HIV-1 Reverse Transcriptase: Inhibition by 2′,5′-Oligoadenylates†

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Received March 10, 1993; Revised Manuscript Received July 7, 1993®

ABSTRACT: 2′,5′-Oligoadenylates (2-5A) and derivatives are noncompetitive inhibitors of primer/HIV-1 reverse transcriptase complex formation. The mechanism and specificity of this inhibitory action of 2-5A and 2-5A derivatives have been evaluated with 2-5A molecules modified in ribosyl moiety, chain length, extent of 5′-phosphorylation, and 2′,5′-phosphodiester linkage. UV covalent cross-linking of preformed complexes of p66/p66 homodimer or p66/p51 heterodimer recombinant HIV-1 reverse transcriptase and the primer analog $pd(T)_{16}$ allowed analysis of the initial step in HIV-1 reverse transcriptase-catalyzed DNA synthesis. Utilizing this primer binding assay, it is demonstrated that 2-5A and 2-5A derivatives inhibit the binding of $pd(T)_{16}$ to HIV-1 reverse transcriptase. This inhibition is specific for the 2′,5′-internucleotide linkage in that the corresponding 3′,5′-adenylate derivatives do not exhibit inhibitory activity. Enhanced inhibitory properties were observed following modifications of the 2-5A molecule which result in an increase in hydrophobicity. Replacement of the D-ribosyl moiety of 2-5A with the 3′-deoxyribosyl moiety increased the inhibition of primer/HIV-1 reverse transcriptase complex formation 15-20%. 2′,5′-Phosphorothioate substitution yielded the most effective inhibitors, with K_i's of 7–13 μM. In all cases, inhibition of primer/HIV-1 reverse transcriptase complex formation showed a preference for the 5′-triphosphate moiety. Nonphosphorylated derivatives were not inhibitory; 5′-monophosphate derivatives exhibited little or no inhibition. The inhibition of primer binding to HIV-1 reverse transcriptase correlated well with the inhibition of DNA-directed DNA synthesis. Introduction of R_P chirality into the 2′,5′-phosphodiester bond, i.e., 2′,5′-$p_3A_3\alpha S$, resulted in the most potent noncompetitive inhibitor of primer/HIV-1 reverse transcriptase complex formation. This 2-5A-mediated inhibition of HIV-1 reverse transcriptase may represent part of the anti-HIV-1 activity of interferon.

Human immunodeficiency virus type 1 (HIV-1)[1] is clearly documented as the causative agent of acquired immunodeficiency syndrome (AIDS) and is further implicated in the pathogenesis of Kaposi's sarcoma (Barre-Sinoussi et al., 1983; Popovic et al., 1984; Fauci, 1988; McCune, 1991). As a retrovirus, the HIV-1 reverse transcriptase (RT) converts the viral genomic RNA into a double-stranded DNA form capable of integration into the host genomic DNA, in which $tRNA^{Lys-3}$ is utilized as the primer for the synthesis of the first strand of complementary DNA. Therefore, an important reaction in the retroviral life cycle is primer recognition [reviewed in Wilson and Abbotts (1992)]. Currently, drugs in use for the treatment of AIDS function by inhibiting reverse transcriptase. However, many antiviral agents under investigation are designed to inhibit different stages of this complex viral life cycle (Mitsuya et al., 1990). In particular, interferon has been reported to act as an indirect antiviral agent to stimulate cells to develop resistance to HIV infection (Ho et al., 1985).

The interferons are effective against a wide variety of viral infections. With respect to HIV-1, interferon protects both T-cells and monocytes/macrophages against HIV-1 infection [reviewed by Pitha (1991)]. The interferon-induced antiviral state is mediated by at least two cytoplasmic enzyme activities: (1) a dsRNA-dependent protein kinase (p68 kinase) and (2) a dsRNA-dependent oligoadenylate synthetase (2-5A synthetase) (Lengyel, 1982; DeMaeyer & DeMaeyer-Guignard, 1988; Sen & Lengyel, 1992). 2-5A synthetase is an allosterically regulated enzyme, which when activated by dsRNA results in the polymerization of ATP to yield 2-5A, 2′,5′-linked oligoribonucleotides [for reviews see Pestka (1981), Lengyel (1982), and DeMaeyer and DeMaeyer-Guignard (1988)]. Primarily, 2-5A exerts its biological effect by binding to and activating RNase L (EC 3.1.27), a 2′,5′-oligoadenylate-dependent endoribonuclease, which is specific for single-stranded RNA (Floyd-Smith et al., 1981; Wreschner et al., 1981); however, 2-5A has also been implicated in mechanisms different from RNase L activation.

