$(s^4dU)_{35}$: a novel, highly potent oligonucleotide inhibitor of the human immunodeficiency virus type 1 reverse transcriptase

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Abstract Oligodeoxycytidylates were converted to s^4dUMP-containing oligomers by treatment with liquid H_2S. The inhibitory potency of the modified oligonucleotides on human immunodeficiency virus type 1 reverse transcriptase depended on the chain length and on the percentage of modification. The most potent reverse transcriptase inhibitor was $(s^4dU)_{35}$. The inhibitory pattern was competitive, when either poly(A)·$(dT)_{16}$ or poly(C)·$(dG)_{16}$ was used as template-primer (variable substrate), suggesting that the free enzyme interacts with $(s^4dU)_{35}$. The K_i values were 3.0 and 2.2 nM in the presence of poly(A)·$(dT)_{16}$ and poly(C)·$(dG)_{16}$, respectively.

Key words: Inhibition; Reverse transcriptase; Human immunodeficiency virus; Oligo 4-thio-2′-deoxyuridylate; Antitemplate

1. Introduction

Reverse transcriptase (EC 2.7.7.49) represents one of the main targets in the development of the chemotherapy against HIV, the etiological agent of the acquired immunodeficiency syndrome [1]. Therefore, a large number of nucleoside and non-nucleoside RT inhibitors have been studied recently as potential agents against AIDS [2–5].

Chemically modified oligo- and polynucleotides have also been studied and characterized as inhibitory template (or primer) analogs for DNA polymerases including RT. They have been termed antitemplates [6]. The antitemplates interact with proteins (i.e. template dependent nucleic acid polymerases) rather than nucleic acids (antisense effect). Their activities are not strictly dependent on specific nucleic acid sequences. In addition to the nucleoside and non-nucleoside type inhibitors the modified oligonucleotides are another class of RT inhibitors.

The most thoroughly studied inhibitory template analogs are 5-mercaptopyrimidine-containing polynucleotides [7–9]. They are potent inhibitors of the replication of HIV in primary human lymphocytes [10]. The poly(U_{60},hs^5U_{40}) proved to be a potent competitive inhibitor of RT [11]. The phosphorothioate-linked and phosphorodithioate-linked oligodeoxycytidylate analogs are also potent competitive inhibitors of viral DNA polymerases [12–15]. A pentose modified oligonucleotide, β-4′-thio-oligouridylate, has been shown recently to interact with HIV-1 RT [16]. It was shown earlier that poly(s^4U), a base modified polyribonucleotide, inhibits the virion-associated transcriptase of influenza A [17].

In this paper we report the HIV-1 RT inhibitory activity of oligonucleotides containing s^4dUMP.

2. Materials and methods

2.1. Materials

The unlabeled deoxyribonucleotides dTTP, dGTP ('Ultrapure') as well as poly(A) and poly(C) were Pharmacia products. The labelled nucleotides [^{3}H]dTTP and [^{3}H]dGTP were from Amersham. All other reagents were of the highest quality commercially available.

HIV-1 RT was purchased from Worthington (Freehold, NJ). Its concentration was 8 U/μl, Lot No. 63E792N. The alkaline phosphatase (grade I) and phosphodiesterase (snake venom nuclease from *Crotalus durissus*) were purchased from Boehringer Mannheim (Germany).

The template-primers were poly(A)·$(dT)_{16}$ and poly(C)·$(dG)_{16}$, prepared as described earlier [11].

The oligodeoxycytidylates were synthesized on a Pharmacia Gene Assembler Plus DNA synthesizer in our laboratory and purified by two precipitations with *n*-butanol.

