2′-O-methyl-modified phosphorothioate antisense oligonucleotides have reduced non-specific effects in vitro

Byong Hoon Yoo, Elena Bochkareva1, Alexey Bochkarev1, Tung-Chung Mou2 and Donald M. Gray*

Department of Molecular and Cell Biology, Mail Stop FO31, The University of Texas at Dallas, PO Box 830688, Richardson, TX 75083-0688, USA, 1Department of Biochemistry and Molecular Biology, The University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190, USA and 2Howard Hughes Medical Institute, The University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75390, USA

Received January 19, 2004; Revised March 1, 2004; Accepted March 9, 2004

ABSTRACT
Antisense oligodeoxynucleotides (ODNs) have biological activity in treating various forms of cancer. The antisense effects of two types of 20mer ODNs, phosphorothioate-modified ODNs (S-ODNs) and S-ODNs with 12 2′-O-methyl groups (Me-S-ODNs), targeted to sites 109 and 277 of bcl-2 mRNA, were compared. Both types were at least as effective as G3139 (Genta, Inc.) in reducing the level of Bcl-2 protein in T24 cells following a 4 h transfection at a dose of 0.1 μM. Circular dichroism spectra showed that both types formed A-form duplexes with the complementary RNA, and the melting temperatures were in the order of Me-S-ODN-RNA > normal DNA-RNA > S-ODN-RNA. In comparison with the S-ODN, the Me-S-ODN had reduced toxic growth inhibitory effects, was less prone to bind the DNA-binding domain A of human replication protein A, and was as resistant to serum nucleases. Neither type of oligomer induced apoptosis, according to a PARP-cleavage assay. Hybrids formed with Me-S-ODN sequences were less sensitive to RNase H degradation than those formed with S-ODN sequences. Despite this latter disadvantage, the addition of 2′-O-methyl groups to a phosphorothioate-modified ODN is advantageous because of increased stability of binding and reduced non-specific effects.

INTRODUCTION
Phosphorothioate oligodeoxynucleotides (S-ODNs) were the most common type of the first generation antisense ODNs. These oligomers have resistance to nuclease and the ability to activate RNase H. The major shortcomings of S-ODNs are (i) the relatively low stability of duplexes with complementary RNA and (ii) the toxic effects, possibly related to non-specific protein–S-ODN interactions (1–8). Photocrosslinking experiments by others have shown that human replication protein A (RPA) is one of the major protein products crosslinked with single-stranded DNA oligomers (9).

A prevalent type of second-generation antisense ODN carries 2′-O-sugar modifications, which increases the stability of duplexes with complementary RNA (8). Duplexes formed with the 2′-O-sugar-modified S-ODNs themselves do not have the structure needed to activate RNase H cleavage of the target RNA, and such modified S-ODNs are routinely interrupted with a gap of phosphorothioate nucleotides that do not carry the sugar modification. Monia et al. (10) showed that 2′-O-modifications (such as pentyl, propyl, methyl and fluoro) in chimeric gap-mer S-ODNs had antisense effectiveness that correlated with the Tm values of hybrids formed with the target RNA, which was an Ha-ras oncogene in a reporter gene system. Stability of those gap-mers also correlated with the ability to inhibit Ha-ras mRNA expression in T24 cells, at a dose of 100 nM (11).

McKay et al. (12) found that a gap size of eight was required in 2′-O-methyl chimeras for optimal reduction of PKCα mRNA in A549 cells. These authors advocated the use of 2′-O-methoxyethyl-modified sugars, because a 20 nt gap-mer with this modification had increased thermal stability when hybridized with the target mRNA and enhanced antisense effectiveness in reducing PKCα mRNA (~85% at 100 nM dose), when compared with an analogous 2′-O-methyl chimera (~60% at 100 nM dose). In addition, maximum resistance to degradation by snake venom phosphodiesterase was shown to require that chimeras with either 2′-O-methyl or 2′-O-methoxyethyl sugars have a uniform phosphorothioate-modified backbone (12). The 2′-O-methyl modification remains one of the most valuable 2′-sugar modifications because it is cost-effective. In contrast, 2′-O-methoxyethyl-modified oligomers are not commercially available.

