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Investigation of some properties of oligodeoxynucleotides containing 4′-thio-2′-deoxynucleotides: duplex hybridization and nuclease sensitivity

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ABSTRACT

The thermal stabilities of the duplexes formed between 4′-thio-modified oligodeoxynucleotides and their DNA and RNA complementary strands were determined and compared with those of the corresponding unmodified oligodeoxynucleotides. A 16mer oligodeoxynucleotide containing 10 contiguous 4′-thiothymidylate modifications formed a less stable duplex with the DNA target (ΔT_m/modification –1.0°C) than the corresponding unmodified oligodeoxynucleotide. However, when the same oligodeoxynucleotide was bound to the corresponding RNA target, a small increase in T_m was observed (ΔT_m/modification +0.16°C) when compared with the unmodified duplex. A study to identify the specificity of an oligodeoxynucleotide containing a 4′-thiothymidylate modification when forming a duplex with DNA or RNA containing a single mismatch opposite the modification found the resulting T_ms to be almost identical to the wild-type duplexes, demonstrating that the 4′-thio-modification in oligodeoxynucleotides has no deleterious effect on specificity. The nuclease stability of 4′-thio-modified oligodeoxynucleotides was examined using snake venom phosphodiesterase (SVPD) and nuclease S1. No significant resistance to degradation by the exonuclease SVPD was observed when compared with the corresponding unmodified oligodeoxynucleotide. However, 4′-thio-modified oligodeoxynucleotides were found to be highly resistant to degradation by the endonuclease S1. It was also demonstrated that 4′-thio-modified oligodeoxynucleotides elicit *Escherichia coli* RNase H hydrolysis of the RNA target only at high enzyme concentration.

INTRODUCTION

The inhibition of gene expression mediated by the binding of an antisense oligonucleotide to complementary mRNA offers great potential for therapeutic intervention in many human diseases (1–3). To be of therapeutic use *in vivo*, an antisense oligonucleotide needs to remain intact sufficiently long to be able to exert an effect and must therefore be stable to nucleases. It must also be able to penetrate cellular membranes and reach the target mRNA. Once in a position to interact with the mRNA, the antisense oligonucleotide needs to bind to the mRNA with sufficient affinity and specificity for subsequent translational arrest or degradation of the RNA by RNase H to occur. Unmodified oligonucleotides are limited in their usefulness for such applications, primarily due to rapid degradation of the oligonucleotide by extra- and intracellular nucleases.

In an attempt to impart nuclease resistance, while simultaneously minimizing deleterious effects on duplex stability and other properties, many chemically modified oligonucleotides have been synthesized (4,5). Among these, many sugar-modified oligonucleotides have been studied, despite the fact that a considerable amount of synthetic effort is required, due to the need first to synthesize the modified nucleoside before incorporation into oligonucleotides (6). Recently, more fundamental modifications of the sugar moiety have been achieved by replacing the 4′-furanose oxygen atom by a different atom. This has given rise to 4′-methylene- (7,8), 4′-aza- (9) and 4′-thio-modified oligonucleotides (10–12).

Convenient syntheses of 4′-thio-2′-deoxynucleosides was reported independently, in 1991, by Walker and co-workers (13,14) and Secrist and co-workers (15). Subsequent studies found that these nucleoside analogues showed considerable resistance to nucleoside phosphorylase (16), which gave rise to much research into 4′-thio-2′-deoxynucleoside analogues as potential antiviral agents (16,17).

A previous study into some properties of oligodeoxynucleotides containing 4′-thiothymidine demonstrated their resistance to hydrolysis by the restriction endonuclease *Eco*RV when the 4′-thio-modification was placed in an oligodeoxynucleotide within the sequence recognized by the enzyme (12). Conformational studies comparing thymidine with 4′-thiothymidine showed only very minor differences. X-Ray crystallography confirmed that the base was in the *anti*-conformation and the modified sugar adopted the South-type (C2′-endo/C3′-exo) pucker conformation. More relevant studies by ^{1}H-NMR into the dynamic equilibrium of the sugar pucker in solution found that 4′-thiothymidine adopts the identical equilibrium ratio of North/South conformers as does the natural nucleoside (18).