2.2. Synthesis and characterization of s^4dU containing oligonucleotides, $(dC_x,s^4dU_y)_n$

The thiolated oligonucleotides were prepared by treating 1.5–3.5 mg of $(dC)_n$ (n = 10, 20, 25, 30, 35, 40), dissolved in aqueous pyridine, with liquid H_2S [18,19] for 2–10 days at 35°C or 50°C, depending on the desired degree of thiolation. The modified oligonucleotides were isolated by ethanol precipitation as described for modified 5S rRNA [20], or were isolated on a DEAE Sephacell (Pharmacia) column, prepared in a 1 ml pipette tip (Cl^- form, volume about 0.3 ml). A stepwise elution with increasing concentration of KCl was performed to isolate the oligonucleotides.

The base ratio of the modified oligonucleotides was determined by HPLC, after hydrolysis to nucleosides with snake venom diesterase and alkaline phosphatase [21]. The deoxycytidine and 4-thiodeoxyuridine contents of aliquots were analyzed on a C_{18} column (Nova-Pak, 3.9×150 mm) using a 15-ml linear gradient of 0–10% acetonitrile in 0.1 M sodium phosphate (pH 5.8) at a flow rate of 1.0 ml/min. Only two peaks were detected. The chromatography was monitored at 254 nm. The following elution times were observed: 5 min for dC, 12.1 min for s^4dU. The areas under each peak were determined by integration and the amounts of each nucleoside present were determined using the following extinction coefficients (M^{-1} cm^{-1}): $dC(6.2\times10^3)$, $s^4dU(3.3\times10^3)$. The composition of the oligonucleotides according to the HPLC analysis were in accordance with their observed UV spectra. In order to determine the exact concentration of the modified oligonucleotides their organic phosphate contents were measured [22].

The following oligonucleotides were prepared and used in this study: $(dC)_{35}$, $(s^4dU)_{10}$, $(s^4dU)_{20}$, $(s^4dU)_{25}$, $(s^4dU)_{30}$, $(s^4dU)_{35}$, $(s^4dU)_{40}$, $(dC_{0.50},s^4dU_{0.50})_{35}$, $(dC_{0.58},s^4dU_{0.42})_{35}$, $(dC_{0.66},s^4dU_{0.34})_{35}$, $(dC_{0.73},s^4dU_{0.27})_{35}$.

2.3. RT assays

Reaction mixtures were incubated for 45 min at 37°C. In a final volume of 100 μl they contained the following: 100 mM Tris-HCl (pH 8.0), 50 mM KCl, 6 mM $MgCl_2$, 100 μg/ml bovine serum albumin, 5 mM dithiothreitol, 10 μM [^{3}H]dTTP or [^{3}H]dGTP (specific activity 4400 dpm/pmol), and 0.1 μM template-primer (expressed as 3′-primer

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Abbreviations: RT, reverse transcriptase; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; TCA, trichloroacetic acid; Na-PPi, Na-pyrophosphate

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termini) and 0.008 U of enzyme. To study the inhibitory activity of the thiolated oligonucleotides the concentration of the template-primer was changed as indicated in the legends of the figures. The reactions were terminated by the addition of 10% cold TCA containing 100 mM Na-PP_i. The radioactive product was collected by filtration on Whatman GF/C filter, washed four times with 1 ml of 5% TCA containing 50 mM Na-PP_i and finally with ethanol. Filter disks were dried and nucleotide incorporation was quantified by scintillation counting.

2.4. Kinetic analysis

Kinetic results were evaluated graphically by plotting 1/V against inhibitor concentration (Dixon equation). The data points shown for the determination of kinetic constants are the average from triplicate determinations. All lines were obtained from unweighted least squares fit to the data points.

3. Results and discussion

In preliminary experiments it was shown in our laboratory that oligonucleotides containing s^4dU inhibit HIV-1 RT.