Recently, it has been documented that the 2-5A synthetase/RNase L pathway plays a vital role in the control of cellular and viral gene expression in cells infected with HIV (Schröder et al., 1989). The HIV-1 LTR-driven expression of interferon-α or 2-5A synthetase and the subsequent production of 2-5A also result in the selective inhibition of HIV-1 replication (Bednarik et al., 1989; Schröder et al., 1990), implicating the interferon-induced antiviral mechanism, specifically the 2-5A synthetase/RNase L pathway, as critical in the cellular response to retroviral (HIV) infection.

In recent studies, we have demonstrated that nuclease-resistant 2-5A analogs were potent inhibitors of HIV-1 reverse transcriptase (Montefiori et al., 1989; Suhadolnik et al., 1989; Sobol et al., 1990; Müller et al., 1991). Similarly, Schröder

† This study was supported in part by research grant DMB-9004139 from the National Science Foundation (R.J.S.) and from federal work study awards (R.W.S.).

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® Abstract published in *Advance ACS Abstracts*, October 15, 1993.

0006-2960/93/0432-12112$04.00/0

et al. (1992) reported that several 2-5A analogs inhibit $tRNA^{Lys-3}$/HIV-1 RT complex formation in assays using lysates of HIV-1-infected H9 cells. Kinetic studies of HIV-1 RT show that the reaction pathway for DNA synthesis is ordered, with template/primer and free enzyme combining to form the first complex in the reaction sequence (Majumdar et al., 1988, 1989; Kedar et al., 1990). In an effort to evaluate the mechanism of 2-5A-mediated inhibition of RT, homogeneously pure recombinant HXB2-derived p66 homodimer form of RT (Becerra et al., 1991) was covalently cross-linked using UV cross-linking assays (Sobol et al., 1991). A V8 proteolytic peptide, p12 (residues 195–300), contained the ^{32}P-labeled covalently cross-linked primer pd(T)$_{16}$. More recently, Wilson and co-workers have mapped the nucleic acid binding domains within HIV-1 RT by controlled proteolytic digestion (Kumar et al., 1993). Following digestions of p66, p66/p51 heterodimer, and p51 HIV-1 RT and Northwestern or Southwestern blotting assays, they showed that the nucleic acid binding occurred in two distinct peptide regions located in the N-terminal half of p66. These data are in excellent agreement with the model of template/primer bound to the p66/p51 HIV-1 RT heterodimer (Kohlstaedt et al., 1992).

The present studies utilize UV cross-linking/d(T)$_{16}$ binding and classical enzyme inhibition assays with homodimer (p66/p66) and heterodimer (p66/p51) forms of HIV-1 RT to evaluate the mechanism of 2-5A-mediated inhibition of HIV-1 RT. The structural specificity of inhibition was evaluated with 2-5A derivatives modified in ribosyl moiety, chain length, extent of 5′-phosphorylation, and 2′,5′-phosphodiester linkage.

MATERIALS AND METHODS

Materials. 5′-OH d(T)$_8$, 5′-OH d(T)$_{16}$, ribonucleotides, and deoxyribonucleotides were from Pharmacia; [γ-^{32}P]ATP was from ICN (>7000 Ci/mmol) or Amersham (>5000 Ci/mmol); [α-^{32}P]dTTP (3000 Ci/mmol) was from New England Nuclear; T4 polynucleotide kinase was from USB; recombinant HIV-1 RT p66/p66 homodimer and p66/p51 heterodimer was purified from *Escherichia coli* as described in Becerra et al. (1991).

