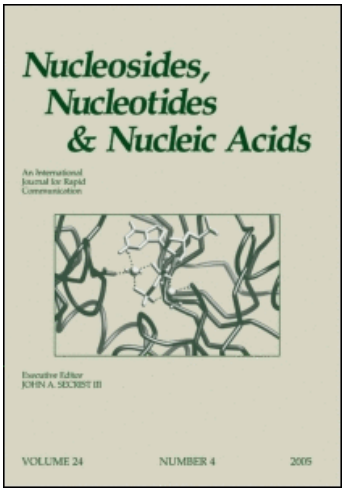

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Influence of P-Chirality of Phosphorothioate Oligonucleotides on the Activity of Amv-Reverse Transcriptase

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INFLUENCE OF P-CHIRALITY OF PHOSPHOROTHIOATE OLIGONUCLEOTIDES ON THE ACTIVITY OF AMV-REVERSE TRANSCRIPTASE

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ABSTRACT: The inhibition of reverse transcriptase by phosphorothioate oligonucleotides of predetermined absolute configuration at each internucleotide centre (PS-oligos) was studied in a cell - free system, composed of an RNA template, a primer oligodeoxynucleotide (PO-oligo) and the AMV or HIV reverse transcriptase. It was found that the *non-antisense* inhibition of the RT enzymes caused by their direct interaction with PS-oligos depends upon a nucleoside sequence of oligonucleotide as well as upon the absolute configuration at phosphorus atoms of internucleotide phosphorothioate bonds.

INTRODUCTION

Antisense oligonucleotides complementary to viral RNA inhibit viral replication in cells cultured with *Rous sarcoma* virus,[1] human immunodefficiency virus,[2,3] vesicular stomatitis virus,[4] herpes simplex virus[5] and influenza virus.[6] However, the mechanisms by which the antisense oligonucleotides inhibit reverse transcriptase activity and retroviral protein synthesis have not been fully elucidated.[7] It was demonstrated that phosphorothioate oligonucleotides (PS-oligos) could inhibit *in vitro* reverse transcription *via* the antisense mechanism which involves the cleavage of the RNA template by the RT RNase H activity.[8] On the other hand, phosphorothioate oligonucleotides can inhibit cDNA synthesis by direct interaction with reverse transcriptase.[9] It was postulated that the enzyme binds a DNA primer not-annealed to the template, while the RNA template alone is not bound. Moreover, phosphorothioate oligonucleotides bind to the enzyme with much higher affinity

than unmodified oligomers and, therefore, can competitively inhibit the synthesis of cDNA, which is directed by a heterologous template/primer complex. The direct interaction of phosphorothioate oligonucleotides with reverse transcriptase was postulated to be sequence independent.[9]

We have examined the influence of P-chirality of PS-oligos on the activity of reverse transcriptase from avian myeloblastosis virus (AMV RT). Our data indicate that the inhibition depends both upon a base sequence and the absolute configuration at phosphorus atoms involved in the internucleotide phosphorothioate linkages in PS-oligos.

MATERIALS AND METHODS

All reagents were of analytical grade. T7 RNA polymerase (EC 2.7.7.6) was purchased from Fermentas (Vilnius, Lithuania). AMV RT, ribonuclease inhibitor (RNasin), DNase I (RNase-free) and HIV RT were obtained from Amersham.

Synthesis of oligonucleotides

Unmodified oligonucleotides PO-**1** - PO-**4** were prepared by the phosphoramidite method on an ABI 391 synthesizer. PS-oligos **1a-b**, **1d-g** and **2a-b** were synthesized using the oxathiaphospholane method as described elsewhere.[10] Oligonucleotides **1c**, **1h** and **2c** were synthesized by the phosphoramidite method with sulfurization of internucleotide phosphites by means of S-Tetra.[11] Purification of all oligonucleotide constructs was carried out by two-step RP-HPLC (DMT-on and DMT-off).[12]

Polyribonucleotide PO-**5** (475 nt) used as a template for RT was obtained by *in vitro* transcription using plasmid PT7-7* containing the interleukin-2 (Il-2) gene.[13] Polyribonucleotide PO-**6** (286 nt) was obtained analogously using plasmid pT7-7 tPA containing the tissue plasminogen activator (tPA) gene (M.Sierant - unpublished results).

