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Inhibition of human immunodeficiency virus type 1 replication by P-stereodefined oligo(nucleoside phosphorothioate)s in a long-term infection model

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Abstract Oligo(nucleoside phosphorothioate)s (S-ODNs), if prepared by conventional methods, consist of a mixture of diastereomers by virtue of the asymmetry of the phosphorus atom involved in the internucleotide linkages. This may affect the stability of the complexes formed between S-ODNs and complementary oligoribonucleotides, which is commonly accepted as the most important factor in determining the efficacy of an antisense approach. Using HIV-1-infected MOLT-4 cells via a long-term culture approach, we studied the influence of the P-chirality sense of stereodefined 28mer oligo(nucleoside phosphorothioate)s, [All-Rp]-S-ODN-*gag*-28-AUG and [All-Sp]-S-ODN-*gag*-28-AUG, complementary to the sequence starting at the AUG initiation codon of the *gag* mRNA of HIV-1, upon the anti-HIV-1 activity. The [All-Sp]-S-ODN-*gag*-28-AUG at a low concentration of 0.5 μM can completely suppress HIV-1^{gag} p24 antigen expression in HIV-1-infected MOLT-4 clone 8 cells for 32 days. Cells treated with [All-Rp]-S-ODN-*gag*-28-AUG (0.5 μM) showed a high level of the antigen expression at day 16. Furthermore, satisfactory suppression could not be achieved from a random [Mix]-S-ODN-*gag*-28-AUG, consisting of a diastereomeric mixture of the oligonucleotides. Our results suggest that chemotherapy based upon the use of stereodefined antisense [All-Sp] S-ODN may be a more effective method for reducing the viral burden in HIV-1-infected individuals. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Antisense therapy; Oligo(nucleoside phosphorothioate)s (S-ODNs); Diastereomerically pure S-ODN; Nuclease resistance; Anti-HIV-1 activity; Long-term assay; *gag*, *rev* and *tat* genes

1. Introduction

Oligonucleotides with a phosphorothioate backbone (S-ODNs) exhibit several advantages over natural ODNs, including enhanced nuclease resistance and the ability to induce the degradation of the targeted RNA sequence by RNase H [1–8]. Numerous S-ODNs are under evaluation as potential drugs against viruses, cancer and other diseases. However, all of the oligo(nucleoside phosphorothioate)s being studied, including [Mix]-S-ODNs, consist of random mixtures of diastereomers, by virtue of the asymmetry of the phosphorus atom involved in each internucleotide phosphorothioate linkage [9]. As such, they undergo nuclease-assisted degradation, primarily by plasma 3′-exonucleases. We have demonstrated that a human plasma 3′-exonuclease is highly stereoselective towards internucleotide [Rp]-phosphorothioates [10], and cleaves them at a rate comparable to that of natural oligonucleotides. Therefore, all fractions of a diastereomeric mixture with 3′-terminal [Rp]-phosphorothioates undergo hydrolysis. Moreover, the stereoselective action of numerous endonucleases on S-ODNs cannot be excluded [11]. The consequences of this degradation are numerous, since in addition to losing the integrity of the antisense construct, which should work in a catalytic manner, the released nucleoside 5′-phosphorothioates contaminate the cell with unpredictable effects [12]. Therefore, several researchers have focused upon either the separation of diastereomers via reverse phase–high-performance liquid chromatography [13,14], or the design of stereocontrolled methods of the synthesis of oligo(nucleoside phosphorothioate)s with a predetermined sense of chirality at the phosphorus of each internucleotide phosphorothioate linkage [15,16]. Beyond the elaborate enzyme-assisted methods providing [All-Rp]-S-ODNs [17,18], so far the most effective seems to be the oxathiaphospholane method designed by our groups [10,19].

In the absence of a practical technology providing stereodefined oligo(nucleoside phosphorothioate)s, examples of biological studies performed with stereodefined compounds are not abundant [20,21]. On the other hand, several publications have documented the suitability of S-ODNs for anti-HIV-1[22–26] and anti-influenza virus [27–31] applications. In particular, we first succeeded in the treatment of influenza in an experimental infectious mouse model of influenza A virus, using S-ODNs [30,31]. We also demonstrated their effectiveness as inhibitors of HIV-1 replication in an acute-infection assay [24,32].

