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Inhibition of Friend Murine Leukemia Virus activity by guanosine/thymidine oligonucleotides

Joshua Ojwang [a], Kevin M. Okleberry [b], Helene B. Marshall [a], Huynh M. Vu [a], John H. Huffman [b], Robert F. Rando [a,*]

[a] *Triplex Pharmaceutical Corporation, 9391 Grogan's Mill Road, The Woodlands, TX 77380, USA*
[b] *Utah State University, Institute for Antiviral Research, Logan, UT 84322, USA*

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Abstract

Oligonucleotides consisting of only deoxyguanosine and deoxythymidine were stable in culture and were able to significantly inhibit Friend Murine Leukemia Virus (FMLV) production in acute cell culture assay systems. The oligonucleotides did not share homology with, or possess any complementary (antisense) sequence motifs to the FMLV genome. The guanosine/thymidine-containing oligonucleotides (GTOs) which demonstrated anti-FMLV activity in acute infection assays were synthesized with natural phosphodiester (PD) linkages (backbones). The observed antiviral activities of these oligonucleotides increased significantly when the PD backbone was replaced with a phosphorothioate (PT) backbone. Experiments designed to investigate a potential antiviral mechanism of action demonstrated that oligonucleotides tested were capable of blocking virus adsorption. In addition, GTOs with PD backbones were competitive inhibitors of FMLV reverse transcriptase (RT). When the same experiments were performed using oligonculeotides with PT backbones, all compounds tested demonstrated significant competitive inhibition of FMLV RT. The measured inhibitory activity of all compounds tested in culture assays was enhanced by at least a factor of 10 when the PD linkages were replaced with PT. The enhanced antiviral activity exhibited by the sulfur group on the oligonucleotide backbone, and the lack of any designed, sequence-specific interactions, suggest that a large percentage of the reported antiviral activity of oligonucleotides containing a phosphorothioate backbone is due to factors other than rationally designed, sequence-specific interactions. The ability of GTOs to inhibit FMLV in culture, potentially via a number of different mechanisms, makes this a class of compounds which warrants investigation as therapeutic agents to be used against retroviral infections.

Key words: Oligonucleotide; Therapeutic; Antiviral; Friend Murine Leukemia Virus (FMLV)

* Corresponding author. Fax: +1 713 363 1168.

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1. Introduction

Rationally designed oligonucleotides such as antisense molecules have been reported to inhibit specific viral gene expression and hence decrease viral production in culture assay systems (Cohen, 1991; Milligan et al. 1993; Zamecnik and Stephenson, 1978). One common feature of many antisense antiviral oligonucleotides which have been studied recently is that the naturally-occurring phosphodiester (PD) linkages (backbone) of the molecules have undergone chemical modifications. Chemical modification of the backbone is performed ostensibly to confer stability to nucleases on these molecules. When the PD backbone is modified (e.g., phosphorothioate or methylphosphonate), the antiviral activity invariably increases for the antisense molecule as well as for oligonucleotides designed as controls (Milligan et al. 1993). These findings suggest that a portion of an oligonucleotide's antiviral activity occurs via mechanisms other than the code-blocking "antisense" mechanism, and that the addition of a sulfur group to the backbone of an oligonucleotide enhances the alternative, as yet undetermined, mechanism(s) of action.

Originally, the central dogma relating to "antisense" oligonucleotide molecules was that they inhibit viruses by interfering with the process of mRNA translation via an RNA : DNA duplex structure. Recent reports, however, indicate that there are a variety of possible mechanisms by which oligonucleotides inhibit viral infections. For example, oligodeoxycytidine with a phosphorothioate (PT) backbone (poly SdC) inhibits HIV-1 replication in culture (Marshall et al. 1992; Matsukura et al. 1987). One potential mechanism for this antiviral activity, competitive inhibition of HIV-1 reverse transcriptase (RT), was postulated by Marshall et al. (Marshall et al. 1992). Poly SdC also was reported to inhibit AMV RT, Pol I (Klenow fragment) and human Polymerase α (Marshall et al. 1992; Gao et al. 1989), β and γ (Gao et al. 1989). Matsukura et al. (Matsukura et al. 1987) showed that the inhibition of HIV-1 in culture was dependent on the size, in nucleotides, of the poly SdC molecule. When Marshall and Caruthers (Marshall and Caruthers, 1993) reported the use of antisense diphosphorothioate oligonucleotides against HIV-1 RT in vitro, their random sequence control oligonucleotides were very similar in both affinity (Ki) for HIV-1 RT and median inhibitory dose (ID_{50}). Other potential mechanisms of antiviral activity were postulated by Boiziau et al. (Boiziau et al. 1992), whereby the "antisense" oligonucleotide promoted RNAse H activity.

Reports on alternative mechanisms of action of "antisense" compounds against HIV-1 follow earlier studies by Gao et al. (Gao et al. 1990a; Gao et al. 1989; Gao et al. 1990b) in which poly $(SdC)_{28}$ was used to inhibit herpes simplex viruses. In this series of experiments, a number of different antiviral mechanisms were determined for poly SdC, including adsorption blocking and inhibition of HSV2 polymerase. The largest contribution to the antiviral effects described in these studies was made by blockage of adsorption and/or penetration of the virus into the cell. More recently, Stein et al. (Stein et al. 1993) have characterized the interaction of poly SdC with the V3 loop of HIV-1 gp120. It was reported in these experiments that poly $(SdC)_{28}$ specifically interacted with the positively charged V3 loop, with an equilibrium dissociation constant of poly SdC for rgp120 of approximately 5×10^{-7} M. Stein et al. (Stein et al. 1993) postulate

that the specific interaction of poly SdC with the HIV-1 V3 loop may be a mechanism by which a PT-containing oligonucleotide could inhibit HIV-1 in vivo.