In vitro transcription

The incubation mixture (50 μl) containing 40 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol (DTT), 0.4 mM of each nucleoside 5'-triphosphate (NTP) (N = A,U,C,G), [α-^{32}P]CTP, bovine serum albumin (5 μg), ribonuclease inhibitor (RNasin) (75 units), 1.72 μg of DNA template (the plasmid pT7-7*

containing Interleukin-2 gene between Eco RI and Hind III sites or the plasmid pT7-7 tPA containing tPA gene) and T7 RNA polymerase (20 units) was incubated at 37°C for 1.5 h. Then, for digestion of DNA template, DNase I (2 units) was added and the reaction mixture was incubated for 0.5 h at 37°C. After the reaction resulting RNA was phenol-extracted, ethanol-precipitated and dissolved in water.

Primer extension

RNA template (PO-**5** or PO-**6**) obtained by *in vitro* transcription (40 ng, 0.25 pmol), primer PO-**3** or PO-**4** (15 pmoles) and the desired amount of appropriate oligonucleotide (**1-2**) (total volume 9 μl) were heated for 2 min at 95°C, cooled for 3 min at 0°C and then pre-incubated for 15 min at 37°C. After adding 4 μl 5x AMV RT buffer (250 mM Tris-HCl, pH 8.3, 40 mM $MgCl_2$, 250 mM NaCl, 5 mM DTT) to the reaction mixture were added: RNasin (11 units), 7.5 nmol of each dNTP, [α-^{32}P] dCTP (2 μCi) and AMV RT (2.5 units). This reaction mixture (total volume 20 μl) was incubated for 1 h at 37°C. The products were analyzed on a 7% polyacrylamide gel. The autoradiograms were scanned using an LKB Ultrascan XL densitometer.

Primer extension catalyzed by HIV RT was performed as above using 5x HIV RT buffer (250 mM Tris-HCl, pH 8.3, 40 mM $MgCl_2$, 250 mM KCl and 5 mM DTT) and 0.5 unit of HIV reverse transcriptase.

RESULTS AND DISCUSSION

The oligonucleotides listed in Table 1 were used for the studies on the inhibition of AMV RT by PS-oligos. We have chosen the oligonucleotide d[AGA TGT TTG AGC TCT] (PO-**1**) and its isosequential phosphorothioate analogues (PS-**1a-c**), which are complementary to the central fragment of mRNA (160-175) transcribed from the IL-2 gene inserted into plasmid PT7-7*.[13] These oligomers were used as potential inhibitors of the AMV RT, while unmodified oligomer (PO-**3**) of the sequence d[AAA GGT AAT CCA TCT GTT CA] (361-381) was used as a primer for the enzyme. Reverse transcription of RNA template (PO-**5**) catalyzed by AMV RT gave the predicted cDNA fragment of 437 nucleotides (Scheme 1a). Inhibitory effect of the oligonucleotides **1** was studied at their concentration ranging from 65 nM to 2.5 μM. The stereoregular oligonucleotide PS-**1b** containing all phosphorothioate linkages of S_P-configuration was stronger inhibitor of the

TABLE 1 Oligonucleotide constructs **1-2** used for AMV RT studies

Compound no.	Sequence 5'→3'	Characteristics of internucleotide bonds
PO-**1**	d[AGA TGT TTG AGC TCT]	$[PO]_{14}$[a]
PS-**1a**	d[AGA TGT TTG AGC TCT]	$[R_P]_{14}$[b]
PS-**1b**	d[AGA TGT TTG AGC TCT]	$[S_P]_{14}$[c]
PS-**1c**	d[AGA TGT TTG AGC TCT]	$[mix]_{14}$[d]
PS-**1d**	d[AGA TGT TTG AGC TCT]	$[R_P]_{12}[S_P]_2$[e]
PS/PO-**1e**	d[AGA TGT TTG AGC TCT]	$[R_P]_{12}[PO]_2$[e]
PS-**1f**	d[AGA TGT TTG AGC TCT]	$[S_P]_{12}[R_P]_2$[f]
PS/PO-**1g**	d[AGA TGT TTG AGC TCT]	$[S_P]_{12}[PO]_2$[f]
PS/PO-**1h**	d[AGA TGT TTG AGC TCT]	$[mix]_{12}[PO]_2$[g]
PO-**2**	d[AAG CAT ACG GGG TGT]	$[PO]_{14}$[a]
PS-**2a**	d[AAG CAT ACG GGG TGT]	$[R_P]_{14}$[b]
PS-**2b**	d[AAG CAT ACG GGG TGT]	$[S_P]_{14}$[c]
PS-**2c**	d[AAG CAT ACG GGG TGT]	$[mix]_{14}$[d]

