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Inhibition of human immunodeficiency virus (HIV-1) replication by synthetic oligo-RNA derivatives

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ABSTRACT

Several synthetic 2'-O-methyl-RNA oligomers and their derivatives have been evaluated for inhibitory effect against HIV-induced cytopathic effect and expression of the virus specific antigen in cultured MT-4 cells. In this study, oligo(2'-O-methyl)ribonucleoside phosphorothioates showed a potent inhibitory activity with size dependency (25-mer showed it at 1 μM), but by contrast both 2'-O-methylribo- and deoxy-oligomers with normal phosphate linkages failed to inhibit. However, it should be noted that the patched oligo(2'-O-methyl)ribonucleotide (20-mer), in which five linkages at 5'- and three linkages at 3'-ends of normal phosphates were replaced with thiophosphates, has recovered the substantial inhibitory effect. These results show that the size of oligomer and phosphorothioate linkages, probably resistant to exolytic nucleases, are essential for exhibiting antiviral activity.

INTRODUCTION

Anti-sense oligodeoxynucleotides and their several modified analogs are widely used as tools for regulating specific gene expression (1), for blocking splicing and translation of m-RNA (2-5) and for inhibiting viral replication (6-9). Recently the antiviral activities against HIV using oligodeoxynucleotides (10,11) and their phosphorothioate analogs (12,13) have been independently reported, representing that those abilities to exhibit antiviral activities seem to be strongly concerned with resistance to some kinds of deoxynucleases. But no one has yet examined "ribo-type oligomer" for this activity.

Synthetic oligo(2'-O-methyl)ribonucleotides have been utilized in the form of chimeric oligomers with DNA oligomers for the site-directed cleavage of RNA with RNase H (14,15) and the unidirectinal deletion of DNA by Bal 31 nuclease (16). These were accomplished on the bases of their characteristic

properties: less susceptibility to several nucleases (17), formation of a stable heteroduplex with the complementary RNA (18) and easy preparation on an automated DNA synthesizer (15).

In this report, we describe the syntheses of oligo(2'-O-methyl)ribonucleotides, their phosphorothioate analogs and also homooligo(2'-O-methyl)ribonucleoside phosphorothioates, and their antiviral effects against HIV in cultured human T cells.

MATERIALS AND METHODS

Syntheses of oligomers

The structures and the sequences of oligomers used in this study were designed by referring to the sequence of HIV genome previously reported (19) and shown in figure 1.

For the syntheses of oligo(2'-O-methyl)ribonucleotides and their phosphorothioate derivatives, 3'-O-methylribonucleosides-bounded CPGs (described below) were used as starting materials. Oligo(2'-O-methyl)ribonucleotides and oligodeoxynucleotides having normal phosphodiester linkages were prepared by the published phosphoramidite method (15,20-22) and purified by C-18 HPLC (YMC-Pak ODS, 6mmϕ x 300mm, Yamamura Chemical Laboratories, Kyoto, Japan) with a linear gradient of acetonitrile (5% - 25% for 40 min) in 0.1 M triethylammonium acetate buffer (pH7.0). Oligo(2'-O-methyl)ribonucleoside phosphorothioates as well as oligodeoxynucleoside phosphorothioate were synthesized by H-phosphonate method (23-26) as follows. The chain elongation reactions were performed on Applied Biosystems Synthesizer model 380A. For each coupling reaction, 20 equiv. of nucleoside H-phosphonate and 100 equiv. of pivaloyl chloride in acetonitrile and pyridine (1:1) were used for 3 min, and 3% trichloroacetic acid in dichloromethane for 3 min was used for each detritylation step. After the chain elongation cycles, the resin was treated with 0.2M S_8 in a mixture of CS_2-pyridine-triethylamine (12:12:1) at room temperature for at least 1 hr, and then with c-NH_4OH at 55°C for 6-12 hr. After 80% acetic acid treatment, the deprotected oligomers were purified by C-18 HPLC (YMC-Pak ODS, 6mmϕ x 300mm, Yamamura Chemical Laboratories, Kyoto, Japan) with a linear gradient of acetonitrile (10% - 50% for 40 min) in 0.1 M

triethylammonium acetate (pH 7.0). ^{31}P-NMR spectra of oligo(2'-O-methyl)ribonucleoside phosphorothioates were measured by JEOL GX-400FT-NMR at 161.83 MHz in D_2O-10 mM triethylammonium bicarbonate (pH 7.5) using trimethylphosphate as an external standard, and multiplet peaks were observed between 51-55ppm.