In this paper we describe the influence of the P-chirality sense of stereodefined oligo(nucleoside phosphorothioate)s ([All-Rp]-S-ODN-*gag*-28-AUG) and ([All-Sp]-S-ODN-*gag*-28-AUG) on the anti-HIV-1 activity in long-term cultures using HIV-1-infected MOLT-4 clone 8 cells.

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2. Materials and methods

2.1. Oligonucleotides

Random [Mix]-S-ODNs, consisting of a diastereomeric mixture of the phosphorothioate oligonucleotides, was purchased from Boston Biosystems, Boston, MA, USA. The synthesis of stereodefined oligo(nucleoside phosphorothioate)s was performed manually [10,19]. The first nucleoside units were anchored to the solid support by a sarcosinyl linker. Appropriately protected monomers possessing a 3′-*O*-(2-thio-'spiro'-4,4-pentamethylene-1,3,2-oxathiaphospholane) moiety were synthesized and separated chromatographically into pure diastereomers. The positions of the selected oligonucleotides along the HIV-1 sequence were 786–813 (S-ODN-*gag*-28-AUG-as), 5809–5836 (S-ODN-*tat*-28-sa-as), and 6006–6033 (S-ODN-*rev*-28-AUG-as) (Fig. 1).

2.2. Cells and virus

The human T lymphotropic virus type I (HTLV-I)-positive human T-cell line, MT-4, and the HTLV-I-non-infected T-cell line, MOLT-4 clone 8, were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μU/ml penicillin, and 100 μg/ml of streptomycin. A strain of HIV-1_{IIIB}, was obtained from the culture supernatant of chronically HIV-1_{IIIB}-infected MOLT-4 clone 8 cells, MOLT-4 clone 8/HIV-1_{IIIB} cells, and was stored in a small volume at −80°C until use. The titer of the virus stocks was determined by 50% tissue culture infectious doses ($TCID_{50}$).

2.3. Anti-HIV assay

The $CD4^+$ T-cell line, MOLT-4 clone 8 (3×10^5 ml^{-1}), was infected with HIV-1_{IIIB} at a MOI of 0.01. After a 2 h infection, the cells were then washed to remove the virus from the medium, and treated with the synthetic oligonucleotides at 0.5–2.5 μM concentrations in the culture medium. After 2 days the medium was removed and fresh medium containing the oligonucleotides at 0.5–2.5 μM concentration was added. Virus replication was monitored at the cellular level by syncytia formation and in the culture supernatants by a p24 enzyme-linked immunosorbent assay (Cellular Products). At the time points indicated, an aliquot of the culture supernatant was removed for p24 antigen analysis and was replaced by fresh medium. Every 4 days, viable cells were counted and passed at 3×10^5 cells per ml [33].

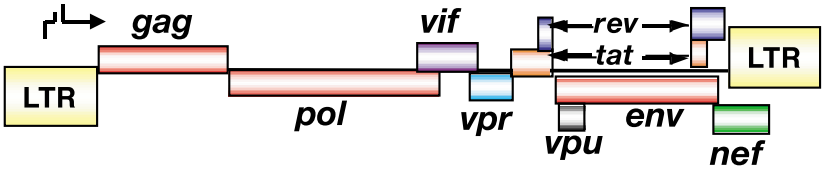
2.4. The indirect immunofluorescence (IF) assay

The number of HIV-1-specific antigen positive cells was counted by the indirect IF assay every 4 days. The methanol-fixed cells were reacted with anti-HIV-1 human serum for 30 min and then with fluorescing isothiocyanate-conjugated anti-human immunoglobulin G (Santa Cruz Biotech., USA) 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 [34].

