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ACCELERATED COMMUNICATION

Phosphorothioate Oligonucleotides Are Inhibitors of Human DNA Polymerases and RNase H: Implications for Antisense Technology

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SUMMARY

Phosphorothioate oligodeoxycytidine (S-dC$_n$) was used as a model compound to examine the impact of the number of phosphorothioate linkages and their position on the inhibition of human DNA polymerases and RNase H *in vitro*. S-dC$_n$ with a chain length longer than 15 could inhibit human DNA polymerases and RNase H activities, in a linkage number-dependent manner. Longer oligomers were more potent inhibitors than shorter ones. Kinetic studies indicated that S-dC$_{28}$ was a competitive inhibitor of DNA polymerase α and β with respect to the DNA template, whereas it was a noncompetitive inhibitor of polymerases γ and δ. S-dC$_{28}$ was also a competitive inhibitor of RNase H1 and H2 with respect to RNA-DNA duplex. Susceptibility of these enzymes to inhibition by S-dC$_{28}$ was in the order of $\delta \approx \gamma > \alpha > \beta$ and RNase H1 > RNase H2. Structural-activity relationships were explored with a group of S-dC$_{28}$ analogs that have phosphorothioate internucleotide linkages at various positions. The inhibitory effect depended on the total number of thioate linkages, rather than the position of the linkages within the oligomer or the chain length itself. No sequence specificity was found. In the presence of the complementary RNA, antisense phosphorothioates (S-oligos) exerted a biphasic effect on RNase H activity. At low concentrations S-oligos could enhance the cleavage of the RNA portion of S-oligo-RNA duplex, whereas at high concentrations (in excess of the complementary RNA) S-oligos could inhibit RNase H and protect the complementary RNA from degradation. Together, these results suggest that the non-sequence-specific inhibitory effect of S-oligos should be taken into consideration in designing antisense inhibitors. This inhibitory activity could be avoided by decreasing the number of phosphorothioate linkages at the backbone, and S-oligos of 15–20 residues are preferable in antisense molecule design.

S-oligo, one of the chemical entities of antisense inhibitors, has been the focus of numerous studies aimed at designing antisense oligonucleotides (1). The modification in which one of the nonbridging oxygen atoms is replaced by a sulfur atom renders S-oligos resistant to various nucleases without reducing their aqueous solubility. S-oligos are able to form stable hybrids with their complementary RNA sequences, although their melting temperatures are lower than those of their oxygen congeners (2). The formation of S-oligo-RNA hybrids not only could interfere with the process of protein translation (3, 4) but also may cause a degradation of the complementary RNA by RNase H (5). S-oligos could also hybridize to double-stranded DNA and form a DNA triplex (6, 7). This structure may prevent the recognition or binding of transcriptional factors, thereby inhibiting gene expression.

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In addition to the sequence-dependent activity, S-oligos could also inhibit herpes virus DNA polymerase and HIV reverse transcriptase *in vitro*, in a sequence-independent manner (8–10). In cell culture studies, S-dC$_{28}$ was found to protect ATH8 cells against HIV infection (8) and to inhibit HSV replication in virus-infected cells (11). Recent studies have shown that S-dC$_{28}$ may also interfere with cellular uptake of HSV (12).

Ideally, S-oligo as an antisense molecule should not interfere with genomic DNA replication. However, it was observed by Majumdar *et al.* (10) that S-dC$_{28}$ could inhibit DNA polymerase α (from calf thymus) and γ (from pig liver) *in vitro*. Our studies indicated that S-oligos, but not their oxygen congeners, could inhibit human DNA polymerases α and γ (9). These observations suggest that certain S-oligos may not fulfill the criteria necessary for an antisense inhibitor. In order to design more selective antisense S-oligos, an understanding of the structural

ABBREVIATIONS: S-oligo, phosphorothioate oligodeoxynucleotide; S-dC$_{28}$, 28-mer phosphorothioate oligodeoxycytidine; S-dC$_n$, phosphorothioate oligodeoxycytidine; HIV, human immunodeficiency virus; HSV, herpes simplex virus; BSA, bovine serum albumin; P(O)S$^-$, phosphorothioate linkage; P(O)O$^-$, phosphodiester linkage; bp, base pairs; TEAB, triethylammonium bicarbonate; DHFR, dihydrofolate reductase.

requirements for the inhibitory effect of S-oligos on human DNA polymerase and RNase H could be beneficial. In the present studies, S-dC_n were used as model compounds to explore the interactions between S-oligos and human DNA polymerases α, β, γ, and δ, as well as RNase H1 and H2. A series of S-dC_n with different chain lengths and a group of S-dC_{28} analogs with mixed phosphorothioate linkages at different positions have been synthesized. These compounds have been used to investigate the role of the total number of phosphorothioate linkages, as well as their position, in the inhibition of human DNA polymerases and RNase H. A dose-dependent activity of antisense S-oligos on the degradation of their complementary RNA by RNase H was also reported.

Materials and Methods

Chemicals. All chemicals were of reagent grade or purer. Deoxynucleoside triphosphates, dithiothreitol, BSA, and DNA polymerase I were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Calf thymus DNA was purchased from Sigma Chemical Co. (St. Louis, MO) ^{3}H-labeled dTTP was purchased from ICN Radiochemicals (Irvine, CA). Pancreatic DNase I was purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ).