A large scale preparation of HS-2b (see figure 1) for plaque forming assay was performed by hand in the filter funnel packed with 88μmol of 3'-O-methylribonucleoside-loaded CPG (described below) on the same conditions for the chain elongation and detritylation steps as described above, and 9.1μmol of pure HS-2b was obtained by a middle pressure liquid chromatography using Lobar LiChroprep RP-18 column (25mmϕ x 310mm, Merck, Darmstadt, F. R. Germany) with a linear gradient of acetonitrile (10%-50% for 3.8 hr) in 0.1 M triethylammonium acetate (pH 7.0).

The patched oligomer HSOS-2 (20-mer, see figure 1) was synthesized manually as described above from 3.3μmol of 3'-O-methylribonucleoside-loaded CPG except that oxidation reactions were performed on the way of chain elongation cycles operating as following order: 1) five cycles of coupling reactions, 2) sulfer treatment, 3) nine cycles of coupling reactions, 4) 0.1 M I_2 oxidation for 40 min at room temperature, 5) five cycles of coupling reactions, 6) sulfer treatment. The deprotection and purification were performed by the same method as oligo(2'-O-methyl)ribonucleoside phosphorothioates and 0.28μmol of HSOS-2 was obtained. ^{31}P-NMR analysis of HSOS-2 indicated that it had seven phosphorus atoms assigned to thiophosphates and twelve phosphorus atoms to phosphates. Considering NMR data and the order of oxidation process because of I_2 treatment making reconversion of P-S bonds to P-O, we determined the structure of HSOS-2 in which two phosphates on the average within five from 3'-end of HO-2 were substituted by thiophosphates whose exact positions could not be identified, and on the other hand five phosphates from 5'-end were completely substituted.

As materials for H-phosphonate method, the protected 2'-O-methyl-ribonucleoside-H-phosphonates were prepared according to the published procedure (26). ^{31}P-NMR spectra of them were recorded on Varian XL-300 operating at 121.42 MHz using

phosphoric acid as an external standard in d_5-pyridine. The chemical shifts of them were as follows: 5'-O-dimethoxytrityl-N^6-benzoyl-2'-O-methyladenosine-3'-H-phosphonate (DBU salt), -2.20 ppm; 5'-O-monomethoxytrityl-N^2-isobutylyl-2'-O-methylguanosine-3'-H-phosphonate (DBU salt), -2.04 ppm; 5'-O-dimethoxytrityl-N^4-benzoyl-2'-O-methylcytidine-3'-H-phosphonate (DBU salt), -1.79 ppm; 5'-O-dimethoxytrityl-2'-O-methyluridine-3'-H-phosphonate (triethylammonium salt), -0.73 ppm; 5'-O-dimethoxytrityl-2'-O-methylinosine-3'-H-phosphonate (triethylammonium salt), -0.40 ppm. 5'-O-dimethoxy- or monomethoxytrityl, N-acyl-3'-O-methylnucleosides bounded CPGs (long chain alkylamine controlled pore glass support, purchased from Pierce Chemical, Rockford, IL.) were prepared according to published procedure (27,28) and used as starting materials for coupling reactions (loading contents, 27-30 µmol/g).

Every HPLC purified oligomer was used after lyophilization and every phosphorothioate oligomer was used as a mixture of stereoisomers at phosphorus for biological experiments.

Cells

The HTLV-I-carrying cell line, MT-4 (29), and the HIV-producing cell line, MOLT-4/$HIV_{HTLV-IIIB}$ (30), were used in this study. The cells were maintained in RPMI 1640 medium supplemented with 10 % fetal calf serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin at 37°C in a CO_2 incubator.