Various antisense modalities need to be carefully compared to determine their relative effectiveness (13). In this work, we...
compared 2′-O-methyl-modified and unmodified S-ODN sequences that are effective in reducing the level of Bcl-2 protein in T24 cells. The anti-apoptotic protein Bcl-2 is localized in the outer mitochondrial membrane, the endoplasmic reticulum and the nuclear envelope (14). Heterodimerization between pro- and anti-apoptotic members of the Bcl-2 family regulate the release of cytochrome c and subsequent apoptosis (15). Because of the Bcl-2 protein’s anti-apoptotic properties and abilities to inhibit tumor cell apoptosis induced by numerous drugs and radiation, the bcl-2 mRNA is an attractive antisense target for cancer treatment (7,15,16).

We show that non-specific effects of phosphorothioates on growth of T24 cells are reduced by the 2′-O-methyl modification. The 2′-O-methyl modification also reduces the binding of S-ODNs to domain A of RPA. Due to reduced non-specific effects, 2′-O-methyl-modified phosphorothioate ODN chimeras may be especially useful, in addition to S-ODNs, for comparative studies.

**MATERIALS AND METHODS**

**Oligonucleotides and protein**

Two oligomer sequences were chosen for the present study. Oligomers-109 (5′-CCGGCATCCCACTCAGGCC-3′) and oligomers-277 (5′-CCGGCCGAGCGCAGGCCCC-3′) were 20 nt long and were targeted at starting locations 109 and 277, respectively, of human bcl-2 mRNA (GenBank accession no. M14745). Negative control oligos-1C (5′-CTCA-TTACTACCCAGACCC-3′) were also 20 nt long and consisted of a scrambled sequence that has no homology in the human genome and that was used as a control sequence by others (17). In preliminary experiments involving 29 S-ODNs targeted to various locations on bcl-2 mRNA, S-ODN-109 was the most effective in reducing Bcl-2 protein in A549 human lung cancer cells by the western blot protocol described below, but using an S-ODN concentration of 0.5 μM. S-ODN-277 was predicted to have the highest duplex stability among the tested oligomers and was as effective as S-ODN-109 in reducing the Bcl-2 level in T24 cells at a concentration of 0.1 μM. The positive control, compound G3139 of Genta, Inc. (Berkeley Heights, NJ), was 18 nt long and was targeted at starting location 32 of human bcl-2 mRNA [reviewed by Dias and Stein (7)].

The 11 DNA or chimeric oligomers used for this study and the modifications they contained are detailed in Table 1. For convenience, we denote the chimeric oligomers that contain 2′-O-methyl-modified nucleotides as ODNs, although they contain modified RNA nucleotides. Except for the unmodified ODN-109, all were 100% phosphorothioates. The unmodified ODN, S-ODNs, Me-S-ODN chimeras (with 12 2′-O-methyl sugars), CmeG-S-ODN chimera (with two 5-methyl cytosines in CpG sequences), and unmodified 20mer RNAs complementary to oligomers-109 and oligomers-277 were purchased from Midland Certified Reagent Co. (Midland, TX). The Me-S-ODN chimeras were HPLC-purified, and the unmodified RNA oligomers were purified using trityl selection. Other oligomers were purified by gel filtration. The unmodified RNAs were dissolved in water treated with diethyl pyrocarbonate as in previous work to reduce RNase degradation (18). Absorption spectra at 90°C (corrected for volume expansion due to heating) were used together with ε(260) values (M⁻¹ cm⁻¹, per mole of strand) to determine concentrations of the oligomers dissolved in water (19,20). Extinction coefficients at 260 nm were calculated as the sums of monomer extinction coefficients; see footnote to Table 1.

Residues 181–291 of the 70 kDa RPA subunit were expressed in *Escherichia coli* and purified as described for other RPA domains (21).

**Circular dichroism (CD) spectra and melting temperatures (T_m)**

CD spectra and melting profiles were measured as in previous work (18–20). To form double-stranded hybrids for T_m
measurements, the ODNs and complementary RNAs, at equimolar strand concentrations (~2 μM) were mixed in 0.15 M K+ (phosphate buffer, pH 7.0) at room temperature for 2 h. Then, the mixtures were heated to 90°C for 90 s and allowed to cool at room temperature for 24 h. CD spectra were collected at 20°C using a Jasco Model J715 spectropolarimeter (Jasco, Easton, MD). To obtain melting profiles of absorbance as a function of temperature, the OD(260) (optical density at 260 nm) of each mixture was monitored in an Olis-modified Cary 14 (On-Line Instrument Co., Bogart, GA) as the temperature was increased from 20 to 95°C in increments of 1°C. Samples were incubated for 3 min at each temperature to allow for equilibration before taking OD readings. Reported $T_m$ values were the maxima in first-derivative plots of the melting profiles.