We report in this study the inhibition of FMLV activity in culture and in vitro achieved using PD- and PT-containing oligonucleotides composed entirely of guanosine and thymidine. These molecules were designed to be nuclease-resistant and therefore allow for the analysis of antiviral activity of non-sequence-dependent oligonucleotides containing a PD backbone. The use of these oligonucleotides allowed for a measure of the contribution of a PT backbone to the antiviral profile of these molecules. We found that PD-containing molecules are capable of inhibiting FMLV production in culture and FMLV RT in vitro, and that all measured activities increased the PD backbone was replaced with a PT backbone when in a dose-dependent manner.

2. Materials and methods

Enzymes and biochemicals. Radioactive nucleotides ([γ-^{32}P]ATP) were purchased from New England Nuclear. T4 polynucleotide kinase was purchased from New England Biolabs. Hexadimethrine bromide (polybrene) was purchased from Sigma.

Cells, plasmids and virus. NIH/3T3, Vero and HeLa cells were obtained from ATCC. The plasmid pLRB215 containing the FMLV (clone 57) genome and NIH/3T3 cells chronically infected with this FMLV clone (pLRB215 cells) were kindly provided by S. Goff (Columbia University). These cell lines were maintained on Delbecco's Modified Eagle Medium DMEM supplemented with 10% calf serum, penicillin and streptomycin. The *Mus dunni* cell line was maintained as previously described (Morrey et al. 1991).

Oligonucleotide synthesis and modification. All oligodeoxynucleotides used in this study were synthesized on an Applied Biosystems (ABI) DNA synthesizer model 380B or 394, using standard phosphoramidite methods at 0.2 or 1.0 μmol scales. 5′-protected nucleoside phosphoramidite monomers and other reagents were obtained from Milligen, with the exception of acetonitrile, which was obtained from Baxter. All oligonucleotides were synthesized with a 3′-Amino Modifier (Glen Research), which results in the covalent attachment of a propanolamine group to the 3′-hydroxyl group (Durland et al. 1991; Nelson et al. 1989). PT-containing oligonucleotides were prepared using the sulfurizing agent TETD (Vu and Hirschbein, 1991). Standard conditions for synthesis were employed with minor modifications. Crude phosphodiester oligonucleotides were purified using Waters high pressure liquid chromatography (HPLC) system by anion-exchange chromatography on a Q-Sepharose column (1.5 cm $\times$ 10 cm), as described by Murphy et al. (Murphy et al. 1993). The oligonucleotides were desalted on an Amicon TCF cell containing an appropriate size membrane filter (Murphy et al. 1993). The purity of the oligonucleotide was confirmed by analytical HPLC, base composition analysis, electrophoresis of ^{32}P-labeled oligonucleotide on a 20% polyacrylamide gel containing 7 M urea, and by capillary electrophoresis, as described by Vu et al. (Vu et al. 1993). The overall yield of oligonucleotides was 5 to 20%, depending on the sequence, length and modification.

Cytotoxicity and stability assays. Oligonucleotides were assayed for cytotoxicity using a CellTiter 96™ Aqueous Non-Radioactivity Cell Proliferation Assay (Promega). Cytotoxicity assays were performed using a 4-day incubation of oligonucleotide with NIH/3T3, Vero or HeLa cells (500 cells/assay). Each assay was performed in quadruplicate. The resultant data were averaged, graphed and then used to calculate the TC_{50} (median toxic concentration) for the oligonucleotides tested. Another measure of cytotoxicity was performed using a neutral red uptake assay. In this assay, one tenth ml of neutral red (0.034% in PBS) was added to *Mus dunni* cells treated with oligonucleotides. Cells were incubated for 2 h at 37°C, at which time the culture medium was aspirated and the cells rinsed twice with PBS. The plates were drained well and neutral red removed from cells by adding an equal volume of ethanol and Sorensen citrate, pH 4, to each well. The plates were placed in the dark for 30 min at room temperature. The contents of each well were mixed and the neutral red quantitated at 540 nm.

For stability studies, oligonucleotides (10 μM) were added to culture medium maintaining NIH/3T3 cell growth. Aliquots were taken after 10 min, 1, 2, 3 or 4 days. The aliquots at each time point were immediately extracted twice with a 50:50 mixture of phenol and chloroform. The whole sample was end-labeled using [γ-^{32}P]ATP and polynucleotide kinase. The integrity of each oligonucleotides was determined by analysis on a 20% polyacrylamide gel (7 M urea).

Inhibition of Acute FMLV Infection. The pLRB215 cell line was established by transfecting NIH/3T3 cells with proviral FMLV DNA and culturing the transfected cells until all cells in the culture became infected. In acute assays, NIH/3T3 cells were plated onto 96-well culture dishes at 1×10^4 cells/well and allowed to incubate at 37°C overnight in 200 μl of complete medium. The culture medium was then removed and replaced with 100 μl of complete medium containing 2 μg/ml polybrene and 10 μl of stock FMLV. The virus stock consisted of medium obtained from confluent pLRB215 cells in which the medium had not been changed for 4 days. The virus was allowed to adsorb onto NIH/3T3 cells for 4 h at 37°C without (Protocols 1 and 3) or with (Protocols 2 and 4) oligonucleotides added to the culture medium. The cells were washed once with PBS and then incubated in 100 μl of complete medium containing various concentrations of oligonucleotides. After 4 days the culture medium was removed and tested for the presence of FMLV RT (Protocol 1 and 2). Cells were incubated for 3 additional days in complete medium without oligonucleotide (Protocol 3 and 4), at which time the presence of FMLV in the culture medium was again determined by monitoring the level of viral RT. Results obtained are presented as percentage of RT activity compared to enzyme levels obtained from untreated FMLV infected cells.

