Biochemical properties of phosphonoacetate and thiophosphonoacetate oligodeoxyribonucleotides

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ABSTRACT

Phosphorus-modified phosphonoacetate and thiophosphonoacetate oligodeoxyribonucleotides were chemically synthesized and their biochemical properties evaluated. Under physiological pH, these DNA analogs possess negative charge and form stable, complementary A-like DNA:RNA heteroduplexes when analyzed via circular dichroism spectroscopy. Phosphonoacetate and thiophosphonoacetate oligomers were found to stimulate RNase H activity and to be completely resistant to degradation by snake venom phosphodiesterase, DNase I and HeLa cell nuclear extract. Further research has demonstrated that neutral, esterified forms of these analogs can be taken up by cells. Phosphonoacetate and thiophosphonoacetate oligomers therefore represent a new class of oligodeoxyribonucleotide analogs having phosphorus–carbon bonds with considerable potential for use in biological research.

INTRODUCTION

Over the years, oligodeoxyribonucleotides (ODNs) and their analogs have become indispensable research tools for studying various biological processes, diagnosing diseases and identifying single nucleotide polymorphisms. More recently, modified ODNs have been used to modulate and control gene expression, which has broad implications from basic research to the use of these oligomers as therapeutic agents (1,2).

Most ODNs useful for modulating gene expression function by specifically inhibiting the production of a targeted gene product. Generally this process involves forming a duplex of mRNA with a complementary oligomer (hence antisense ODNs) followed by inhibition of mRNA expression or its degradation. Because natural DNA is inherently sensitive to cellular nucleases, a useful antisense ODN usually is modified so as to render it resistant to enzymatic degradation. In addition, an antisense ODN must generally activate RNase H which selectively degrades the mRNA strand of an ODN–mRNA duplex and thus eliminates expression of the targeted gene product (3). There are, however, certain nuclease-resistant antisense ODNs that effectively block gene expression by forming a stable hybrid with mRNA, which leads to inhibition of expression (4).

Among the many proposed antisense ODNs, the phosphorothioate and methylphosphonate derivatives have been the most widely studied (1,4). These analogs have either a sulfur atom or a methyl group in place of one non-bridging oxygen at each internucleotide linkage. Both are P-chiral but differ as the phosphorothioate derivative is isoelectronic with natural DNA whereas the methylphosphonate is electronically neutral. Relative to their use as antisense compounds, both have a combination of useful and undesirable features. For example, while phosphorothioate ODNs effectively stimulate RNase H activity and have increased resistance to cellular nucleases (when compared with natural DNA), they are still degraded (1,5,6). Additionally, phosphorothioate ODNs have relatively high affinity for certain proteins, which obscures the antisense mode of action and can lead to cellular toxicity (7). In contrast, methylphosphonate ODNs are stable towards nucleases but, when hybridized to RNA, they do not direct RNase H activity and therefore cannot inhibit gene expression by an RNase H-mediated mechanism (8). Loss of RNase H activity has been attributed to the lack of charge at the internucleotide linkage. Other analogs that possess RNase H activity and nuclease resistance also suffer from various limitations. These include phosphorothioate ODNs (6,9), cyclohexene nucleic acids (10) and 2′-fluoro-2′-deoxycytidinofuranosyl nucleic acids (11).

One of the requirements for RNase H activity is that the ODN be charged, which creates a problem since polyanionic oligomers do not readily diffuse across cell membranes. In order to overcome this problem for cultured cells, cationic lipids when combined with anionic ODNs are generally used to assist uptake. Unfortunately, this complex is generally toxic to cells, which means that both the exposure time and concentration of cationic lipid must be carefully controlled to insure transfection of viable cells.

Because none of the currently popular analogs satisfies all the criteria expected of an antisense ODN, we have continued searching for a superior derivative. These studies have led us to the phosphonoacetate- and thiophosphonoacetate-modified ODNs (Fig. 1) (12). These analogs, which have an acetic acid moiety in place of oxygen at a non-bridging position of an internucleotide linkage, were expected to possess many of the

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desirable antisense properties observed for phosphorothioate and methylphosphonate ODNs. Here we report our initial observations regarding their biophysical and biochemical properties. We have found that these analogs as heteroduplexes with RNA form A-type helices which have reduced melting temperatures (compared with natural heteroduplexes) and that the carboxylate moiety possesses a $pK_a$ of approximately $3.8$ (phosphoroacetate) and $3.9$ (thiophosphoroacetate). They were found to be nuclease resistant, capable of stimulating RNase H activity and, as neutral esters, to be taken up by cells via passive diffusion. The latter observation may lead to a new prodrug approach for uptake of RNase H-active ODNs by cells in culture.