**Cell culture and transfections**

T24 human bladder cancer cells were used because they have a relatively high concentration of Bcl-2 for detection by western blotting. The T24 cells were purchased from the American Type Culture Collection (Rockville, MD) and maintained in McCoy’s 5A medium (Mediatech, Inc., Herdon, VA), supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), in 5% CO$_2$ at 37°C. For transfections, T24 cells were plated 24 h prior to treatment in 25 cm$^2$ flasks at a density of 8 × 10$^5$ cells per flask. A volume of 8 μl of the delivery reagent lipofectin (Invitrogen) was diluted in 1350 μl of McCoy’s 5A medium and incubated for 45 min at room temperature. ODNs were diluted into 650 μl of McCoy’s 5A media to give the required concentration and incubated for 15 min at room temperature. These two solutions were gently mixed and further incubated for 15 min. The concentrations of lipofectin and ODNs were 3 and 0.1 μM, respectively, in the final mixture. This gave a lipofectin/nucleotide (+/−) charge ratio of 1.5. (To determine whether the optimal lipofectin/charge ratio might differ for the methylated and unmethylated oligomers, electrophoretic mobility shift assay (EMSA) experiments were performed with S-ODN-109 and 5’-Me-S-ODN-109 mixed with increasing amounts of lipofectin to give (+/−) charge ratios of 0, 0.5, 1.0, 2.0 and 4.0. Both types of oligomer were partially neutralized at a charge ratio of 1.0 and were fully neutralized at a charge ratio of 2.0. Transfections in this work were therefore all performed with lipofectin/oligomer mixtures at the intermediate charge ratio of 1.5, which, in our experience with S-ODNs, was sufficient to maximize the antisense effect while minimizing non-specific effects of the lipofectin.) Cells were overlaid with this mixture after prewashing with McCoy’s 5A medium. Following a 4 h transfection of cells at 37°C, cells were washed and incubated in McCoy’s 5A medium supplemented with 10% FBS for an additional 24 h. Untreated (i.e. mock-treated) controls were identical except that during the treatment period they were overlaid with media without lipofectin or ODNs.

**Western blot analysis**

Cells treated with ODNs were washed with TBS (0.05 M Tris–HCl, pH 7.4, 0.1 M NaCl), and extracted at 0°C for 25 min in lysis buffer [50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.02% (w/v) sodium azide, 1 mM DTT and 1% (v/v) Triton X-100] with 5% (v/v) protease inhibitor cocktail (Sigma, St Louis, MO). Cell debris was removed by microcentrifugation at 4°C. Protein concentrations were determined using the Bio-Rad protein assay (Richmond, CA). Cell extracts (20 μg) were subjected to SDS–polyacrylamide gel electrophoresis and then transferred to PVDF membranes (Millipore, Bedford, MA). The portions of the membranes containing Bcl-2 (MW 26 kDa) and Raf-1 (MW 73 kDa) were blocked with 5% non-fat dry milk in TTBS [TBS containing 0.1% (v/v) Tween-20], and probed with corresponding primary antibodies at room temperature overnight (for Bcl-2: Cat. sc-509, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; for Raf-1: Cat. 610151, BD Transduction Laboratories, San Diego, CA). After washing in TTBS, the membranes were incubated for 1 h at room temperature with 5% non-fat dry milk in TTBS containing a 1:15 000 dilution of an alkaline phosphatase-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA). The immunocomplexes were visualized by enhanced chemifluorescence reagent (ECF substrate; Amersham Pharmacia Biotech, Piscataway, NJ), followed by scanning with a phosphoimager (Storm 860, Molecular Dynamics) and quantitation using ImageQuant version 5.0 (Molecular Dynamics). The portion of the gel containing actin (MW 42 kDa) was stained by a modification of the Fairbanks Coomassie staining method (22). To calculate the reduction of Bcl-2 protein levels, actin band densities of untreated control samples (at 50 and 100% concentrations) and the treated samples were used to calculate the loading ratios of the treated cell lysates. Bcl-2 band intensities of untreated control samples (at 50 and 100% concentrations), with background subtracted, were fitted with a second-order equation from which the apparent percentages of Bcl-2 protein in the treated samples were determined. These percentages were finally corrected for any unequal loading, using the loading ratios from the actin bands.

