable.

As seen in Figure 3(B), RS485 cells respond to treatment by all control PS ODNs at a concentration as high as 30 μM. Although containing four mismatches, the PS undecamer still causes a 35% inhibition of Ha-*ras* p21 expression and 28% inhibition of cell growth. Another PS undecamer, with an inverted sequence, has no effect on p21 synthesis, while a scrambled PS undecamer markedly stimulates p21 synthesis and minimally inhibits cell growth.

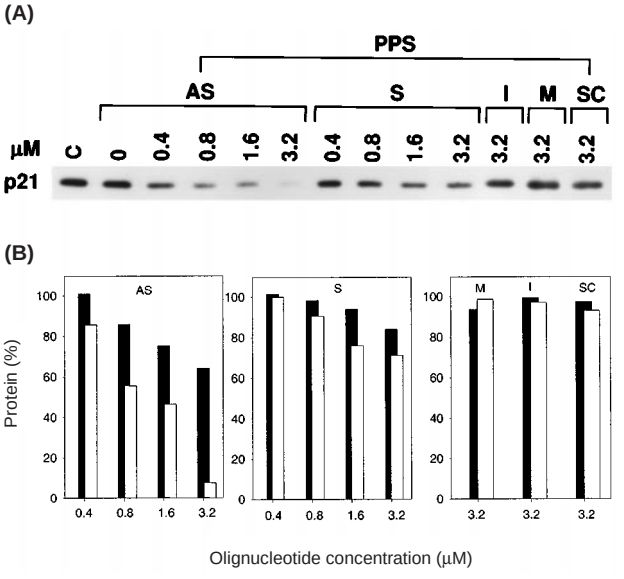

Figure 4. Effect of Lipofectin-bound PPS ODNs on intracellular Ras p21 synthesis (A and white bars in B) and on RS485 cell growth (black bars in B) as monitored by measurement of total protein. (A) represents results of Western blot analysis. The PPS undecamers with antisense (AS), sense (S), mismatch (M), inverted (I) or scrambled (SC) sequence were incubated with RS485 cells for 48 h. At a fixed ODN concentration, three experiments were run in parallel in three separate wells. Before quantitation, lysates from the wells were combined to obtain an average value.

Inhibition of RS485 cell growth and intracellular p21 synthesis by Lipofectin-bound ODNs

Cell growth was also shown to decrease when RS485 cells were treated with the antisense PPS ODN–Lipofectin complex (Figure 4). At concentrations of 0.8, 1.6 and 3.2 μM PPS antisense ODN, protein synthesis levels drop to 86, 75 and 64%, respectively (Figure 4B), as compared to 66% protein synthesis at 30 μM ODN concentration in the absence of Lipofectin (Figure 3B). The sense PPS ODN–Lipofectin complex has no effect on protein synthesis at concentrations up to 1.6 μM. Only when the sense ODN concentration reaches 3.2 μM does the protein synthesis level drop to 84% of control.

Figure 4 shows that when antisense PPS ODN is delivered to RS485 cells with Lipofectin, then p21 expression is inhibited by more than 90% at a concentration of 3.2 μM. In contrast, at this same ODN concentration, the sense PPS–Lipofectin complex shows only weak inhibition. We have examined different types of control oligonucleotides, protected as the antisense PPS ODN, with respect to their inhibitory effect at the highest concentration when complexed with Lipofectin. None of these control ODNs, inverted ODN, mismatch ODN or scrambled ODN, shows any noticeable inhibition at 3.2 μM (Figure 4).

Discussion

In the RNA pool consisting of 10^4 different mRNA species, a 17 mer, containing seven internal different and consecutive 11 mers, might match about 33 different sites of 11 consecutive bases since any given 11 mer is expected to occur about 4.8 times.[10] This illustration shows that increasing the length of an antisense oligonucleotide beyond the minimum length that can hybridize would be expected to decrease rather than increase its specificity in the RNase H-mediated degradation of an intended target mRNA. Knowing the critical antisense length is especially valuable when inhibition of the mutation-carrying gene is desirable without affecting expression of its normal counterpart, which is essential for cell survival. Experiments done[36] with unmodified ODNs suggest that dodecamers are capable of eliciting efficient RNase H cleavage of perfectly matched hybrids and little cleavage of mismatched hybrids. The discrimination was lost when longer ODNs (16 mers) were used.

