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Antiviral Effect of Phosphorothioate Oligodeoxyribonucleotides Complementary to Human Immunodeficiency Virus

Sang-Gug Kim, a Toshifumi Hatta, a Satoru Tsukahara, a Hideki Nakashima, b Naoki Yamamoto, b Yoko Shoji, c Kazuyuki Takai a and Hiroshi Takaku a

"Department of Industrial Chemistry, Chiba Institute of Technology, Tsudanuma, Narashino, Chiba 275, Japan
b Department of Microbiology, Tokyo Medical and Dental University School of Medicine, Yushima, Bunkyo-ku, Tokyo 113, Japan

Abstract—Modifications of oligodeoxyribonucleotides include the replacement of the backbone phosphodiester groups with phosphorothioate (S-ODNs) groups and the substitution of phosphorothioate (SO-ODNs) groups at both the 3'- and 5'-ends. In assays for HIV, oligomers (S-ODNs) were more active at the micromolar range than were SO-ODNs of the same sequence. Furthermore, the abilities of antisense-, sense-, random-, and mismatched-oligomers, or homo-oligomers containing internucleotidic phosphorothioate linkages to inhibit HIV-1 replication were examined. Antisense oligonucleotides inhibit the replication and the expression of HIV-1 more efficiently than random-, sense-, mismatched-, and homo-oligomers of the same length or with the same internucleotide modification. Five different target sites (gag, pol, rev, tat, and tar) within the HIV genes were also studied with regard to the inhibition of HIV replication by antisense oligonucleotides. Antisense oligomers complementary to the sites of initiation sequences and to certain splice sites were most effective. The effect of antisense oligomer length on inhibiting viral replication was also investigated. Of particular interest was the S-ODNs-rev 15 mer, which possessed higher anti-HIV activity than the sense-, random-, mismatched-, and homo-20 mers.

Introduction
Antisense oligonucleotides and their analogues have been shown to inhibit viral replication, 1-3 to block the splicing and translation of mRNA, 4,5 and to regulate specific gene expression. 6,7 An oligonucleotide complementary to a viral genome or to its mRNA may interfere with the expression of that genetic segment by hybridization. 1,2

The stability of the oligonucleotide in the culture medium and within the cell will influence its inhibitory effect. It has been demonstrated that unmodified oligonucleotides have limited use in vitro as well as in vivo, because of their sensitivity to cellular nucleases, which are present in the culture medium and serum. 8-15 To overcome this problem, the backbone phosphates of some antisense oligonucleotides have been modified to methylphosphonates 11,12 or phosphorothioates (S-ODNs), 9 and S-ODNs have proven anti-HIV activity in acute infections in vitro. 9,13-16 Matsukura et al. reported that the inhibition of de novo infection by S-ODNs is both composition- and length-dependent, for example, homo-oligo S-dC20 is a better inhibitor than either S-dC20 or S-ODNs (20mer, encoding exon I of the art/tar gene in HIV-1). 9 However, in chronically infected T cells, S-dC20 did not inhibit p24 gag expression, whereas S-ODNs complementary to the initiation sequence of HIV-rev inhibited the production of several viral proteins. 17 On the other hand, Lisziewicz et al. 18 have reported that chemotherapy based on specifically targeted antisense oligonucleotide phosphorothioates is an effective means of reducing the viral burden in HIV-1 infected individuals, at clinically achievable oligonucleotide concentrations. In a previous paper, we reported the inhibition of HIV-1 replication in acutely HIV-1 infected cells by phosphorothioate oligonucleotides. 19 To define the dependence upon both the target sequences and the length of the phosphorothioate oligonucleotides for optimal anti-HIV activity, we synthesized phosphorothioate oligonucleotide analogues. In this paper, we extend those observations with a comparison of oligonucleotides with either backbone of phosphorothioate groups or those capped with a phosphorothioate group at both the 3' and 5'-ends, in sense, random, mismatched, homo-oligomeric, or antisense sequences, with five different target sites (gag, pol, rev, tat, and tar) within the HIV-1 gene (Fig. 1).

Results and Discussion
Sequence-specific inhibition of viral expression in HIV-1 infected cells
To examine the selective antisense inhibition of HIV with a phosphorothioate oligomer, we selected targets...
Figure 1. DNA sequences of the leader site of gag, the splice acceptor sites of pol, rev, and tat, and the translation start site of rev. The random sequence has the same base content as antisense rev-sa, but has <70% homology with any portion of the HIV sequence, as either antisense or sense. Homo-oligomers of the four bases (A, C, G, and T) were synthesized in four lengths (10, 15, 20, and 28 mer). The phosphorothioate derivatives are denoted by ‘S’.