[a] Oligomer PO-**1** contains only unmodified phosphodiester bonds (PO).

[b,c] PS-oligos **1a** and **1b** contain internucleotide phosphorothioate linkages of R_P or S_P configuration, respectively.

[d] This oligomer consists of a mixture of all possible diastereomers.

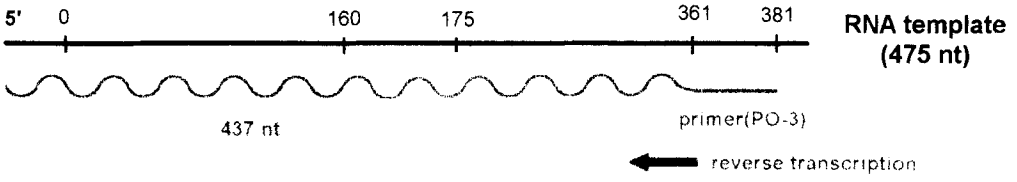
[e] Oligomers PS-**1d** and PS/PO-**1e** contain twelve R_P-phosphorothioate bonds and two-S_P- or two PO-bonds, respectively, located at the 3'-end.

[f] Compounds PS-**1f** and PS/PO-**1g** have twelve S_P-linkages and two R_P- or PO-bonds, respectively.

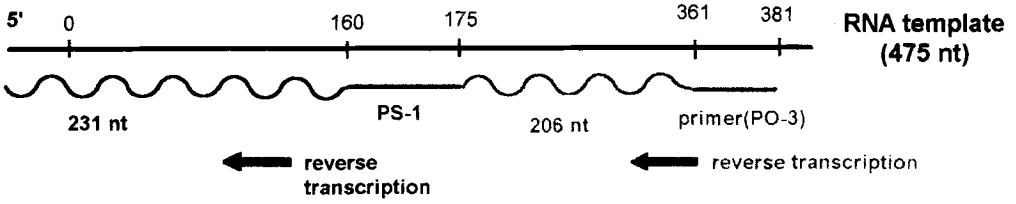
[g] This oligomer consists of a mixture of 2^{12} diastereomers, since it has two achiral phosphodiester bonds at the 3'-end.

TABLE 2 Oligo- and polynucleotide constructs **3-6** used for RT studies.

Compound no.	Sequence 5'→3'	Characteristics of internucleotide bonds[a)]
PO-3	d[AAA GGT AAT CCA TCT GTT CA]	$[PO]_{19}$
PO-4	d[TTG AGC AGA TGG GTT TGG]	$[PO]_{17}$
PO-5	Polyribonucleotide (475 nt)	$[PO]_{474}$
PO-6	Polyribonucleotide (286 nt)	$[PO]_{285}$



SCHEME 1a



SCHEME 1b

AMV RT than its [all-R_P]-counterpart: PS-**1b** at the concentration 400 nM inhibited the cDNA synthesis in 85%, while PS-**1a** containing only R_P-linkages showed comparable inhibitory effect (70%) at the concentration 2.5 μM. Unexpectedly, the oligomer PS-**1c** consisting of a mixture of all possible diastereomers appeared to be weak inhibitor (40% inhibition at 2.5 μM concentration) (Fig.1).

