Nuclease Resistance and Antisense Activity of Modified Oligonucleotides Targeted to Ha-ras*

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We have previously described structure-activity studies on a 17-mer uniform phosphorothioate antisense sequence targeted to human Ha-ras. In an effort to further improve the pharmacological properties of antisense oligonucleotides, structure-activity studies on this 17-mer sequence were expanded to examine both the effects of replacing phosphorothioate backbone linkages with phosphodiester linkages and the effects of incorporating various 2'–sugar modifications into phosphorothioate and phosphodiester oligonucleotides on oligonucleotide stability against nucleases in vitro and on antisense activity in cells. Replacement of three or more phosphorothioate linkages with phosphodiester linkages greatly compromised both nuclease resistance and antisense activity, and these effects correlated directly with the number of phosphodiester linkages incorporated into the oligonucleotide. However, substantial nuclease resistance sufficient for obtaining potent antisense effects in cells, was conferred to phosphodiester oligonucleotides by incorporation of appropriate 2'-alkoxy sugar modifications. Nuclease stability and antisense activity imparted by these sugar modifications in phosphodiester backbones correlated with the size of the 2'–alkoxy substituent (pent oxy > prop oxy > meth oxy > deoxy). Furthermore, antisense activity mediated by oligonucleotides that exhibit partial resistance to nucleolytic degradation was dependent on both oligonucleotide concentration and the duration of oligonucleotide treatment.

Susceptibility of unmodified phosphodiester oligodeoxynucleotides to nucleolytic degradation by intracellular and extracellular nucleases has made them unattractive molecules for oligodeoxynucleotide therapeutics. Reports on the stability of unmodified oligodeoxynucleotides in biological fluids have demonstrated half-lives for these molecules as short as 5 min in serum and 30 min in living cells (1–4). The primary mechanism of oligodeoxynucleotide degradation in serum has been reported to be 3'-exonuclease activity, whereas both endonuclease and exonuclease activity have been reported to play significant roles in the degradation of these molecules in cells (2–7).

To alleviate the problem of nucleolytic degradation, chemical modifications of the natural phosphodiester backbone have been introduced into oligonucleotides to increase their stability in biological systems (8, 9). The most commonly employed synthetic modification designed to reduce oligonucleotide sensitivity toward nucleases is the phosphorothioate analog, created by replacing one of the nonbridging oxygen atoms of the internucleotide linkage with sulfur (10). Stein and co-workers (11) have reported that the stability of phosphorothioate oligonucleotides against purified nucleases in vitro varies greatly depending on oligonucleotide sequence and the type of nuclease examined. Studies have also been performed demonstrating that uniform modification of oligonucleotides with nuclease-resistant linkages is not required to confer enhanced stability. For example, increased resistance to degradation in vitro can be achieved by substitution of one or more phosphodiester linkages at the 3'-end of an oligonucleotide with phosphorothioate modifications (2–5). Alternating phosphorothioate modifications with phosphodiester linkages has also been shown to increase the stability of these molecules against purified nucleases in vitro (5).

Despite the fact that phosphorothioate oligonucleotides display many attractive features, some potential limitations do exist with these compounds. For example, high concentrations of phosphorothioates have been shown to competitively inhibit a variety of nucleases and polymerases (6, 7, 12–14), interact with and potentially abrogate the activity of heparin-binding growth factors (13, 15), induce immune stimulatory effects in rodents (13, 16), cause complement activation and hypotension in monkeys, and induce clotting abnormalities in monkeys as a result of direct interactions with thrombin (17, 18). Although these potential limitations have not proven to be problematic in clinical trials to date, evaluation of novel oligonucleotide modifications that reduce phosphorothioate content but maintain stability against nucleolytic degradation is obviously warranted.

Enhanced nuclease stability of phosphodiester oligonucleotides containing modified nucleosides has been investigated with some success. Incorporation of α-anomers into oligonucleotides has been shown to dramatically increase their stability against nuclease degradation (19, 20). Significant enhancement of nuclease resistance has also been demonstrated in oligonucleotides that contain a methylene group in place of the oxygen in the ribose ring (21). Replacement of the 3'-sugar deoxy substituent with 2'-O-methyl and 2'-O-allyl modifications has been reported to increase oligonucleotide stability toward various nucleases under cell-free conditions (22, 23).