The crystal structure analysis of an oligodeoxynucleotide duplex dodecamer, containing four 4′-thiothymidine replacements for

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thymidine, has recently been published (19). A comparison of the structure with that of the native dodecamer revealed that the major differences between the two structures is a small change in the conformation of the sugar-phosphate backbone in the regions at and adjacent to the positions of the modified nucleosides. It was also observed that the modified sugars adopted a different conformation when compared with the sugars in the native structure. However, overall, the differences are very slight.

Bellon *et al*. published the synthesis of 4′-thio-oligo-β-**ribo**-nucleotides and reported the initial results of nuclease resistance and base-pairing properties of β-4′-thio-oligouridylates (10,11). The work carried out by the authors demonstrated that the 4′-thio-oligoribonucleotides were considerably more stable towards nucleases when compared with the corresponding unmodified oligoribonucleotides. Studies investigating the binding affinity of 4′-thio-oligoribonucleotides with complementary RNA indicated that a more stable duplex was formed. These results demonstrate that such oligonucleotides may be of use for antisense applications, although recent studies have shown that duplexes formed between 4′-thio-ribo-modified oligonucleotides and complementary RNA are not substrates for RNase H. This lack of RNase H activity is not surprising, as natural RNA/RNA duplexes have no substrate affinity for this enzyme either.

It can be conjectured that if the 2′-hydroxyl group were to be removed, giving rise to 4′-thio-2′-deoxyoligonucleotides, such a modification might retain the benefits of nuclease resistance and good binding affinity, as well as inducing RNase H when bound to complementary RNA. Such properties would have obvious potential for antisense applications, when compared with the desired properties previously discussed.

This paper reports on the hybridization properties of 4′-thio-modified oligodeoxynucleotides when forming a duplex with DNA or RNA, their stability towards degradation by some nucleases and the ability of these modified oligodeoxynucleotides to induce the cleavage of bound RNA by the enzyme RNase H.

MATERIALS AND METHODS

Oligonucleotide synthesis and 5′-^{32}P-end labelling

Synthesis of the protected phosphoramidite of 4′-thiothymidine was performed as previously published (12) and the protected phosphoramidite of 5-ethyl-4′-thio-2′-deoxyuridine was prepared in a similar manner. Synthesis of the 4′-thio-modified oligodeoxynucleotides was performed using standard automated solid phase phosphoramidite methodology by Oswel DNA service (University of Southampton, Southampton, UK). Purification was achieved using trityl-on/ trityl-off HPLC. Synthesis of all other oligonucleotides was performed by ISIS Pharmaceuticals (Carlsbad, CA, USA). Oligodeoxynucleotides were 5′-end labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase. After the labelling reaction, the T4 polynucleotide kinase was heat inactivated at 95°C for 3 min and oligomers used without any further purification.

Melting temperatures

Absorbance (260 nm) versus temperature curves of duplexes were measured at 8 µM total strand concentration in 100 mM Na^+, 10 mM phosphate, 0.1 mM EDTA, pH 7.0 as described elsewhere (20). The melting temperature (T_m) and free energies of duplex formation were obtained from fits of data to a two state model with a linear sloping base line (21). Free energies of duplex formation are a more valid measure of thermodynamic stability than T_m (22,23). Experimental errors, however, were larger for ΔG_{310} than for T_m. Therefore, we report T_m values in Tables 2, 3, 4 and 5. Similar trends were observed for free energies.