**Growth inhibition**

The effects on cell growth of the antisense ODNs were determined by [3H]thymidine incorporation (23,24). T24 cells were plated 24 h prior to treatment in 24 well plates at a density of 2 × 10$^4$ cells per well. Cells were treated with ODNs using the procedure described above, except that after the 4 h treatment, cells were washed and incubated in McCoy’s 5A medium supplemented with 10% FBS plus 1 μCi of [3H]thymidine for 24 h. After the 24 h incubation, media in the wells was removed by aspiration and cells were incubated in Hanks balanced salt solution with trypsin-EDTA (Invitrogen) for 5 min at 37°C. A cell harvester (Cambridge Technology, Watertown, MA) was used to harvest cells on glass fiber filters. The percentage of growth inhibition was calculated as 100 × [1 − (counts due to [3H]thymidine incorporation by the treated cells)/counts due to [3H]thymidine incorporation by untreated cells)].

**Assay for apoptosis**

As a marker of cells undergoing apoptosis, the percentage of PARP [poly(ADP-ribose) polymerase; MW 116 kDa] that was cleaved to give an 89 kDa cleavage product was determined in treated cell lysates by western blot analysis using a PARP antibody (BD Biosciences, San Diego, CA).
Stability of ODNs in serum

To test the stabilities of ODNs in serum, the ODNs (at 10 μM concentration) were incubated in McCoy’s 5A medium supplemented with 10% FBS at 37°C. At each time point (0, 24, 48 and 72 h), 12 μl of each mixture was taken and the reaction was terminated by addition of 12 μl of loading buffer (0.1 M Tris–HCl pH 8.4, 0.09 M boric acid, 7 M urea, 0.1% bromophenol blue, 0.01% xylene cyanol). Samples were subjected to electrophoresis in a denaturing 20% polyacrylamide gel with 7 M urea. After electrophoresis, the gels were stained with SYBR Green II (Molecular Probes, Eugene, OR) prepared in TBE (0.089 M Tris, 0.089 M boric acid and 1 mM EDTA pH 8.0) and scanned with a phosphoimager.

EMSA of RPA70-A binding to ODNs

Two types of EMSA experiments were used to investigate the binding of RPA70-A to S-ODN-109 versus 5′-Me-S-ODN-109. ODNs were end-labeled with T4 polynucleotide kinase (Promega, Madison, WI). (i) Competition experiments were performed to compare the binding affinities of RPA70-A to S-ODN-109 and 5′-Me-S-ODN-109. RPA70-A was added to saturate the 32P end-labeled S-ODN-109 at 1 μM. After 15 min incubation at room temperature, unlabeled competitor, either S-ODN-109 or 5′-Me-S-ODN-109 was added, and the mixtures were incubated for an additional 15 min. After addition of competitor, the concentration of ODN that was initially saturated with RPA70-A was 0.5 μM in all mixtures. (ii) Further competition experiments were performed to compare binding affinities of RPA70-A to S-ODN-109 versus 5′-Me-S-ODN-109 in the presence of the complementary 20mer RNA. RPA70-A was added to complementary RNA, and, after 15 min incubation, either 32P-labeled S-ODN or 32P-labeled Me-S-ODN was added and incubated for an additional 5 h at room temperature. The ODN and complementary RNA concentrations were each 1 μM in the final mixtures. The final [P]/[N] ratios (molar protein monomer/molar nucleotide ratios) were 0.2, 0.2 and 0.5, where the nucleotide concentrations were the total of ODN plus RNA nucleotides.

In each of the above cases, the mixtures were subjected to 20% non-denaturing polyacrylamide gel electrophoresis in TBE buffer, the gels were scanned with a phosphoimager, and the data were analyzed using ImageQuant 5.0.