Determination of the minimal length at which PS or PPS ODNs can still hybridize *in vivo* is not simplified by the measurement of melting temperatures (T_m) for corresponding hetroduplexes or duplexes under some model conditions. At a concentration of 9 μM in 0.1 M NaCl, pH 7, the antisense PS undecamer and the complementary non-modified undecamer form a duplex whose T_m is 24.5 ± 2.5°C.[12] Having 40% phosphodiester linkages, the antisense PPS undecamer should yeild a duplex with T_m higher than 24.5°C but still below 34°C, which is characteristic for a duplex formed by undecamers with all natural internucleoside linkages.[12] Thus, the profound effect of the antisense PS or PPS undecamers seen in the cell-based assay (37°C, Figures 3 and 4) and in the cell-free assay (30°C, Figure 1) seem to be produced at temperatures that are unfavorable for stable interaction of these ODNs and Ha-*ras* mRNA. However, the actual thermostability of the heteroduplexes in cell extracts or in live cells may be substantially higher due to biogenic polyamines (like spermine or spermidine) that are present at high concentrations in eukaryotic nuclei,[37] and have also been proven to stabilize duplexes and triplexes.[38,39]

Further, because of the involvement of RNase H in translation arrest, one needs to take into account some specific features of this enzyme. It has been shown recently that the *E. coli* RNase H1 not only digests an RNA strand in a preformed hybrid, but actively participates in the formation of hybrids using a matching deoxynucleotide fragment from the stable DNA hairpin or duplex.[40] There are also data indicating an inverse relationship between the thermostability of heteroduplexes and the rate of their RNase H hydrolysis.[41,42] It is thought that dissociation of the enzyme–product complex is a rate-limiting step in the overall reaction and that less stable heteroduplexes allow antisense molecules to act in a more catalytical manner.[43] Therefore, with the above considerations, there exists sufficient interest for performing trials using comparatively short antisense oligonucleotides.

Using a full set of control ODNs, we observed a strong inhibitory influence of sense PS undecamer on Ha-*ras* expression in the cell-free system (Figure 1), as well as in RS485 cells (Figure 3). In the latter case at 30 μM, the extent of inhibition (82%) is more than twice the inhibition caused by the mismatch control PS ODN, which contains four mismatched bases (Figure 3). In contrast, the sense PPS undecamer has no effect on Ha-*ras* mRNA translation in the cell-free protein synthesizing system (Figure 1) and it minimally affects Ras p21 synthesis in the more sensitive cell-based assay (Figures 3 and 4). Reproducing the functionally significant part of Ha-*ras* mRNA, sense ODN can inhibit translation by possibly competing with some component(s) of the 80S translation initiation complex in a non-antisense sequence-specific mode. Due to a uniform phosphorothioate stretch, sense PS ODN might have a higher affinity to component(s) than the corresponding PPS ODN. Whether this is the case or not, among controls the difference between the two sense ODNs (Figures 1, 3 and 4) is the largest difference observed in this comparative study of the PS and PPS undecamers, and this difference is in apparent favor of PPS ODNs.

As established, the nuclease resistance is practically identical for the PS and the minimally protected PPS 11 mers in medium with RS485 cells (Figure 2A). At relatively low ODN concentrations (15 μM or below), undecamers coexist in medium with decamers made by 3′-exonuclease(s) which are shown to be stereoselective with regard to phosphorothioate configuration.[44,45] Placing two to three phosphorothioate linkages with the S_p configuration at the 3′-end of ODN will prevent this type of degradation and may further improve the design of antisense PPS ODNs. In accordance with our previous observations,[8] we saw an increase in inhibition of serum nucleases at increasing concentrations of the ODNs (Figure 2A) and increased inhibition of intracellular Ras p21 synthesis (Figure 3). Being bound by Lipofectin, the antisense PS and PPS 11 mers remained intact at all tested concentrations in medium with RS485 cells even after 48 h of incubation (Figure 2B). Thus, unlike cellular uptake of free ODNs, Lipofectin-mediated uptake was not complicated by a heterogeneity of the ODNs in the medium.