Synthesis of oligodeoxynucleotides. Solid-phase oligodeoxynucleotide synthesis was carried out on an Applied Biosystems model 380B DNA synthesizer, using standard β-cyanoethyl chemistry; capping was performed after sulfurization/oxidation (13). All reagents pertaining to the automated synthesis of oligonucleotides, with the exception of the sulfurizing reagent, were purchased from Applied Biosystems, Inc., and used according to the manufacturer's protocol. Sulfurization at the desired oligonucleotide locations was accomplished using a 0.2 M solution of [^{3}H]1,2-benzodithiol-3-one-1,1-dioxide in acetonitrile, as described (13); the sulfurizing reagent could be delivered from ports 13 or 16 of the 380B DNA synthesizer.

The base-deprotected oligonucleotides bearing the 5′-dimethoxytrityl group were purified by reverse phase high performance liquid chromatography, using a Hamilton PRP-1 column (10 mm × 270 mm), under the following conditions: linear gradient of 5 to 27.5% acetonitrile in 0.1 M TEAB, pH 7.4, for 20 min, followed by a 27.5 to 50% gradient of acetonitrile in TEAB for 5 min (flow rate of 2.5 ml/min). Purified oligomers were detritylated using 80% acetic acid (30 min), dried, and rehydrated in water. The solution was extracted three times with an equal volume of ethyl acetate. The aqueous phase was evaporated to dryness under reduced pressure and repurified by reverse phase high performance liquid chromatography, using a Hamilton PRP-1 column, under the following conditions: 0 to 17.5% gradient of acetonitrile in 0.1 M TEAB for 20 min, followed by a 17.5 to 50% gradient of acetonitrile in 0.1 M TEAB for 5 min (flow rate of 2.5 ml/min).

The $P(O)S^-$ to $P(O)O^-$ ratios were checked by ^{31}P NMR spectroscopy under conditions where there were no differential relaxation effects between the two phosphorus functionalities. The NMR spectra were recorded on a JEOL GSX-500 NMR spectrometer at a ^{31}P frequency of 202.45 MHz. In accordance with previous observations (13), <1% $P(O)O^-$ linkages were observed in the compound intended to have only $P(O)S^-$ linkages. The observed $P(O)S^-$ to $P(O)O^-$ ratios were in accord with the number of sulfur atoms present in a given S-dC_{28} analog; this is in accordance with previous observations (13) that sulfur is not washed out by subsequent oxidations with I_2.

Enzymes. Human DNA polymerase α, β, and γ were purified from cells obtained from a patient with chronic lymphocytic leukemia, using the procedure described previously (14). After single-strand DNA-cellulose chromatography, the specific activities of polymerases α, β, and γ were approximately 1500, 2000, and 1080 units/mg of protein, respectively. One unit of DNA polymerase activity is defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of dTMP into gapped DNA per hour at 37°. Human DNA polymerase δ was purified from K-562 cells, a chronic myelogenous leukemia cell line, as previously described (15). The specific activity of polymerase δ was approximately 1300 units/mg of protein. Human RNase H was also purified from K-562 cells. The methods for purification and characterization were similar to those described by Vonwirth *et al.* (16).

Enzyme assays. All enzyme assays were performed at 37°. The standard DNA polymerase reaction mixture contained 25 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.25 mg/ml BSA, 100 μg/ml activated calf thymus DNA, 0.1 mM each dATP, dCTP, and dGTP, 10 μM [^{3}H]dTTP (2 Ci/mmol), and 100 nM S-oligos, in a volume of 0.05 ml. All the DNA polymerase reaction mixtures were the same as the standard assay mixture, except for the KCl concentration. No KCl was used in the human DNA polymerase α and δ assays, and 100 mM KCl was used in the human DNA polymerases β and γ assays. After incubation for 30 min, aliquots of the samples were spotted on 2.4-cm Whatman GF/A glass fiber filter discs, and the trichloroacetic acid-insoluble radioactivity was measured. One unit of DNA polymerase activity is defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of dTMP into activated DNA per hour at 37°.

The standard RNase H reaction mixture contained 50 mM Tris·HCl (pH 8.0), 2 mM dithiothreitol, 100 μg/ml BSA, 4 mM $MgCl_2$, and 0.8 nmol of [^{3}H]poly(rA)·poly(dT) (1.4×10^4 cpm/nmol; P-L Biochemicals, Milwaukee, WI). All the RNase H reaction mixtures were the same as the standard assay mixture, except for the KCl concentration. KCl was used at 50 mM in RNase H1 assays, and 120 mM KCl was used in RNase H2 assays. After incubation for 30 min, aliquots of the samples were spotted on 2.4-cm Whatman GF/A glass fiber filter discs, and trichloroacetic acid-insoluble radioactivity was measured. One unit of RNase H activity is defined as the amount of enzyme that catalyzes the degradation of 1 nmol of [^{3}H]poly(rA)·poly(dT) per hour at 37°.