A focal immunoenzyme assay (FIEA) was also used to determine the antiviral activity of oligonucleotides. In this assay, *Mus dunni* cells were infected in triplicate with the Lilly-Steeves B-tropic strain of Friend virus complex for 3 h at 37°C as previously described (Morrey et al. 1991). After viral infection, the virus-containing medium was replaced with fresh medium and various concentrations of oligonucleotide, in triplicate. The infected cells were then incubated for 4 days, at which time the presence of FMLV gp70 in these cells was monitored using MAb 48 in the indirect immunofluorescent assay (Chesebro et al. 1983; Morrey et al. 1991).

2.1. In vitro reverse transcriptase inhibition assays

RT assays were performed using an enzyme linked oligonucleotide sorbent assay, ELOSA, RT-Detect Kit (New England Nuclear), according to the manufacturer's instructions. The ELOSA assays were performed in a 96-well microtitre plate, using 30 μl of assay mix (4 × buffer, template RNA, oligonucleotide primer, dNTPs, DTT and RNAse inhibitor) and 10 μl of a solution containing RT enzyme. The source of RT was either culture medium obtained from pLRB215 cells, from acutely infected NIH/3T3 cells or the commercially available Moloney murine leukemia virus (MMLV) RT. When required, in enzyme kinetic experiments, the reaction mix was manipulated to vary the dNTP concentrations. The ID_{50} was defined as the amount of oligonucleotide required to inhibit FMLV RT present in 10 μl of culture medium by 50%. The results are the average of two or more experiments, performed in duplicate each time. In kinetic experiments, the amount of FMLV RT used in each assay was estimated to be 0.5 units when compared with known activities of MMLV RT.

3. Results

Experimental design. Modification of the 3′ hydroxyl group of oligonucleotides has been shown to greatly reduce degradation by both cellular and extracellular exonucleases (Orson et al. 1991; Stein et al. 1988; Birg et al. 1990). All oligonucleotides used in this study were therefore modified at their 3′ terminus with a propylamine moiety in order to enhace stability. In addition, oligonucleotides consisting of only deoxyguanosine and deoxythymidine, with blocked 3′ termini, have been shown to be stable both in vitro and in vivo (Durland et al. 1991; Orson et al. 1991; Zendegui et al. 1991). We have also detected potent antiviral activity with this particular class of oligonucleotides for a wide range of viruses (Fennewald et al. 1994; Ojwang et al. 1994). Therefore, to assess the contribution of sequence, length and the chemical nature of the internucleoside linkage to anti-retroviral activity in culture, oligonucleotides consisting of only deoxyguanosine and deoxythymidine bases (GTOs) were synthesized (Table 1). The analytical HPLC profile and base composition analysis of one oligonucleotide synthesized for this study (I100–00) is presented in Fig. 1.

The stability profile for the synthesized oligonucleotides in culture was determined by the additon of these compounds to confluent NIH/3T3 cell cultures as described in Section 2. The results of this experiment indicate that a full-length GTO (I100–00, with PD backbone) was present in the culture medium after 4 days at 37°C (Fig. 2). In the same assay after 1 day in culture there was no detectable level of full-length 1231, a PD oligonucleotide containing a random mixture of all four bases (Fig. 2). The data indicate that 3′-modified GTOs with PD backbones had enhanced stability against both endonuclease and exonuclease degradation relative to oligonucleotides containing a random mixture of all four deoxynucleotides. In Fig. 2 (lane 8), I100–00 appears as a doublet even though only a sharp peak was observed in the analytical HPLC profile of this compound (Fig. 1). This observation has been previously reported by other investigators using 3′ amine tailed oligonucleotides (Reed et al. 1991). It is also interesting to note

Table 1
Guanosine/thymidine and control oligonucleotide sequences synthesized for stability and antiviral efficacy studies

Oligo	Length	Backbone [a]	Sequence	TC_{50} [b]
I100-07	45 mer	PD	5′ gtggtgggtgggtgggtggtgggtggtggttgtgggtgggtggtg 3′	> 50 μM
I100-21	45 mer	PT	5′ gtggtgggtgggtgggtggtgggtggtggttgtgggtgggtggtg 3′	ND [c]
I100-00	26 mer	PD	5′ ggtggtggggtggttgttgggggttg 3′	37 μM
I100-12	26 mer	PT	5′ ggtggtggggtggttgttgggggttg 3′	18 μM
I100-01	45 mer	PD	5′ ggtggtggggtggttgttgggggttgttgggggtgtgtgggtggt 3′	ND
1171	18 mer	PT	5′ gggtggttgggtggttgg 3′	ND
1172	18 mer	PD	5′ gggtggttgggtggttgg 3′	ND
1173	18 mer	PD	5′ gggtgggtgggtgggtgg 3′	ND
1174	18 mer	PT	5′ gggtgggtgggtgggtgg 3′	ND
I100-11	45 mer	PD	5′ gatccatgtcagtgacactgcgtagatccgatgatccagtcgatg 3′	46.5 μM
1208	36 mer	PT	5′ gatccatgtcagtgacactgcgtagatccgatgatc 3′	ND
1231	18 mer	PD	5′ gatccatgtcagtgacac 3′	ND
1232	18 mer	PT	5′ gatccatgtcagtgacac 3′	ND
1229	18 mer	PD	5′ cccccccccccccccccc 3′	ND
1230	18 mer	PT	5′ cccccccccccccccccc 3′	ND