Virus and virus infection

HTLV-IIIB, one of the HIV strains, was obtained from the culture supernatant of MOLT-4/$HIV_{HTLV-IIIB}$ cells (30). The titer of this virus preparation was 10^6 plaque forming units (PFU)/ml. Infection of MT-4 cells with HTLV-IIIB was made at a multiplicity of infection of 0.002 as described previously (31,32). Briefly, MT-4 cells were exposed to HIV and incubated for 60 min at 37°C for virus adsorption. After adsorption, infected cells were washed and resuspended in fresh medium to make a concentration of 3 x 10^5 cells/ml. This infected cell suspension was then cultured in the presence of various concentrations of oligomers in a CO_2 incubator. The number of

cells were counted by the trypan blue dye exclusion method on day 3 after infection, half medium was changed with fresh medium containing the same concentration of oligomer, and then culture was continued for more 3 days.

Assay for HIV-induced cytopathic effect

HIV-induced cytopathic effect was analyzed by measuring the decrease in the number of viable cells 6 days after infection. The viable cells were counted by the trypan blue dye exclusion method.

Assay for HIV-specific antigen expression

The number of HIV-specific antigen positive cells was counted by the indirect immunofluorescence (IF) method on day 6 after infection. For this, methanol-fixed cells were reacted with 1:1000-diluted anti-HIV human serum (31) (He-1; IF titer, 1:4096) for 30 min and then with fluorescein isothiocyanate-conjugated rabbit anti-human immunoglobulin G (Dakoppatts A/S, Copenhagen, Denmark) for 30 min at 37°C. More than 500 cells were counted under a fluorescence microscope, and the percentage of IF-positive cells was calculated.

Plaque-forming assay

To investigate the effect of HS-2b (figure 1) on the viral production from chronically infected MOLT-4/$HIV_{HTLV-IIIB}$ cells, we performed a plaque-forming assay. The viruses were obtained from supernatants of MOLT-4/$HIV_{HTLV-IIIB}$ cells which were cultured with various concentrations of HS-2b (figure 1) for 3 days and 100 µl of one thousand-diluted viral preparation was added to 35 x 10mm tissue culture dishes. The plaque-forming assay has been done as described previously (31,32).

HIV-antigen enzyme immunoassay (EIA)

HIV-specific antigen (p24) was measured by an EIA (Abbott Laboratories, Chicago, IL.) (33). HIV-producing cell line, MOLT-4/$HIV_{HTLV-IIIB}$ cell was adjusted to a concentration of 3 x 10^5 cells/ml and cultured. Serially diluted supernatants of 3 days-cultured MOLT-4/$HIV_{HTLV-IIIB}$ cells with various concentrations of HS-2b (figure 1) were incubated with polystyrene beads coated with antibody to HIV. Rabbit antibody to HIV was added to react with those beads, and then goat

antibody to rabbit IgG conjugated with horseradish peroxidase was added. *o*-Phenylenediamine solution containing hydrogen peroxide was added to the beads and the intensity of color was read using a spectrophotometer at 492 nm.

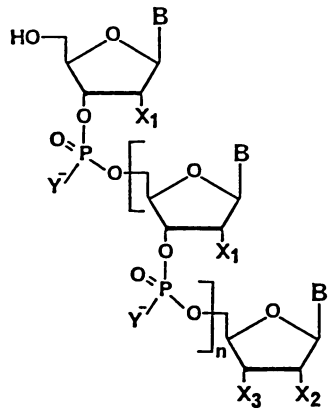
RESULTS AND DISCUSSION

Anti-HIV effects of various oligomers containing different phosphate backbones

We selected the splicing acceptor site as a target region of oligo(2'-O-methyl)ribonucleotide (HO-2), its phosphorothioate analog (HS-2b), and also oligodeoxynucleotide (DO-2) and its phosphorothioate analog (DS-2) to compare the inhibitory effects against HIV-induced cytopathic effect and the expression of HIV-specific antigen in MT-4 cells (figure 1).

Phosphorothioate analog of oligo(2'-O-methyl)ribonucleotide (HS-2b) showed the inhibitory effect similar to that of oligodeoxynucleotide (DS-2) (see figure 2a and 2b), but HS-2b did not affect the growth of HIV-uninfected MT-4 cells at the concentration of 120 μM (data not shown). On the other hand we could not detect any inhibitory effects of both oligo(2'-O-methyl)ribonucleotide (HO-2) and oligodeoxynucleotide (DO-2) even at the highest concentration of 120 μM, tested in this assay (see figure 2c and 2d about HO-2 and data not shown about DO-2). Similar results to the above experiment were observed, when tRNA primer binding site was selected as a different target region, showing that phosphorothioate analog of oligo(2'-O-methyl)ribonucleotide (HS-1) had also antiviral activity (see figure 2a and 2b for HS-1, data not shown for oligo(2'-O-methyl)ribonucleotide (HO-1) and their structures were shown in figure 1).