Cummins et al. have extended these studies by demonstrating that the sensitivity of a variety of 2'-alkoxy phosphodiester oligonucleotides toward snake venom phosphodiesterase under cell-free conditions is dependent on the size of the 2'-substituent with nuclease resistance correlating directly with 2'-alkoxy chain length (24). In addition, it has been reported that fluorescently labeled 2'-O-methyl and 2'-O-allyl modified phosphodiester oligonucleotides are detectable in mammalian cells for greater periods of time following microinjection, as com-
pared with fluorescently labeled unmodified oligodeoxynucleotides (25).

Despite the progress achieved investigating the stability of modified oligonucleotides toward nucleases under cell-free conditions, reports directly demonstrating a relationship between the level of nuclease resistance observed for a modified oligonucleotide under cell-free conditions and the degree of antisense activity obtained in cells are rare. Unfortunately, conclusions regarding the level and duration of antisense activity that will be obtained by a modified, "nuclease-resistant," oligonucleotide based on the extrapolation of results from cell-free nuclease assays can be misleading. One reason for this is that the level of nuclease activity that must be overcome by an effective antisense oligonucleotide prior to, during, and after cell uptake is unknown. Furthermore, different cell types and intracellular compartments contain different types of nucleases and levels of nuclease activity (26). Additionally, sequence and secondary structure can greatly affect the sensitivity of an oligonucleotide to nuclease degradation (24). Thus, it is essential to directly compare cell-free nuclease results with antisense effects in cells when drawing conclusions regarding the utility of a nuclease-resistant modification for the purpose of antisense exploitation.

We have previously described a 17-mer phosphorothioate antisense oligonucleotide targeted to the codon 12 region of mutant H-a-ras expression, relative to wild type, in cells (27). Additionally, structure-activity studies have been performed on this phosphorothioate in which various 2'-sugar modifications were evaluated for their ability to direct RNase H cleavage of the target mRNA in vitro, to affect target affinity, and to modulate antisense activity against the Ha-ras message in cells (28). In that report, it was demonstrated that antisense activity can be significantly enhanced through the use of certain 2'-sugar modifications that hybridize to complementary RNA with a relatively high affinity, relative to unmodified DNA, provided that the oligonucleotide is designed as a chimera in which the 2'-sugar-modified region of the oligonucleotide, which is unable to activate RNase H, is fused to an RNase H-sensitive deoxy gap region of the appropriate length.

We now describe a systematic study in which modified chimeric oligonucleotides were characterized for both their relative susceptibility to degradation by purified nucleases in vitro and their ability to elicit antisense effects in cells. The antisense target for these studies was again the Ha-ras oncogene containing a GGC → GTC point mutation at codon 12 (27). Our results indicate that replacement of as few as three phosphorothioate linkages with phosphodiester linkages in an oligonucleotide greatly compromises both nuclease resistance and antisense activity and that substantial nuclease resistance, sufficient for obtaining antisense activity in cells, can be conferred to phosphodiester oligonucleotides through the use of appropriate 2'-alkoxy modifications. Furthermore, our findings demonstrate that the antisense activity observed for oligonucleotides that exhibit partial resistance to nuclease degradation is dependent on both the employed oligonucleotide concentration as well as the duration of oligonucleotide treatment.

**MATERIALS AND METHODS**

**Cells and Reagents**—The human bladder carcinoma cell line T24 was obtained from the American Type Culture Collection (Bethesda, MD). T24 cells were grown in McCoy's 5a medium supplemented with 10% fetal bovine serum at 37 °C with 5% CO₂. This cell line contains and expresses oncogenic Ha-ras containing a homozygous point mutation at codon 12 (GGC → GTC) (29, 30). DOTMA:DOPE (Lipofectin) solution (N-[1-(2,3-dioleyloxy-propyl)-N,N,N-triethylammonium chloride) was purchased from Life Technologies, Inc. (Gaithersburg, MD). Opti-MEM was purchased from Life Technologies, Inc. Snake venom phosphodiesterase was purchased from U.S. Biochemical Corp. S1 nuclease was purchased from Life Technologies, Inc.

**Oligonucleotide Synthesis**—2'-alkoxy and 2'-fluoro monomers were synthesized as described previously (31, 32). Synthesis of phosphorothioate and phosphodiester oligonucleotides (deoxy and 2'-modified) were performed using an Applied Biosystems 380B automated DNA synthesizer as described previously (27). Purification of oligonucleotide products was also as described previously (27). Purified oligonucleotide products were greater than 90% full-length material as determined by polyacrylamide gel electrophoresis analysis.