[a] The backbone modifications are denoted as PD for phosphodiester and PT for phosphorothioate.
[b] Median inhibitory (toxic) concentration in tissue culture.
[c] Not determined (ND).

that we observed a buildup of specific breakdown products for I100–00 (Fig. 2, lanes 9–13). These breakdown products may correspond to internal cleavage of this oligonucleotide at the positions where there are two contiguous pyrimidines in the sequence (Fig. 2). Under these same assay conditions, 4 days post-addition of oligonucleotide to the culture medium, a larger percentage of full-length molecules was observed for oligonucleotides containing PT backbones than for PD GTOs (data not shown). The use of stable phosphodiester oligonucleotides allowed us to separate out effects due to stability of the oligonucleotides from changes in antiviral activity related to other modifications such as sequence or backbone modification.

The toxicity of selected compounds, based on the MTS assay, was determined in NIH/3T3 cells and the TC_{50} results are presented in Table 1. The toxicity of selected compounds using the neutral red assay are presented in Table 3.

Inhibition of FMLV production in an acute assay system. A portion of antiviral activity attributed to oligonucleotides is due to the blockage of viral adsorption to cells (Gao et al. 1990a). Therefore, to obviate antiviral effects attributable to initial blocking of virus adsorption and to monitor the contribution of this effect to the overall antiviral activity, we performed the following experiments by adding the oligonucleotide either after (protocol 1) or during (protocol 2) the initial viral infection. All oligonucleotides with PD backbones tested using protocol 1 were able to significantly reduce FMLV RT levels in the culture medium, except the control oligonucleotide I100–11 (Fig. 3A). This PD control oligonucleotide, which contains an equal proportion of all four bases,

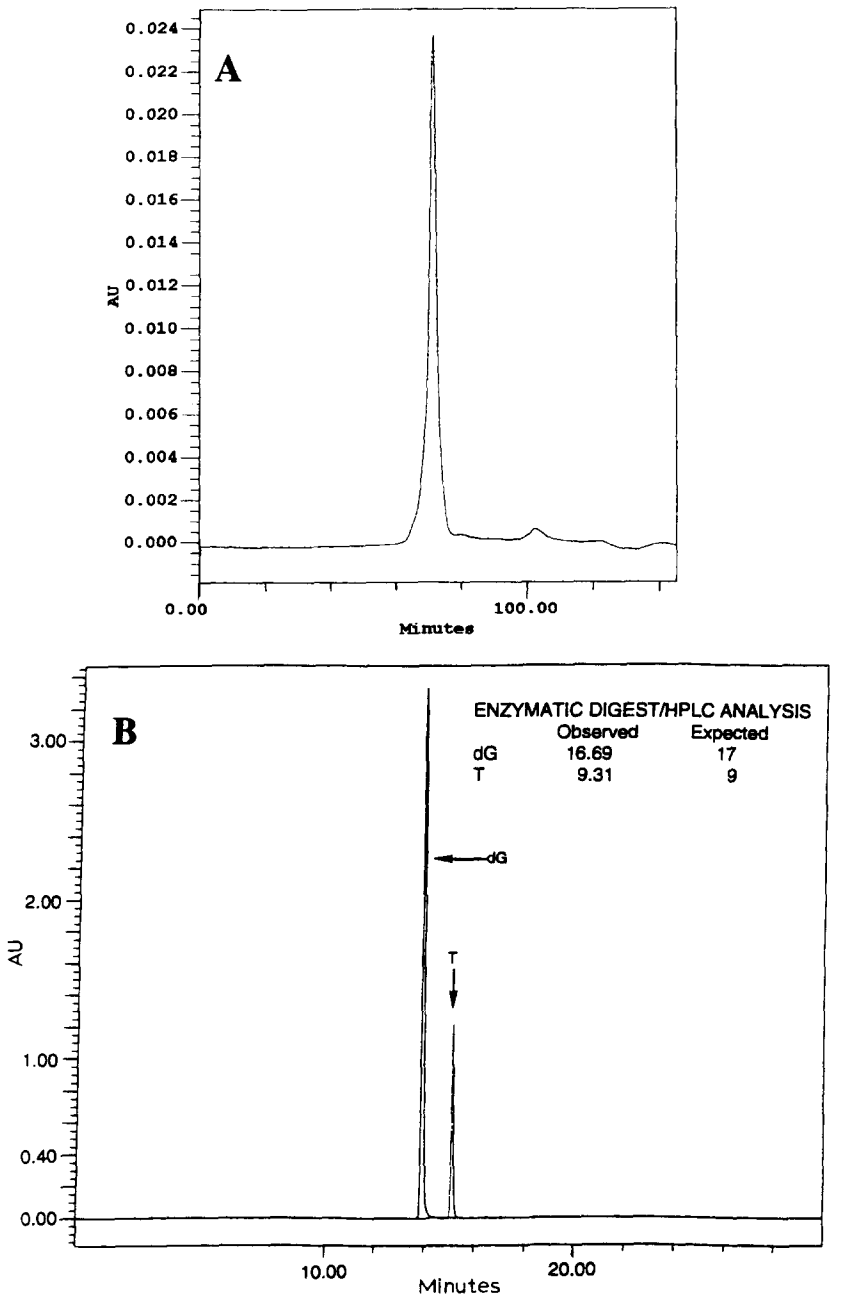

Fig. 1. (A) Analytical ion-exchange HPLC of purified oligonucleotide I100–00 (26 mer with 3'-amine modification). Buffer A: 0.5 M NaCl, 10 mM NaOH; buffer B: 1.5 M NaCl, 10 mM NaOH. Column: high-performance Q Sepharose, flow rate: 2.5 ml/min, gradient: 0–55 min, 80–30% A and 20–70% B. (B) Base composition analysis of I100–00 degraded by P1 nuclease/bacterial alkaline phosphatase treatment. The resulting deoxynucleosides were separated on a reverse phase column (Customsil ODS, 4.6×250 mm). Buffer A: water, buffer B: acetonitrile, flow rate: 1.0 ml/min, gradient: 0–35 min, using an isocratic system with 98% A and 2% B.