In a series of phosphorothioate analogs of oligodeoxynucleotides having anti-HIV activity, it has been pointed out that oligomers having G+C rich sequences are preferable to show potent activities (12,34). On the other hand, we obtained a result which was unaccountable for on G+C content alone. Namely, HS-1 and HS-2b, whose G+C contents were 71 % and 40 %,



type of oligomer	2'-O-methyl	deoxy
X_1=	OCH_3	H
X_2=	OH	H
X_3=	OCH_3	OH

Y= S , O n=8-23

B=Adenine, Guanine, Uracil, Thymine, Cytosine and Hypoxanthine

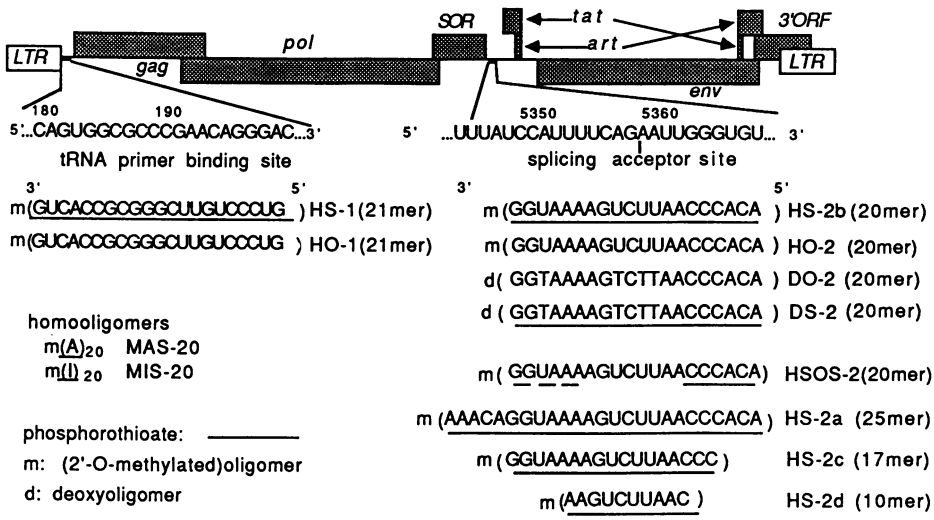


Figure 1. Structures of oligomers. The chemical structures of oligomers were shown at the top and their sequences were shown corresponding to complementary regions of HIV genome (19) illustrated in the middle of the figure. The phosphorothioate regions were underlined. A broken line of HSOS-2 exhibited two of five phosphates were substituted by thiophosphates and details were mentioned under "MATERIALS AND METHODS".