**In Vitro Stability Studies**—Oligonucleotides were purified by polyacrylamide gel electrophoresis and desalted using Poly Pak Cartridges (Glen Research, Sterling, VA). Labeling was carried out using [γ³²P]ATP and T4 polynucleotide kinase. After the labeling reaction, the samples were heated at 95 °C for 2 min to inactivate the T4 polynucleotide kinase for snake venom phosphodiesterase assays. Nuclease stability of the oligonucleotides was assayed at 0.1 µM oligonucleotide using 5 × 10⁻³ units/ml snake venom phosphodiesterase (U.S. Biochemical Corp.) in a buffer of 50 mM Tris-HCl, pH 8.5, 72 mM CaCl₂, and 14 mM MgCl₂ in a final volume of 50 µl. For Bal31 nuclease assays, nuclease stabilities of the oligonucleotides were assayed at 0.1 µM oligonucleotide using 2 × 10⁻² units/ml Bal31 nuclease (Boehringer Mannheim) in a buffer of 20 mM Tris-HCl, pH 7.5, 30 mM NaCl, 5 mM CaCl₂, 5 mM MgCl₂, 5 mM EDTA (final volume = 100 µl). For both nuclease assays, 5-µl reaction aliquots were removed at the indicated times, added to an equal volume of 80% formamide containing bromphenol blue and xylene cyanol dye tracking dyes, and then heated for 2 min at 95 °C. Aliquots were then stored at −20 °C until analysis by denaturing polyacrylamide electrophoresis. Quantiﬁcation was performed on a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Oligonucleotide Treatment of Cells**—T24 cells growing in 10-cm plates at a density of 50–75% confluency were used for oligonucleotide treatments and mRNA analysis. Cells were washed one time with phosphate-buffered saline, prewarmed to 37 °C, and Opti-MEM containing 5% fetal bovine serum, prewarmed to 37 °C, and Opti-MEM containing 10% fetal bovine serum, and the cells were incubated 37 °C. Northern Blot Analysis—Total RNA was prepared from cells by the guanidinium isothiocyanate procedure (33) 24–72 h (as indicated under “Results”) following initiation of oligonucleotide treatment. Total RNA was isolated by centrifugation of the cell lysates over a cesium chloride cushion (33). RNA samples were electrophoresed through 1.2% agarose-formaldehyde gels and transferred to Zeta-Probe hybridization membrane (Bio-Rad) by capillary diffusion overnight, a 12-14 h period. The RNA was cross-linked to the membrane by exposure to ultraviolet light in a Stratalinker (Strategene) and hybridized to random-primed ³²P-labeled full-length cDNA probes corresponding to human Ha-ras or human glyceraldehyde-3-phosphate dehydrogenase. RNA was quantitated using a Molecular Dynamics PhosphorImager as described previously (34).

**RESULTS**

**Phosphorothioate/Phosphodiester Chimeras**—In previous reports, oligonucleotide phosphorothioates (P=S) of varying lengths were tested for antisense activity and selectivity for Ha-ras mRNA containing a G → T transversion at codon 12 (27, 28). In those reports, antisense activity was shown to correlate directly with relative affinity of an oligonucleotide for its RNA target and on the ability of the oligonucleotide to activate RNase H cleavage in vitro using HeLa cell extracts (22, 23). The oligonucleotide that conferred the greatest mutant selectivity in those reports was a 17-mer, the sequence of which is shown in Fig. 1.

Based on the sequence of the mutant selective 17-mer oligonucleotide, a series of chimeric P=S oligonucleotides were synthesized using 2'-sugar modification.
Modified Oligonucleotides as Antisense Inhibitors of Ha-ras