For more detailed studies on the influence of P-chirality of PS-oligos on the AMV RT activity we have prepared chimeric PS-oligonucleotides containing at their 3'-ends two

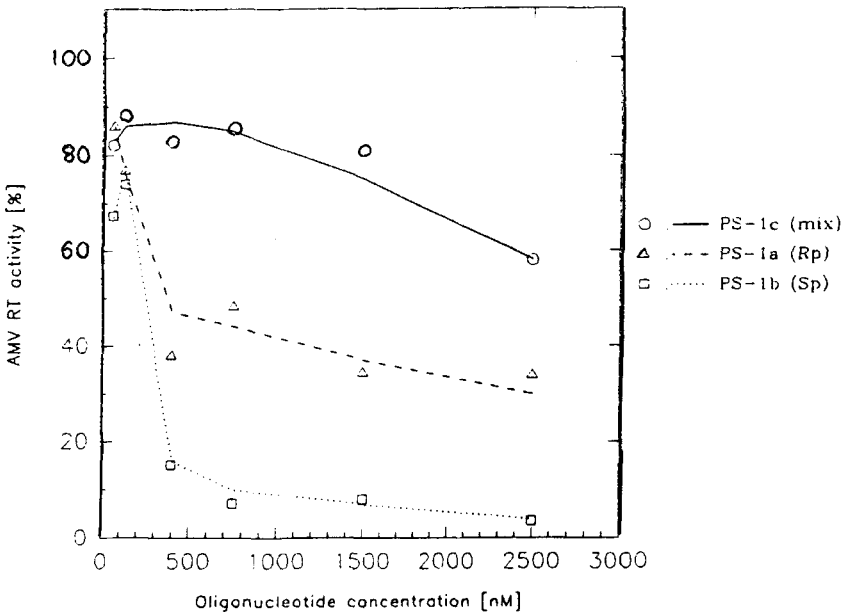


Fig. 1. Inhibition of AMV RT by oligomers PS-**1a**, PS-**1b** and PS-**1c**.

phosphodiester bonds (**1e**, **1g**, **1h**), or two phosphorothioate linkages of the absolute configuration opposite to the configuration within dodecameric phosphorothioate cluster (**1d**, **1f**) (Table 1). We suspected that the 3'-end of phosphorothioate oligonucleotides may be especially important for RT/PS-oligo interaction.[14] The oligonucleotides **1d-1h** were tested as inhibitors of cDNA synthesis, which was primed with the oligomer PO-**3** (Scheme 1).

The oligomers PS-**1f** and PS/PO-**1g**, possessing at 3'-ends two R_P- or two phosphodiester linkages, did not show any inhibitory effect (Fig. 2). These results indicate that the presence of two R_P-PS or two PO-bonds in 3'-terminal positions eradicates the inhibitory effect of the PS-oligomer **1b** containing only S_P-linkages.

Supposedly, two S_P-linkages at the 3'-end of PS-oligo are important for its inhibitory action, but it was found that the presence of two S_P-linkages at the 3'-end of oligonucleotide PS-**1d** did not result in stronger inhibitory effect, as compared to PS-**1a** (Fig. 3).