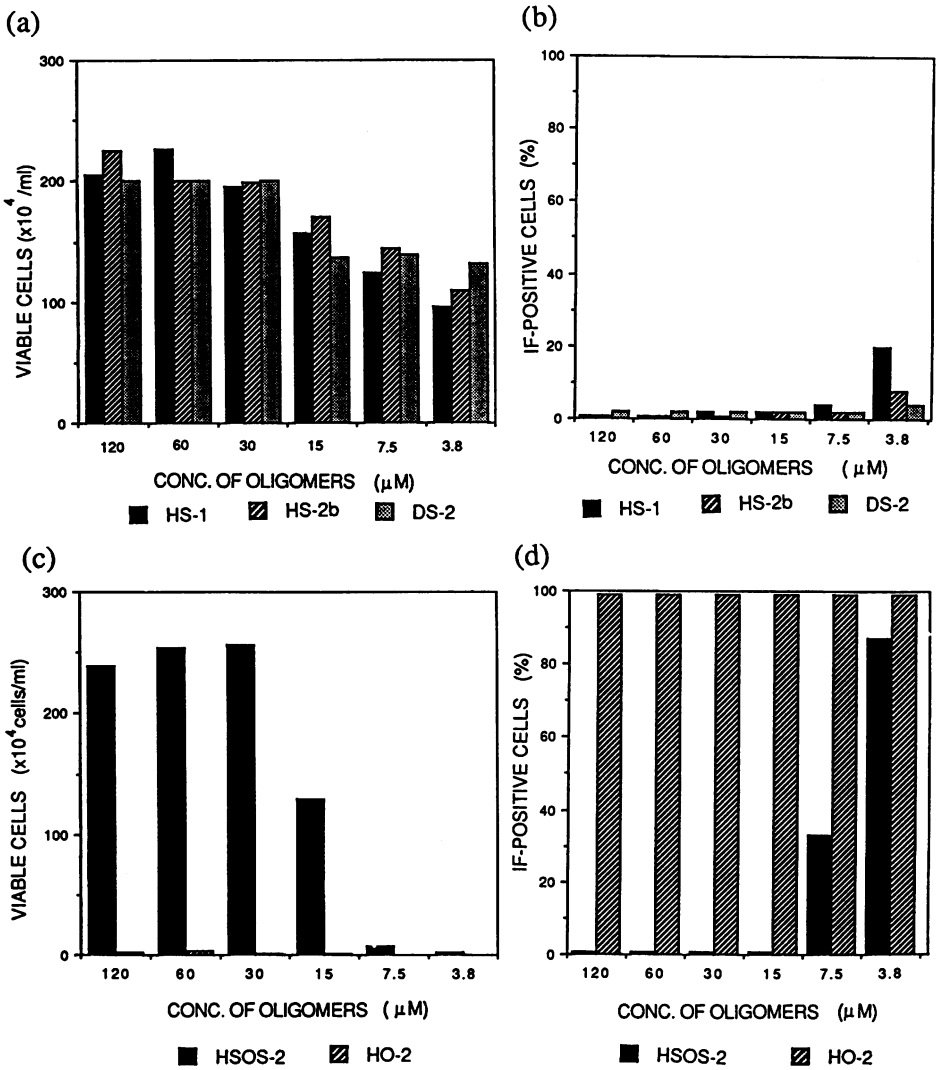


Figure 2. Inhibitory effects of various oligomers. (a) and (c) inhibitory effect on HIV-induced cytopathic effect in MT-4 cells, (b) and (d) inhibitory effect on the expression of HIV-specific antigen in MT-4 cells.

respectively, exhibited the anti-HIV activities in the same degree (figure 2a and 2b).

Oligo(2'-O-methyl)ribonucleotide is known to be more stable against several nucleases than deoxy- and ribo-oligonucleotides

(17 and unpublished data) and can form a stable hetero duplex with its complementary oligoribonucleotide (18). In the above results, however, several oligomers of normal phosphodiester (HO-2 and HO-1) have not shown any anti-HIV activity in our experimental condition, while phosphorothioate analogs of them with the same sequences (HS-2b and HS-1) have exhibited strong anti-HIV effect which is also similar level to that of oligodeoxynucleotide (DS-2). The result obtained above using oligo(2'-O-methyl)ribonucleotide series is coincident with that reported in the case of oligodeoxynucleotide series (12). We consider this is due to the instability of normal phosphodiester linkages in our experimental condition, even in the case of oligo(2'-O-methyl)ribonucleotides. This suggests that a part of characters of "nucleases" might be involved in degradation of both deoxy- and ribo-type-oligomers.

Anti-HIV activity of HSOS-2

In order to know the functional structure of active phosphorothioate oligomers, namely the role of whole phosphorothioate linkages of active materials, a patched derivative (HSOS-2), in which twelve phosphorothioate linkages in the middle of HS-2b were replaced by phosphate linkages, was prepared and served for the same assays. This also exhibited substantial anti-HIV effect which was slightly less than HS-2b but remarkably recovered from HO-2 (figure 2a, 2b, 2c and 2d). This fact shows that all their thiophosphate linkages are not necessary for this kind of oligomers to exhibit anti-HIV activity. This result also suggests that the suspected nucleases could break down phosphodiester bonds mainly in the manner of exonucleolytic rather than endonucleolytic cleavage on oligo(2'-O-methyl)ribonucleotides.

Effect of oligomer length on anti-HIV activity

To investigate the influence of oligomer length on the anti-HIV activities, additional phosphorothioate analogs of oligo(2'-O-methyl)ribonucleotides; HS-2a (25-mer), HS-2c (17-mer) and HS-2d (10-mer), which were complementary to splicing acceptor region (figure 1), were prepared and tested on the same assays with HS-2b (20-mer). Figure 3a and 3b show a remarkable length dependency of these on the anti-HIV activity.