**MATERIALS AND METHODS**

Phosphonoacetate and thiophosphonoacetate ODNs with the structures shown in Figure 1 were synthesized and characterized as described (12). ODNs with various combinations of phosphorothioate and phosphonate/thiophosphonate internucleotidic linkages were synthesized via the cycle shown in Figure 2. Appropriate synthons (standard deoxynucleoside 3'-phosphoramidites and deoxynucleoside 3'-O-diisopropylaminophosphonoacetic acid dimethyl-β-cyanoethyl esters) were introduced according to the synthetic plan in order to generate chimeric ODNs. Deoxynucleoside 3'-O-diisopropylaminophosphonoacetic acid dimethyl-β-cyanoethyl esters were prepared as described (12) or obtained from MetaSense Technologies (Des Moines, IA). Control ODNs (DNA, phosphorothioate DNA) were synthesized by solid phase phosphoramidite chemistry on controlled pore glass (CPG) (13) using an automated synthesizer (Applied Biosystems Model 394, Foster City, CA). Oligoribonucleotides and 2'-O-methyl oligoribonucleotides were obtained from Dharmacon Research Inc., Louisville, CO. 5'-32P-labeled oligomers were prepared by standard procedures (14) followed by purification using gel electrophoresis and ethanol precipitation.

Chimeric ODNs having phosphorothioate β-cyanoethyl esters and thiophosphonate methyl and butyl esters were synthesized on 3'-phosphate-CPG (Glen Research) using procedures as published previously (12). 5'-Fluorescein phosphoramidite (Glen Research) and standard sulfurization (13) were used to generate a 5'-fluorescein label joined to these ODNs through a nuclease-stable phosphorothioate linkage. To avoid hydrolysis of the acetate esters, it was necessary to remove the β-cyanoethyl group and cleave DNA from the support under non-nucleophilic conditions. Post-synthesis, the CPG for each ODN synthesis was transferred to an oven-dried 2.0 ml screw-cap vial. Diazabicyclo[5.4.0]-undec-7-ene (DBU, 1.5%) in anhydrous acetonitrile was then added to selectively remove the β-cyanoethyl group from phosphorothioate linkages and cleave the ODN from the support. After 30 min, the DBU was quenched with excess acetic acid (relative to DBU) and solvents were removed under reduced pressure. The sample was then diluted with triethylammonium acetate buffer (50 mM, pH 8.0) and filtered through a 0.45 μm microcentrifuge tube (Millipore). The buffer was then removed under reduced pressure, and the ODN was dissolved in 0.5 ml of water and purified by reverse phase HPLC utilizing the 5'-fluoroscein (FITC) group for hydrophobic affinity. Preparative HPLC was carried out on a 25 cm Hypersil BDS-C18pm column with a 9.4 mm ID. Gradient eluents were (A) 50 mM triethylammonium bicarbonate (pH 8.0) and (B) acetonitrile. A gradient of 8–90% B over 50 min at a flow rate of 1.2 ml/min was used to elute the ODN. Product fractions were pooled, concentrated under reduced pressure, and diluted in a buffer of 10 mM Tris–HCl pH 8.0 and 1 mM EDTA (TE buffer).

Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry was performed on a PerSeptive Biosystems Voyager Biospectrometry Workstation (Framingham, MA). ODNs were concentrated to dryness and dissolved in isopropanol/water (1:1) to a final concentration of 200 μM. Samples were prepared as described in the Sequenase Oligonucleotide Sequencing Kit Manual (PerSeptive Biosystems) with the following modifications: 1 μl of ODN, 1 μl of 25mer DNA standard, 1 μl of ammonium citrate buffer and 7 μl of matrix were combined on a layer of parafilm coated with ammonium cation exchange beads. After pipetting the solution over the beads for 60 s, 5 μl was transferred to a gold-plated 100-well sample plate. The MALDI-TOF measurements were observed in the positive-ion mode.

Ionization of phosphonoacetate and thiophosphonoacetate linkages was examined by monitoring HPLC retention time as a function of HPLC buffer pH for thymidine–thymidylate dimers. Analytical HPLC utilized a 1 ml Resource RPC Reverse-Phase Column (6.4 × 30 mm). Gradient eluents were (A) 100 mM sodium formate adjusted to the appropriate pH with formic acid and (B) acetonitrile. The following gradient was used to evaluate dimer retention time: 2–22% B in 30 min at a flow rate of 1.0 ml/min followed by a 10 min wash with 100% B. Dimer retention time was monitored using HPLC buffers at pH values between 1 and 8 (one unit increments). To achieve a buffer pH of 1, HCl was added to aqueous formic acid. A 20 μl aliquot of a 100 μM dimer solution (in water) was used per sample injection. Dimer retention time in minutes at a given pH was determined from the average of three separate chromatograms. Approximate $pK_a$ values were obtained from the midpoint of the titration curve for plots of retention time versus HPLC buffer pH. A dimer containing a natural phosphodiester linkage was used as a control.

Melting points ($T_m$) were determined on a Varian Cary 1E UV-Visible Spectrometer. The absorbance at 260 nm was

![Figure 1. Chemical structure of phosphonoacetate (X = O) and thiophosphonoacetate (X = S) ODNs. B represents adenine, guanine, cytosine or thymine.](https://academic.oup.com/nar/article-abstract/31/14/4109/2904333)
measured while the temperature of the sample was increased at a rate of 1.0°C/min. Phosphonoacetate, thiophosphonoacetate and phosphorothioate oligonucleotides were mixed separately with complementary RNA or DNA in a 1 ml cuvette and the T_m determined as the maximum of the first derivative of the melting curve. Control experiments were performed using unmodified DNA duplexes. Typical concentrations were 1 mM for each strand.