3. Results and discussion

In a previous study, we demonstrated the effects of a stereodefined S-ODN [35]. The results of the RNase H-catalyzed cleavage of RNA indicated that the DNA–RNA duplexes containing All-Rp-S-ODN are better substrates for RNase H than those containing [Mix]-S-ODN or All-Sp-S-ODN, although the extent of degradation seems to be dependent upon the length and the primary and secondary structures of the RNA substrate [35]. Furthermore, the affinity of the All-Rp-S-ODN towards the complementary RNA was higher (T_m) than those of the All-Sp-S-ODN and [Mix]-S-ODN, although it was still lower than that of the ODN. With these observations in mind, it was tempting to study the influence of the P-chirality sense of the S-ODNs upon their antiviral activity.

In previous studies, the random mixtures of diastereomers S-ODNs complementary to the *gag* mRNA containing the AUG initiation codon sequence were more effective inhibitors of HIV-1 replication than those targeted to the splice-acceptor site of the *tat* gene and the AUG initiation codon of the *rev* gene in acutely infected cells [32]. Thus, we evaluated the anti-HIV-1 activity by the inhibition of virus-specific antigen expression in long-term HIV-1_{IIIB}-infected MOLT-4 clone 8 cells treated with three different target oligonucleotides ([Mix]-S-ODN-*gag*-28-AUG-as, [Mix]-S-ODN-*tat*-28-sa-as, and [Mix]-S-ODN-*rev*-28-AUG-as). We also chose a random sequence as the control oligonucleotide ([Mix]-S-ODN-28-ran). The MOLT-4 clone 8 (3×10^5 ml^{-1}) cells were incubated with HIV-1_{IIIB} at an MOI of 0.01 for 2 h to allow absorption [33]. The cells were then washed to remove the virus from the medium, and the oligonucleotides (1 μM) were added with fresh medium. After 2 days, new medium supplemented with the oligonucleotides was added. The virus production in the culture supernatant was monitored by the HIV-1^{gag} p24 antigen assay (Fig. 2). In this assay system, the phosphorothioate-modified oligonucleotides cannot interfere with retroviral binding at the CD4-receptor, because the cells were incubated with an infectious HIV-1 supernatant for 2 h before the application of the oligonucleotides. The expression of HIV-1^{gag} p24 antigen was not detectable during the first 6 days after HIV-1 infection. However, Fig. 2 shows that 30 days after infection, a high level of HIV-1^{gag} p24 antigen



(diastereomeric mixture oligomers)
[Mix]-S-ODN-*gag*-28-AUG-as: 5'-CGCTCTCGCTCCCATCTCTCTCCTTCTA-3'
[Mix]-S-ODN-*tat*-28-sa-as: 5'-TGTCGACACCCAATTCTGAAAATGGATA-3'
[Mix]-S-ODN-*rev*-28-AUG-as: 5'-TCTCCGCTTCTTCCTGCCATAGGAGATG-3'
[Mix]-S-ODN-28-ran: 5'-TATTCATTACGCTGCTCAGTCTACAGTG-3'

(the pure diastereomeric stereoregular oligomers)
[All-Rp]-S-ODN-gag-28-AUG-as: 5'-CGCTCTCGCTCCCATCTCTCTCCTTCTA-3'
[All-Sp]-S-ODN-gag-28-AUG-as: 5'-CGCTCTCGCTCCCATCTCTCTCCTTCTA-3'

Fig. 1. Sequences of antisense and control oligo(nucleoside phosphorothioate)s used in this study and the target genes in the genomic structure of HIV-1. The positions of the selected oligonucleotides along the HIV-1 sequence were 786–813 (S-ODN-*gag*-28-AUG-as), 5809–5836 (S-ODN-*tat*-28-sa-as), and 6006–6033 (S-ODN-*rev*-28-AUG-as).