2′,5′-Oligonucleotides. 2′,5′-p$_3$A$_3$, 2′,5′-p$_3$A$_4$, and 2′,5′-A$_3$ were from Pharmacia; 2′,5′-p$_3$A$_2$ was a generous gift from Dr. J. Justesen; R_P- and S_P-2′,5′-phosphorothioate trimer and tetramer cores and their 5′-monophosphates were synthesized, purified, and characterized as described by Kariko et al. (1987), Charachon et al. (1990), and Charubala et al. (1991a); 2′,5′-p$_3$(3′-dA)$_2$ and 2′,5′-p$_3$(3′-dA)$_3$ were synthesized chemically as described in Montefiori et al. (1989); 2′,5′-(3′-dA)-R_P- and S_P-phosphorothioate trimer cores and their 5′-monophosphates were synthesized as described by Sobol et al. (1993). 3′,5′-A$_3$, 3′,5′-pA$_3$, and 3′,5′-p$_3$A$_3$ were synthesized chemically and enzymatically (Flockerzie et al., 1983; Charubala et al., 1991b).

5′-Phosphorylation of d(T)$_n$. d(T)$_8$ and d(T)$_{16}$ were phosphorylated at the 5′-terminus with T4 polynucleotide kinase (USB) in a reaction mixture (total volume = 50 μL) containing [γ-^{32}P]ATP (0.5 mCi), d(T)$_8$ or d(T)$_{16}$ (110 μM), and T4 polynucleotide kinase (10 units) in 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, and 5 mM DTT as described by Sobol et al. (1991). This reaction mixture was incubated at 37 °C for 1 h, followed by the addition of ATP (final concentration = 0.16 mM) and T4 polynucleotide kinase (10 units) with a second incubation at 37 °C for 30 min. [^{32}P]-5′-pd(T)$_8$ or [^{32}P]5′-pd(T)$_{16}$ was separated from unreacted ATP by passing the mixture over a DuPont NENsorb 20 column following the manufacturer's suggested protocol.

Photochemical Cross-Linking. Photochemical cross-linking, V8 protease hydrolysis, and data analysis for competition experiments with 2′,5′-oligonucleotides were completed as previously described (Sobol et al., 1991).

Inhibition of DNA-Dependent HIV-1 RT Activity by p$_3$A$_3$$\alpha$S. HIV-1 RT activity was determined using a standard reaction mixture (20 μL) containing 50 mM Tris-HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, and the indicated concentrations of substrates and inhibitors. Reactions were initiated by the addition of 19.4 nM p66/p51 (expressed as the concentration of dimer), incubated at 25 °C for 10 min and stopped by the addition of 10 μL of 0.5 M EDTA, pH 8. Quenched reaction mixtures were spotted on Whatman DE-81 filter discs and dried. Unincorporated [α-^{32}P]dTTP was removed by four washes with 0.3 M ammonium formate, pH 8, followed by two washes with 95% ethanol and one wash with acetone. The filters were dried, and radioactivity was determined by scintillation spectrometry. Apparent kinetic constants (k_{cat} and K_m) were determined from initial velocity