Circular dichroism (CD) spectra were recorded using a JASCO J-720 Spectropolarimeter. All CD experiments were performed at 25°C in 1 mm path length cuvettes with a buffer (pH 7.4) containing 25 mM sodium phosphate and 100 mM NaCl. The concentration was 10 μM for each strand. Prior to CD analysis, each sample was incubated briefly at 95°C, equilibrated to room temperature, chilled on ice for 30 min and finally re-equilibrated to 25°C.

Exonuclease digestion experiments were carried out using snake venom phosphodiesterase (SVP) I from Crotalus adamanteus (USB). The assays were performed using a mixture of 5'-32P-labeled ODN (100 000 c.p.m.) and unlabeled ODN (40 pmol) in a buffer (pH 8.5) containing 50 mM Tris–HCl, 72 mM NaCl and 14 mM MgCl_2. Enzyme was added to a final concentration of 1 U/ml (40 μl total reaction volume). The reaction mixture was overlaid with 25 μl of mineral oil and incubated at 37°C. Aliquots (3.5 μl) were removed, quenched by adding 12 μl of 7 M urea in TBE buffer, and heated to 95°C for 5 min. Samples were taken at 0, 1, 3, 7 and 18 h after the addition of enzyme. The reaction products were analyzed by PAGE (20% containing 7 M urea). Autoradiographic imaging was performed on a Molecular Dynamics Phosphorimager (Storm 820). Gel bands were quantitated using ImageQuant software (version 5.1).

Endonuclease digestion experiments were carried out using DNase I (Boehringer-Mannheim). The assays were performed using a mixture of 5'-32P-labeled ODN (100 000 c.p.m.) and unlabeled ODN (40 pmol) in a buffer (pH 7.5) containing 40 mM Tris–HCl and 6 mM MgCl_2. Enzyme was added to a final concentration of 1 U/ml (40 μl total reaction volume). The reaction mixture was overlaid with 25 μl of mineral oil and incubated at 37°C. Aliquots (3.5 μl) were removed, quenched by adding 12 μl of 7 M urea in TBE buffer, and heated to 95°C for 5 min. Samples were taken at 0, 1, 3, 7 and 18 h after the addition of enzyme. The reaction products were analyzed by PAGE (20% containing 7 M urea). Autoradiographic imaging was performed on a Molecular Dynamics Phosphorimager (Storm 820). Gel bands were quantitated using ImageQuant software (version 5.1).

Phosphonoacetate and thiophosphonoacetate ODNs were tested for stability toward HeLa cell nuclear extracts using the following assay. HeLa cell nuclear extracts were prepared by methods described previously (6). ODNs (500 pmol) in a buffer containing 50 mM Tris–HCl pH 8.0, 20 mM KCl, 10 mM MgCl_2 and 3 mM dithiothreitol (DTT) were incubated with 20 μg of HeLa cell nuclear extract (1 μl) at 37°C for 5 h (40 μl total volume). A second 20 μg aliquot of cell extract was added and the reaction proceeded for another 5 h. This step was repeated once more for a total of 15 h exposure to HeLa cell nuclear extract. The reaction was quenched by adding 3 vols of 8.3 M urea in TBE buffer and heating the samples at 95°C for 5 min. The reaction products were analyzed by ion-exchange HPLC using a 1 ml Resource Q Dynamics Phosphorimager (Storm 820). Gel bands were quantitated using ImageQuant software (version 5.1).

Figure 2. Synthesis cycle for phosphonoacetate and thiophosphonoacetate ODNs. The open circle represents controlled pore glass support; B is an appropriately protected purine or pyrimidine base (12).
column of 6.4 mm ID obtained from Amersham/Pharmacia. Gradient eluents were (A) 10 mM NaOH/80 mM NaBr and (B) 10 mM NaOH/1.5 M NaBr. The gradient was 0% A to 100% B in 45 min at a flow rate of 1.5 ml/min.

*Escherichia coli* RNase H1 kinetic experiments were done as follows. 5°-32P-labeled RNA (100 000 c.p.m/reaction), unlabeled RNA (100 pmol) and complementary ODN (100 pmol) were added to a buffer (pH 7.8) containing 20 mM HEPES-KOH, 50 mM KCl, 10 mM MgCl2 and 1 mM DTT. The duplexes were hybridized by heating briefly to 95°C and then incubated at 4°C for 30 min. *Escherichia coli* RNase H1 (Promega) was added (8 U) and the reactions were allowed to proceed for various times at 25°C (40 µl total reaction volume). Aliquots of the reaction mixture (3.5 µl) were quenched with 6.5 µl of 7 M urea and 20 mM EDTA and stored on ice until analysis by gel electrophoresis (20%, 19:1 cross-link). All reactions were performed in triplicate. Data were analyzed by KaleidaGraph using a linear curve fit.