***In vitro* transcription.** To examine the effects of complementary S-oligos on RNase H, a transcript of HIV-1 *rev* was synthesized by *in vitro* transcription. Briefly, cloned HIV-1 proviral DNA (clone BH10) (17) was restricted by *Eco*RI and *Kpn*I at bp 5101 and 5702, respectively. A 0.6-kilobase fragment was isolated and inserted into pBluescriptII at the site 70 bp downstream of the T3 promoter sequence. The pBluescriptII recombinants were then propagated, purified, and linearized by *Kpn*I at the site upstream of the T7 promoter sequence (see Fig. 6). The linearized plasmid was then transcribed by T3 RNA polymerase in the presence of [α-^{32}P]GTP (Amersham, Arlington Heights, IL). The DNA template was then removed by RNase-free DNase, and the transcript was isolated. A 28-mer antisense S-*rev* (5′-TCGTCGCTGTCTCCGCTTCTTCCTGCCA-3′) complementary to HIV-1 *rev* RNA at bp 5329–5356 was synthesized by the method previously described (18).

Results

Effect of S-dC_n with different numbers of phosphorothioate linkages on human DNA polymerases and RNase H. Human DNA polymerases and RNase H were purified and characterized according to the methods described previously (14–16). The enzyme activities were examined in the presence of 100 nM S-dC_n with different chain lengths. DNA polymerases α, γ, and δ were found to be more susceptible to inhibition by S-dC_n than was polymerase β (Fig. 1A). The inhibitory effects of S-dC_n directly depended on the number of phosphorothioate linkages in the backbone, with more thioate linkages causing stronger inhibition (Fig. 1A). For DNA polymerase α, when the chain length increased from 15 to 28 residues the inhibition increased from 0 to 60% of control; it reached a plateau at the 28-mer (Fig. 1A). For DNA polymerases γ and δ, when the chain length increased from 15 to 28 residues the inhibition increased significantly from 20 to 80% of control; it reached a plateau at the 28-mer (Fig. 1A).

The RNase H1 and H2 activities were examined under con-

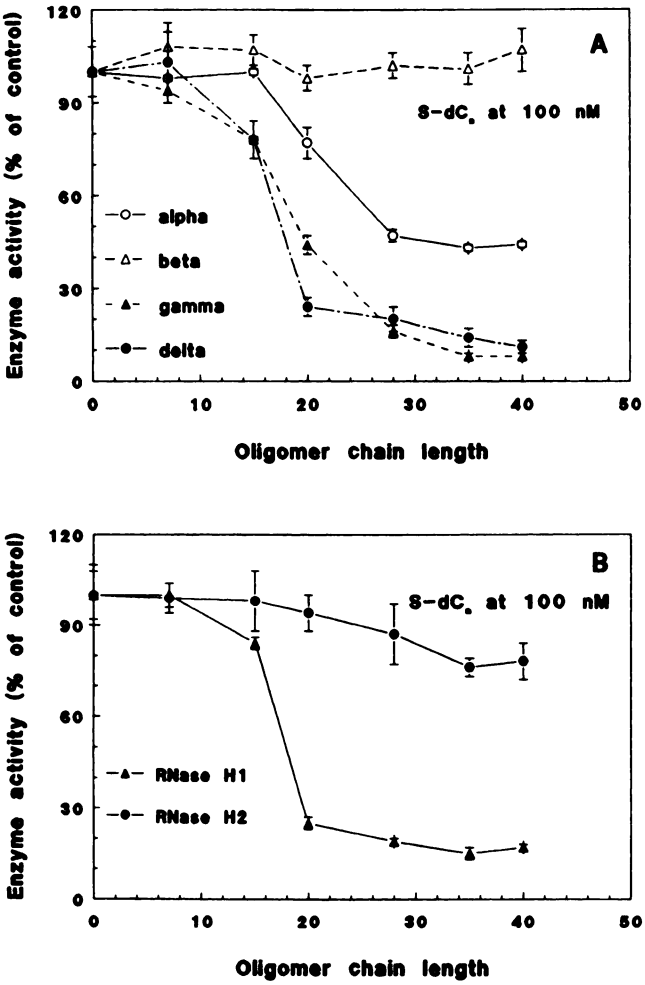


Fig. 1. Effect of phosphorothioate linkage number on human DNA polymerases (A) and RNase H (B). A, The standard DNA polymerase reaction mixture contained 25 mM Tris·HCl (pH 8.0), 100 μg/ml activated calf thymus DNA, 100 μM each dATP, dCTP, and dGTP, 10 μM [^{3}H] dTTP, and 100 nM S-dC$_n$ (S-dC$_7$ to S-dC$_{40}$). All the DNA polymerase reaction mixtures were the same as the standard assay mixture, except for the KCl concentration, as described in Materials and Methods. B, The standard RNase H reaction mixture contained 0.8 nmol of [^{3}H] poly(rA)·poly(dT) (1.4 × 10^4 cpm/nmol). All the RNase H reaction mixtures were the same as the standard assay mixture, except for the KCl concentration. KCl was used at 50 mM in RNase H1 assays, and 120 mM KCl was used in RNase H2 assays. The data represent the mean ± standard deviation (two experiments) of duplicate determinations.

ditions similar to the DNA polymerase assay, except that [^{3}H] poly(rA)·poly(dT) was used as the substrate. RNase H1 activity was found to be more susceptible to inhibition by S-dC$_n$ than was RNase H2. When the chain length increased from 15 to 20 residues, the inhibition of RNase H1 increased from 20 to 75% at 100 nM (Fig. 1B).