[1] Abbreviations: 2-5A, 2′,5′-oligoadenylates (p$_3$A$_n$); 3-5A, 3′,5′-oligoadenylate; 2′,5′-phosphorothioate dimer (p$_3$A$_2$$\alpha$S), 5′-*O*-($S_P$)-1-*P*-thiotriphosphoryl-(R_P)-*P*-thioadenylyl-(2′,5′)-adenosine; 2′,5′-phosphorothioate trimer (p$_3$A$_3$$\alpha$S), 5′-*O*-($S_P$)-1-*P*-thiotriphosphoryl-(R_P)-*P*-thioadenylyl-(2′,5′)-(R_P)-*P*-thioadenylyl-(2′,5′)-adenosine; 2′,5′-phosphorothioate tetramer (p$_3$A$_4$$\alpha$S), 5′-*O*-($S_P$)-1-*P*-thiotriphosphoryl-(R_P)-*P*-thioadenylyl-(2′,5′)-(R_P)-*P*-thioadenylyl-(2′,5′)-(R_P)-*P*-thioadenylyl-(2′,5′)-adenosine; A$_2$, A$_3$, and A$_4$, 5′-dephosphorylated p$_3$A$_2$, p$_3$A$_3$, and p$_3$A$_4$; AIDS, acquired immunodeficiency syndrome; AMPS, adenosine 5′-*O*-phosphorothioate; AMV, avian myeloblastosis virus; BAP, bacterial alkaline phosphatase; BSA, bovine serum albumin; d(C)$_{19-24}$, mixture of 19–24 residue long normal oligodeoxycytidylate; DEAE, (diethylamino)-ethyl; D-MEM, Dulbecco's modified Eagle medium; dNTP, deoxynucleoside triphosphate; dsRNA, double-stranded RNA; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIV-1, human immunodeficiency virus type 1; HPLC, high-performance liquid chromatography; IFN, interferon; kDa, kilodalton; mRNA, messenger RNA; NP40, Nonidet P-40; d(T)$_8$ and d(T)$_{16}$, oligomers of deoxythymidylate; pCp, cytidine 3′,5′-bisphosphate; p$_3$A$_2$, p$_3$A$_3$ and p$_3$A$_4$, dimer, trimer and tetramer, respectively, of adenylic acid with 2′,5′-phosphodiester linkages and a 5′-triphosphate; pA$_3$ and pA$_4$, trimer and tetramer of adenylic acid with 2′,5′-phosphodiester linkages and a 5′-monophosphate; PEI, poly(ethylenimine); RNase, endoribonuclease; RNase L, 2-5A-dependent endoribonuclease; (R_P)- and (S_P)-ATPαS, the Rp and Sp diastereomers of adenosine 5′-*O*-(1-thiotriphosphate); Rp and Sp dimer core, phosphorothioate analogs of 2′,5′-A$_2$ with R_P and S_P stereoconfigurations in the internucleotide linkages; RpRp, SpRp, RpSp, and SpSp trimer cores, phosphorothioate analogs of 2′,5′-A$_3$ with R_P and S_P stereoconfigurations in the two chiral centers with assignment of configuration from the 5′ to the 2′ terminus; RpRp, SpRp, RpSp, and SpSp (3′-dA) trimer cores, phosphorothioate analogs of 2′,5′-(3′-dA)$_3$ with R_P and S_P stereoconfigurations in the two chiral centers with assignment of configuration from the 5′ to the 2′ terminus; RpRp, SpRp, RpSp, and SpSp 3-5A trimer cores, phosphorothioate analogs of 3′,5′-A$_3$ with R_P and S_P stereoconfigurations in the two chiral centers with assignment of configuration from the 5′ to the 2′ terminus; RpRpRp, RpSpSp, SpRpSp, SpSpRp, SpRpRp, RpSpRp, RpRpSp, and SpSpSp tetramer cores, phosphorothioate analogs of 2′,5′-A$_4$ cores with R_P and S_P stereoconfigurations in the three chiral centers with assignment of configuration from the 5′-terminus to the 2′ terminus; pRpRp, pSpRp, pRpSp, and pSpSp trimers, 5′-monophosphorylated trimer phosphorothioate analogs of 2′,5′-A$_3$; pRpRp, pSpRp, pRpSp, and pSpSp (3′-dA) trimers, 5′-monophosphorylated trimer phosphorothioate analogs of 2′,5′-(3′dA)$_3$; pRpRp, pSpRp, pRpSp, and pSpSp 3-5A trimers, 5′-monophosphorylated trimer phosphorothioate analogs of 3′,5′-A$_3$; pRpRpRp, pRpSpSp, pSpRpSp, pSpSpRp, pSpRpRp, pRpSpRp, pRpRpSp, and pSpSpSp tetramers, 5′-monophosphorylated tetramer phosphorothioate analogs of 2′,5′-A$_4$; rRNA, ribosomal RNA; RT, reverse transcriptase; SCP, specific cleavage products; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TAR, trans-activating region; TCA, trichloroacetic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; tRNA, transfer RNA; VSV, vesicular stomatitis virus. Enzymes: reverse transcriptase (EC 2.7.7.7); T4 polynucleotide kinase (EC 2.7.1.78).

data fitted to the Michaelis–Menten equation by nonlinear least-squares methods.

Radioactive Measurements. Beckman LS-100C, Beckman LS-9000, and Tm Analytics Model 8420 scintillation spectrometers were used for radioactive measurements. The scintillation solutions used were BCS (Amersham) and BioSafe II (RPI) (counting efficiencies, 99% for ^{32}P; for Cerenkov methods, 52%).