### Table

| Oligo | Sequence |
|-------|----------|
| 1     | Cs Cs As Cs As Cs Cs Cs Gs As Cs Gs Gs Cs Gs Cs Cs C |
| 2     | Cs Cs As Cs As Cs Cs Cs Gs Ao Cs Gs Gs Cs Gs Cs Cs C |
| 3     | Cs Cs As Cs As Cs Cs Gs Ao Cs Gs Gs Cs Gs Cs Cs C |
| 4     | Cs Cs As Cs As Cs Cs Gs Ao Cs Gs Gs Cs Gs Cs Cs C |
| 5     | Cs Cs As Cs As Cs Cs Gs Ao Gs Gs Gs Cs Gs Cs Cs C |
| 6     | Cs Cs As Cs As Cs Cs Gs Ao Gs Gs Gs Cs Gs Cs Cs C |
| 7     | Cs Cs As Cs Cs Cs Cs Gs Ao Gs Ao Gs Gs Cs Gs Cs Cs C |
| 8     | Cs Cs As Cs Cs Cs Cs Gs Ao Gs Ao Gs Gs Cs Gs Cs Cs C |
| 9     | Cs Cs As Cs Cs Cs Cs Cs Gs Ao Cs Gs Gs Gs Gs Cs Gs Cs Cs C |

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**Fig. 1.** Design of phosphorothioate/phosphodiester chimeric antisense oligonucleotides. The 17-mer antisense oligonucleotide sequence is targeted to the mutated (GGC → GUC) codon 12 region of human Ha-ras mRNA expressed in the bladder carcinoma cell line T24 (27, 29, 30). Phosphorothioate backbone linkages are indicated by a lowercase s between bases; phosphodiester backbone linkages are indicated by a lowercase o between bases and are underlined. Oligonucleotide sequence is shown 5’ to 3’.

The uniform P=5 and the chimera containing a single P=O linkage (oligos 1 and 2) were both totally resistant to endonuclease degradation. Additionally, nuclease sensitivity correlated directly with the number of P=O linkages (“P=O content”) for chimeras containing 2 or more P=O linkages. The greatest increase in nuclease sensitivity occurred when P=O content was increased from three linkages to four (oligos 4 and 5, respectively). Finally, all of the chimeras were less sensitive to nuclease degradation as compared with the uniform P=O oligonucleotide, with the exception of oligo 7 which contains 10 consecutive P=O linkages.

To determine the ability of P=5/P=O chimeric oligonucleotides to elicit antisense effects in cells, Ha-ras transformed T24 cells were treated in culture with oligonucleotides 1–8 (Fig. 1) at a final oligonucleotide concentration of either 0.1 μM or 1.0 μM and antisense activity was assessed by analysis of Ha-ras mRNA expression. As shown in Fig. 3, oligonucleotide-mediated inhibition of Ha-ras mRNA expression was dose-dependent. At the high dose (1.0 μM), complete inhibition of Ha-ras mRNA expression was observed for oligonucleotides 1–6 (Fig. 3C). However, at the low dose (0.1 μM), the relative antisense activity between oligonucleotides containing different amounts of P=O linkages could be readily distinguished. Chimeras containing one or two P=O linkages displayed activity equal to that of the parent uniform P=5. However, as P=O content was increased beyond two linkages, antisense activity gradually decreased. Antisense activity was not observed at either dose for the chimera containing 10 consecutive P=O linkages (oligo 7) nor with the uniform phosphodiester (oligo 8).

The kinetics of antisense inhibition of Ha-ras mRNA was also determined for the P=5/P=O chimeric oligonucleotide series (Fig. 4). In this analysis, T24 cells were treated with a relatively low concentration of antisense oligonucleotide (0.1 μM) and Ha-ras mRNA levels were determined at 4, 10, and 24 h following initiation of oligonucleotide treatment. The degree of antisense activity induced by the chimeric oligonucleotides was found to be highly time-dependent (Fig. 4). Oligonucleotides containing 0, 1, or 2 P=O linkages all displayed the greatest activity, which was equal and maintained throughout the analysis (up to 24 h). Oligonucleotides containing 3–5 consecutive P=O linkages displayed good to moderate activity at the early time point (4 h) but substantially diminished activity over time. This loss in activity over time for oligonucleotides 4–6 correlated well with the loss of intact oligonucleotide over time in these cells as determined by capillary gel electrophoresis.2 Oligonucleotide 7, which contains 10 consecutive P=O linkages, as well as oligonucleotide 10, which contains eight alternating P=O linkages, displayed no significant antisense activity at any time following oligonucleotide administration.