Nuclease stability studies

Snake venom phosphodiesterase (USB, Cleveland, OH) assays were performed using 100 nM oligomer at 37°C in a buffer of 50 mM Tris–HCl, pH 8.5, 75 mM NaCl and 14 mM $MgCl_2$ at an enzyme concentration of 5×10^{-3} U/ml. Nuclease S1 (Gibco-BRL, Gaithersberg, MD) assays were performed using 100 nM oligomer at 37°C in 30 mM NaOAc, pH 4.5, 50 mM NaCl, 1 mM $ZnCl_2$ (24). Nuclease S1 was used at a concentration of either 1.93×10^{-3}, 1.93×10^{-4} or 1.93×10^{-5} U/µl. Aliquots of the nuclease stability reactions were removed at the indicated times, quenched by addition to an equal volume of 80% formamide gel loading buffer containing tracking dyes, heated for 2 min at 95°C and then stored at –20°C for analysis by denaturing polyacrylamide electrophoresis (24). Quantitation was performed on a Molecular Dynamics Phosphorimager (Molecular Dynamics, Sunnyvale, CA), using a linear relationship between the signal and the image intensity.

Table 1. Oligonucleotide sequences used for T_m studies

No.	Oligonucleotide sequence (5′–3′)
1	d(GCGTTTTTTTTTGCG)
2	d(GCGTTT**TTTT**TTTGCG)[a]
3	d(GCG**TTTTTTTTT**GCG)[a]
4	d(GCG**EEEEEEEEE**GCG)[b]
5	d(CGCAAAAAAAAAACGC)
6	r(CGCAAAAAAAAAACGC)
7	d(CTCGTACC**T**TTCCGGTCC)[a]
8–**12**[c]	d(GGACCGGAA**X**GGTACGAG)
13–**17**[d]	r(GGACCGGAA**Y**GGUACGAG)

[a]**T**, 4′-thiothymidylate.
[b]**E**, 5-ethyl-4′-thio-2′-deoxyuridylate.
[c]**8**, X = A; **9**, X = G; **10**, X = C; **11**, X = T; **12**, X = none[e].
[d]**13**, Y = A; **14**, Y = G; **15**, Y = C; **16**, Y = U; **17**, Y = none[e].
[e]None refers to residue X or Y being absent (i.e., 17mer oligonucleotide).

RNase H digestion studies

The complementary RNA target sequence **24**, 2′-*O*-methyl-modified at the CGC ends to prevent degradation by exonucleases, was 5′-end labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase and subsequently purified by polyacrylamide electrophoresis. The ^{32}P-labelled RNA at a concentration of ~625 pM was hybridized with the complementary oligodeoxynucleotide at a concentration of 250 nM in a buffer of 20 mM Tris–HCl, pH 7.5, 20 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA and 0.1 mM DTT, in the presence of an RNase inhibitor. The mixture was incubated at 65°C, then left for 24 h at 37°C. *Escherichia coli* RNase H (USB, Cleveland, OH) assays were performed by the addition of RNase H to the hybridization mixture to give an enzyme concentration of 6.34×10^{-2}, 6.34×10^{-3}, 6.34×10^{-4} or 6.34×10^{-5} U/µl. Aliquots of the RNase H digestion reactions were removed at the indicated times, quenched by addition to an equal volume of 80% formamide gel loading buffer containing tracking dyes and then stored at –80°C for analysis by denaturing polyacrylamide electrophoresis.

Table 2. T_m results for DNA/DNA duplexes

duplex	modification	no. of mods	T_m/°C	ΔT_m/°C	ΔT_m/mod^{-1} °C
1/5	unmodified	0	53.4	–	–
2/5	4′-thioT	4	50.4	–3.0	–0.75
3/5	4′-thioT	10	43.7	–9.7	–0.97
4/5	5-Et-4′-thio-2′-dU	10	31.0	–22.4	–2.24