First we studied the effect of the chain length on the RT inhibitory activity of $(s^4dU)_n$ ($n = 10$, 20, 25, 30, 35, 40). A dramatic increase in the inhibitory activity was observed with increasing chain length of the modified nucleotide (Fig. 1). While the 10-mer showed very little inhibitory activity the 25–40-mers were potent inhibitors of HIV-1 RT, with practically the same IC_{50} values (results for the 40-mer are not shown). Further increase in the chain length did not significantly affect the inhibitory activities of the modified oligonucleotides. The size dependence of the inhibitory potential of the modified polynucleotides is in good agreement with the structure and mechanism of HIV-1 RT. While data, based on 3.5 Å resolution electron density map of HIV-1 RT, demonstrate that the number of nucleotides between the polymerization site and RNA cutting site is 20 [23], enzymatic footprinting suggests that an even larger portion of the template-primer is encompassed by the replicating enzyme [24].

In this experiment the template-primer was poly(A)·$(dT)_{16}$. Interaction between the template-primer and the inhibitor (which is an oligouridylate analog) cannot be ruled out completely. However, the significant differences found in the inhibitory potential of the 10-mer, 20-mer and 25-mer suggest an interaction between RT and the inhibitor.

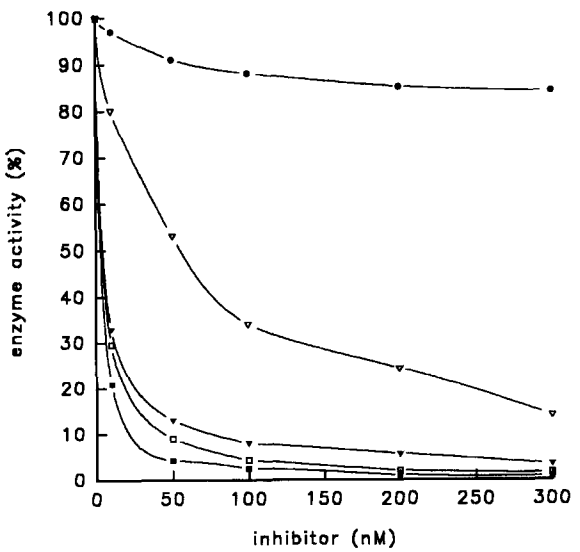

Fig. 1. Effect of $(s^4dU)_n$ on HIV-1 reverse transcriptase. The assays were carried out as described in Section 2; template-primer: poly(A)·$(dT)_{16}$. $n = 10$ (●), 20 (▽), 25 (▼), 30 (□), 35 (■).

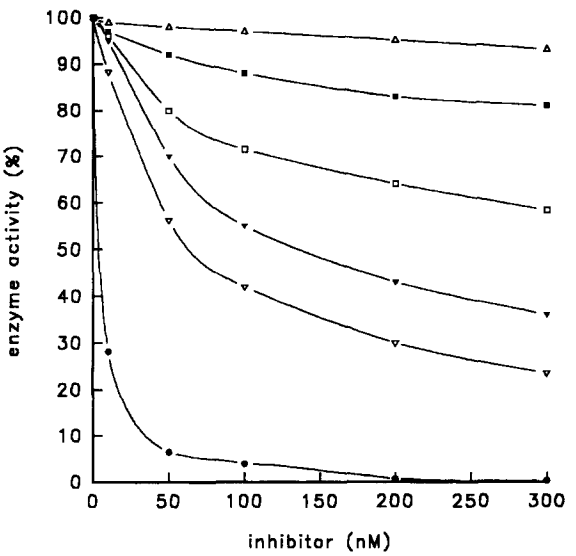

Fig. 2. Effect of $(dC_x,s^4dU_y)_{35}$ on HIV-1 reverse transcriptase. The assays were carried out as described in Section 2; template-primer: poly(A)·$(dT)_{16}$. $x = 1.00$ $y = 0.00$ (△), $x = 0.73$ $y = 0.27$ (■), $x = 0.66$ $y = 0.34$ (□), $x = 0.57$ $y = 0.43$ (▼), $x = 0.50$ $y = 0.50$ (▽), $x = 0$ $y = 1.00$ (●).