All the antisense oligomers inhibited HIV-1 replication without toxicity in acutely HIV-1 infected cells (Table 1) and, moreover, the antisense S-ODNs of the targeted internal splice sites (rev and tat splice acceptor sites) and the initiator codon (AUG) were very active, causing more than 95% inhibition at 0.02-0.5 μM. The S-ODNs-pol-sa, however, failed to satisfactorily prevent HIV-1 replication (up to 64% inhibition at 0.02 μM). On the other hand, the S-ODNs-gag-ls contains a sequence complementary to the leader sequence of p24 (CCU), which may play an important role in mRNA processing, and its activity (>92% inhibition at 0.02-0.5 μM) was as good as those of the internal, valid splice sites or the initiator codon (Table 1).

On the other hand, we could not detect any inhibitory effects of the 3′,5′-capped, phosphorothioate-substituted oligomer (SO-ODNs-rev-sa) at a concentration of 0.5 μM (Table 1). The weaker anti-HIV activity of the SO-ODNs oligomer than that of the S-ODNs oligomer was supported by the uptake studies.22 Figure 2 shows the effect of time on the uptake of the 3′-[32P] labeled SO-ODNs-tat-sa and S-ODNs-tat-sa by MOLT-4 cells. The uptake reached a plateau after 60 min, and the SO-ODNs oligomer yielded about 10 times less cell-associated counts than the S-ODNs oligomer. This 3′,5′-capped phosphorothioate group (SO-ODNs) was weakly assimilated by MOLT-4 cells in cooperation with the phosphorothioate oligomers (S-ODNs), and decreased the antiviral efficiency of the phosphorothioate oligomers in our assay. Thus, in a defined concentration, the 3′,5′-capped, phosphorothioate-substituted oligomers seemed to remain on the cell surface, and they may be digested by nucleases in an endonucleolytic manner, rather than by exonucleolytic cleavage. In addition, the complete phosphorothioate oligomers (S-ODNs-rev or tat) showed anti-HIV activity, and which was probably due mainly to the relative resistance of the S-ODNs to nuclease, which kept them intact relative to the SO-ODNs and allowed them to reach and to remain at the target site. The anti-HIV activity of the antisense oligomers is influenced by the resistance of the oligonucleotides to nucleases, rather than to the stability of the RNA-DNA duplexes.22

The tat protein requires a specific sequence, in the 5′-end LTR promoter, termed the TAR element, for HIV-1 replication.23,24 The target sequences of the antisense S-ODNs-TAR-1, -TAR-2, and -TAR-3 are 1-20, 15-34, and 40-59 in the 5′-terminal LTR region, respectively, but
Table 1. Comparison of anti-HIV activities of the phosphorothioate oligodeoxyribonucleotides

| Oligomer          | Inhibitory effect (%) | Cytotoxicity (%) |
|-------------------|-----------------------|------------------|
|                   |                       |                  |
| X 10^-3μM         | X 10^-3μM             |                  |
|                   |                       |                  |
| SO-ODNs-rev-sa    | 0 0 0 0 0 2           | 0 0 0 0 0 5      |
| S-ODNs-rev-ts     | 10 58 100 100 93      | 0 0 0 0 0 0      |
| S-ODNs-rev-sa     | 8 24 100 100 100      | 6 0 0 0 0 0      |
| S-ODNs-tat-sa     | 10 15 95 100 99       | 1 0 0 0 0 0      |
| S-ODNs-pol-sa     | 7 66 64 92 66         | 40 8 34 6 26     |
| S-ODNs-gag-ls     | 15 60 93 92 100       | 0 0 4 4 21       |
| S-ODNs-rev-ran    | 38 59 70 71 0         | 0 0 3 8 95       |
| S-ODNs-rev-sa-sen | 31 50 70 69 65        | 0 0 0 0 0 24     |
| S-ODNs-rev-sa-8mis| 25 60 65 71 70        | 0 0 0 0 0 0      |
| S-ODNs-rev-sa-16mis| 38 59 62 67 73       | 0 0 0 0 0 20     |
| S-ODNs-TAR-1      | 6 14 88 93 67         | 6 0 0 0 0 30     |
| S-ODNs-TAR-2      | 19 30 59 78 76        | 6 20 31 14 29    |
| S-ODNs-TAR-3      | 6 88 74 77 82         | 0 0 0 11 16      |

a) The antisense sequences are described in Fig. 1.
b) The inhibitory effect percentage of HIV-1 infected cells represents the complete protective effect by the oligomer, without cytotoxicity to MT-4 cells.
c) Cytotoxicity of oligomers represents the percentage reduction of viable cell numbers in mock-infected MT-4 cells. Both inhibition and cytotoxicity assays were performed with the MTT assay described in the Experimental Section. Values are means of duplicate determinations.