Cell transfection experiments were performed as follows. SKBR-3 cells (American Type Culture Collection) were propagated in RPMI 1640 containing 10% (v/v) fetal bovine serum. Cells (100 000 per well) were plated onto 4-well chambered coverglass slides (Nalge Nunc) and grown over-night. Following removal of medium, transfections were carried out at 10 µM ODN in serum-free Opti-MEM. All cells were viewed live in culture medium or phosphate-buffered saline at various times on an Olympus IMT2-RFC reflected light fluorescence inverted microscope. Propidium iodide quenched with 6.5 µl of 7 M urea and 20 mM EDTA and stored on ice until analysis by gel electrophoresis (20%, 19:1 cross-link). All reactions were performed in triplicate. Data were analyzed by KaleidaGraph using a linear curve fit.

RESULTS

The chemical synthesis of phosphonoacetate and thiophosphonoacetate ODNs was carried out in the solid phase using the scheme outlined in Figure 2. Briefly, appropriately protected deoxynucleoside 3′-O-diisopropylaminophosphonoacetic acid dimethyl-β-cyanoethyl esters were condensed to a deoxynucleoside linked to CPG (12). The phosphonite linkage was then oxidized to phosphate using (1S)-(−)(10-camphorsulfonyl)-oxaziridine. Following capping with acetic anhydride and treatment with trichloroacetic acid to remove the 5′-O-dimethoxytrityl group, the cycle was repeated an appropriate number of times to yield the phosphonoacetate ODN. By substituting 3H-1,2-benzodithiol-3-one-1,1-dioxide (BDT) as the oxidant, a thiophosphonoacetate internucleotide linkage was generated. Using this approach, coupling yields of >97% were observed for oligomers up to 18 nucleotides in length. Isolated yields of 18mers (single peaks, ion exchange HPLC) were 32–33%. 31P-Nuclear magnetic resonance (NMR) analysis for these P-chiral oligomers gave single, broad peaks as expected at 28 and 94 p.p.m., respectively, for the phosphonoacetate and thiophosphonoacetate derivatives. The lack of any phosphate signal indicated that these oligomers were free of phosphate internucleotide linkages. Using the same synthesis cycle, chimeric ODNs containing phosphate, phosphorothioate and thiophosphoroacetate/thiophosphonoacetate internucleotide linkages were also prepared from standard deoxynucleoside phosphonoacetate/thiophosphoroacetate internucleotide linkages were all prepared from standard deoxynucleoside 3′-phosphoramidites and deoxyoligonucleotide 3′-O-diisopropylaminophosphonoacetic acid dimethyl-β-cyanoethyl esters. Mass characterization for phosphonoacetate, thiophosphonoacetate, chimeric ODNs and control ODNs used for various studies gave the expected results (Table 1).

For transfection experiments, oligodeoxythymidine 12mers were synthesized that had variable ratios of phosphorothioate and esterified thiophosphonoacetate internucleotide linkages. These oligomers were prepared using standard 5′-O-dimethoxytrityl deoxyoligonucleotide 3′-O-diisopropylaminophosphonoacetate/thiophosphoroacetate internucleotide linkages were all prepared from standard deoxynucleoside 3′-phosphoramidites and deoxyoligonucleotide 3′-O-diisopropylaminophosphonoacetic acid dimethyl-β-cyanoethyl esters. Mass characterization for phosphonoacetate, thiophosphonoacetate, chimeric ODNs and control ODNs used for various studies gave the expected results (Table 1).

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Structure</th>
<th>Calculated mass</th>
<th>Observed mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>d(CaTaCaAaAaGtaGtaGtaGtaGtaGtaGtaAaC)</td>
<td>6270.2</td>
<td>6271.3</td>
</tr>
<tr>
<td>ACE*</td>
<td>d(CaTaCaAaAaGtaGtaGtaGtaGtaGtaGtaGtaAaC)</td>
<td>6227.9</td>
<td>6228.8</td>
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<tr>
<td>S-ACE</td>
<td>d(CsTsAsAsGsTsGsGsCsTsGsGsTsGsAsC)</td>
<td>6542.1</td>
<td>6540.5</td>
</tr>
<tr>
<td>S-ACE*</td>
<td>d(CsTsAsAsGsTsGsGsCsTsGsGsTsGsAsC)</td>
<td>6525.9</td>
<td>6524.3</td>
</tr>
<tr>
<td>P</td>
<td>d(C-T-C-A-A-G-T-G-C-T-G-T-G-A-C)</td>
<td>5855.8</td>
<td>5555.1</td>
</tr>
<tr>
<td>ACE EO</td>
<td>d(CaTaCaAaGtaGtaGtaGtaGtaGtaGtaAaC)</td>
<td>5933.8</td>
<td>5933.1</td>
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<tr>
<td>S-ACE EO</td>
<td>d(CsTsAsAsGsTsGsGsCsTsGsGsTsGsAsC)</td>
<td>6205.8</td>
<td>6208.2</td>
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<tr>
<td>ACE Cap</td>
<td>d(CaTaCaAaA-A-G-T-G-G-C-T-G-GaTaGaAaC)</td>
<td>6036.2</td>
<td>6038.0</td>
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<tr>
<td>S-ACE Cap</td>
<td>d(CsTsAsAsA-G-T-G-G-C-T-G-GaTaGaAaC)</td>
<td>6163.8</td>
<td>6165.5</td>
</tr>
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</table>