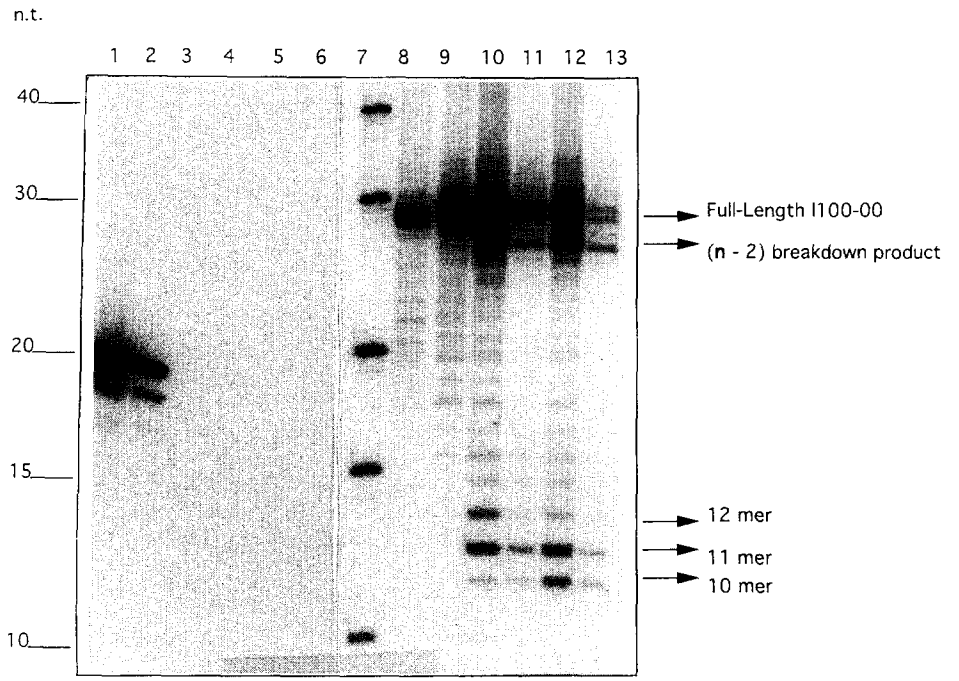

Fig. 2. Stability of PD oligonucleotides under cell culture conditions. Oligonucleotides I100–00 and 1231 were placed in cell culture medium supporting the growth of NIH/3T3 cells. Oligonucleotide was extracted from the culture medium at various times, ^{32}P-end labeled and analysed using a 20% acrylamide gel. Lanes 1–6 contain 1231 samples obtained at times 0, 10 min, 1, 2, 3 or 4 days post-addition to the culture medium, respectively. Lane 7 contains oligodeoxythymidine nucleotide markers. The size of each marker band in nucleotides (n.t.) is shown to the left of the panel. Lanes 8–13 contain I100–00 samples obtained at times 0, 10 min, 1, 2, 3, or 4 days. The position of full-length I100–00 and the observed accumulated breakdown products are indicated on the right side of the panel.

increased measured RT levels above the viral control samples. The lack of viral inhibition observed when I100–11 was added to the culture post-viral infection may be due to the rapid degradation of this compound under culture conditions. Culture samples taken from cells treated under protocol 2 conditions had the same or lower levels of detectable FMLV RT for all oligonucleotides tested (Fig. 3B, Table 2). The ability of I100–11 to inhibit virus under protocol 2 conditions suggests that a portion of the antiviral activity observed was due to direct blockage of the initial viral infection. The inhibitory effects of the PT analogs of all oligonucleotides tested were always better than the PD version for protocols 1 and 2. The median effective doses (ED_{50}s) of the compounds under the different protocol conditions are presented in Table 2. The least variation in activity between oligonucleotides having the same sequence but with different backbones was between 1173 and 1174 (Table 2).

In our assay conditions, full-length PD and PT GTOs were still detected after 4 days in culture. This information, coupled with the observation that oligonucleotides with PT backbones directly inhibit HIV-1 RT in vitro (Marshall and Caruthers, 1993) suggests

Table 2
Measurement of FMLV RT levels as an indicator of antiviral activity of oligonucleotides in culture

Oligonucleotide	Length	Backbone [a]	ED_{50} [b] (μM)			
			Prot. 1	Prot. 2	Prot. 3	Prot. 4
I100-07	45 mer	PD	0.150	0.090	ND [c]	ND
I100-21	45 mer	PT	0.025	0.020	2.00	1.50
I100-00	26 mer	PD	0.220	0.150	> 20	> 20
I100-12	26 mer	PT	0.006	0.003	0.75	0.15
1173	18 mer	PD	0.550	0.450	ND	ND
1174	18 mer	PT	0.170	0.110	ND	ND
I100-11	45 mer	PD	> 20	2.000	> 20	> 20
1208	36 mer	PT	0.002	0.004	0.40	0.55