The degree of chemical modification was one of the important factors affecting the inhibitory potential of the s^4dUMP containing oligonucleotides. A series of 35-mers was prepared by shorter or milder H_2S treatment of $(dC)_{35}$ as described in Section 2. It is evident from the results in Fig. 2 that the inhibitory activity of the oligonucleotides depends on the degree of modification. The inhibitory potential was increased when the modified nucleotide content was higher. Due to the nature of the chemical modification the distribution of the thiolated bases within the oligonucleotide must be random as was found in the case of partially 5-thiolated poly(C) [7].

These results indicated that the oligonucleotides composed exclusively of s^4dU, with a chain length of at least 30, are the most potent inhibitors of this type on HIV-1 RT.

The sulfur-containing oligo- and polynucleotides studied thus far have proved to be competitive inhibitors of the RT with respect to the functioning template-primers [11–13,15]. $(s^4dU)_{35}$ was expected to be a new member of this family of inhibitors. Therefore the template-primers poly(A)·$(dT)_{16}$ and poly(C)·$(dG)_{16}$ were chosen as variable substrates in the Dixon plot experiments shown in Figs. 3 and 4. The results clearly indicated that $(s^4dU)_{35}$ was a competitive inhibitor of RT with respect to the template-primers. The graphically determined K_i values were essentially the same: 3.0 nM for the poly(A)·$(dT)_{16}$-directed reaction and 2.2 nM for the poly(C)·$(dG)_{16}$-directed reaction, verifying our earlier conclusion that the inhibitory activity of the modified oligonucleotide was due to a protein-oligonucleotide interaction and the template-primer function was not affected by $(s^4dU)_{35}$.

It must be noted that a chemical modification is usually not complete. Although the HPLC analysis of our 'fully modified' oligonucleotides could not detect any unmodified cytidine a minute amount of unmodified base may be present in the inhibitory oligonucleotide, not significantly affecting its in-

hibitory potential. A difference between the chemically produced and enzymatically synthesized poly(s^4U) was found when the modified polynucleotides were used in protein synthesizing systems [25,26]. While the chemically produced poly(s^4U) could not direct the synthesis of polyphenylalanine in an in vitro protein synthesizing system [25], the enzymatically produced polymer served as an artificial mRNA [26]. It should be pointed out that the two systems, protein synthesis and inhibition of nucleic acid synthesis, cannot be compared. The protein synthesis is strictly dependent on the sequence of the mRNA. A minute amount of unmodified nucleotide (in this case cytidylate) can stop the in vitro incorporation of phenylalanine (for example CUU is a code for Leu), but the inhibitory potential of an oligonucleotide is barely affected by a very low amount of unmodified base.

The chemically modified oligonucleotides with potent inhibitory potential on HIV RT are physico-chemically distinct compounds, with the same inhibitory patterns [11,13,15]. They are competitive inhibitors. In accordance with the antitemplate mode of action [6], it can be presumed that the inhibitory oligonucleotide (which must be a 30-mer for full activity) interacts with a large number of physico-chemically distinct side chains of amino acids of the RT. This multisite and multi-mode interaction may explain why the inhibitory patterns are the same on RT for physico-chemically distinct modified oligonucleotides [11]. The dependence of the inhibitory activity on the chain length found in the present study verifies the multi-site interaction.

The hydrophobic character of (s^4dU)$_{35}$ (see the retention time for 4-thiouridine compared to cytidine in Section 2) must be one of the reasons for its increased affinity for the enzyme, as it was presumed in the case of the phosphorothioate and phosphorodithionate oligonucleotides [12,15]. The 4-thiono group has a propensity toward tautomeric conversion to form the -SH group at position 4. The 4-SH group may interact with mercapto-groups of the protein to form temporary disulfide bridges. Both of these interactions might contribute to the inhibitory activity of (s^4dU)$_{35}$.