*a*Nomenclature: a, phosphonoacetate; s, thiophosphonoacetate; -, phosphorothioate; the absence of a symbol between nucleoside letters corresponds to a normal phosphate linkage (i.e. CT).
dimers indicated an increase in hydrophobic character due to ionic charge neutralization. Approximate pK<sub>a</sub> values for the phosphonoacetate and thiophosphonoacetate linkages were 3.8 and 3.9, respectively. While only estimates, these values indicated that phosphonoacetate and thiophosphonoacetate ODNs were likely to be charged near physiological pH. As expected due to the presence of hydrophobic sulfur, the thiophosphonoacetate dimer had a greater retention time than the corresponding phosphonoacetate compound. This relative enhanced hydrophobicity remained irrespective of the dimer charge state.

Owing to the presence of a carbon−phosphorus bond, we expected the phosphonoacetate and thiophosphonoacetate ODNs to be nuclease resistant. In order to test this possibility, nuclease studies were completed using DNase I, SVP and HeLa cell nuclear extract. Attempts to monitor these studies using 5′-32P-labeled, fully modified ODNs was not possible as T4 kinase failed to phosphorylate these oligomers. With methylphosphonate DNA, similar problems were overcome by adding one phosphodiester linkage at the 5′ end of an oligomer (15). This proved to be a successful strategy for the phosphonoacetate and thiophosphonoacetate oligomers as well. This type of ODN was therefore synthesized, purified and characterized by 31P NMR and mass spectra (Table 1, ACE* and S-ACE*). Phosphorylation of these modified ODNs required a 4-fold excess of [γ-32P]ATP with respect to the conditions used for unmodified DNA.

When tested with SVP, these oligomers generated the profiles shown in Figure 4. Under conditions where natural DNA has undergone considerable degradation in 1 h and complete hydrolysis within 3 h (lanes 8 and 9), neither the phosphonoacetate nor the thiophosphonoacetate oligomers were degraded, even after 18 h (lanes 1−6 and 13−18, respectively). Lanes 19−24 show the hydrolysis products from incubation of phosphorothioate DNA with SVP. As expected from previous results (6), this analog degraded, but at a slower rate than natural DNA (65 and 40% full-length oligomer remains after 8 and 18 h, respectively). Chimeric ODNs when analyzed by HPLC were essentially resistant towards degradation by SVP. For example, oligomers with phosphonoacetate/phosphate (ACE EO) or thiophosphonoacetate/phosphate (S-ACE EO) at every other linkage were stable toward degradation. Similarly, phosphorothioate oligomers with the 3′ and 5′ ends capped with either phosphonoacetate (ACE Cap) or thiophosphonoacetate (S-ACE Cap) were also stable (see Table 1 for the sequences of these oligomers).

In order to characterize the stability of these ODNs towards endonucleases, the radiolabeled oligomers having one 5′-internucleotide phosphate linkage were incubated with DNase I under conditions similar to those used with SVP. Results are shown in Figure 5. Analysis of lanes containing phosphonoacetate (lanes 1−6) and thiophosphonoacetate (lanes 13−18) ODNs showed no degradation even after 18 h. Again, as expected from previous results (6), the...
phosphorothioate oligomer was partially degraded (78% full-length product remained after 18 h, lanes 19–24). Under these conditions, full-length natural DNA was degraded even after 1 h to a predicted series of smaller oligomers.

To further examine the stability of phosphonoacetate and thiophosphonoacetate ODNs towards nucleases, fully modified oligomers were incubated with HeLa cell nuclear extract and analyzed by ion-exchange HPLC (5′-32P-labeling could not be used for reaction product analysis because this extract was rich in phosphatases). Under conditions where the unmodified oligomer was completely degraded, both the phosphonoacetate and thiophosphonoacetate ODNs were unchanged and there were no degradation products (data not shown).