[a] Oligonucleotide backbones are either phosphodiester (PD) or phosphorothioate (PT).
[b] The assay protocols, described in Section 2, included: (1) virus infection followed by addition of oligonucleotide, RT assay on day 4; (2) virus infection in the presence of oligonucleotide, RT assay on day 4; (3) same as protocol 1 above, additional 3 day incubation in media without oligonucleotide; and (4) same protocol as 2, additional 3 day incubation in media without oligonucleotide. The results are the average of two or more experiments performed in duplicate.
[c] Not determined (ND).

that a portion of the anti-FMLV activity observed in the 4 day post-infection assays may have been due to residual oligonucleotide, in the culture medium, directly interfering with the enzyme assay. To determine whether the presence of an oligonucleotide in the culture medium was suppressing the production of virus, or the ability to monitor the production of virus, the oligonucleotides were removed from the culture medium and the cells were incubated for 3 additional days. At this time point, reduced FMLV RT levels were observed only in culture medium taken from cells treated with PT oligonucleotides (Fig. 4, Table 2).

To further confirm that the observed antiviral activity was a result of an inhibition of virus production and not inhibition of viral RT, we performed the detection assay using a monoclonal antibody (MAb48) specific for the FMLV gp70 to determine the number of cells infected with FMLV. The results of this focal immunoenzyme assay experiment

Table 3
Detection of FMLV glycoprotein gp70 as an indicator of antiviral activity of oligonucleotides in culture

Oligonucleotide concentration μM	I100-07		I100-21	
	Antiviral [a] % reduction	NR uptake [b] % of control	Antiviral [a] % reduction	NR uptake [b] % of control
5	22	> 100	100	84
0.5	23	> 100	100	92
0.1	16	98	36	91

[a] Focal immunoenzyme assay using Mab 48 to detect envelope gp70 after FMLV infection of *Mus dunni* cells. The results presented are the average of an experiment performed in triplicate.
[b] Percent of neutral red uptake measured when compared to untreated control cells.

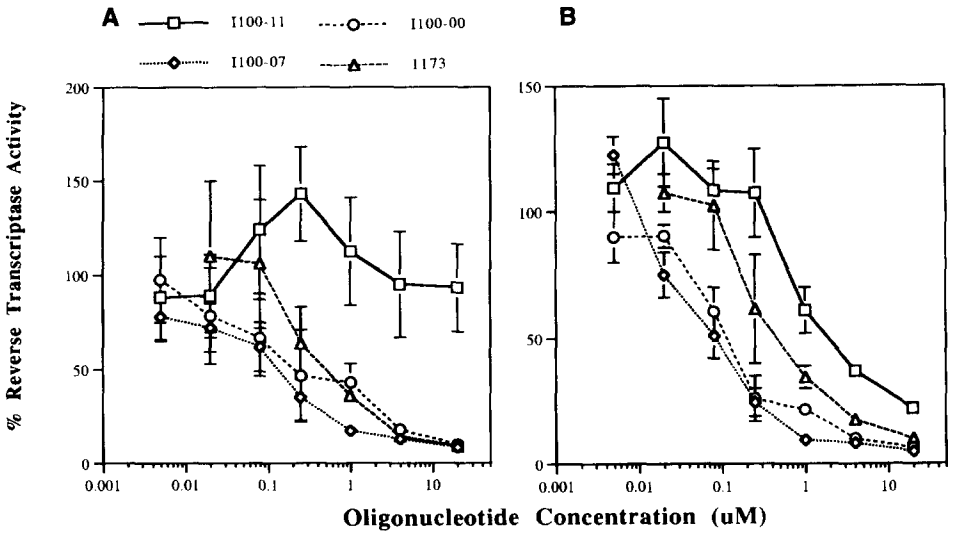

Fig. 3. Detection of FMLV RT in infected cell culture medium using protocol 1 and protocol 2 conditions. (A) PD-containing oligonucleotides were added to FMLV-infected cell cultures after virus infection and the level of viral RT was measured 4 days post-infection (protocol 1). (B) Viral RT was measured 4 days post-infection when PD oligonucleotides were added during viral infection of cell cultures (protocol 2). The data presented are the average of two experiments, each performed in duplicate.

indicated that after 4 days in culture, both PD (I100–07) and PT (I100–21) versions of the same oligonculeotide were able to inhibit the expression of viral gp70 (Table 3). The enhanced antiviral activity conferred by the PT backbone supports the observations of prolonged suppression of FMLV by the PT-containing oligonucleotides (Fig. 4).

In vitro inhibition of FMLV RT. The ability of oligonucleotides to inhibit HIV-1 RT in vitro has been well documented. Marshall et al. (Marshall et al. 1992; Marshall and Caruthers, 1993) have described a competitive interaction at the active site as the mechanism by which mono- or diphosphorothioate-containing oligonucleotides inhibit HIV-1 RT, independent of whether the molecule tested was antisense, a random sequence or poly SdC.