Table 3. T_m results for DNA/RNA duplexes

duplex	modification	no. of mods.	T_m/°C	ΔT_m/°C	ΔT_m/mod^{-1} °C
1/6	unmodified	0	48.0	–	–
2/6	4′-thioT	4	48.3	+0.3	+0.08
3/6	4′-thioT	10	49.6	+1.6	+0.16
4/6	5-Et-4′-thio-2′-dU	10	42.5	–5.5	–0.55

RESULTS AND DISCUSSION

Hybridization studies

The oligonucleotide sequences synthesized for the hybridization studies are shown in Table 1. The sequences were chosen because of the availability of data from other modified oligonucleotides of the same sequence which could be used as a control (25). The duplexes formed between the 4′-thiothymidylate-modified oligodeoxynucleotides and the DNA complement showed a decrease of ~1°C per modification (Table 2), suggesting a slightly unfavourable change in the structure of the duplex when compared with the natural duplex. This result is in agreement with the results of crystal structure analysis of an oligodeoxynucleotide duplex dodecamer containing four 4′-thiothymidylate replacements for thymidine, which showed a slight perturbation of the sugar-phosphate backbone in the region of the modification (19).

Duplex hybridization studies were also performed on an oligodeoxynucleotide analogue containing 5-ethyl-4′-thio-2′-deoxyuridylate modifications. A study by Ueda and co-workers (26), showed that a self-complementary DNA duplex containing two 5-ethyl-2′-deoxyuridylate modifications depressed the T_m by 2°C per modification when compared with the natural duplex. Therefore, the result reported herein, of a decrease in T_m of 2.2°C per 5-ethyl-4′-thio-2′-deoxyuridylate modification, is in agreement with the previously reported result when allowance is made for the difference in sequence, as well as the effect which the presence of a 4′-thio-sugar is causing on the T_m.

In contrast, the results of the melting temperatures for 4′-thiothymidylate-modified oligodeoxynucleotides bound to the RNA complement (Table 3) demonstrate a very slight increase in T_m. These results suggest that if the 4′-thiothymidylate modifications do have an effect on oligodeoxynucleotide structure, it is not detrimental to the formation of a duplex with complementary RNA.

The result for the 5-ethyl-4′-thio-2′-deoxyuridylate-modified oligodeoxynucleotide bound to complementary RNA (Table 3) showed a significant drop in T_m of 0.55°C per modification. However, from the previous results for 5-ethyl-4′-thio-2′-deoxyuridylate-modified oligodeoxynucleotides bound to DNA, where a decrease of ~2°C per modification was observed, the depression in T_m would appear to be entirely due to the 5-ethyl-uracil modification. The 4′-thio-modification is probably not contributing towards the decrease in T_m and may even be providing a slight beneficial effect.

It has been seen on previous occasions (27,28) that there is often no direct correlation between the effect on T_m of modified DNA/DNA duplexes and the corresponding modified DNA/RNA duplexes, which are more relevant for antisense purposes. Our observations suggest that the backbone torsion angles of a DNA/RNA duplex are better able to accommodate 4′-thiothymidine substitutions than a DNA/DNA duplex, which has smaller gamma and delta dihedral angles along the sugar-phosphate backbone.

Mismatch studies (29) were performed to identify whether the 4′-thio-modification was having a detrimental effect on specificity. One 4′-thiothymidylate modification was placed in the centre of an oligodeoxynucleotide and duplexes formed with the DNA and RNA complements with mismatches placed opposite the modification.

The T_m of the matched duplex containing the 4′-thiothymidylate modification, bound to the complementary DNA oligonucleotide was almost identical to the natural duplex (Table 4). Previously, it was observed that a decrease of 1°C per modification occurred in a 4′-thio-DNA/DNA duplex, suggesting that the near neighbour sequence may be an important factor in determining the effect of 4′-thio-modifications. Previously determined T_m values for mismatched duplexes containing an unmodified thymidylate in the mismatch position, show the same, or a slightly lower, specificity when compared with the 4′-thiothymidylate modification in the mismatch position (i.e., the decrease in T_m for each of the three possible mismatches was the same for thymidine and 4′-thiothymidine). The duplex **7/12** requires the 4′-thiothymidine, or thymidine in the unmodified duplex, to bulge out of the duplex so that the remaining 17 residues can perfectly base-pair. The destabilization caused by this bulge is again identical whether the bulged residue be 4′-thiothymidine or thymidine.