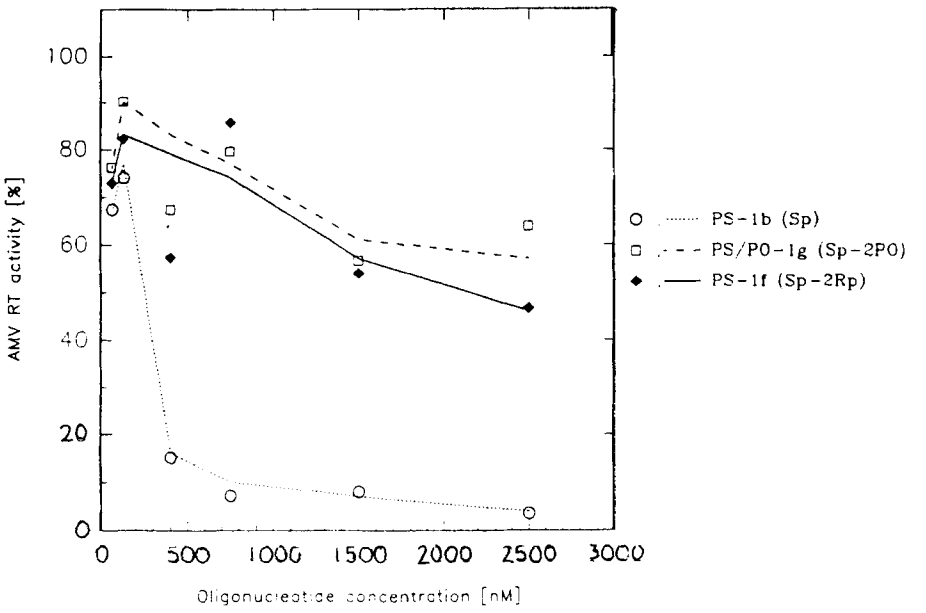


Fig. 2. Inhibition of AMV RT by oligomers PS-**1b**, PS-**1f** and PS/PO-**1g**.

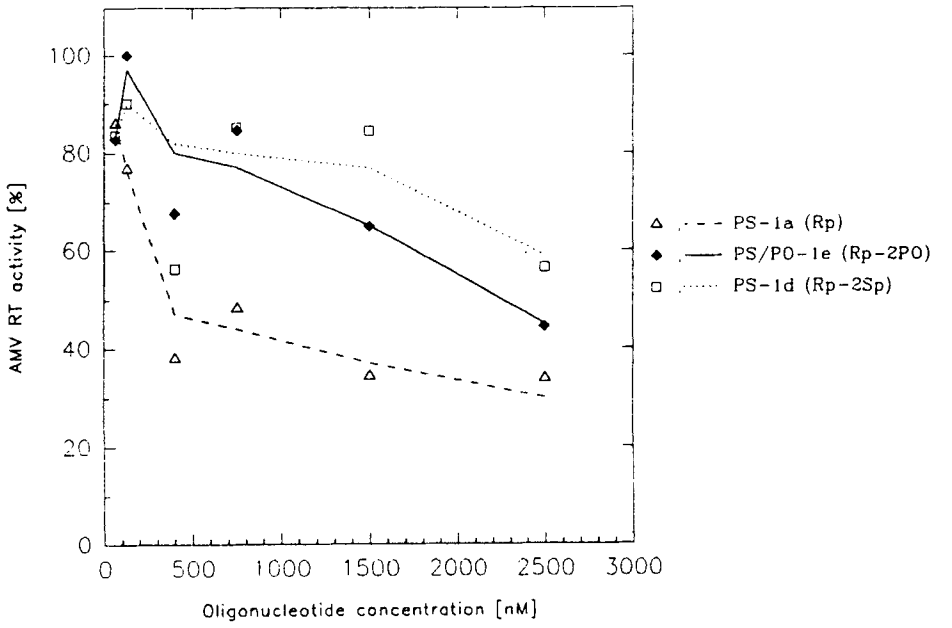


Fig. 3. Inhibition of AMV RT by oligomers PS-**1a**, PS-**1d** and PS/PO-**1e**.

It should be noted that in these experiments the oligonucleotides **1** can hybridize to the polyribonucleotide PO-**5** and serve as the second primer for polymerization reaction, although the resulting cDNA will be shorter (231 nt, Scheme 1b). On the other hand DNA-RNA duplexes containing oligomers **1** can be hydrolyzed by RNase H (activity inherent to the RT enzyme). Therefore, in our model system oligomers **1** can be involved in the three processes:

1) binding to the RT enzyme (*non-antisense interaction*)
2) binding to the RNA template and the synthesis of shortened cDNA (*RT activity*)
3) binding to the RNA template and cleavage of the RNA strand mediated by RNase H activity (*antisense inhibition*).

PAGE analysis of resulting products indicates that all these processes take place simultaneously. We have found a band corresponding to expected full-length cDNA (437 nt, Scheme 1a) and two bands related to truncated DNA fragments (Scheme 1b). One of them (231 nt) corresponds to the product of reverse transcription initiated by the oligonucleotides **1** (process 2) while another (206 nt) results from the reverse transcription initiated by the primer PO-**5** in the presence of the shortened RNA template (process 3). The direct binding of PS-oligos to the enzyme causes the decrease of total cDNA (full-length and truncated fragments) as judged from decreased intensity of the relative bands compared to the intensity of the bands in control experiment (with PO-, but without PS-oligo, Fig. 4).