The difference in the ED_{50} values obtained for I100–07 under protocol 1 conditions using the FMLV RT detection assay (0.08 mM, Table 2) and the FIEA test (> 5.0 mM, Table 3) suggests that a portion of the observed inhibition in the RT detection assay may have been due to a direct inhibition of the FMLV RT enzyme by residual oligonucleotides in the culture medium. To investigate this possibility, FMLV RT activity was assayed, as described in Section 2, in the presence of both PD- and PT-containing oligonucleotides. A kinetic analysis of enzyme inhibition was conducted to determine the mechanism by which these oligonucleotides inhibit FMLV RT. Dixon plots of inhibitor concentration verus $1/v$ (where v is the reaction velocity) were used at a variety of substrate concentrations. For competitive inhibitors, this type of analysis will result in a family of lines all intersecting at the point $(-K_i, 1/V_{max})$, were K_i is the inhibition constant and V_{max} is the maximum velocity. Noncompetitive inhibitors give a

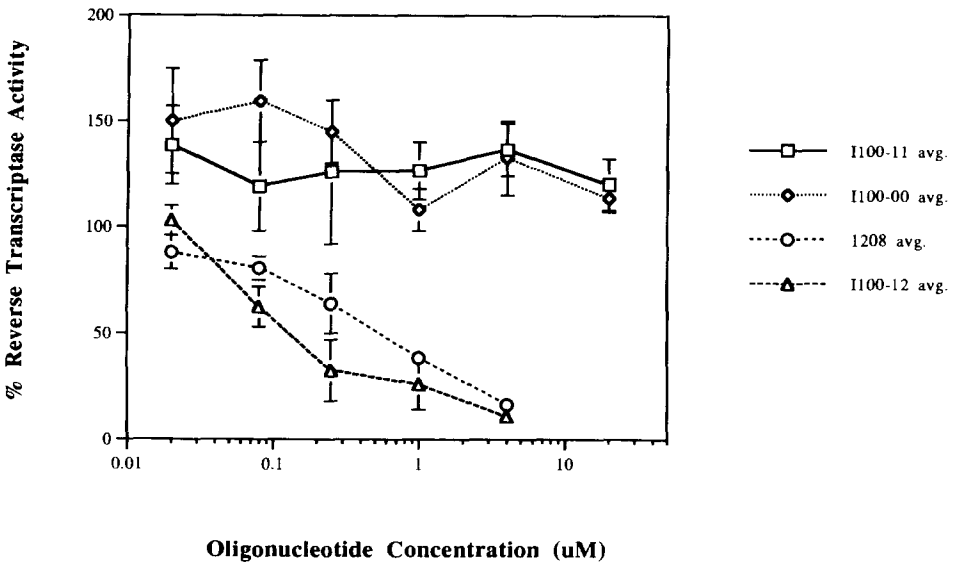

Fig. 4. Detection of FMLV RT 3 days post-removal of oligonucleotides from acutely infected cell culture medium. Matched PD and PT oligonucleotides were added to cells during viral infection (protocol 4) and incubated with infected cells for 4 days. The oligonucleotides were then removed and infected cells were incubated for 3 additional days, at which time the level of RT in the culture medium was assayed. I100–00 (PD) and I100–12 (PT) have the same sequence, while 1208 (PT, 36 mer) is a shorter version of I100–11 (PD, 45 mer). The data presented are the average of two or more experiments.

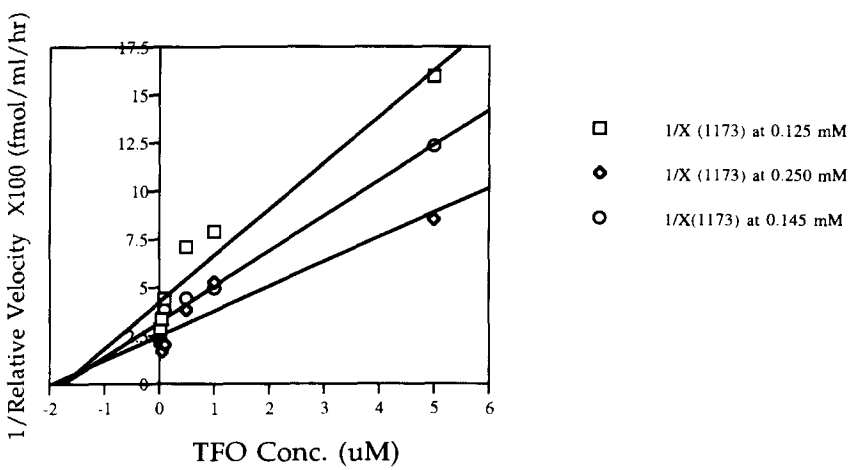

Fig. 5. Dixon plot of oligonucleotide 1173 obtained from kinetic analysis of inhibition of FMLV RT with respect to dNTP concentration. The inhibition constant (K_i) was determined by simultaneously varying the concentrations of the dNTPs and of the inhibitor (1173). The K_i determination was performed at 0.125 mM, 0.145 mM and 0.25 mM dNTP concentrations with constant primer-template concentrations. Ten μl of supernatant from pLRB215 cells was used in each reaction. The reported values are the average result of simultaneous independent duplicate determinations.

Table 4
Inhibition of FMLV RT in vitro

Oligonucleotide	Backbone [a]	Inhibition of viral RT (μM)	
		K_i [b]	ID_{50} [b]
I100-00	PD	1.24 (0.020)	0.9 (0.01)
I100-12	PT	0.0038 (0.0003)	0.007 (0.0002)
I100-07	PD	2.5 (0.20)	0.06 (0.01)
I100-21	PT	0.0018 (0.0004)	0.001 (0.0)
1172	PD	6.59 (0.04)	1.00 (0.5)
1171	PT	0.293 (0.003)	0.05 (0.01)
1173	PD	1.769 (0.01)	0.65 (0.1)
1174	PT	0.105 (0.005)	0.04 (0.005)
1229 poly dC	PD	> 5.0 (0.50)	> 5.0 (0.5)
1230 poly SdC	PT	0.015 (0.003)	0.05 (0.01)
1231 (GATC) [c]	PD	> 5.0 (0.0)	0.75 (0.2)
1232 (GATC)	PT	0.094 (0.002)	0.017 (0.001)