Effect of S-dC$_{28}$ analogs with mixed phosphorothioate and phosphodiester linkages on human DNA polymerases and RNase H. Because S-dC$_{28}$, but not dC$_{28}$ is an inhibitor of DNA polymerases (9), relationship between this effect and the structure of the phosphorothioate linkage was examined using a series of S-dC$_{28}$ analogs with different amounts of phosphorothioate linkage at different positions (Fig. 2). Fig. 3 shows that, at 100 nM, S-dC$_{28}$ analogs with the mixed thioate linkage could inhibit DNA polymerases α, γ, and δ, but not polymerase β. The inhibitory effect of these analogs depended

1) SOO	+ + + + + + + + + - - - - - - - - - - - - - - - - - -
2) OOS	- + + + + + + + + +
3) OSO	- - - - - - - - - + + + + + + + + - - - - - - - - -
4) SSO	+ + + + + + + + + + + + + + + + + - - - - - - - - -
5) OSS	- - - - - - - - - + + + + + + + + + + + + + + + + +
6) SOS	+ + + + + + + + + - - - - - - - - - + + + + + + + + +
7) SSS	+ +
8) DHFR	+ +
9) S,O	+ - + - + - + - + - + - + - + - + - + - + - + - + - +
10) O,S	- + - + - + - + - + - + - + - + - + - + - + - + - + -

* +, Phosphorothioate linkage.

-, Phosphodiester linkage.

Fig. 2. Structures of S-dC$_{28}$ analogs modified at the backbone or the base moiety. *S* and +, phosphorothioate linkage; *O* and –, phosphodiester linkage. *DHFR*, a 28-mer S-oligo (5′-CGAACCAACCATGA-CAGCAGCGGGAGGA-3′) complementary to the junction of 5′ leading sequence and exon I of human DHFR RNA at the position from bp 1307 to 1334 (28).

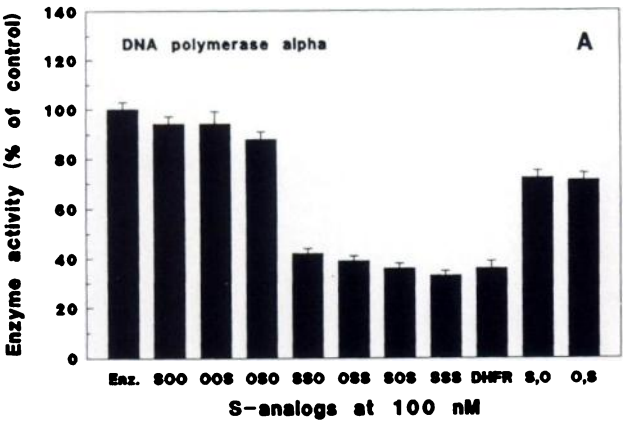
on the total number of thioate linkages rather than their position or their chain length (28-mer). For example, the analogs with 18 thioate linkages (the SSO, the OSS, and the SOS) were found to be more potent inhibitors than the analogs with nine thioate linkages (the SOO, the OOS, and the OSO), whereas with the same number of thioate linkages the SOS was equally potent as the SSO and the OSS in the inhibition of polymerases α, γ, and δ (Fig. 3, A, B, and D). For polymerases α and δ, the inhibitory effect appeared to reach a plateau at 18 thioate linkages (Fig. 3, A and B) and an increase of the thioate linkages from 18 to 27 did not result in increased inhibition, even at lower drug concentrations (data not shown). For polymerase γ, however, 18 thioate linkages did not reach a plateau and the SSS could cause more potent inhibition than the SSO, the OSS, or the SOS at the same concentration (Fig. 3D).

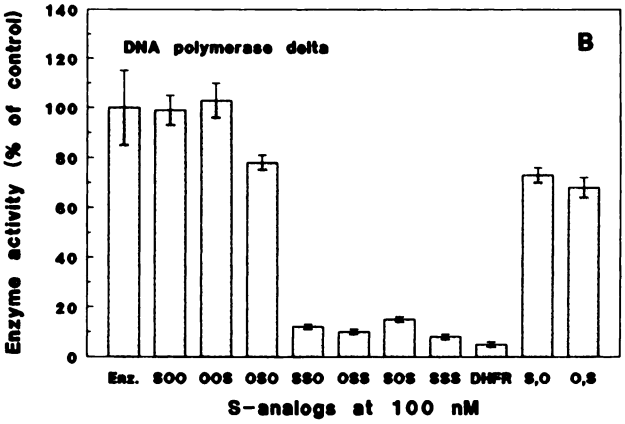
The position of phosphorothioate linkages did not appear to play an important role in the inhibition of polymerases α and δ (Fig. 3, A and B); however, it was important in the inhibition of DNA polymerase γ, and the order of potency was OSS > SSO > SOS, with 24%, 36%, and 65% inhibition at 100 nM, respectively (Fig. 3D).