RNase H elicitation

To examine the ability of RNase H to degrade hybrids formed with modified ODNs, ~5 pmol of each modified ODN and complementary 32P 5′-end-labeled RNA were hybridized in 60 mM Tris–HCl (pH 7.8) containing 2 mM dithiothreitol, 60 mM KCl and 2.5 mM MgCl2. The desired hybrid duplexes were formed by incubating at 90°C for 2 min and slow cooling to room temperature for at least 1 h. Then, solutions of hybrids were treated with 0.05 units of Escherichia coli RNase H (Invitrogen) at 37°C for 20 min. Samples were analyzed as in the EMSA experiments described above.

RESULTS

Reductions in Bcl-2 level

In order to determine whether antisense effectiveness correlates with hybridization stabilities of the different ODN modifications, T24 human bladder cancer cells were transfected with the modified ODNs targeted to sites 109 and 277 on bcl-2 mRNA. The ODNs were used at a concentration of 0.1 μM. (In preliminary experiments, we found that there were no substantial decreases of Bcl-2 level in T24 cells when the concentrations of S-ODNs or Me-S-ODNs targeted to these two sites were increased above 0.1 μM under our conditions.) As shown in Figure 1, the S-ODN-109 and 5′-Me-S-ODN-109 resulted in 57 ± 5 and 70 ± 2% (SD) reductions of Bcl-2 level, respectively, with the Me-S-ODN having the greater effect. The analogous oligomers targeted to site 277, S-ODN-277 and 5′-Me-S-ODN-277, resulted in 63 ± 4 and 45 ± 5% reductions of the Bcl-2 level, respectively. In that case, the Me-S-ODN had the lesser effect. The S-ODN-109 and S-ODN-277 had the same effect, within error, as an effective positive control, G3139, directed against the first six codons of the bcl-2 mRNA (7). The S-ODN and Me-S-ODN controls averaged 3 ± 10 and 12 ± 10%, respectively, and were not significantly different in their effects on the Bcl-2 level (Fig. 1).

The level of Raf-1 protein, a protein in a pathway separate from the Bcl-2 pathway, was monitored as an additional indication of non-specific cellular effects of the oligomer treatments. For the three Me-S-ODNs used to obtain data for Figure 1, the level of Raf-1 protein was reduced by an average of 21%, no different from the average of 24% reduction found after treatments by the three 20mer S-ODNs (data not shown).

The antisense effectiveness of three different Me-S-ODN chimeras, all targeted to site 109, were compared to study the effect of placing the 12′-O-methyl groups in one block at the 5′ end, in one block at the 3′ end or in two equal blocks at both ends to form a gap-mer. The Me-S-ODN chimeric sequences (5′-Me-S-ODN, 3′-Me-S ODN and gap-Me-S-ODN) are shown in Table 1. The hybrids of these chimeras paired with the target RNA had Tm values that differed by only 4°C (Table 1), and the reductions of Bcl-2 level caused by treatments with the three chimeras were all in the range
73 ± 4% (data not shown). Therefore, for this 20mer sequence, the location of the one or two blocks of 2'-O-methyl nucleotides was unimportant.

Because additional work (below) focused on the oligomers targeted to site 109, we examined whether the reduction of the Bcl-2 level by S-ODN-109 was influenced by the presence of CpG sequences. Synthetic DNAs containing unmethylated CpG dinucleotides can stimulate an immune response in vertebrates, and CpG islands in antisense ODNs can cause non-sequence-specific effects (7,25,26). Since sequence 109 contained two CpG islands, an ODN was prepared (CmeG-S-ODN; see Table 1) in which C-5-methyl groups were added to cytosines in these CpG sequences to suppress any non-specific effects. In simultaneous experiments, the reductions in the Bcl-2 level by S-ODN-109 and CmeG-S-ODN-109 were essentially identical (62 ± 6 and 60 ± 11%, respectively) demonstrating that the reduction of Bcl-2 level by the S-ODN-109 was not affected by CpG islands. This was not unexpected, since T24 cells are not immune system cells.

From all the data above, we take the reductions in Bcl-2 levels by the S-ODN and Me-S-ODN sequences targeted to sites 109 and 277 to denote primarily the antisense effects of these oligomers.

Although the relative effectiveness of the two types of ODN depended on the target site sequence, the oligomers targeted to site 109 show that an Me-S-ODN can have an apparent antisense effectiveness greater than that of a cognate S-ODN. The actual specific activity of the Me-S-ODN may be even greater due to decreased non-specific effects, such as a reduced toxic effect on cell growth (see below).