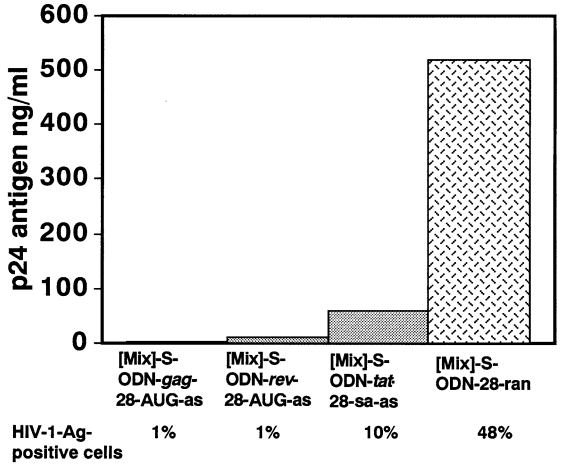


Fig. 2. Comparison of the antiviral activities of three different target oligonucleotides ([Mix]-S-ODN-*gag*-28-AUG-as, [Mix]-S-ODN-*tat*-28-sa-as, and [Mix]-S-ODN-*rev*-28-AUG-as, (mixture of diastereomers)) prepared via non-stereocontrolled methods, at a 1 μM concentration in a long-term assay. Sequence-specific anti-HIV-1 activities of the [Mix]-S-ODN-*gag*-28-AUG-as, [Mix]-S-ODN-*tat*-28-sa-as, and [Mix]-S-ODN-*rev*-28-AUG-as, (mixture of diastereomers), compared to the random oligonucleotide, [Mix]-S-ODN-28-ran, 32 days after infection. Indirect IF method.

expression was detected in the cultures treated with the random oligonucleotide ([Mix]-S-ODN-28-ran), demonstrating that this control oligonucleotide failed to inhibit HIV-1 replication. The inhibition of HIV-1^{gag} p24 antigen expression in cultures treated by the other oligonucleotides was >90% as compared with that of the random oligonucleotide. In particular, the suppression of HIV-1^{gag} p24 antigen expression by the [Mix]-S-ODN-*gag*-28-AUG-as and [Mix]-S-ODN-*rev*-28-AUG-as oligonucleotides in long-term HIV-1_{IIIB}-infected MOLT-4 clone 8 cells for 30 days was >99% as compared with that of the random oligonucleotide. In contrast, the long-term treatment (32 days) with [Mix]-S-ODN-*tat*-28-sa-as still permitted the HIV-1^{gag} p24 antigen expression. The number of HIV-specific antigen-positive cells was counted by the indirect IF method at 4 day intervals [34]. The IF assay showed similar results to those of the HIV-1^{gag} p24 antigen assay, 32 days after infection.

Next, we investigated the antiviral activity effects of the stereodefined S-ODNs, composed either of [All-Rp]- or [All-Sp]-diastereomers. In this assay, we chose the [All-Rp]-S-ODN-*gag*-28-AUG-as, and the [All-Sp]-S-ODN-*gag*-28-AUG-as, which encode sequences directly adjacent to the complementary target sequence on the *gag* mRNA (776–803) containing the AUG initiation codon from HIV-1_{IIIB}. The synthesis of stereodefined oligo(nucleoside phosphorothioate)s was performed manually [10,19]. The first nucleoside units were anchored to the solid support by a sarcosinyl linker. Appropriately protected monomers possessing a 3′-*O*-(2-thio-'spiro'-4,4-pentamethylene-1,3,2-oxathiaphospholane) moiety were synthesized and separated chromatographically into pure diastereomers. We also chose [Mix]-S-ODN-*gag*-28-AUG-as, consisting of the random mixture of diastereomers, and a random sequence ([Mix]-S-ODN-28-ran), as the control oligonucleotide. The virus production in the culture supernatant was monitored by the HIV-1^{gag} p24 antigen assay (Fig. 3A–C).