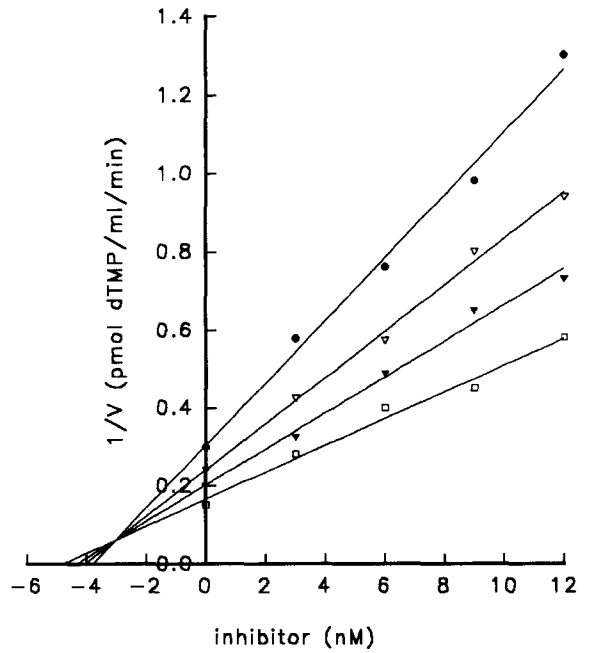

Fig. 3. Dixon plot for inhibition of HIV-1 reverse transcriptase by (s^4dU)$_{35}$ using poly(A)·(dT)$_{16}$ as template-primer and variable substrate. Substrate concentrations (expressed as 3′-primer termini of template-primers): 45 nM (●), 60 nM (▽), 75 nM (▼), 90 nM (□).

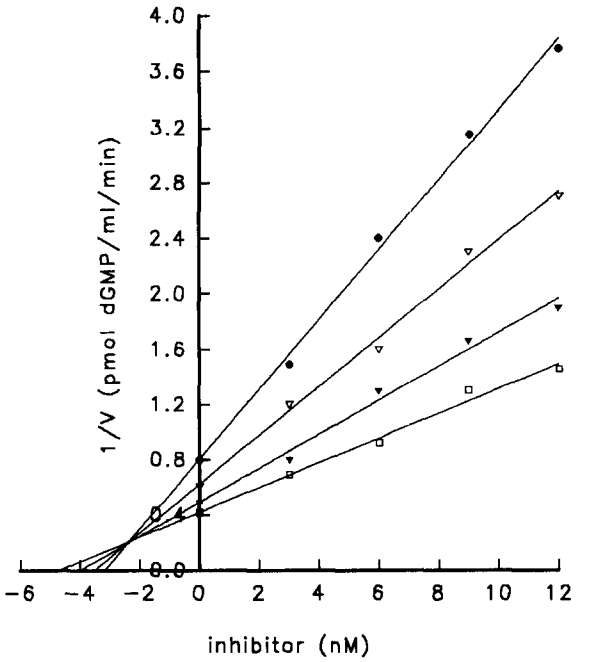

Fig. 4. Dixon plot for inhibition of HIV-1 reverse transcriptase by (s^4dU)$_{35}$ using poly(C)·(dG)$_{16}$ as template-primer and variable substrate. Substrate concentrations (expressed as 3′-primer termini of template-primers): 45 nM (●), 60 nM (▽), 75 nM (▼), 90 nM (□).

RT catalyzes DNA synthesis in an ordered sequential manner. First, the template-primer binds to the free enzyme. The deoxynucleoside triphosphate to be incorporated then binds to the binary complex. This relatively simple mechanism was specified recently [27,28], suggesting a two-step process in both cases. After initial binding of the template, the conformation of the enzyme changes and a more stable complex is formed. The antitemplates, being close structural analogs of the natural templates, may induce the conformation change of RT similarly to the functional counterparts.

The (s^4dU)$_{35}$ was a potent inhibitor of the enzyme, thus being a new member of the antitemplate inhibitors of HIV-1 RT. The antitemplate mode of action involves a multi-site and multi-mode interaction between the enzyme and modified oligonucleotides. The development of resistance to such a complex multi-site interacting drug is not very likely. We believe that the further evaluation of the antitemplates (including s^4dU-containing oligonucleotides) as potential agents for the treatment of HIV infection is warranted.

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