The T_m of the matched DNA/RNA duplex (d**T**-rA) showed an increase in T_m of ~3°C when compared with the natural duplex (Table 5). However, the melting temperatures for the 4′-thiothymidylate mismatched DNA/RNA duplexes were very similar to the corresponding unmodified duplexes. These results suggest that, although one 4′-thiothymidylate modification appears to increase the T_m of the DNA/RNA duplex by 3°C, when the modification is placed opposite a mismatch the specificity is as good as for the unmodified counterpart.

Table 4. Summary of T_m data for the specificity against DNA mismatches

Duplex	Mismatch base pair	4′-thio-dT T_m/°C	4′-thio-dT ΔT_m/°C	Unmodified dT T_m/°C	Unmodified dT ΔT_m/°C
7/8	d**T**–dA	61.8	0	61.7	0
7/9	d**T**–dG	54.7	–7.1	54.9	–6.8
7/10	d**T**–dC	45.9	–15.9	46.8	–14.9
7/11	d**T**–dT	55.3	–6.5	55.7	–6.0
7/12	d**T**–none	51.9	–9.9	51.9	–9.8

Table 5. Summary of T_m data for the specificity against RNA mismatches

Duplex	Mismatch base pair	4′-thio-dT T_m/°C	4′-thio-dT ΔT_m/°C	Unmodified dT T_m/°C	Unmodified dT ΔT_m/°C
7/13	d**T**–rA	66.1	0	63.3	0
7/14	d**T**–rG	61.3	–4.8	61.6	–1.7
7/15	d**T**–rC	55.1	–11.0	54.4	–8.9
7/16	d**T**–rU	56.0	–10.1	55.8	–7.5
7/17	d**T**–none	59.2	–6.9	59.4	–3.9

Table 6. Oligonucleotide sequences used for nuclease degradation studies and RNase H induction studies

No.	Oligonucleotide sequence (5′–3′)
18	d(TTTTTTTTTTTTTTTTTTT)
19	d(TsTsTsTsTsTsTsTsTsTsTsTsTsTsTsTsT)[a]
20	d(TTTTTTTTTTTTTT**TTTT**T)[b]
21	d(**TTTTTTTTTTTTTTTTTT**T)[b]
22	d(GsCsGsTsTsTsTsTsTsTsTsTsGsCsG)[a]
23	d(GCGT$_f$T$_f$T$_f$T$_f$T$_f$T$_f$T$_f$T$_f$T$_f$T$_f$GCG)[c]
24	r(C$_m$G$_m$C$_m$AAAAAAAAAAC$_m$G$_m$C$_m$)[d]

[a]Ts, Gs, Cs = phosphorothioate linkage.
[b]**T**, 4′-thiothymidylate.
[c]T_f, 2′-deoxy-2′-fluorothymidylate.
[d]C_m, G_m = 2′-*O*-methyl modified.

For an oligonucleotide modification to be of any use for an antisense application, it is important for the modified oligonucleotide to hybridize well to the complementary RNA and to be specific for the target sequence. The 4′-thio-modification to oligodeoxynucleotides appears to meet these requirements and even improve upon the natural oligodeoxynucleotide, if only slightly. The 4′-thio-modification performs slightly worse with DNA when compared with the unmodified oligodeoxynucleotide, although this is of no importance for antisense applications and may even be beneficial by not favouring undesirable interactions with DNA.