**CD spectra of ODN-RNA hybrids**

The ODN-RNA duplexes were characterized by CD spectral measurements. CD measurements were performed with the samples in 0.15 M K+ (phosphate buffer, pH 7.0), 20°C. The spectra of the ODN-RNA duplexes were significantly different from the averaged spectra of the individual strands (Fig. 2). Spectra of the hybrids showed an increase in the magnitude of the long wavelength positive band above 260 nm, an increase in the negative band at ~210 nm, and a shift in the crossover near 250 nm to shorter wavelengths, relative to the averaged spectra. These CD characteristics are typical features of DNA-RNA duplexes (18,20) and confirm the formation of the desired duplexes.

**Tm values of ODN-RNA hybrids**

The S-ODN and Me-S-ODN were further characterized by the stabilities (Tm values) of the hybrids they formed with complementary RNAs. The Me-S-ODN-RNA hybrids had relatively high Tm values (69°C to above 82°C), but the corresponding S-ODN-RNA hybrids had lower Tm values (55 and 66°C). The Tm difference between the three chimeric Me-S-ODN-109-RNA hybrids and the S-ODN-109-RNA hybrid was 14–18°C and that between one chimeric Me-S-ODN-277-RNA and the S-ODN-277-RNA was >16°C (Table 1). The Me-S-ODNs-RNA hybrids presumably have higher Tm values than the corresponding hybrids formed with the S-ODNs because the 2'-O-methyl group results in an RNA-like conformation, and RNA duplexes have higher Tm values than DNA-RNA hybrids (6,18,27,28).

![Figure 2. Measured CD spectra of ODN-RNA hybrids compared with the averaged spectra of the individual strands. Hybrids were formed by pairing the following ODNs with the complementary RNA-109 strand: (A) Unmodified ODN-109, (B) S-ODN-109, (C) 5'-Me-S-ODN-109. (Solid line) Measured spectra; (dashed line) averaged spectra of the individual, unpaired strands. Spectra were taken at 20°C with samples in 0.15 M K+ (phosphate buffer, pH 7.0).](image)

![Figure 3. Growth inhibition of T24 cells following treatments with the S-ODN and the 5'-Me-S-ODN targeted to site 109 on bcl-2 mRNA. Growth inhibition was determined as the decrease in [3H]thymidine incorporation, relative to the incorporation in mock-treated cells. Error bars are ±1 SD of three determinations.](image)
were minimal for the Me-S-ODN oligomer. There was no effect on [3H]thymidine incorporation by treatment with lipofectin alone (data not shown). We conclude that the effects on growth inhibition by this assay were essentially caused by the cytotoxic effects of the ODN modifications and were not secondary to a reduction of the Bcl-2 level.

Apoptosis

We tested whether a reduction in the level of the Bcl-2 anti-apoptotic protein was correlated with apoptosis of T24 cells. PARP is one of the main cleavage targets of caspase-3, which is activated in the apoptosis cascade following release of cytochrome c, so that the cleavage of PARP serves as a marker of cells undergoing apoptosis (29). Cells were treated with ODNs as described in Materials and Methods for reducing the Bcl-2 protein level. The percentage of the 89 kDa PARP cleavage product after treatments with S-ODN-109, 5′-Me-S-ODN-109, and the two negative controls, ranged from 2 to 6%, not detectably different from the 2% cleavage product found in lysates of untreated controls (data not shown). Thus, according to this assay, there was no detectable apoptotic effect with either the S-ODN-109 or 5′-Me-S-ODN-109, although these oligomers reduce the Bcl-2 level by 57 and 70%, respectively, and they have differing effects on cell growth according to the [3H]thymidine incorporation assay.

Stability of ODNs in serum

An important consideration in the design of antisense ODN modifications is the degree to which the ODN is rendered nuclease resistant. The resistance to degradation in 10% FBS of the unmodified ODN-109 (phosphodiester) and three cognate modified ODNs (the S-ODN, 5′-Me-S-ODN and Gap-Me-S-ODN) was examined as described in Materials and Methods. The results are shown in Figure 4. While little of the unmodified ODN remained intact after 24 h, all three of the modified ODNs exhibited a half-life of >72 h in 10% FBS. This was in agreement with results by Grünweller et al. (30), who reported the half-life of an S-ODN as being >48 h under similar conditions. Thus, the S-ODN and Me-S-ODN were completely stable over the time course of the transfections and incubations to reduce the level of Bcl-2, and the difference in antisense effect (Fig. 1) was not obviously due to a difference in nuclease stability.