RESULTS

Photochemical Cross-Linking of p66/p66 or p66/p51 HIV-1 RT to [^{32}P]5′-pd(T)$_n$. Recently, we reported on the UV cross-linking of preformed complexes of the p66/p66 homodimer form of HIV-1 RT and the primer analogs d(T)$_8$ and d(T)$_{16}$ (Sobol et al., 1991). Radioactive primer molecules covalently attached to the 66 000 molecular weight HIV-1 RT (p66) are quantified by excising the radiolabeled p66-RT/d(T)$_n$ complex from the gel followed by scintillation spectrometry with maximal covalent cross-linking of pd(T)$_8$ or pd(T)$_{16}$ occurring after 150 s of UV treatment (3.75×10^4 erg/mm^2) (Sobol et al., 1991). We have previously demonstrated that covalent cross-linking with bench-top UV irradiation does not significantly perturb the equilibrium binding of RT and oligo(dT) when compared to rapid laser irradiation (Sobol et al., 1991). This allows the measurement of the relative amount of enzyme/oligo(dT) complexes formed under equilibrium conditions and the estimation of the equilibrium dissociation constant (K_d).

Inhibition of oligo d(T) binding to the p66/p66 homodimer and the p66/p51 heterodimer forms of HIV-1 RT by 2-5A and 2-5A derivatives and localization of binding/cross-linking were compared. Previously, it was determined that binding of the p66/p66 homodimer form of RT to d(T)$_8$ or d(T)$_{16}$ occurred with a K_d of 2 and 0.2 μM, respectively (Sobol et al., 1991). Further, the site of UV cross-linking of d(T)$_8$ to RT was localized to a V8 protease peptide that was identified as residues 195–~300 (Sobol et al., 1991). However, due to the presence of two identical subunits in the RT preparation, the location of this binding region in one or both subunits was not determined. UV cross-linking analysis of the complex between d(T)$_{16}$ and the p66/p51 form of RT indicates that cross-linking of d(T)$_{16}$ is more abundant for the p66 subunit than the p51 subunit (Figure 1A,B). The proteolysis patterns at 120-min digestion of homodimer and heterodimer are different since the labeling intensities at time zero in p66 for the two preparations are quite different. The digestion time course studies suggest that the two preparations are not structurally equivalent, which is consistent with the 10-fold difference in dimerization dissociation constants (Becerra et al., 1991). A similar observation was reported by Cheng et al. (1991). Binding of d(T)$_{16}$ to p66/p51 RT is saturable with a K_d of 0.65 μM (Figure 1B). V8 protease digestion of cross-linked primer/HIV-1 RT complexes suggests that d(T)$_{16}$ binds to identical sites in both homodimer and heterodimer forms of HIV-1 RT (Figure 1A). Basu et al. (1992) reported a K_d of 300 nM for p(dT)$_{15}$ binding to recombinant RT derived from the same viral source as that used here (HXB2) by nitrocellulose filter binding. Using HIV-1 RT derived from a different proviral DNA (BRU), Painter et al. (1991) have measured an equilibrium dissociation binding constant of 1500 nM using a different technique (fluorescence).

Inhibition of Primer/HIV-1 RT Complex Formation by 2′,5′-Oligoadenylates and Derivatives. 2′,5′-Oligoadenylates with a 5′-triphosphate moiety exhibited marked inhibition of RT/d(T)$_{16}$ complex formation (Figures 2 and 3). The