[a] PD = phosphodiester backbone, PT = phosphorothioate backbone.
[b] The standard deviation for each value is presented in parenthesis.
[c] Compounds 1231 and 1232 are composed of a random mixture of guanosine, adenosine, thymidine and cytidine (GATC).

family of lines intersecting at ($-K_i$, 0) (Segal, 1975). Data from the inhibition profile of FMLV RT by compound 1173 is presented in Fig. 5. This result clearly indicates that the oligomer is binding to FMLV RT in a manner that precludes binding of natural substrate, suggesting a competitive mechanism of inhibition with respect to the template. The K_i value for all of the oligonucleotides tested and the ID_{50}'s in the RT assays are presented in Table 4. The data demonstrate that the presence of the sulfur group in the backbone enhanced the interaction between oligonucleotides and enzyme with the degree of enhancement between one to three orders of magnitude (Table 4). In addition, the ID_{50}'s revealed that the presence of the sulfur group in the backbone increased the observed inhibitory effect of the matched compounds 20- to 1000-fold (Table 4).

4. Discussion

In this report we demostrated that oligonucleotides containing only guanosine and thymdine, synthesized with naturally-occurring phosphodiester backbones, were more resistant to nuclease digestion than oligonucleotides containing all four bases, and were capable of inhibiting FMLV production in cultured cells. The oligonucleotides tested were designed to maximize their stability in culture conditions and were previously empirically determined to exhibit profound antiviral activity in culture for a number of different target viruses (Ojwang et al. 1993; Fennewald et al. 1994). As such, they were synthesized without rational antiviral design specifications and represent an entirely new

mechanism for oligonucleotide antiviral activity. We refer to this unique class of oligonucleotide-based antiviral therapeutic agents as GTOs. At this time we can only speculate as to the structure-activity relationship observed in GTOs. It is evident, however, that sequence and length variations in the guanosine/thymidine motifs can have profound effects on activity for any particular virus and that the optimal antiviral motif varies for different viruses (Fennewald et al. 1994; Ojwang et al. 1994). Experiments are underway to further investigate the essential elements in the sequences studied which impart the antiviral activity. In all experiments reported, the inhibitory effect of the PT version of a GTO was greater than the matched PD version. In addition, persistent suppression of FMLV was observed only for the PT-containing oligonucleotides (Fig. 4, Table 2). It is interesting to note that the PT-containing random sequence compound (1208) was able to suppress FMLV production for at least 3 days after the removal of oligonucleotide from the culture medium (Fig. 4). This compound was designed as a control and does not contain any DNA sequence homology with FMLV. This observation further exemplifies the degree of antiviral activity imparted to an oligonucleotide by the PT backbone linkage.

The use of matched PD and PT oligonucleotides in analysis of the inhibition of FMLV RT allowed for a quantitative analysis of the differences in K_i and ID_{50}. These experiments determined that all oligonucleotides tested, with PD backbones, were competitive inhibitors of the RNA-dependent DNA polymerase activity of FMLV RT, although the PD random oligonucleotide 1231 and the PD poly dC were only weakly so (Table 4). The level of inhibition increased 20- to 1000-fold, however, when the matched oligonucleotide contained a PT backbone (Table 4). The ID_{50} values for all PT oligonucleotides tested were also much better than the analogous PD versions. The differences in actual ID_{50} values between the PT-containing compounds with different sequences (up to 50-fold) were smaller than some of the differences between sequence-matched compounds with different backbones (Table 4). This observation suggests that the backbone modification is important for the enzyme inhibition. In addition, the large difference (162-fold) in the K_i between I100–21 (0.0018 μM) and 1171 (0.293 μM) was not reflected in their respective ID_{50}'s (5-fold difference). This result suggests that a second mechanism of inhibition, which is not monitored in our assay system, may be involved in the inhibition of FMLV RT. This possibility is supported by the recent work of Cheng et al. (1991) in which it was demonstrated that poly SdC_{28} was able to inhibit all three enzyme activities of HIV-1 RT (RT, DNA polymerase and RNAse H). Of these three enzymatic acivities it was found that the RNAse H activity of RT was the most susceptible to poly SdC_{28} (Cheng et al. 1991).

It is possible that the inhibition of FMLV RT observed in culture assays was only due to the presence of residual oligonucleotides in the culture medium (Figs. 3 and 4). This possibility is unlikely however for several reasons. First, I100–11 was able to inhibit FMLV when co-incubated with the virus (Fig. 3B). Second, reduced RT levels were observed 3 days post-removal of oligonucleotides from the culture medium and third, the MAb based immunofluorescence assay detection scheme registered reduction in the number of cells producing viral glycoprotein in cultures treated with oligonucleotides under protocol 1 conditions. Taken together this information indicates that there was a reduction in FMLV in cultures treated with oligonucleotides.

It is likely that several different mechanisms are involved in the inhibition of FMLV by oligonucleotides. One such mechanism involves the blocking of virus/cell interactions, as was observed for I100–11 under protocol 2 conditions. Another potential mechanism of action may be the inhibition of intracellular viral RT activity. In addition, we have observed in vitro inhibition of HIV-1 integrase enzyme using GTOs (Ojwang et al. unpublished observations). Experiments are underway to further elucidate these and other potential mechanisms of action.

The therapeutic antiviral potential of oligonucleotides is not decreased due to the nature of their mechanism of action as long as a large selective index is maintained. While the further characterization of their mechanism(s) of action should help in the rational use and design of future generations of antiviral oligonucleotides, the observations reported here and by others in the field demonstrate the efficacy of this class of compounds.

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