For further studies we have used simplified model where cDNA synthesis is primed with the oligonucleotide d[TTG AGC AGA TGG GTT TGG] (PO-**4**) (125-142) on polyribonucleotide template (PO-**6**, 286 nt) coding tPA protein (Scheme 2). This template does not contain any fragment complementary to oligonucleotides **1** and therefore is suitable for testing their ability to block the reverse transcription only by binding to the RT enzyme. The result of this experiment confirmed that at conc. 400 nM PS-**1b** was stronger inhibitor than its [all-R_P]-counterpart (PS-**1a**) (70% inhibition versus 30%), while chimeric constructs **1d-1h** exerted only weak inhibitory effect (data not shown). These results suggest that the binding of phosphorothioate oligonucleotides to RT enzyme, which mainly affects RT activity, depends strongly upon subtle changes in conformation and/or shape of PS-oligos.

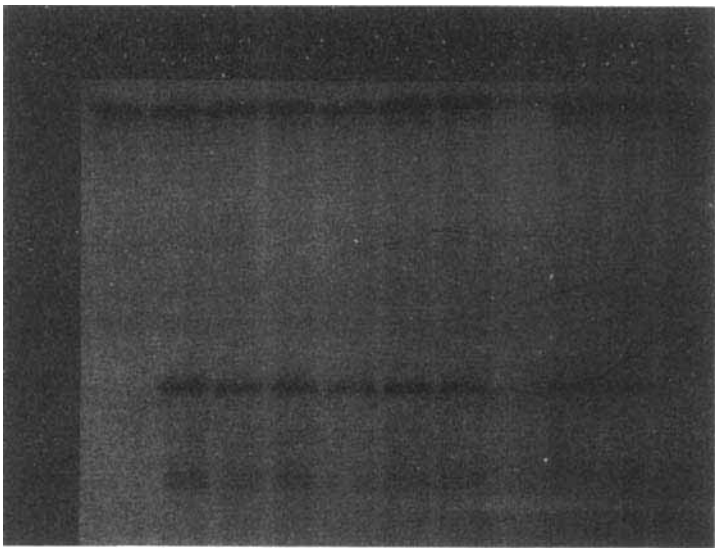
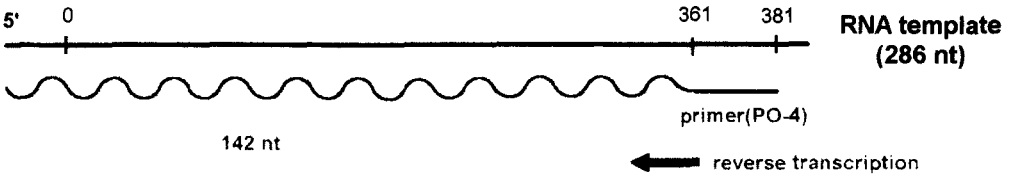


Fig. 4. Reverse transcription inhibition by oligonucleotides **1**.
Autoradiograph of reverse transcription products initiated by primer PO-**3**, in the absence (C_1 and C_2) or in the presence of 400 nM of the oligonucleotide; where numbers *1-9* correspond to experiments with oligomers **1** as follows:
PO-**1** (*1*), PS-**1c** (*2*), PS/PO-**1h** (*3*), PS-**1a** (*4*), PS/PO-**1e** (*5*), PS-**1d** (*6*), PS-**1b** (*7*), PS/PO-**1g** (*8*), PS-**1** (*9*).