each antisense S-ODNs-TAR elicited weak anti-HIV activity (88% inhibition by TAR-1, 59% by TAR-2, and 74% by TAR-3 at 0.02 μM, but toxic) (Table 1). This suggests a marked decrease in the ability of the antisense oligonucleotides, S-ODNs-TAR-1, -TAR-2, and -TAR-3, to bind to the TAR RNA, because of the spanning sequences on the three bulges (GGUCUCU, 1-7; UUAGACCAG, 12-20; AGAUCUCUG, 19-27) and the very short loop sites (CUGGGA, 29-35). The former binds to the tat protein and the latter to a 68 kD cellular protein. Perhaps the secondary or tertiary RNA structures would not readily be susceptible to the antisense hybridization at the initiation of transcription or during mRNA processing.

Included in this series were three different phosphorothioate derivatives containing the rev sense sequence (S-ODNs/sen), a random sequence (S-ODNs/ran), and mismatched sequences (S-ODNs/8 mis and S-ODNs/16 mis). These compounds showed less activity than the antisense phosphorothioates at 0.02-0.1 μM, and at 0.5 μM were relatively toxic (Table 1). This finding differs from those of Matsukura et al. However, only the antisense sequence of the phosphorothioate oligomers inhibited viral expression without toxicity, supporting the notion of the sequence-specific regulation of viral expression of HIV in chronically infected cells.
splice acceptor site of the HIV-1 rev gene was used (Fig. 3). The phosphorothioate oligonucleotide with a chain length of 15 (S-ODNs-rev-sa-15) was as active as the 20-mer phosphorothioate oligomer (S-ODNs-rev-sa-20), but the phosphorothioate 10-mer (S-ODNs-rev-sa-10) had no activity. Of particular interest was S-ODNs-rev-sa-15, which had activity similar to that of the phosphorothioate 20-mer (S-ODNs-rev-sa-20). However, oligomer chain length had a greater effect on antiviral activity, since longer compounds were more potent. Specifically targeted antisense-phosphorothioate oligonucleotides, such as rev or tat, may be useful for treating HIV-infected patients.

Non-sequence-specific inhibition of viral expression in HIV-1 infected cells

The adenosine, thymidine, and guanosine 20 mers and cytidine 15, 20, and 28 mers were tested for their comparative anti-HIV activity in acutely HIV-1 infected cells (Fig. 4). In this series, the homooligonucleotide phosphorothioates (S-dC28 and S-dC20) had activity of the same order as the antisense phosphorothioate 20-mer (S-ODNs-rev-sa-20) at a concentration of 0.1 μM. However, at 0.5 μM, it showed low activity and some toxicity. On the other hand, S-dC15 was less active than the 20-mers (S-dC28 and S-dC20). This observation is somewhat different from a previous description of homooligonucleotide phosphorothioates.9 Furthermore, the anti-HIV activity of the homooligomers S-dA20 and S-dT20 was also tested in acutely HIV-1 infected cells. S-dA20 and S-dT20 were both somewhat more active at all concentrations, and they inhibited up to 75% at concentrations from 0.1 to 0.5 μM without toxicity. These results did not indicate any correlation of the inhibitory activity with the G + C content.

Thus, homooligomers appear to behave differently from heterooligomers, and may inhibit HIV by some mechanism other than by antisense competitive hybridization. Homopolymers are better substrates than heteropolymers for reverse transcriptase.26 We also looked for similar replication inhibition by binding to the reverse transcriptase rather than to the mRNA.27,28 Consequently, the phosphorothioate homooligomers might be competitive inhibitors of reverse transcriptase or DNA polymerase. While the antiviral properties of the homooligomers are worth further consideration, they were less selective in their anti-HIV activity and were potentially more toxic than the antisense oligonucleotides.