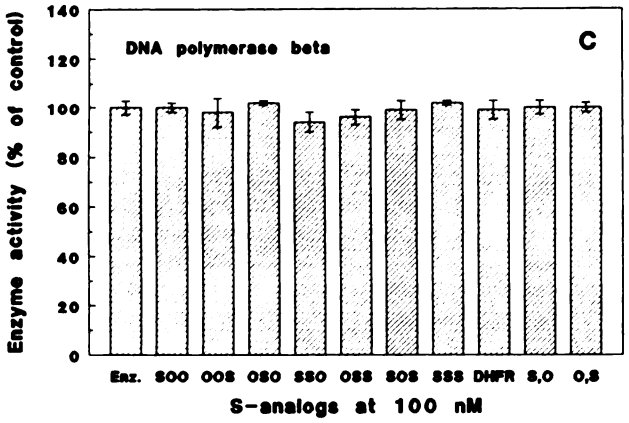
The analogs S,O and O,S, with alternate phosphorothioate linkages, were found to be less potent than the SOS in the inhibition of DNA polymerases α, γ, and δ (Fig. 3, A, B, and D).

The 28-mer S-oligo complementary to the 5′ leading sequence of the human DHFR RNA (the DHFR) (19) was found to have a similar potency as S-dC$_{28}$ in the inhibition of human DNA polymerases (Fig. 3).

The RNase H1 activity was found to be more susceptible to inhibition by S-dC$_{28}$ analogs than was the RNase H2 activity. The inhibitory effect depended on the number of phosphorothioate linkages rather than their position (Fig. 4). The analogs S,O and O,S did not inhibit human RNase H1 and H2 (Fig. 4). The DHFR was as potent as the SSS in the inhibition of RNase H1 (Fig. 4).







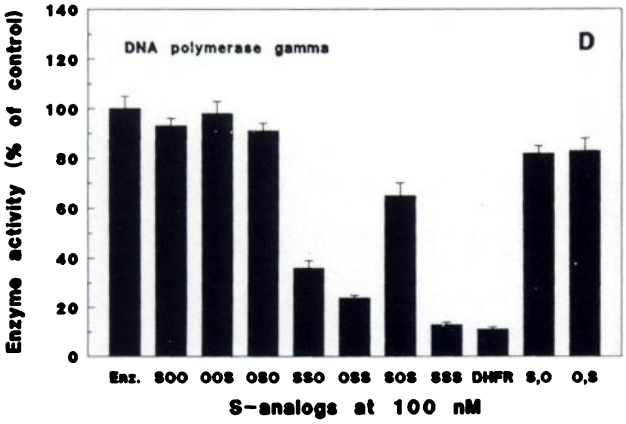
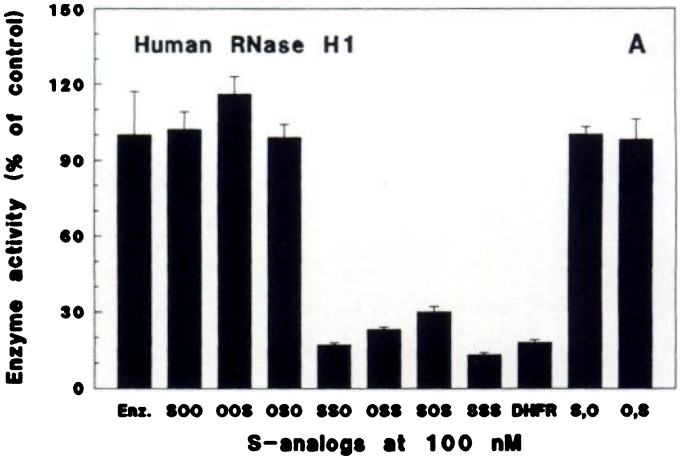


Fig. 3. Effect of phosphorothioate linkage position on human DNA polymerases. The DNA polymerase assays were performed in the presence of 100 nM S-dC_{28} analogs, as described in the legend to Fig. 1. The data represent the mean ± standard deviation (two experiments) of duplicate determinations.



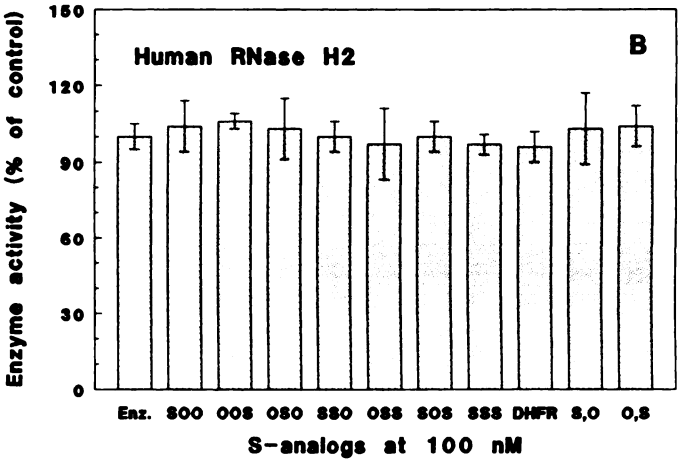


Fig. 4. Effect of phosphorothioate linkage position on human RNase H. The RNase H assays were carried out in the presence of 100 nM S-dC_{28} analogs. The data represent the mean ± standard deviation (two experiments) of duplicate determinations.