RPA70-A binding affinities of S-ODN-109 and Me-S-ODN-109

One non-specific effect of ODNs is their binding to cellular proteins (1–9). The most abundant single-stranded DNA binding protein in human cells is RPA (31), and domain A of the 70 kDa subunit of RPA is the site of initial binding to single-stranded DNA (32–34). The binding site of RPA70-A accommodates ~5 nt, similar to the binding site size of the Ff g5p, which has been used to assess the effects of different nucleotide modifications (6,32). Therefore, we determined the relative binding affinity of RPA for S-ODN-109 and 5′-Me-S-ODN-109 in both the absence and presence of the complementary RNA sequence.

To compare the RPA70-A binding affinities for the S-ODN and the Me-S-ODN, saturated S-ODN-109:RPA70-A complexes were prepared with 32P-labeled S-ODN and various amounts of unlabeled competing S-ODN-109 or Me-S-ODN-109 were added (in phosphate, pH 7.0, 37°C). The EMSA results after 15 min of incubation with the competitor are shown in Figure 5A and B. At an 8-fold excess of unlabeled competing S-ODN, ~61% of the labeled S-ODN:RPA70-A complex was completely dissociated (Fig. 5A). The same results were obtained after up to 4 h of incubation with competing S-ODN-109, indicating that equilibrium had been reached (data not shown). The band of complex became more diffuse as the labeled S-ODN was dissociated because RPA70-A does not bind with high cooperativity, and up to five protein molecules can be bound per ODN 20mer. The same 8-fold excess of competing Me-S-ODN dissociated a maximum of 15% of the S-ODN:RPA70-A complex, 4-fold less than the same amount of competing S-ODN (Fig. 5B). Additional experiments in which the labeled S-ODN and unlabeled Me-S-ODN were premixed before adding the protein demonstrated that even at a 15-fold excess of Me-S-ODN the amount of completely dissociated labeled S-ODN was still only ~15%. Finally, the ratio of 5′-Me-S-ODN-109 to S-ODN-109 needed for 50% dissociation of an unmodified ODN:RPA70-A complex was determined to be 9.9 ± 0.1 (range) in competition experiments (data not shown). In summary, the binding affinity of RPA70-A was estimated to be ~10-fold greater for the S-ODN than for the 5′-Me-S-ODN, even though the latter has a stretch of eight phosphorothioate nucleotides.
compared for their ability to recruit RNase H. RNase H and 5¢-Me-S-ODNs are important criteria for antisense effectiveness. The S-ODNs of the target mRNA is considered to be one of the most important criteria for antisense effectiveness. The S-ODNs and 5¢-Me-S-ODNs targeted to RNA sites 109 and 277 were compared for their ability to recruit RNase H. RNase H degradation of the target RNAs (unmodified 20mer RNAs complementary to oligomers-109 and oligomers-277) was higher for hybrids formed with the S-ODNs than with the Me-S-ODNs (Fig. 6). For example, ~80% of the hybrid formed with S-ODN-109 was degraded but only ~30% of the hybrid formed with Me-S-ODN-109 was degraded under the same conditions. The lowered ability of RNase H to degrade hybrids with the Me-S-ODN was not unexpected. The Me-S-ODNs are chimeric, with 12 nt that are 2¢-O-methyl and RNA-like. Therefore, a major portion of the hybrid formed with the Me-S-ODN sequesters the target RNA in an RNA-RNA duplex that is not recognized by RNase H. The most effective hybrid in recruiting RNase H was that formed by S-ODN-109, which interestingly had the lowest Tm.