Nuclease sensitivity studies

Nuclease stability of the 19mer series, consisting of an unmodified oligodeoxynucleotide **18**, a fully phosphorothioate-modified oligomer **19**, a partially 4′-thio-modified oligodeoxynucleotide containing four modifications **20** and a completely 4′-thio-modified oligodeoxynucleotide apart from one unmodified residue at the 3′-end **21**, was examined using the exonuclease, snake venom phosphodiesterase. Visualisation of the gels demonstrated that whereas the phosphorothioate-modified oligodeoxynucleotide **19** was virtually intact after 15 min, the 4′-thio-modified oligodeoxynucleotides **20** and **21** had been degraded to a similar extent as the unmodified oligodeoxynucleotide **18**. Due to the 3′-end thymidylate residue of the 4′-thio-modified oligodeoxynucleotides **20** and **21** being unmodified, the quantitative degradation of the oligodeoxynucleotides was monitored by the loss of the full length oligodeoxynucleotide plus the corresponding *n*–1 degradation product. Loss of *n*–1 would then be due to the hydrolysis of the first 4′-thio-modification and therefore any difference in degradation due to the 4′-thio-modification can be identified. The results, Figure 1, demonstrate no significant difference between the degradation of the unmodified oligodeoxynucleotide **18** and the 4′-thio-modified oligodeoxynucleotides **20** and **21**, with half-lives of ~7, 7 and 9 min, respectively.

The stabilities of the oligodeoxynucleotides **18**, **19** and **21** were also investigated using nuclease S1; a single-strand-specific endonuclease. With this enzyme, degradation was monitored by the loss of the full length oligodeoxynucleotide, as the presence of the unmodified residue on the 3′-end of the 4′-thio-modified oligodeoxynucleotide **21** is far less important in the resulting degradation. At an enzyme concentration of 1.93×10^{-4} U/µl, no significant degradation of the 4′-thio-modified oligodeoxynucleotide **21** was observed over a 60 min interval, whereas under the same conditions, the unmodified oligodeoxynucleotide **18** was found to have a half-life of 6.5 min and the phosphorothioate-modified oligodeoxynucleotide **19** had a half-life of 15.5 min. In an attempt to observe significant degradation of the 4′-thio-modified oligodeoxynucleotide, the enzyme concentration was increased 10-fold to 1.93×10^{-3} U/µl. The results, shown in Figure 2, demonstrate that the 4′-thio-modified oligodeoxynucleotide **21** (half-life ~50 min) is >100-fold more resistant to degradation by nuclease S1 than the corresponding unmodified oligodeoxynucleotide **18** (half-life <30 s). In comparison, the 4′-thio-modified oligodeoxynucleotide **21** was also found to be almost 4-fold more

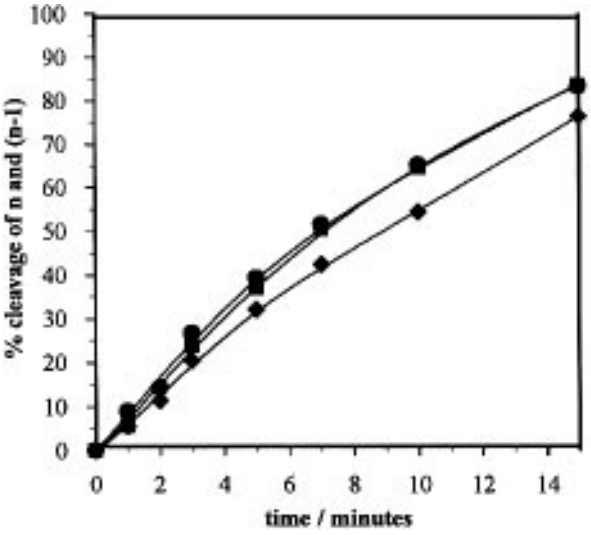

Figure 1. SVPD assay showing percent cleavage of full length oligonucleotide (n) and n–1 as a function of time. ●, $d(T)_{19}$ **18**; ■, $d(T)_{14}(\underline{T})_4T$ **20**; ♦, $d(\underline{T})_{18}T$ **21**. Numbered compounds refer to entries in Table 6.