2-Sugar-modified Chimeras—A series of 2'-alkoxy and 2'-fluoro, sugar-modified oligonucleotides were analyzed for their ability to confer both resistance to snake venom phosphodiesterase exonuclease activity in vitro and antisense activity

2 B. P. Monia, unpublished experiments.
against Ha-ras in intact cells. Since these modifications have been reported to be unable to support RNase H cleavage in vitro (28), and since three or more consecutive deoxy/P O linkages greatly compromises the antisense activity observed in cells (Figs. 2–4), the Ha-ras mutant-selective 17-mer test sequence was designed as a chimera containing 2'-sugar-modified/P O regions flanking a centered deoxy/P S "gap" that is of sufficient length to support RNase H activity in vitro and antisense activity in cells (28). In addition, oligonucleotides of identical design but containing a uniform P S backbone were synthesized and tested for comparative antisense activity. The sugar modifications included in this series were methoxy, propoxy, pentoxy, and fluoro. These modifications have previously been reported to markedly affect affinity for a complementary RNA sequence with a rank order (high to low affinity) fluoro > methoxy > propoxy > pentoxy = deoxy (28). The design of these chimeric oligonucleotides along with the chemical structures of the sugar modifications examined in this study are illustrated in Fig. 5.

Significant exonuclease resistance, relative to the unmodified deoxy/P = O chimera, was observed for all of the 2'-alkoxy-modified oligonucleotides (Fig. 6). However, the degree of resistance conferred by a given 2'-alkoxy-modification was dependent on the length of the alkoxy chain. The pentoxy modification clearly conferred the greatest exonuclease resistance, displaying stability to degradation equal to that of the uniform deoxy/phosphorothioate (t1/2 > 5 h). The 2'-propoxy chimera displayed resistance dramatically less than that of the pentoxy chimera but slightly better than that displayed by the methoxy chimera (propoxy t1/2 = 60 min; methoxy t1/2 = 30 min).
The 2'-fluoro-modified chimera displayed no enhanced exonuclease resistance, as compared with the unmodified deoxy/P5O chimera (t1/2, 5 min).

The results described above indicate that the rank order of resistance to exonucleolytic degradation conferred by the 2'-sugar modifications tested in this study are pentoxy > propoxy > methoxy > fluoro = deoxy. To determine whether these in vitro results correlate with antisense activity in intact cells, these same oligonucleotides were tested for inhibition of Ha-ras mRNA expression at two doses (0.1 μM and 1.0 μM). As shown in Fig. 7, antisense activity of the 2'-modified chimeric series was dose-dependent. As previously reported (28), all of the chimeras containing a uniform P=S backbone displayed potent activity at both low and high oligonucleotide concentrations. However, the level of activity achieved for these chimeras differed, depending on the particular modification, with the most potent modification in the uniform P=S backbone being the 2'-fluoro followed by the 2'-methoxy and 2'-propoxy. The 2'-pentoxy chimera displayed activity equal to that of the parent deoxy P=S oligonucleotide. This rank order potency for these 2'-modified chimeras (fluoro > methoxy > propoxy > pentoxy = deoxy) correlates directly with the affinity of these molecules for their complementary RNA sequence (28).

The relative activity of chimeras containing 2'-modifications in a P=S backbone was markedly different from that of the uniform P=S 2'-modified chimeras (Fig. 7). In this case, activity did not correlate with relative affinity for the complementary RNA sequence but instead correlated with the snake venom phosphodiesterase exonuclease resistance conferred by the particular 2'-modification (Fig. 6). The exonuclease-resistant 2'-alkoxy-modified chimeras all displayed dose-dependent activity. However, the most active oligos in this series contained 2'-pentoxy or 2'-propoxy modifications. The 2'-methoxy chimeras displayed intermediate activity, and the 2'-fluoro and unmodified deoxy chimeras displayed no antisense activity at either of the employed concentrations.

The kinetics of antisense inhibition of Ha-ras mRNA expression by the 2'-modified P=S/P = O chimeras was also examined.
in T24 cells treated with low oligonucleotide doses (0.1 μM), (Fig. 8). Duration of antisense activity for a particular 2'-modified oligonucleotide correlated directly with the relative resistance to snake venom phosphodiesterase-mediated exonuclease degradation in vitro (Fig. 5). For example, at 4 h following oligonucleotide treatment, the extent of activity for pentoxy, prooxy, and methoxy chimeras was virtually indistinguishable. However, at 48 h following initiation of oligonucleotide treatment, a clear rank order activity was apparent (pentoxy > prooxy > methoxy). At no time point did the 2'-fluoro-modified P=S/P=O chimera display significant activity. These results demonstrate that, in a P=O backbone, relative activity of a modified oligonucleotide is strongly dependent on the degree of nucleotide stability conferred by oligonucleotide modifications regardless of relative affinity for the target mRNA.