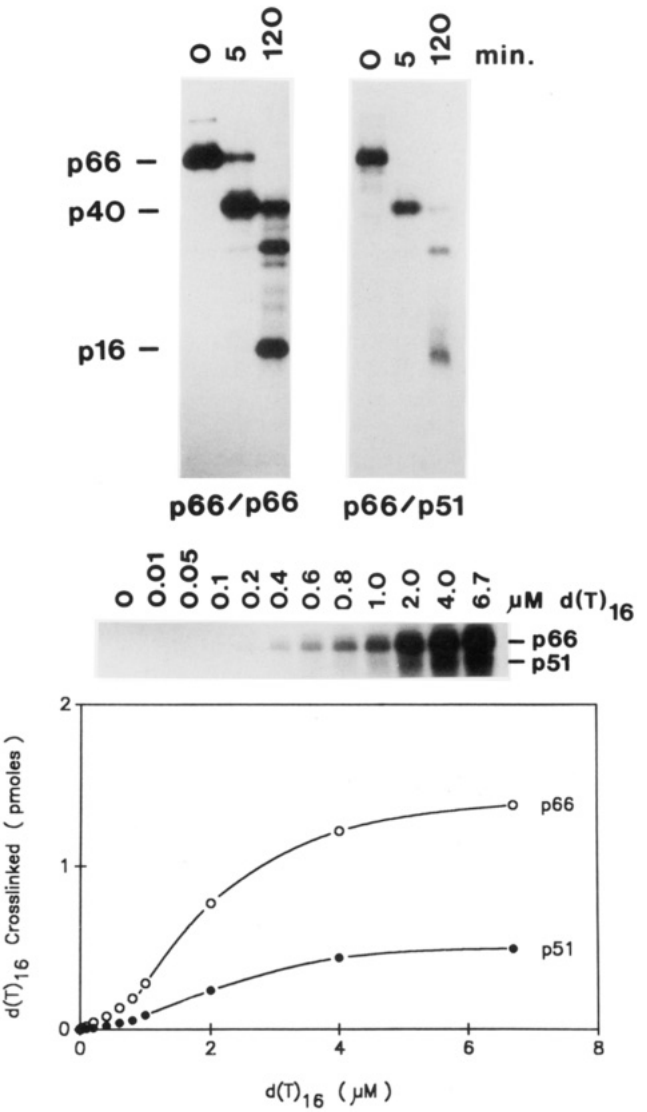

FIGURE 1: (A, Top) V8 protease hydrolysis of [^{32}P]pd(T)$_{16}$ cross-linked to p66/p66 or p66/p51 HIV-1 RT. The d(T)$_{16}$ cross-linked RT was hydrolyzed with V8 protease at 25 °C for 0, 5, or 120 min, as indicated (Sobol et al., 1991). Hydrolysis products were analyzed by 15% SDS–PAGE as described under Materials and Methods. Autoradiograms of SDS–polyacrylamide gels are shown. (B, Bottom) Saturation photochemical cross-linking of the p66/p51 form of HIV-1 RT with [^{32}P]pd(T)$_{16}$. The pd(T)$_{16}$/HIV-1 RT reaction mixture, with increasing concentrations of pd(T)$_{16}$ (expressed as 3′-termini), was UV-irradiated and analyzed by 12.5% SDS–PAGE as described under Materials and Methods. An autoradiogram of the SDS–polyacrylamide gel is shown in the inset. Incorporation of [^{32}P]5′-p-d(T)$_n$ into p66-RT and p51-RT was determined by excising the radiolabeled RT/d(T)$_n$ complex from the gel followed by scintillation spectrometry.

authentic 2′,5′-oligoadenylate 5′-triphosphates, 2′,5′-p$_3$A$_2$, p$_3$A$_3$, and p$_3$A$_4$, inhibited d(T)$_{16}$/RT complex formation with the p66/p66 homodimer and the p66/p51 heterodimer forms of HIV-1 RT. Authentic 2′,5′-p$_3$A$_4$ exhibits one-half competition of pd(T)$_{16}$ binding to the p66/p66 homodimer at 73 μM, whereas the one-half competition for 2′,5′-p$_3$A$_4$ with the p66/p51 heterodimer was 55 μM (Table I). The inhibition of primer/HIV-1 RT complex formation was increased with the increased chain length of 2-5A.

Replacement of the ribosyl moiety of 2-5A with the 3′-deoxyribosyl moiety resulted in 2-5A derivatives that had a slight inhibitory effect (Table II). However, much more effective inhibition was observed with phosphorothioate substitution within the 2′,5′-internucleotide linkages. The