The control-infected cells (maintained in the absence of oligonucleotides) showed a high level of HIV-1 replication at 12 days after infection (Fig. 3A–C). In the cells treated with the [All-Sp]-S-ODN-*gag*-28-AUG-as, [Mix]-S-ODN-*gag*-28-AUG-as, and [Mix]-S-ODN-28-ran (1 μM), the HIV-1^{gag} p24 antigen expression was completely suppressed, as compared to the untreated control cells (Fig. 3B). However, the cells treated with [All-Rp]-S-ODN-*gag*-AUG-28-as showed a high level of HIV-1^{gag} p24 antigen expression at 16 days after infection. In contrast, the cells treated with [All-Sp]-S-ODN-*gag*-AUG-28-as completely suppressed the HIV-1^{gag} p24 antigen expression for 32 days. At day 28, [Mix]-S-ODN-*gag*-28-AUG-as showed even greater inhibition of the HIV-1^{gag} p24 antigen expression, by 99.9%; however, at 32 days after infection, a very low level of HIV-1^{gag} p24 antigen was detected, indicating that [Mix]-S-ODN-*gag*-AUG-28-as was less effective than [All-Sp]-S-ODN-*gag*-28-AUG-as. Somewhat higher activity was also observed for the diastereomeric mixture oligonucleotide, [Mix]-S-ODN-*gag*-28-AUG-as, than for [All-Rp]-S-ODN-*gag*-AUG-28-as. In this case, the effect of the nuclease stability prevailed over the effect of the enhanced RNase H activation. We observed earlier that the rate of RNase H-assisted cleavage of mRNA involved in the duplex formed by oligo(nucleoside phosphorothioate)s and the complementary mRNA depends upon the chirality sense of the involved phosphorothioates [35]. The present results suggest that the stereodifferentiated stability of the phos-

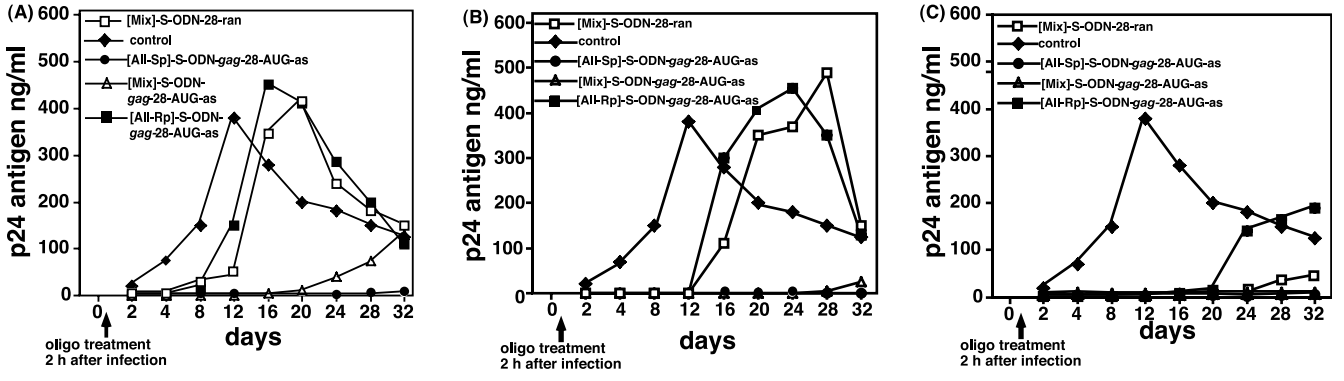


Fig. 3. Antiviral activities of stereoregular S-ODNs ([All-Rp]-(S-ODN-*gag*-AUG-28-as) and [All-Sp]-28mer) at different concentrations (A: 0.5 μM, B: 1.0 μM, and C: 2.5 μM) in long-term cultures using HIV-1-infected MOLT-4 clone 8 cells. Differences in antiviral activities between antisense phosphorothioate oligonucleotides composed either of a mixture of 2^{27} diastereomers ([Mix]-S-ODN-*gag*-AUG-28-as) at each position, or of [All-Rp]-S-ODN-*gag*-AUG-28-as or [All-Sp]-S-ODN-*gag*-AUG-28-as diastereomers, 32 days after infection.