These results encouraged us to examine whether modified oligomers having phosphonoacetate and thiophosphonoacetate internucleotide linkages would form duplexes with natural, complementary DNA and RNA. This was important as our initial objective was to examine these modified oligomers with enzymes that required duplexes for biochemical activity (RNase H and DNA polymerases). Heteroduplexes were prepared using equimolar amounts of phosphonoacetate, thiophosphonoacetate and phosphorothioate ODNs in combination with an unmodified, complementary DNA or RNA strand. Natural duplexes with the same sequences were used as controls. Experiments with RNA–DNA duplexes 18 nucleotides in length were carried out with buffers of variable ionic strength and pH (Table 2). One of the buffers (buffer B) was also used for RNase H studies. As expected for oligomers with multiple P-chiral centers, the melting curves, although cooperative, were not as sharp as for natural DNA, but more similar to results observed previously with phosphorothioate DNA (3). The \( T_m \) values of some duplexes were lower than expected and had reduced stability. Further analysis confirmed that the presence of sulfur depressed the \( T_m \) for the thiophosphonoacetate-containing duplex relative to phosphonoacetate. For RNA–DNA duplexes, lower \( T_m \) values were observed with buffer B, which has reduced ionic strength and higher pH than buffer A. The observation that duplexes with phosphonoacetate and thiophosphonoacetate oligomers were still formed but with reduced \( T_m \) values relative to the unmodified control was encouraging. As was the case with VII and VIII, the RNA–DNA hybrid duplex having sulfur (IV) displayed a lower \( T_m \) than the phosphonoacetate duplex (III).

To further characterize duplexes III and IV (Table 2), CD spectra were recorded at 25°C and compared with duplex I (Fig. 6). The CD spectra of duplex I exhibited the characteristics of A-form rather than B-form as expected for an RNA–DNA hybrid (16). For example, the positive band at 266 nm and the negative band at 211 nm are characteristic of the A-form pattern. Similarly, the phosphonoacetate–RNA

Table 2. \( T_m \) for modified duplexes

<table>
<thead>
<tr>
<th>Duplex</th>
<th>Oligomer linkage</th>
<th>RNA ( ^a )</th>
<th>Buffer A</th>
<th>Buffer B</th>
<th>DNA ( ^a )</th>
<th>Buffer A</th>
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<tbody>
<tr>
<td>I</td>
<td>P</td>
<td>70.7</td>
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<td>48.8</td>
<td>45.7</td>
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<tr>
<td>IV</td>
<td>S-ACE</td>
<td>40.6</td>
<td>36.7</td>
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<tr>
<td>VIII</td>
<td>S-ACE</td>
<td>52.7</td>
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\( ^a \) \( T_m \) values are reported in °C as the average of six experiments. Buffer A: 10 mM sodium phosphate, 1 M NaCl, 1 mM EDTA pH 7.2. Buffer B: 20 mM HEPES-KOH, 50 mM KCl, 10 mM MgCl\(_2\), 1 mM DTT pH 7.8. 5′-d(CTCAAGTGGGCTGGTGAC) with various internucleotide linkages combined with RNA (duplexes I–IV) or DNA (duplexes V–VIII). 5′-d(GUCACCAGCCACUUGAG) for duplexes I–IV. 5′-d(GTCAACCAGCCCAGTTG) for duplexes V–VIII.
(III) and thiophosphonoacetate–RNA (IV) heteroduplexes form A-like conformations. This A-like form appears to be important for the recognition and cleavage of substrates by RNase H (17).

Because these analogs appear to be anionic at physiological pH, nuclease stable and form A-like duplexes with complementary RNA, their ability to stimulate E.coli RNase H1 activity was examined. In order to monitor for this activity, the RNA component (an 18mer) was 5' labeled with [32P]phosphate using T4 kinase and γ-32P]ATP. RNA substrate was then annealed with various phosphonoacetate- and thiophosphonoacetate-modified, complementary ODNs and tested for cleavage with RNase H1. Results are summarized in Figure 7 and Table 3. For each modification, three oligomers were examined (Table 1 contains the sequences, order of internucleotide modifications and appropriate abbreviations for phosphonoacetate, ACE, and thiophosphonoacetate, S-ACE, oligomers). In all cases, these oligomers stimulated degradation with RNase H1. There were, however, major differences. For example, the completely modified ODNs (ACE and S-ACE) were the least active and the capped oligomers (ACE Cap and S-ACE Cap) were even more efficient at stimulating RNase H1 than unmodified DNA. The remaining oligomers were of intermediate activity, with ACE EO and S-ACE EO having rates comparable with the phosphorothioate control ODN.

Because of encouraging results with RNase H1, experiments were carried out to determine the transfection ability of these ODNs. The approach was based upon earlier work with AZT and oligodeoxyribonucleotide derivatives where charge neutralization was shown to improve cell uptake over fully ionized species. These compounds were designed to initially diffuse across cell membranes and then be converted to biochemically active components by endogenous cellular carboxy esterases (18,19). Since the converted species were anionic, they were unable to diffuse out of the cell, which resulted in cellular enrichment. We speculated that ODNs with neutral thiophosphonoacetate esters would be hydrolyzed in a similar manner and that the ionized species would prevent diffusion out of cells.