SCHEME 2

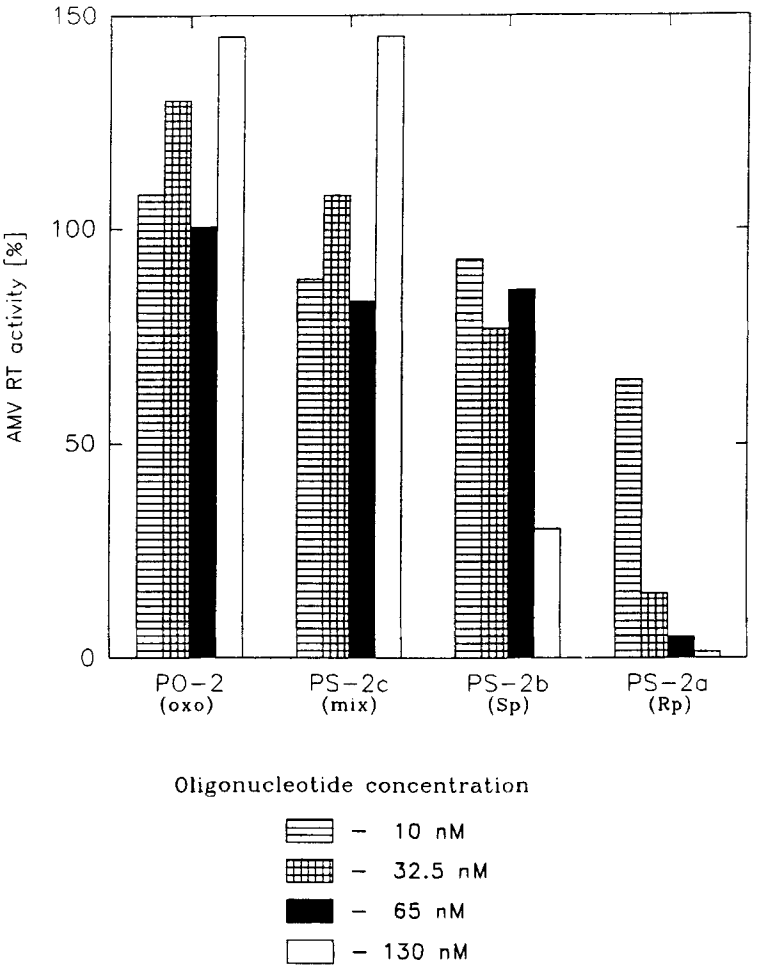


Fig. 5. Influence of oligonucleotides PO-**2**, PS-**2a**, PS-**2b**, and PS-**2c** on the activity of AMV RT.

Although the AMV RT activity was more vividly affected by [all-S_P]-isomer **1b** than by its [all-R_P]-counterpart, further studies did not confirm the S_P-selectivity of the enzyme. Stereoregular analogues of another pentadecamer d[AAG CAT ACG GGG TGT] (PS-**2**) showed that AMV enzyme is strongly inhibited by its [all-R_P]-isomer (PS-**2a**, 90% inhibition at 60 nM concentration, Fig. 5). Interestingly, our data indicate that PO-**2** and PS-**2c**, if used at conc. 130 nM, act as an inhibitor of this enzyme. To the similar extent (80%) HIV RT was also inhibited by PS-**2a** but at conc. 130 nM. HIV RT seems to be less

sensitive towards phosphorothioate inhibitors than AMV reverse transcriptase (A.Krakowiak, M.Koziołkiewicz, W.J.Stec - manuscript in preparation). One has to notice, however, that the sequence d[AAG CAT ACG GGG TGT] (PS-**2**) does contain contiguous four G bases which can be responsible under *in vitro* conditions for the formation of quadruplex structures of oligonucleotides.[15,16] It was reported that the oligonucleotides possessing such structures act as aptamers for thrombin and HIV integrase.[17,18] Further investigations of sequence-dependent inhibition of reverse transcriptases are in progress.

Conclusions

Earlier reports claimed that the binding of phosphorothioate oligonucleotides to the RT enzymes is sequence - independent.[9,19] It should be noticed that in these studies PS-oligos have been used as mixtures of 2^n diastereomers, where n is a number of internucleotide phosphorothioate linkages). Results presented in this paper indicate that the inhibitory effect of PS-oligos against the RT enzymes depends upon a nucleotide sequence of oligonucleotide (**1** vs **2**) as well as upon the absolute configuration at PS-atoms of internucleotide phosphorothioate bonds. We demonstrate that phosphorothioate oligonucleotides used as "the random mixture of diastereomers" are less effective inhibitors than stereoregular PS-oligos. Supposedly, the search for aptameric stereodefined PS-oligos can lead to more active inhibitors of RT enzymes than those described in this paper.

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