Although taken up by cells in a slightly lower amount and having a decreased hybridization potential, the antisense PS undecamer produced a slightly higher level of Ras p21 inhibition than the PPS undecamer at concentrations up to 30 μM (Figure 3). Therefore, inside RS485 cells the PS ODNs seem to be more nuclease resistant than the PPS ODNs. Alternatively, a PS ODN, 'stuck' to an intracellular RNase H, may possibly direct the enzyme to a fully or partially matched target more effectively than the enzyme can by itself through a random search for a corresponding preformed hybrid. At least, in the case of the *E. coli* RNase H, covalently bound octa- and nonadeoxynucleotides were shown guiding the enzyme. Moreover, the turnover number was seen to be higher with the shorter ODN.[46] This suggestion might also explain non-sequence-specific effects of control PS ODNs (Figure 3) without contradicting the data of Gao *et al.*,[47] which demonstrated inhibition of human RNase H1 by phosphorothioates only longer than a 15 mer.

Lipofectin, effectively protecting undecamers in medium with RS485 cells, povided also on average a 6-fold increased association of the PPS 11 mers with the cells (Table 2). As a result, the Lipofectin-bound antisense PPS ODN at 3.2 μM caused the same 93% inhibition of Ras p21 synthesis as observed with free antisense PS ODN at 30 μM (Figures 3 and 4). This corresponds to a 10-fold increase in antisense efficiency. More significantly, the mean decrease in the p21 and total protein levels in cells treated with the scrambled, inverted or mismatched PPS ODN was less than 4% (Figure 4). It is worth noting that the 3.2 μM concentration of an 11 mer corresponds (when calculated in nucleotides) to 1.6 μM of a 22 mer. It is unlikely that a PPS 22 mer with 40% natural linkages will have the same endonuclease resistance as the minimally protected PPS undecamers used here. As reported by Monia *et al.*,[29] the PPS antisense 17 mer containing five consecutive natural linkages displayed moderate anti-*ras* activity only at the early time point (4 h), while the activity substantially diminished over time because of the loss of intact ODN inside cultured T24 cells.

In conclusion, this study showed that PS and PPS ODNs, as short as undecamers and complementary to the open secondary structure region in target mRNA, may produce a well-defined antisense effect in live cells despite instability of corresponding model complexes *in vitro*. However, even at the critical length, non-antisense effects of uniform phosphorothioates are still noticeable. The end-capped, pyrimidine-protected PPS ODNs represent a better alternative causing a minimum of non-sequence-specific effects and having sufficiently high nuclease resistance. The combined use of the short PPS ODNs and cationic lipids may have a therapeutic impact permitting delivery of intact antisense molecules into cells.

Conclusion

We have compared nuclease resistance, cellular uptake, sequence specificity and antisense potency of uniform phosphorothioated PS and partially phosphorothioated (end-capped and pyrimidine-protected) PPS ODNs against the first 11 bases in the initiation codon region of Ha-*ras* mRNA. Both the PS and PPS antisense ODNs inhibited p21 synthesis in the *in vitro* translation system. However, the PS sense control, but not the PPS sense control, showed non-sequence-specific inhibition of p21 synthesis.

The PPS ODNs are as resistant to nucleases as the PS ODNs in serum suplemented medium with RS485 cells at concentrations of 1–30 μM over 48 h. When complexed with Lipofectin reagent, PPS ODNs are completely stable in medium with RS485 cells and a cellular uptake increased an average of 6-fold.

Both antisense PS and PPS ODNs inhibit p21 synthesis intracellularly, but only the PPS ODN acts in a sequence-specific manner. By delivering the PPS antisense ODNs to the cells as a complex with Lipofectin reagent, its potency for sequence-specific inhibition is enhanced approximately 10-fold. This is demonstrated by the reduction in concentration of the antisense PPS ODN from 30 to 3.2 μM needed to achieve the same level (more than 90%) of inhibition of p21 synthesis. Control inverted, mismatched and scrambled PPS ODNs almost have no effect (about 5% inhibition) on p21 synthesis and RS485 growth.

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