Kinetic effects of S-dC_{28} effect on human DNA polymerases and RNase H. In order to explore the interactions of S-oligo with DNA template and DNA polymerase during DNA synthesis, human DNA polymerases were incubated in reaction mixtures containing three deoxynucleoside triphosphates at saturation concentrations. The concentration of activated DNA template was varied in the presence or absence of S-dC_{28}, and the results were analyzed using Lineweaver-Burk plots. S-dC_{28} was shown to inhibit DNA polymerases through different mechanisms. S-dC_{28} inhibited DNA polymerase α and β competitively with respect to the DNA template. The values of the apparent inhibition constant (K_i) of DNA polymerases α and β were 120 ± 10 nM and 550 ± 70 nM, respectively (Table 1); however S-dC_{28} inhibited DNA polymerases γ and δ in a noncompetitive manner. When S-dC_{28} concentrations increased, the $1/[S]$ axis intercepts remained the same, and all the reciprocal plots intersected at the $1/[S]$ axis (data not

shown). The apparent K_i values of DNA polymerases γ and δ were 47 ± 4 and 31 ± 2 nM, respectively (Table 1).

These results also suggest that the susceptibility of human DNA polymerases to S-dC_{28} is in the order DNA polymerases $\delta \approx \gamma > \alpha > \beta$.

Human RNase H1 and H2 could be inhibited competitively by S-dC_{28}. The RNase H1 activity was found to be more susceptible to inhibition by S-dC_{28} than was RNase H2. The K_i values of RNase H1 and H2 were 70 ± 5 nM and 450 ± 14 nM, respectively (Table 1).

Biphasic effect of S-oligos on human RNase H. Based on the observation that S-dC_{28} is a competitive inhibitor of both RNase H1 and H2, the interactions between complementary S-oligos and RNase H were studied further. In the presence of 0.01–5 nM S-dT_{28} or dT_{28}, [^{3}H]poly(rA) could be digested by both RNase H1 and H2 at 37° (Fig. 5); however, when the concentration increased to 250 nM, S-dT_{28} inhibited >90% of both enzyme activities (Fig. 5). At the same concentration, however, dT_{28} significantly enhanced the enzyme activities. (Fig. 5). These results suggest that the biphasic effect on RNase H is unique for S-oligos.

The unique biphasic effect of antisense S-oligos was further examined using S-oligo and RNA with defined sequence. A 28-mer S-oligo complementary to the initial sequence of HIV-1 *rev* was synthesized and annealed to a ^{32}P-labeled HIV-1 *rev* RNA (a 0.67-kilobase *Eco*RI/*Kpn*I fragment) transcribed *in vitro*. The S-*rev*-RNA duplex was then incubated with RNase H. Fig. 6 shows the dose response of S-*rev* on human RNase H1 and H2. The HIV *rev* transcript could be cleaved by both RNase H1 and H2 in the presence of 0.012 μM (for RNase H1; 2 units/assay) and 0.012–0.12 μM S-*rev* (for RNase H2; 5 units/assay) (Fig. 6, *lanes f, j,* and *k*). Two major degradation products of approximately 0.31 and 0.36 kilobases in size were observed (Fig. 6). When the concentrations of S-*rev* increased from 0.12 to 12 μM, these two bands disappeared and the density of the undigested *rev* transcript increased significantly (Fig. 6, *lanes c, d, e, h,* and *i*). It was also found that the density of the *rev* transcript in the drug-free control (Fig. 6, *lanes b* and *g*) was lower than that in the presence of 1.2–12 μM S-*rev*. This difference could be due to a contamination by RNase, because the density of the undigested *rev* transcript in the drug-free control (Fig. 6, *lanes b* and *g*) also appeared to be lower than that of the enzyme-free control (Fig. 6, *lane a*). S-*rev* at 1.2–12 μM could protect the transcript against RNase degradation.

TABLE 1

Kinetic effects of S-dC_{28} on human DNA polymerases and RNase H

DNA polymerase and RNase H assays were performed as described in Materials and Methods. Kinetic constants were determined by Lineweaver-Burk plots. C, the mode of inhibition is competitive; N, the mode of inhibition is noncompetive. The data represent the mean ± standard deviations of three independent experiments.

Enzyme	K_m	K_i
	μg/ml	nM
DNA polymerase		
α	46 ± 5	120 ± 10 (C)
β	20 ± 2	550 ± 70 (C)
γ	8 ± 4	47 ± 4 (N)
δ	19 ± 2	31 ± 2 (N)
RNase		
H1		70 ± 5 (C)
H2		450 ± 14 (C)