**DISCUSSION**

We have previously identified a 17-mer phosphorothioate antisense oligonucleotide targeted to the Ha-ras codon 12 point mutation (GGC → GTC), which displays point mutation specificity for mutated forms of Ha-ras (27). We have also shown previously that incorporation of high affinity 2'-sugar modifications within this uniform phosphorothioate oligonucleotide increases antisense potency up to 15-fold in a manner that correlates directly with increased target affinity conferred by the particular 2'-sugar modification (28). In that study, relative nuclease stability of the modified oligonucleotides did not contribute significantly to relative antisense activity, since activity was assessed at short times following oligonucleotide treatment and the oligonucleotides were synthesized as stable uniform phosphorothioates.

In this report, we have used this 17-mer sequence to test the effects of replacing P=S backbone linkages with P=O linkages on both nuclease stability in vitro and antisense activity in cells. We also determined the relative stability of oligonucleotides containing various 2'-sugar modifications against nuclease degradation in vitro and their relative activity for inhibiting target gene expression in cells. Our results demonstrate a clear correlation between the in vitro nuclease stability and antisense activity of the tested modified oligonucleotides. Replacement of P=S linkages with P=O linkages was found to greatly reduce both stability toward nucleases in vitro and antisense activity in cells. Furthermore, a direct correlation was observed between the number of P=O linkages introduced into an oligonucleotide ("P=O content") and nuclease sensitivity. However, reduced antisense activity resulting from the introduction of P=O linkages was overcome to some extent through the utilization of higher oligonucleotide concentrations or by examining antisense effects at shorter time periods following the initiation of oligonucleotide treatment.

In agreement with a previous report (24), 2'-alkoxy modifications introduced into a P=O backbone were found to increase stability toward snake venom phosphodiesterase in a manner that correlated directly with 2'-alkoxy chain length. 2'-pentoxy modifications were found to be the most stabilizing sugar modifications examined in this study. This modification, when tested in a P=O backbone, displayed stability toward exostrandic cleavage in vitro and antisense activity in cells equal to that of a uniform deoxy/P=S oligonucleotide of the same sequence. 2'-prooxy/P=O and 2'-methoxy/P=O oligonucleo-
We have observed that combination of nuclease-resistant sugar modifications and nuclease-resistant backbone modifications yields antisense molecules possessing levels of nuclease resistance far greater than that of simple P=S oligonucleotides. These findings suggest that when the level of nuclease resistance required for a particular oligonucleotide application is even greater than that conferred by phosphorothioate oligodeoxynucleotides, combinations of nuclease-resistant backbone modifications with nuclease-resistant sugar modifications may be the design of choice. Such an application has been suggested by Agrawal and colleagues (35, 36), who demonstrated oral bioavailability in rodents with a 2′-methoxy chimeric phosphorothioate. The study reported here suggests that utilization of other 2′-modifications that display even greater nuclease resistance than 2′-methoxy (e.g. propoxy and pentoxy), when incorporated into a P=S backbone, may yield oligonucleotides with even greater bioavailability than 2′-methoxy chimeric P=S oligonucleotides. Studies examining these and other pharmacokinetic parameters for 2′-modified antisense oligonucleotides are in progress.

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REFERENCES

1. Cazenave, C., Chevrier, M., Thuong, N. T., and Helm, C. (1987) Nucleic Acids Res. 15, 10507–10521
2. Tidd, D. M., and Warenius, H. M. (1989) Br. J. Cancer 60, 343–350
3. Shaw, J. P., Kent, K., Bird, J., Fishback, J., and Froehler, B. (1991) Nucleic Acids Res. 19, 747–750
4. Woolf, T. M., Jennings, C. G. B., Rebagliati, M., and Melton, D. A. (1990) Nucleic Acids Res. 18, 1763–1769
5. Hoke, G. D., Draper, K., Freier, S. M., Gonzalez, C., Driver, V. B., Zaunes, M. C., and Eckert, D. J. (1991) Nucleic Acids Res. 19, 5743–5748
6. Crooke, R. M., Graham, M. J., Cooke, M. E., and Crooke, S. T. (1995) J. Pharmacol. Exp. Ther. 275, 462–473
7. Crooke, S. T., Graham, M. J., Zackman, J. E., Brooks, D., Conklin, B. S., Cummins, L. L., Greig, M. J., Guinosso, C. J., Kornbrust, D., Manoharan, M., Saurur, H. M., Schleich, T., Tivet, K. L., and Griffey, R. H. (1996) J. Pharmacol. Exp. Ther., in press
8. Cook, P. D. (1991) Anti-Cancer Drug Des. 6, 585–607