phorothioate oligonucleotides against 3′-exonucleases enhances the potency of the antisense oligonucleotides. The indirect IF assay showed similar results (HIV-1-Ag-positive cells: [Mix]-S-ODN-*gag*-AUG-28-as, 0%; [All-Sp]-S-ODN-*gag*-AUG-28-as, 0%: [All-Rp]-S-ODN-*gag*-AUG-28-as, 49%; [Mix]-S-ODN-28-ran, 51%), 28 days after infection. In contrast, at a low concentration of 0.5 μM, the cells treated with [All-Sp]-S-ODN-*gag*-AUG-28-as completely suppressed the HIV-1^{gag} p24 antigen expression for 32 days (Fig. 3A). However, the cells treated with [Mix]-S-ODN-*gag*-28-AUG-as expressed the HIV-1^{gag} p24 antigen at 24 days after infection, also indicating that [Mix]-S-ODN-*gag*-AUG-28-as was less effective than [All-Sp]-S-ODN-*gag*-28-AUG-as (Fig. 3A). On the other hand, at a high concentration of 2.5 μM, [Mix]-S-ODN-28-ran and [All-Rp]-S-ODN-*gag*-AUG-28-as had inhibitory effects on the HIV-1^{gag} p24 antigen expression, as compared to the untreated control at 32 days (Fig. 3C). These results indicate that at high concentrations, the random sequence phosphorothioate oligonucleotides may have a non-sequence-specific antiviral effect, which delays virus replication, but does not inhibit it effectively. On the other hand, the exonuclease resistance conferred by the presence of internucleotide bonds of the Rp configuration, as demonstrated with [All-Sp]-S-ODN-*gag*-28-AUG-as, enhances the potency of this antisense oligonucleotide.

In conclusion, the 28mer oligo(nucleoside phosphorothioate)s directed to the *gag* and *rev* mRNAs ([Mix]-S-ODN-*gag*-28-AUG-as and [Mix]-S-ODN-*rev*-28-AUG-as), at a 1 μM concentration, can completely suppress the HIV-1^{gag} p24 antigen expression in long-term HIV-1_{IIIB}-infected MOLT-4 clone 8 cells for 30 days (Fig. 2). Essentially, all of the phosphorothioate oligonucleotides applied thus far in the antisense methodology were synthesized either by phosphoramidite or H-phosphonate methods, and thus consisted of 2^n diastereomers, where n is the number of phosphorothioate bonds [9]. We investigated the stability of stereoregular phosphorothioate oligonucleotides in human plasma [10]. The [All-Sp]-phosphorothioate oligonucleotides were the most resistant towards nucleases, followed by the random diastereomeric mixture and the [All-Rp]-phosphorothioate oligonucleotides, with the lowest nuclease resistance [10]. Here we have demonstrated the effect of the P-chirality sense of stereodefined phosphorothioate oligonucleotides ([All-Rp]-S-ODN-*gag*-AUG-28-as- and –[All-Sp]-28mer) on the inhibition of HIV-1 replication in long-term cultures, using HIV-1-infected MOLT-4 clone 8 cells. The cells treated with [All-Sp]-S-ODN-*gag*-AUG-28-as completely suppressed the HIV-1^{gag} p24 antigen expression for 32 days. In contrast, the cells treated with [All-Rp]-S-ODN-*gag*-AUG-28-as showed a high level of HIV-1 replication. The random mixture of diastereomers, [Mix]-S-ODN-*gag*-AUG-28-as, more effectively inhibited the HIV-1^{gag} p24 antigen expression than [All-Rp]-S-ODN-*gag*-AUG-28-as. The protection of the phosphorothioate oligonucleotide from exonucleases by means of stereochemical control, as demonstrated with the compound [All-Sp]-S-ODN-*gag*-28-AUG-as, enhances the potency of this antisense oligonucleotide and thus supports the hypothesis that the stability against exo- and endonucleases is among the most important factors for the efficacy of antisense oligonucleotides in long-term HIV-1_{IIIB}-infected MOLT-4 clone 8 cells. The antisense phosphorothioate oligonucleotides containing the Sp-configuration at the phosphorus of each internucleotide phosphorothioate linkage may yield important theoretical and clinical insights into the regulation of HIV-1 replication.

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