Preliminary studies were carried out with chimeric oligomers having a variable number of phosphorothioate linkages interspersed with either the methyl or butyl thiophosphonoacetate ester linkages. The thiophosphonoacetate modification was chosen over the phosphonoacetate because of its increased hydrophobic properties (Fig. 3). Chimeric ODNs were prepared as oligodeoxythymidine 12mers having a 5'-pivaloyl-protected FITC group. Hydrolysis of pivaloyl-protected FITC generates a green fluorescent signal and served as an indicator for cellular uptake and esterase activity (19). Initial results are presented in Figure 8. As can be seen in Figure 8B, the thymidine oligomer with a charge-to-linkage ratio of 0.5 (anionic phosphate/total number of linkages) was taken up by the cells (compare Fig. 8A and B) after only 6 h. The fluorescent stain did not immediately localize in the nucleus but remained diffuse throughout the cells (not on the cell surface). This was shown by observing internal fluorescence around subcellular structures and the nucleus. Confocal microscopy (not shown) also confirmed internalization of fluorescence. After 24 h, these ODNs began to accumulate in the nucleus (Fig. 8C).

<table>
<thead>
<tr>
<th>ODN</th>
<th>Initial rate (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>0.577</td>
</tr>
<tr>
<td>PS</td>
<td>0.244</td>
</tr>
<tr>
<td>ACE</td>
<td>0.000522</td>
</tr>
<tr>
<td>S-ACE</td>
<td>0.0134</td>
</tr>
<tr>
<td>ACE EO</td>
<td>0.0382</td>
</tr>
<tr>
<td>S-ACE EO</td>
<td>0.0173</td>
</tr>
<tr>
<td>ACE Cap</td>
<td>1.421</td>
</tr>
<tr>
<td>S-ACE Cap</td>
<td>1.266</td>
</tr>
</tbody>
</table>

Figure 6. CD spectra of 18mer ODN–RNA duplexes. Phosphonoacetate–RNA (circles), thiophosphonoacetate–RNA (squares) and DNA–RNA (triangles).

Figure 7. RNA degradation profiles for chimeric phosphonoacetate- and thiophosphonoacetate-containing heteroduplexes. Duplexes having radio-labeled RNA 18mer oligonucleotide with a sequence complementary to the 18mer deoxynucleotides defined in Table 1 were incubated with RNase H1 for the times indicated. Deoxynucleotide structures: open squares, normal DNA; open diamonds, phosphorothioate DNA; open circles, ACE Cap; filled diamonds, S-ACE Cap; filled circles, ACE EO; plus signs, S-ACE EO; filled squares, ACE; crosses, S-ACE.
When the transfection medium (6 h exposure to cells) was replaced with fresh medium lacking ODN, the diffuse stain disappeared within 2 h (Fig. 8D) and left behind a stable punctate pattern. These cells continued to grow after fluorescence had diffused away and remained viable.

The residual punctate pattern (Fig. 8D) suggests that a fraction of the methyl phosphonoacetate esters were hydrolyzed (and trapped) within endosomes and/or lysosomes. To test this theory, ODNs with a charge-to-linkage ratio of 0.75 were incubated for 6 h with cells. The results (Fig. 8E) illustrate a typical punctate pattern, as has been observed many times when negatively charged oligomers, such as phosphorothioates, are exposed to cells in culture. These patterns have typically been interpreted as the irreversible accumulation of oligomers in endosomes and/or lysosomes (20). As also observed with phosphorothioate DNA (20), transfection of these ODNs with cationic lipids such as lipofectin resulted in a nuclear stain (Fig. 8F) that did not diffuse when the medium was replaced. Since lipofection goes through an endosomal pathway, this result indicates that endosomal/lysosomal enzymes are capable of deprotection (20). Results similar to those presented in Figure 8A–D and F were also obtained with the corresponding butyl derivative and with a thymidine 12mer where six phosphorothioates were flanked on each side by esterified thiophosphonoacetate linkages.

**DISCUSSION**

A comparison of melting temperatures ($\Delta T_{m}$ expressed as a change in °C per linkage) showed a loss of heteroduplex stability for these modified ODNs relative to natural DNA–RNA and DNA–DNA duplexes. When hybridized to RNA in high salt buffer (Table 2, buffer A), the average decreases in $T_{m}$s were 1.3 and 1.8°C per linkage (18mer ODNs) for phosphonoacetate and thiophosphonoacetate duplexes (III and IV). This decrease was considerably greater than for the corresponding phosphorothioate duplex (0.5°C per linkage). In the low salt buffer used for experiments with RNase H1 (buffer B), trends were similar but with reduced $T_{m}$s, as expected. With DNA in a high salt buffer (buffer A), these analogs were also less stable than the duplex with natural DNA (V). For example, with the 18mer duplexes, the decrease in stability was ~0.3 and 1.0°C per linkage, respectively, for the phosphonoacetate (VII) and thiophosphonoacetate (VIII) analogs. Interestingly, the $\Delta T_{m}$ per linkage for the phosphonoacetate ODN was less than that observed with phosphorothioate DNA (0.3 versus 0.5°C). Without more data relative to conformational analysis, hydrogen bonding of the acetate group, perhaps the negative carboxylate for both these analogs were also obtained with the corresponding butyl derivative and with a thymidine 12mer where six phosphorothioates were flanked on each side by esterified thiophosphonoacetate linkages.