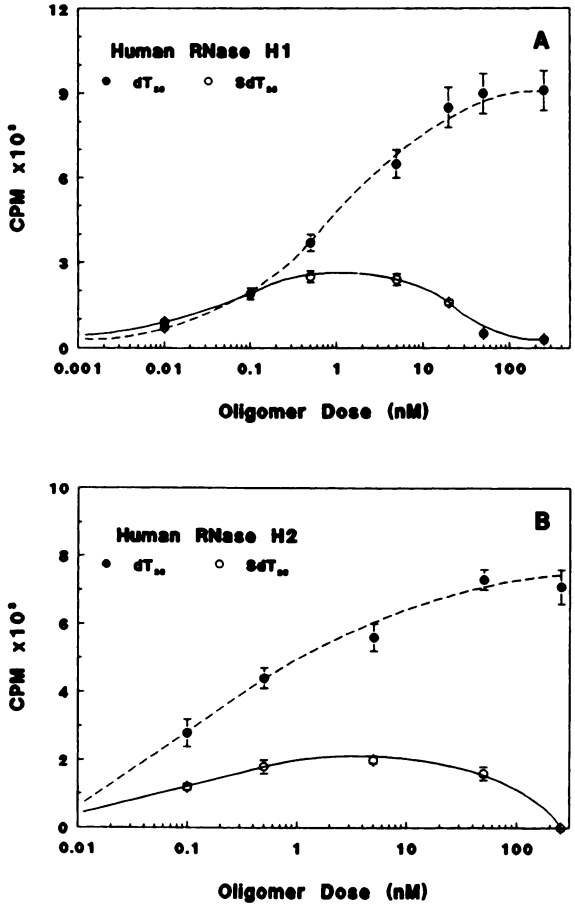


Fig. 5. Different modes of action of S-dT_{28} and dT_{28} on human RNase H. S-dT_{28} and dT_{28} at various concentrations were mixed with [^{3}H]poly(rA) [500 mCi/mmol of nucleoside residue, 900 nucleoside residues/poly(rA) molecule] at 40° for 10 min. An aliquot of the sample was then added to the RNase H reaction mixture, containing 50 mM Tris·HCl, (pH 8.0), 4 mM $MgCl_2$, 2 mM dithiothreitol, 50 μg of BSA, and 50 mM KCl (for RNase H1) or 120 mM KCl (for RNase H2). A, RNase H1 assays. B, RNase H2 assays. The data represent the mean ± standard deviation (two experiments) of duplicate determinations.

Discussion

S-oligos have been shown to be some of the most effective chemical entities in antisense technology (20–22). No studies of their non-sequence-specific inhibitory effects against human metabolic enzymes, however, have been reported thus far. The results of the present study demonstrate that certain S-oligos can inhibit human DNA polymerases α, δ, and γ, as well as RNase H1, *in vitro* in a sequence-independent manner at nanomolar concentrations and may possibly inhibit DNA polymerase β and RNase H2 at higher concentrations. As a result, genomic DNA replication could be interrupted and certain antisense S-oligos could lose their sequence-specific interactions with the target genes. Furthermore, the results from this study demonstrate that this inhibition can be prevented, because it is related to the number of phosphorothioate linkages at the oligomer backbone. The inhibitory effect appears to require a minimum of 15 thioate linkages to initiate and reaches a plateau at 28 thioate linkages in the presence of 100 nM S-oligos for most of the enzymes examined. Because 15-mer S-oligos with minor G/C composition hybridize well with their complementary sequence at 37° (2), to minimize this inhibitory