Antisense oligonucleotides enter MOLT-4 cells, hybridize with complementary segments of RNA or DNA, and selectively inhibit viral replication and expression. In cells infected with HIV, perfectly matched phosphorothioate oligomers were more effective inhibitors than sense-, random-, and mismatched oligomers or homooligomers. A chain length of 15–20 mer units was optimal under our tissue culture conditions. They thus reinforce the concept that specific base pairing is a crucial feature of oligonucleotide inhibition of the human immunodeficiency virus.
Experimental

Abbreviations: HIV, human immunodeficiency virus; S-ODNs, phosphorothioate oligodeoxyribonucleotides; SO-ODNs, 3′,5′-capped phosphorothioate oligodeoxy-ribonucleotides; HTLV-III, human T-cell lymphotropic virus type III.

Synthesis and purification of the phosphorothioate oligonucleotide analogues

The phosphorothioate oligonucleotide analogues (S-ODNs or SO-ODNs) were prepared on a synthesizer using our new phosphate approach, and were purified by HPLC according to published procedures.29

Sequences of the target region in the HIV genome

We used five genes [gag,30,31 pol,32 rev,33,34 tar,35 and tat26] as targets for antisense interruption of viral gene expression. To clarify the sequence specificity, we tested the phosphorothioate oligomers containing the rev sense sequence (S-ODNs/sen), rev mismatched sequences (S-ODNs-rev-sa-mis), six different gene antisense sequences [S-ODNs-gag-leader sequence (is), pol-splice acceptor (sa), rev-sa, rev-translation start site (ts), tat-sa, tat], a random sequence with the same base composition as rev-sa (S-ODNs/ran), and 15–28 mer homo-oligomers [S-dC28, S-dC20, S-dA20, S-dT20, S-dG20, and S-dC15; Fig. 1]

Cell lines

The human T lymphotropic virus type I (HTLV-I)-positive human T cell line, MT-4, was subcultured twice weekly at a density of 3 x 10^5 cells mL^{-1} in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU mL^{-1} penicillin, and 100 mg mL^{-1} of streptomycin.

Virus

The HTLV-IIIB strain was used in the anti-HIV assay. The virus was prepared from the culture supernatants of MOLT-4/HTLV-IIIB cells, which were persistently infected with HTLV-IIIB. HIV stocks were titrated in MT-4 cells as determined by 50% tissue culture infectious doses (TCID_{50}) and plaque forming units, and stored at −80 °C until use.

Anti-HIV assay

The anti-HIV activity of test compounds in a fresh, cell-free HIV infection was determined as protection against HIV-induced cytopathic effects (CPE). Briefly, MT-4 cells were infected with HTLV-IIIB at a multiplicity of infection (MOI) of 0.01. HIV-infected or mock-infected MT-4 cells (1.5 x 10^5 mL^{-1}, 200 mL) were placed into 96 well microtiter plates and incubated in the presence of various concentrations of test compounds. The dilution ranged from one to five-fold and nine concentrations of each compound were examined. All experiments were performed in triplicate. After a 5-day incubation at 37 °C in a CO_2 incubator, the cell viability was quantified by a calorimetric assay that monitored the ability of the viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to a blue formazan product. The absorbances were read in a microcomputer-controlled photometer (Titertek Multiskan^b, Labsystem Oy, Helsinki, Finland) at two wavelengths (540 and 690 nm). The absorbance measured at 690 nm was automatically subtracted from that at 540 nm, to eliminate the effects of non-specific absorption. All data represent the mean values of triplicate wells. These values were then translated into percentage cytotoxicity and percentage antiviral protection, from which the 50% cytotoxic concentration (CC_{50}), the 50% effective concentration (EC_{50}), and the selectivity indexes (SI) were calculated.37,38

3′-End labelling and cellular uptake

The 3′-ends of the S-ODNs-rev-ts and the SO-ODNs-rev-ts were labelled with [α-32P]dATP and terminal deoxynucleotidyl transferase, then purified using a Quick spin column (G-25). MOLT-4 cells were diluted to 1 x 10^5 cells mL^{-1} in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (FCS) and dispensed into 24 multi-well plates. After 48 h at 37 °C in a CO_2 incubator, the RPMI-1640 medium was replaced. The cells were further incubated with the 3′-end labelled oligomers for the stated periods. After washing the cells four times with chilled phosphate buffered saline (PBS), the cells were pelleted by centrifugation and lysed in 0.5 mL of 1% sodium dodecyl sulfate (SDS). The cellular uptake of [α-32P]dATP was measured in a β-scintillation counter.

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