DISCUSSION

Hybrids formed with 20mer S-ODNs and Me-S-ODNs and their cognate RNA sequences were shown to have the CD spectra of DNA-RNA and RNA-RNA A-form structures (Fig. 2). As expected, melting temperatures of the hybrids formed with the Me-S-ODNs were above those formed with the S-ODNs (Table 1). The two S-ODN sequences whose properties were compared in this work were as effective in reducing the level of Bcl-2 protein in T24 cells as the well studied Bcl-2 antisense sequence G3139 (7) (Fig. 1), independent of the presence of CpG islands. Treatments with S-ODN-109 and 5¢-Me-S-ODN-109 did not lead to apoptosis according to a PARP-cleavage assay. G3139 also does not induce apoptosis in all cell lines (35), and Benimetskaya et al. (36) reported that G3139-induced down regulation of Bcl-2 in T24 cells is insufficient to decrease cellular viability. Neither S-ODN-109 nor Me-S-ODN-109 oligomers were significantly degraded by serum over the time course of the antisense treatments (Fig. 4). The latter is important in comparative studies, because without having the nuclease protection of a phosphorothioate backbone the 2¢-O-methyl modification can be less effective in an antisense oligomer than a simple S-ODN (30).
Eight phosphorothioate nucleotides are needed in gap-mers with 2'-O-methyl-modified sugars and locked nucleic acids to fully activate RNase H (12,37). Oligomers containing 12 2'-O-methyl-modified nucleotides at the 5' ends were less able to induce RNase H degradation of hybridized RNA than were the analogous S-ODNs (Fig. 6). Nevertheless, the abilities of different chimeric arrangements of the 12 modified nucleotides, in one block at either end or as a gap-mer (5'-Me-S-ODN, 3'-Me-S ODN and gap-Me-S-ODN), were equally able to reduce the level of Bcl-2 protein. This implies that slight differences in the ability to activate RNase H are not as important as the ability to form stable hybrids with the target RNA and other factors in antisense effectiveness. Although we did not examine mRNA levels by northern blot analysis in this work, others have shown that antisense phosphorothioate oligomers carrying the 2'-O-methyl modification are unable to reduce endogenous PKCα mRNA levels unless there is a gap without the sugar modification so that RNAse H can be activated (12). Thus, we reasonably expect that the main mechanism of action of our oligomers in reducing the level of Bcl-2 protein was by RNAse H inactivation of the encoding mRNA.

We have shown for the first time that the 2'-O-methyl modification results in a reduction of important non-specific effects. [3H]Thymidine incorporation into DNA has been used by others as a means of assaying cellular growth and proliferation, because dead and dying cells do not incorporate thymidine into their DNA (23,24). Under our transfection conditions, oligomers carrying just the phosphorothioate modification were about three times as inhibitory to the growth of T24 cells as oligomers that included 12 2'-O-methyl-modified nucleotides (Fig. 3). Moreover, we have determined that the reduced non-specific effects of Me-S-ODNs could be related to their reduced binding to cellular proteins, such as RPA (Fig. 5A and B). There was a decided decrease in binding affinity for the RPA70-A domain, by a factor of ~10-fold, when 2'-O-methyl sugars were included in the phosphorothioate ODN. This decrease in binding affinity for RPA helps ensure that an ODN-RNA hybrid will remain stable in the presence of this cellular protein (Fig. 5C).

Others have found that S-ODNs and G-tetraplex-forming oligomers bind to nucleolin and that this binding can result in altered cell morphology and apoptosis (38–40). It remains to be determined whether Me-S-ODNs have reduced nucleolin-binding properties, consistent with the possibility that the 2'-O-methyl modification leads to reduced non-specific effects via reduced binding to cellular proteins.

Our previous work with the model Ff g5p single-stranded DNA-binding protein showed that a fully 2'-O-methyl-modified S-dA36 oligomer had about one-half of the binding affinity for g5p as did the simple S-dA36 oligomer (6). The use of a model protein such as the g5p, or the RPA70-A domain used in the present study, to assay relative binding affinities may help establish which nucleotide modifications are likely to have reduced non-specific effects in cells.

In summary, chimeric ODNs carrying 12 2'-O-methyl-modified nucleotides would be a good choice to include, in addition to simple phosphorothioates, in much needed comparative studies (4) of different antisense molecules. Our results also suggest that there could be a reduction in cost of synthesizing oligomers with 2'-O-modified sugars by including all 12 modified nucleotides at the 5' or the 3' end of the oligomers.

ACKNOWLEDGEMENTS
This work was performed by B.H.Y. in partial fulfillment of the requirements for the PhD degree in the Department of Molecular and Cell Biology, The University of Texas at Dallas. Support was provided in part by grants from the Robert A. Welch foundation (AT-503), eXegenics, Inc. (Dallas, TX) and the Texas Advanced Technology Program (009741-0021-1999) to D.M.G. and from the US National Institutes of Health to A.B. (RO1 GM061192).

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