**Figure 8.** Transfection with methyl-protected thiophosphonoacetate ODNs. (A–D) and (F) show results obtained with 5’ FITC-TxTxTxT-TxT-TxT-Tx having a phosphorothioate between the 3’ end having one negative phosphate charge. Phase contrast (A±D) and (F) show results obtained with 5’ FITC-TxTxT-TxT-TxTx where - represents phosphorothioate (including a phosphorothioate linkage between FITC and 5’ thymidine) and x represents methyl-protected thiophosphonoacetate (including a thiophosphonoacetate at the 3’ end having one negative phosphate charge). Phase contrast (A±D) and (F) show results obtained with 5’ FITC-TxTxT-TxT-TxTxT-Tx having a phosphorothioate between FITC and 5’ thymidine and a thiophosphonoacetate with one negative charge at the 3’ end.

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the difference in RNase H1 activity is not likely to be a result of duplex structure. For some ribozyme-mediated reactions, duplex substrates with low $T_m$ lead to increased product release and substrate turnover during the course of the reaction (23). Perhaps the reduced $T_m$ for the thiophosphonoacetate–RNA duplex promoted product release and substrate turnover during the RNase H1 reaction.

Of particular interest is the unprecedented observation that the capped ODNs stimulated RNase H1 catalysis at a rate faster than with unmodified DNA (Fig. 7, Table 3). Although capped phosphorodithioate oligomers have been shown to be more RNase H1 active than natural DNA (6), the results cannot be easily compared with those obtained with the capped chimeras. This is because the experiments with capped phosphorodithioate DNA were carried out in a background rich in nucleases (HeLa cell extracts). As a result, it is difficult to differentiate any intrinsic catalytic rate with capped phosphorodithioate ODNs from a secondary effect due to enhanced stabilization against nuclease degradation. Earlier work involving similar chimeric, antisense ODNs routinely led to reduced catalysis rates. The rationale for these chimeras is to design antisense oligomers having a central region (phosphorothioate or phosphate internucleotide linkages) that stimulates RNase H1 activity flanked by capped analogs that enhance nuclease resistance and, owing to their ability to form A-type heteroduplexes, have increased affinity for RNase H1 (24). So far, the same properties that perhaps enhance affinity of these chimeras for RNA/RNase H1 result in loss of RNase H1 activity. This does not appear to be the case with a chimera having phosphonoacetate or thiophosphonoacetate capped ends and a catalytic phosphorothioate central region (ACE Cap and S-ACE Cap). Without extensive analysis, we can only speculate on the reasons for this enhanced activity. Based upon the CD data with the phosphonoacetate ODN, this chimera probably forms an A-type duplex with RNA, which is the preferred structure recognized by RNase H1 and could lead to a more rapid initial rate. Unlike other modified bases commonly used as capping agents, such as 2′-O-methyl nucleosides, the phosphonoacetate or thiophosphonoacetate analog in complex with RNA forms a less stable duplex. Perhaps, as is the case with certain ribozymes (23), a weaker binding affinity enhanced by cleavage of RNA could destabilize the heteroduplex, which could also increase the enzyme turnover rate. Finally, the phosphonoacetate internucleotide linkage with carboxylic anionic character could form a less stable (relative to phosphate) chimeric duplex with the RNase H1 lysine cluster which is believed to define the enzyme-binding surface (25). This could lead to a more rapid initial rate. Further kinetic analysis of several chimeric constructs may lead to even more enhanced RNase H1 activity.

Because of the unique functionality of these analogs, transfection via a prodrug approach was considered. Others have shown that phosphate-protected S-pivaloylthioethyl ODNs are efficiently transfected into cells in culture via a passive diffusion mechanism (18,19). Following membrane transport, endogenous esterases cleaved the pivaloyl ester and the resulting thio-alcohol eliminated via cyclization. These ODNs were irreversibly trapped and concentrated inside cells, presumably due to an increase in charge character afforded by cell-mediated ester hydrolysis. Furthermore, this approach eliminates the need for toxic cationic lipids during transfection, and the increased hydrophobicity of esterified ODNs has been shown to significantly reduce binding to serum proteins (26).

For the work described here, thiophosphonoacetate-containing ODNs were esterified with either methyl or n-butyl groups and transfection studied as a function of the thioephosphate or anionic content of the oligomers. The results shown in Figure 8 suggest that the relative ratio of anionic charge is important. When half the charge was neutralized via hydrophobic esters, oligomers diffused reversibly into cells and accumulated after removal of the methyl esters. In contrast, oligomers with relatively higher anionic content failed to undergo transfection unless treated with lipofectin. Of particular interest was the reversible diffusion of these esterified analogs. The challenge for future research will be to identify thiophosphonoacetate esters that more clearly resemble natural substrates for esterases and lipases. Perhaps such analogs will undergo hydrolysis more rapidly and thus increase the cellular concentration of the RNase H1-active form of the corresponding ODNs.

Based upon these results, phosphonoacetate- and thiophosphonoacetate-modified ODNs merit further research as these analogs may prove useful for various diagnostic applications and for controlling gene expression via an antisense mechanism. Of particular interest were observations that these ODNs were completely resistant to the nucleases tested, exhibited RNase H1 activity and can be taken up by cells as neutral esters.

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