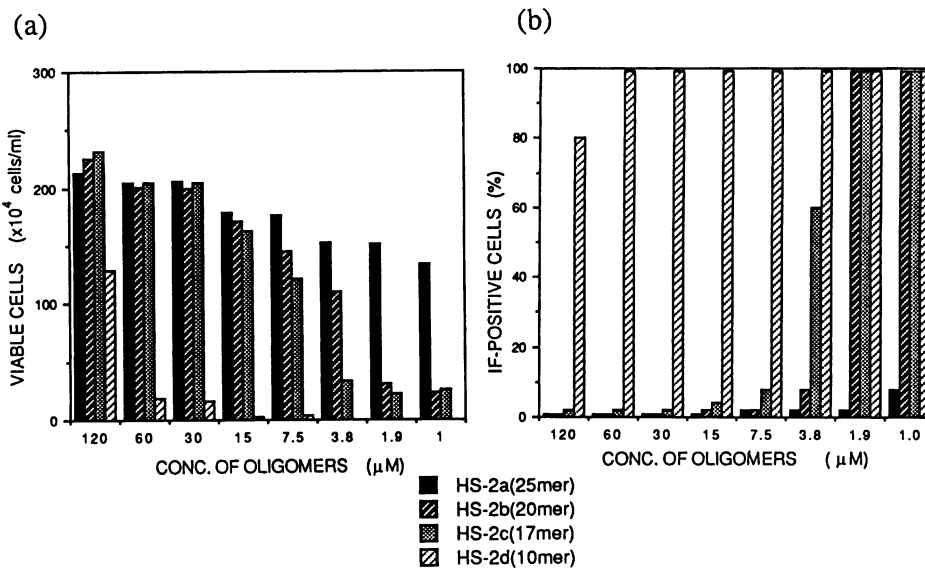


Figure 3. The length effect of phosphorothioate analogs of oligo(2'-O-methyl)ribonucleotides. (a) inhibitory effect on HIV-induced cytopathic effect in MT-4 cells, (b) inhibitory effect on the expression of HIV-specific antigen in MT-4 cells.

HS-2a (25-mer) exhibited a potent inhibitory effect at the concentration of 1 μM, while no effect was observed in HS-2d (10-mer) even at 60 μM. The length of oligomer is more important factor for anti-HIV activity than the numbers of phosphorothioate moieties because HS-2d (10-mer) having nine thioates showed less anti-HIV activity than HSOS-2b (20-mer) which had seven thioates.

Effects of HS-2b on chronically infected MOLT-4 cells with HIV

The effect of HS-2b on the production of viruses in a HIV-producing cell line, MOLT-4, was studied. The viruses were harvested from a culture supernatant of 3-day-old MOLT-4/$HIV_{HTLV-IIIB}$ cells in both the presence and absence of HS-2b (table 1). Under these experimental conditions no difference in cell growth was noted in both cell cultures. Results showed that there was no significant difference in the number of plaques as well as in the production of p24 antigen

Table 1. Effect of HS-2b on viral replication of HIV-persistent infected cell line, MOLT-4/$HIV_{HTLV-IIIB}$

Concn. of HS-2b (μM)	No. of plaque ($x10^4$ PFU/ml)	Production of p24 (pg/ml)
100	73.7 ± 4.9[a]	1033[b]
50	97.0 ± 4.6	1162
25	69.3 ± 5.1	1126
12.5	51.3 ± 4.0	1039
6.2	54.0 ± 2.0	897
3.1	49.3 ± 8.7	1005
0	61.3 ± 4.6	788

[a] Experiments were carried out in triplicate. Number represents the mean ± SD.

[b] The production of p24 in culture supernatants was assayed by Abbott HIV antigen EIA as described in materials and methods.

in the supernatants from the cultures with and without HS-2b.

Recently, however, it has been reported that phosphorothioate analogs of oligodeoxynucleotides with anti-sense sequence have inhibited viral expression in chronically HIV-infected cells, in which 28 mer complementary to *art/trs* gene region has a strong inhibitory effect against the production of p24 (13). About our result on a failure of HS-2b in inhibition of HIV expression in chronically infected MOLT-4 cells, it is unclear why the conflicting result with that on MT-4 cells was obtained. This agent might be acting at the courses from the viral infection to integration in MT-4 cells, or this result on MOLT-4/$HIV_{HTLV-IIIB}$ cells might be due to lack of some factors, for example length dependency or membrane permeability which might be different from the case of MT-4 cells. A further study should be continued to solve this point.