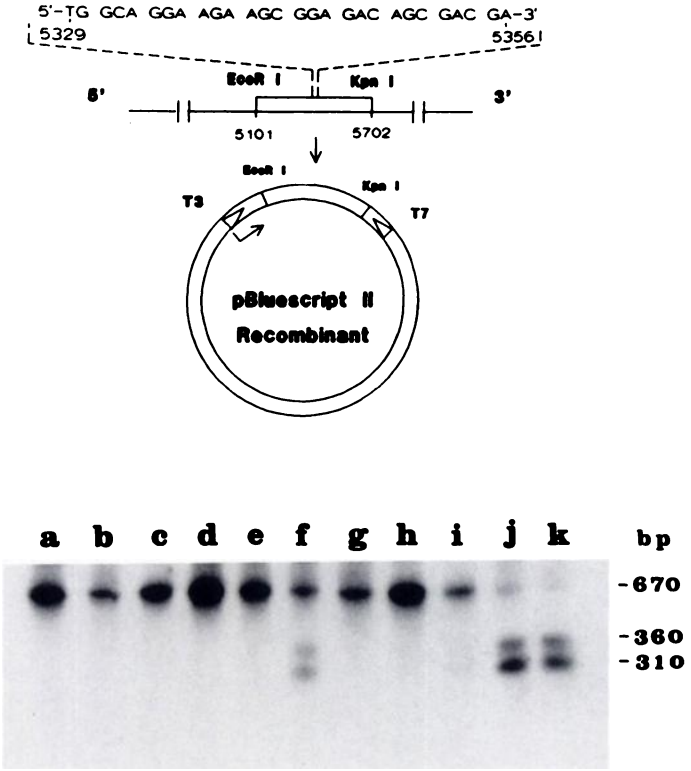


Fig. 6. Biphasic activity of 28-mer S-*rev* against human RNase H1 and H2. HIV-1 *rev* transcript was synthesized by *in vitro* transcription; HIV clone BH10 was restricted by *Eco*RI and *Kpn*I. A 0.6-kilobase fragment (from 5101 to 5702) was isolated and inserted into pBluescriptII. The recombinant was then linearized by *Kpn*I. The transcripts (670 bp) containing HIV-1 *rev* sequence were synthesized by T3 RNA polymerase and were uniformly labeled with [^{32}P]GTP. A 28-mer antisense S-*rev* (5′-TCGTCGCTGTCTCCGCTTCTTCCTGCCA-3′) complementary to HIV-1 *rev* sequence (from 5329 to 5356) was synthesized and annealed to the transcript at various concentrations. The substrate was then incubated with RNase H for 20 min. After RNase H reaction, samples were fractionated on a 6% polyacrylamide-urea gel. *Lane a*, no-enzyme control; *lanes b–f*, samples were cleaved by RNase H1 in the presence of 0 (*lane b*), 12 (*lane c*), 1.2 (*lane d*), 0.12 (*lane e*), and 0.012 μM S-*rev* (*lane f*). *Lanes g–k*, samples were cleaved by RNase H2 in the presence of 0 (*lane g*), 12 (*lane h*), 1.2 (*lane i*), 0.12 (*lane j*), and 0.012 μM S-*rev* (*lane k*).

effect while retaining the hybridization potential 15–20-mer S-oligos may be preferable in the design of antisense inhibitors.

The mechanisms responsible for the inhibition of human DNA polymerases and RNase H by S-oligos are not completely understood. Based on the observation that only S-oligos, but not their oxygen congeners, have inhibitory effects against human metabolic enzymes and that there is no sequence specificity observed, the interaction of DNA polymerase (or RNase H) with S-oligos appears to occur between the DNA (or DNA-RNA duplex) binding site of the enzymes and the phosphorothioate backbone of S-oligos. Thioate modification causes a negative charge delocalization at the internucleotide linkages (23, 24). This change may provide S-oligos a better chance to access the DNA binding site of the enzymes and to form a tight complex. Our kinetic studies suggest that two types of interactions may be involved, competitive and noncompetitive inhibition. In competitive inhibition (e.g., the inhibition of DNA polymerase α, S-dC$_{28}$ competes with the DNA template for binding to the binding domain of the enzyme. The structure of the DNA-binding domain involves a repeated finger structure that could interact with the phosphodiester backbone of DNA, and each motif could only interact with a length of a few base pairs (25, 26). It appears that, to compete with the DNA template, the size of the binding site has to span a length greater than that of 15-base oligomers. Shorter S-oligos may have insufficient interaction with the binding domain and, therefore, compete with the DNA template less efficiently than longer ones. In noncompetitive inhibition (e.g., the inhibition of DNA polymerases γ and δ, the interaction of S-dC$_{28}$ and the enzyme may occur at a site away from the DNA-binding domain and may cause a conformational change at the catalytic center of the enzyme or may inactivate the enzyme-DNA template complex. Alternatively, polymerases γ and δ could also be inhibited through a mechanism of blocking of the processivity of DNA synthesis. DNA polymerases γ and δ have higher processivity than polymerases α and β (distributive) (27, 28) and were found to be more susceptible to inhibition by S-oligos. Speculation upon the precise mechanism of the two types of interaction will require further information about the catalytic center of the enzyme from crystallographic studies.

The experiments with S-dC$_{28}$ analogs with mixed phosphorothioate linkages clearly show that the position of the bonds does not play an important role in the inhibition of DNA polymerases α and δ or RNase H1, whereas it could be important in the inhibition of DNA polymerase γ. From independent experiments, the SOS was consistently found to be less potent than the OSS and the SSO in polymerase γ inhibition (Fig. 3D). This observation suggests that, with an equal number of thioate linkages, S-oligos with continuous thioate linkages are more inhibitory to DNA polymerase γ than are those with discontinuous thioate linkages.

In the mixed linkage studies, it was also found that, with a 28-mer chain length, the S,O and the O,S exhibit only a minor inhibitory effect against human DNA polymerases and RNase H (Figs. 3 and 4), whereas with the same chain length the SSO is significantly more inhibitory than the SOO or the S,O or the O,S (Figs. 3 and 4). This observation suggests that analog chain length alone does not determine the inhibitory effect against the enzymes. Therefore, it is possible to maintain a certain chain length for the specific interaction with the target sequence while minimizing the nonspecific effect by reducing the number of thioate linkages at the backbone. S-oligo analogs with mixed thioate linkages could be candidates for future antisense inhibitors, if they are relatively stable in the presence of nucleases.

Studies by Walder and Walder (5) suggested that the formation of a DNA-RNA duplex alone is not sufficient for blocking of translation and that the cleavage of complementary RNA by RNase H is the major causative factor of hybridization arrest by antisense inhibitors. Our results demonstrated that antisense S-oligos could interact with human RNase H in a biphasic manner. At low concentrations (with an excess of the complementary RNA over antisense S-oligos) antisense S-oligos anneal to the complementary RNA and work as co-substrates for RNase H, whereas at high concentrations the excess antisense S-oligos could become competitive inhibitors of RNase H that protect the RNA sequence from degradation. The biphasic mode of action was found to be unique for antisense S-oligos but not for their oxygen congeners. In antisense technology, the inhibitory phase of S-oligo action should be avoided, and it is reasonable to propose that a decrease in the

number of phosphorothioate linkages in the backbone could reduce the inhibitory effect on RNase H and may increase the specificity of antisense S-oligos.

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