Fig. 8. Kinetic analysis of Ha-ras mRNA reduction in cultured T24 cells by 2′-sugar-modified chimeric antisense oligonucleotides. T24 cells were treated with 0.1 μM of the indicated oligonucleotide (oligonucleotides 10, 12, 14, 16, and 18, as described in Fig. 5), and Ha-ras mRNA levels were determined at the indicated times following initiation of oligonucleotide treatment, as described under "Materials and Methods." Percentage of control was calculated as described in Fig. 3. The results are representative of two independent experiments.

tides were also found to confer significant nuclease resistance relative to a deoxyP=O oligonucleotide. As was the case for the 2′-pent oxyP=O modifications, these 2′-alkoxy P=O modified oligonucleotides also elicited significant antisense effects in cells that were both dose- and time-dependent and in a manner that correlated with relative stability against nuclease degradation in vitro. A 2′-fluoroP=O modified oligonucleotide displayed sensitivity toward snake venom phosphodiesterase equal to that of the unmodified deoxyphosphodiester oligonucleotide and was completely ineffective as an antisense agent in cells. These results demonstrate that appropriate 2′-sugar oligonucleotide modifications are attractive alternatives for the design of effective nuclease-stable antisense molecules with reduced phosphorothioate content.

All of the 2′-sugar modifications described above were also tested as chimeras having a uniform P=S backbone for antisense activity in cells. As we have previously reported (28), all of the 2′-sugar-modified P=S chimeras displayed potent antisense activity in cells. However, the level of activity achieved for these chimeras differed, depending on relative affinity for their target RNA and not on relative nuclease resistance (relative affinity = fluoro > methoxy > propoxy > pentoxy = deoxy). Thus, for 2′-modifiedP=S oligonucleotides, antisense potency is most dependent on the degree of nuclease stability conferred by the particular 2′-sugar modification. However, for oligonucleotides stabilized by uniform P=S incorporation, additional nuclease sensitivity is not an important determinant of antisense activity when tested up to 48 h following oligonucleotide treatment. Under these conditions, antisense activity is primarily determined by the relative affinity of the oligonucleotide for its target RNA, which is conferred by a particular 2′-sugar modification.