Anti-HIV activities of non-sense homooligomers

We also examined antiviral effects of phosphorothioate analogs of (2'-O-methyl)homooligomers (MAS-20, Adenosine derivative; MIS-20, Inosine derivative; MCS-20, Cytidine derivative; MUS-20, Uridine derivative; each was 20-mer) on the

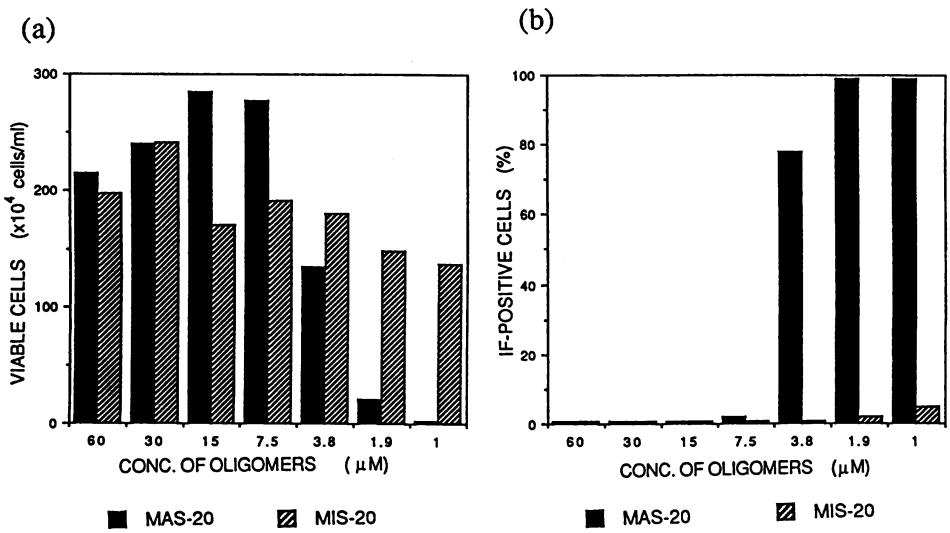


Figure 4. Inhibitory effects of homooligomers. The effects of phosphorothioate analogs of 2'-O-methyl-oligoadenylate (MAS-20, 20 mer) and 2'-O-methyl-oligoinosinate (MIS-20, 20 mer) on HIV-induced cytopathic effect in MT-4 cells (a) and on the expression of HIV-specific antigen in MT-4 cells (b).

expression of HIV-specific antigen and the HIV-induced cytopathic effect in MT-4 cells. The anti-HIV activity was seen in the following order; MIS-20 〉 MAS-20 〉 MUS-20 〉 MCS-20. Noteworthily, MIS-20 exhibited anti-HIV activity at the concentration of 1 μM (figure 4a and 4b, and data not shown for MUS-20 and MCS-20).

The result that non-sense homooligo(2'-O-methyl)-ribonucleoside phosphorothioates exhibited the actual inhibitory effects against HIV as same as anti-sense oligomers mentioned above is similar to that reported in oligodeoxynucleotide series (12), except for the order of activities depending on nucleotide species, or in terms of nucleic acids bases. In our case of oligo(2'-O-methyl)-ribonucleotide series, MIS-20 (inosine derivative) has potent and MCS-20 (cytidine derivative, data not shown) has poor anti-HIV activities, whereas it is reported that oligo-dC has potent in oligodeoxynucleotide series (12). However, it is unclear what are reflected by the species of nucleotide bases, for

example, resistance to degradation, membrane permeability, or non-specific binding ability to the viral genome. Further investigations are required to know the precise mechanism(s) of action of phosphorothioate analogs of homooligo(2'-O-methyl)ribonucleoside, including those of the complementary oligomers.

In conclusion, synthetic oligo(2'-O-methyl)ribonucleotides have anti-HIV activity in cultured cells in the form of phosphorothioate or in the partially phosphorothioate-substituted form, so that these have a potential to become a candidate worthy of further exploration for AIDS-treatment.

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