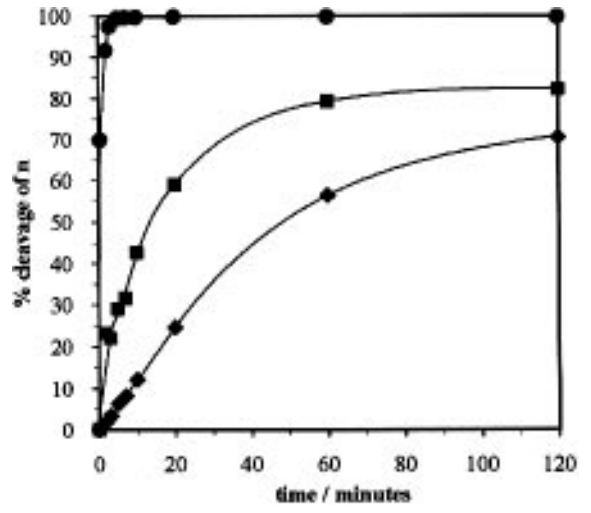

Figure 2. Nuclease S1 assay showing percent cleavage of full length oligonucleotide (n) as a function of time. ●, $d(T)_{19}$ **18**; ■, $d(Ts)_{18}T$ **19**; ♦, $d(\mathbf{T})_{18}T$ **21**. Numbered compounds refer to entries in Table 6.

resistant to degradation than the corresponding phosphorothioate-modified oligodeoxynucleotide **19**, which, under identical conditions, was found to have a half-life of 13.5 min. Only a slight increase in the rate of degradation of the phosphorothioate-modified oligodeoxynucleotide **19** was observed, even though the enzyme concentration had been increased 10-fold. A possible reason for this is that the phosphorothioate oligodeoxynucleotides containing a high proportion of the more easily degraded S_p isomer are degraded rapidly at both enzyme concentrations, whereas the rate of degradation reduces dramatically when only oligodeoxynucleotides containing a high proportion of the more resistant R_p isomer remains (30). Another possibility is that the phosphorothioate oligodeoxynucleotide is a non-competitive inhibitor of the enzyme.

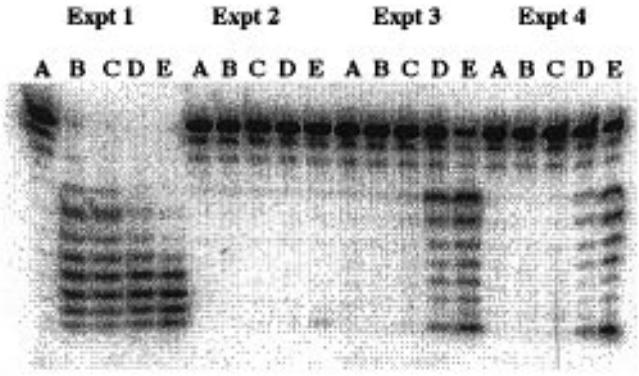

Figure 3. Denaturing PAGE showing degradation of duplexed [^{32}P]RNA by RNase H. Expt 1: [^{32}P]RNA (**24**)/**22** (phosphorothioate); Expt 2: [^{32}P]RNA (**24**)/**23** (2′-fluoro); Expt 3: [^{32}P]RNA (**24**)/**3** (4′-thioT); Expt 4: [^{32}P]RNA (**24**)/**4** (5-Et-4′-thio-2′-dU). Enzyme concentrations: A = 0; B = 6.34×10^{-5} U/µl (1×); C = 6.34×10^{-4} U/µl (10×); D = 6.34×10^{-3} U/µl (100×); E = 6.34×10^{-2} U/µl (1000×). Numbered compounds refer to entries in Tables 1 and 6.