In addition to the studies described above, we have also examined the nuclease sensitivity and antisense activity of 2′-modified chimeras in uniform P=S backbones over very long periods of time following oligonucleotide treatment (>60 h). In these studies, we have observed that combination of nuclease-resistant sugar modifications and nuclease-resistant backbone modifications yields antisense molecules possessing levels of nuclease resistance far greater than that of simple P=S oligonucleotides. These findings suggest that when the level of nuclease resistance required for a particular oligonucleotide application is even greater than that conferred by phosphorothioate oligodeoxynucleotides, combinations of nuclease-resistant backbone modifications with nuclease-resistant sugar modifications may be the design of choice. Such an application has been suggested by Agrawal and colleagues (35, 36), who demonstrated oral bioavailability in rodents with a 2′-methoxy chimeric phosphorothioate. The study reported here suggests that utilization of other 2′-modifications that display even greater nuclease resistance than 2′-methoxy (e.g. propoxy and pentoxy), when incorporated into a P=S backbone, may yield oligonucleotides with even greater bioavailability than 2′-methoxy chimeric P=S oligonucleotides. Studies examining these and other pharmacokinetic parameters for 2′-modified antisense oligonucleotides are in progress.
9. Cook, P. D. (1993) in Antisense Research and Applications (Crooke, S. T., and LeBel, B., eds) pp. 149–187, CRC Press, Inc., Boca Raton, FL
10. Eckstein, F. (1985) Annu. Rev. Biochem. 54, 367–372
11. Stein, C. A., Subasinghe, C., Shinouzuki, K., and Cohen, J. S. (1988) Nucleic Acids Res. 16, 3209–3221
12. Gao, W. Y., Han, F. S., Storm, C., Egan, W., and Cheng, Y. C. (1991) Mol. Pharmacol. 43, 223–230
13. Crooke, S. T. (1995) Therapeutic Applications of Oligonucleotides, pp. 63–79, R. G. Landes Co., Austin, TX
14. Crooke, S. T., Lemonidis, K. L., Neilson, L., Griffey, R., Lesnik, E. A., and Monia, B. P. (1995) Biochem. J. 312, 599–608
15. Guvakova, M. A., Yukubov, L. A., Vladovskiy, I., Tonkinson, J. L., and Stein, C. A. (1994) J. Biol. Chem. 270, 2620–2627
16. Pisetsky, D. S., and Reich, C. F. (1995) Life Sci. 54, 101–109
17. Cornish, K. G., Iversen, P., Smith, L., Becker, D., and Meyer, C. B. (1993) Pharmacol. Res. Commun. 3, 239–244
18. Galbraith, W. M., Holson, W. C., Giclas, D. C., Schechter, P. J., and Agrawal, S. (1994) Antisense Res. Dev. 4, 201–211
19. Morvan, F., Rayner, B., Imbach, J. L., Thenet, S., Bertrand, J. R., Padgett, J., Malvy, C., and Padett, C. (1987) Nucleic Acids Res. 15, 3421–3437
20. Bacon, T. A., Morvan, F., Rayner, B., Imbach, J.-L., and Wickstrom, E. (1988) J. Biomed. Biophys. Methods 16, 311–314
21. Perbost, M., Lucas, M., Chavis, C., Pompon, A., Baumgartner, H., Rayner, B., Griengl, H., and Imbach, J.-L. (1989) Biochem. Biophys. Res. Commun. 165, 742–744
22. Sproat, B. S., Lamond, A. I., Beijer, B., Neuner, P., and Ryder, U. (1989) Nucleic Acids Res. 17, 3373–3386
23. Iribarren, A. M., Sproat, B. S., Neuner, P., Sulston, I., Ryder, V., and Lamond, A. I. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7477–7481
24. Cummins, L. L., Owens, S. R., Risen, L. M., Lesnik, E. A., Freier, S. M., McGee, D., Guinosso, C. J., and Cook, P. D. (1995) Nucleic Acids Res. 23, 2019–2024
25. Fisher, T. L., Terhorst, T., Cao, X., and Wagner, R. W. (1993) Nucleic Acids Res. 21, 3857–3865
26. Linn, S. M., Lloyd, R. S., and Roberts, R. J. (eds) (1993) Nucleases, 2nd Ed., pp. 1–34, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
27. Monia, B. P., Johnston, J. F., Ecker, D. J., Zounes, M. A., Lima, W. F., and Freier, S. M. (1992) J. Biol. Chem. 267, 19954–19962
28. Monia, B. P., Lesnik, E. A., Gonzalez, C., Lima, W. F., McGee, D., Guinosso, C. J., Kawasaki, A. M., Cook, P. D., and Freier, S. M. (1993) J. Biol. Chem. 268, 14514–14522
29. Reddy, E. P., Reynolds, R. K., Santos, E., and Barbacid, M. (1982) Nature 300, 149–152
30. Reddy, E. P. (1983) Science 220, 1061–1063
31. Guinosso, C. J., Hoke, G. D., Freier, S. M., Martin, J. F., Ecker, D. J., Mirabeli, C. K., Crooke, S. T., and Cook, P. D. (1991) Nucleosides & Nucleotides 10, 209–262
32. Kawasaki, A. M., Casper, M. D., Freier, S. M., Lesnik, E. A., Zounes, M. A., Cummins, L. L., Gonzalez, C., and Cook, P. D. (1993) J. Med. Chem. 36, 831–841
33. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1987) Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York
34. Dean, N. M., McKay, R., Condon, T., and Bennett, C. F. (1994) J. Biol. Chem. 269, 16416–16424
35. Agrawal, S., Temsamani, J., and Tang, J. Y. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7905–7909
36. Agrawal, S., Zhang, X., Lu, Z., Zhao, H., Tamburin, J. M., Yan, J., Cai, H., Diasio, R. B., Habus, I., Jiang, Z., Iyer, R. P., Yu, D., and Zhang, R. (1995) Biochem. Pharmacol. 56, 571–576
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