RNase H induction studies

Substrate activities of duplexes formed between the 4′-thio-modified oligodeoxynucleotides **3** or **4**, and their RNA complement **24** were evaluated for susceptibility to *E.coli* RNase H. Duplexes of the fully phosphorothioate-modified oligodeoxynucleotide **22**, or oligodeoxynucleotide **23** containing 2′-fluoro-modified residues, and their RNA complement **24** were used as positive and negative controls respectively. The RNA was ^{32}P-5′-end labelled and the assays were analysed by polyacrylamide electrophoresis. Different concentrations of the enzyme RNase H were used and one time point taken. The result, Figure 3, demonstrates that the controls performed as expected but that the 4′-thio-modified oligodeoxynucleotides elicit *E.coli* RNase H hydrolysis of the RNA target only at high enzyme concentration. Further studies using the 4′-thio-modified oligodeoxynucleotides **3** or **4** and their RNA complement **24** had additional unlabelled RNA added to ensure substrate saturation. The results from these further studies demonstrated that the RNA bound to the 4′-thiothymidylate-modified oligodeoxynucleotide **3** was cleaved ~200–300 times slower when compared with RNA bound to the phosphorothioate-modified oligodeoxynucleotide **22**. It should be noted that RNA bound to phosphorothioate-modified oligodeoxynucleotides is cleaved approximately twice as fast as when it is bound to an unmodified oligodeoxynucleotide. It was also found that the RNA bound to the 4′-thiothymidylate-modified oligodeoxynucleotide **3** was cleaved more than three times faster than when compared with RNA bound to the 5-ethyl-4′-thio-2′-deoxyuridylate-modified oligodeoxynucleotide **4**. Thus the less stable (lower T_m) RNA/DNA duplex is an even poorer substrate for RNase H which indicates that the enzyme needs to recognize a stable RNA/DNA structure before effecting RNA phosphodiester bond hydrolysis.

It is interesting to compare the results obtained for 4′-thio-modified oligodeoxynucleotides with those observed with 4′-methylene-modified (i.e., carbocyclic) oligodeoxynucleotides (Moser,H.E., personal communication). The carbocyclic modification had little effect on T_m or specificity when bound to either DNA or RNA. It was also found that the carbocyclic-modified oligodeoxynucleotides did not elicit the cleavage of bound RNA by RNase H. Therefore, the 4′-thio modification and the carbocyclic

modification appear to impart similar properties to the correspondingly modified oligodeoxynucleotides.

We are beginning to accumulate several examples where enzymes which act in an endo-fashion are inhibited by the presence of 4′-thio-2′-deoxynucleoside residues despite the fact that the presence of the analogue apparently causes little or no detectable alteration to the nucleic acid structure (31). It is probably not too surprising that such enzymes are much more capable of detecting slight imperfections in structure than many of the exo-enzymes which can apparently degrade many analogue-containing structures without too much difficulty.

CONCLUSIONS

Oligodeoxynucleotides having 4′-thio-modifications demonstrated desirable thermal stability and specificity towards target RNA and good resistance to degradation by nuclease S1. However, since, they were degraded by exonuclease SVPD and did not activate RNase H degradation of bound RNA, a more appropriate use of 4′-thio-modified oligodeoxynucleotides in a therapeutic antisense oligonucleotide might be in a chimeric molecule. It has been demonstrated on numerous occasions (32,33) that the use of chimeric modifications can bring about marked improvements in the cleavage of bound RNA by RNase H. It has also been shown (34,35) that the use of exonuclease resistant ‘end-caps’ on an oligonucleotide can provide good resistance to degradation of the oligonucleotide. Therefore, it can be envisaged that an end-capped chimeric 4′-thio-2′-deoxy-modified oligonucleotide may show desirable antisense properties. The good resistance to endonucleases also makes the 4′-thio-2′-deoxy-modification a potentially useful tool for examining protein–nucleic acid interactions.

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