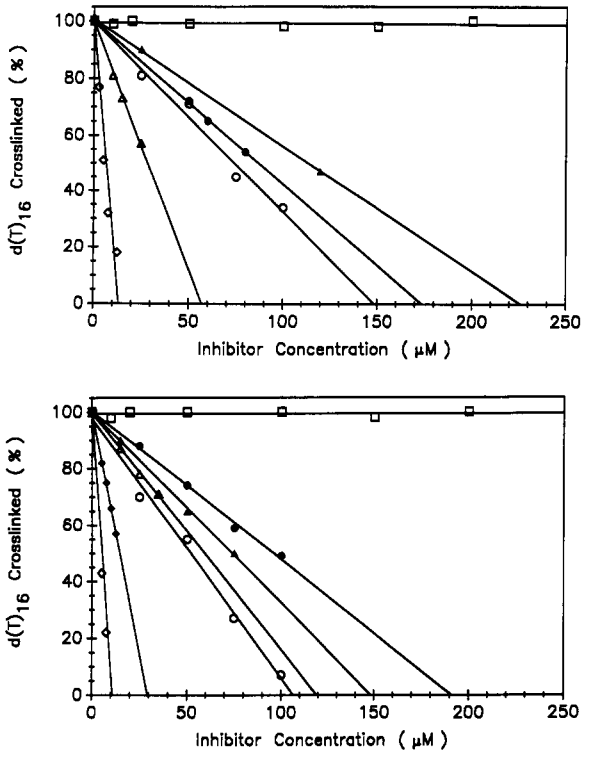

FIGURE 2: Competition for primer binding to HIV-1 RT by 2-5A and 2-5A derivatives. Inhibition of $d(T)_{16}$/RT complex formation by 2-5A and 2-5A derivatives was determined by photochemical cross-linking assays with either the p66/p66 homodimer (A, top) or p66/p51 heterodimer (B, bottom) form of HIV-1 RT, under equilibrium binding conditions. Reaction mixtures contained HIV-1 RT and [^{32}P]5′p-$d(T)_{16}$ (as described under Materials and Methods) with 2′,5′-p_3A_3 (·): p_3A_4 (○); 2′,5′-p_3(3′-dA)$_2$ (▲); 2′,5′-p_3(3′-dA)$_3$ (△); 2′,5′-$p_3A_3\alpha S$ (♦); 2′,5′-$p_3A_4\alpha S$ (◇); 2′,5′-A_3, 2′,5′-pA_3, 3′,5′-A_3, 3′,5′-pA_3, 3′,5′-p_3A_3, or ATP (□). The $pd(T)_{16}$/HIV-1 RT complex formation was quantified as described under Materials and Methods and in the legend to Figure 1.

range of 50% competition values for the phosphorothioate-substituted 2-5A 5′-triphosphate molecules was 7–13 μM (Table III). With either p66/p66 homodimer HIV-1 RT or the p66/p51 heterodimer HIV-1 RT, no inhibition of complex formation was observed with dimer, trimer, or tetramer core 2-5A, phosphorothioate core derivatives (Figure 2A,B), or 3′,5′-oligoadenylates (core, 5′-monophosphates, and 5′-triphosphates). Partial inhibition was observed with several phosphorothioate and 3′-deoxyadenosine-substituted 2-5A 5′-monophosphate derivatives, in agreement with that reported earlier (Montefiori et al., 1989; Müller et al., 1991).

Inhibition of DNA Synthesis by 2′,5′-Oligoadenylates. To evaluate the type inhibition (i.e., competitive or noncompetitive) of 2-5A molecules on the DNA synthetic enzymatic activity of HIV-1 RT, steady-state DNA-directed synthesis reactions were performed with the p66/p51 RT heterodimer. Poly[d(A)-$d(T)_{16}$] as template/primer (T·P) was only weakly inhibited (10%) by 2′,5′-p_3A_3 (80 μM), whereas the phosphorothioate analog, $p_3A_3\alpha S$, inhibited binding by 50% at 24 μM without a significant effect on K_m for T·P or dTTP (Figure 3A,B). In the absence of inhibitor, $K_{m,poly[d(A)-d(T)16]}$ = 40 nM primer, $K_{m,dTTP}$ = 6 μM, and k_{cat} = 0.06 1/s.

These results indicate that inhibition is noncompetitive with respect to both substrates. Inhibitor titration of activity in the presence of 400 nM T·P and 50 μM dTTP was linear at low concentrations of inhibitor with a $K_{i,apparent}$ = 40 μM (Figure 4). At higher concentrations, however, inhibition became complex, as illustrated by the parabolic Dixon plot (Figure 4, inset).

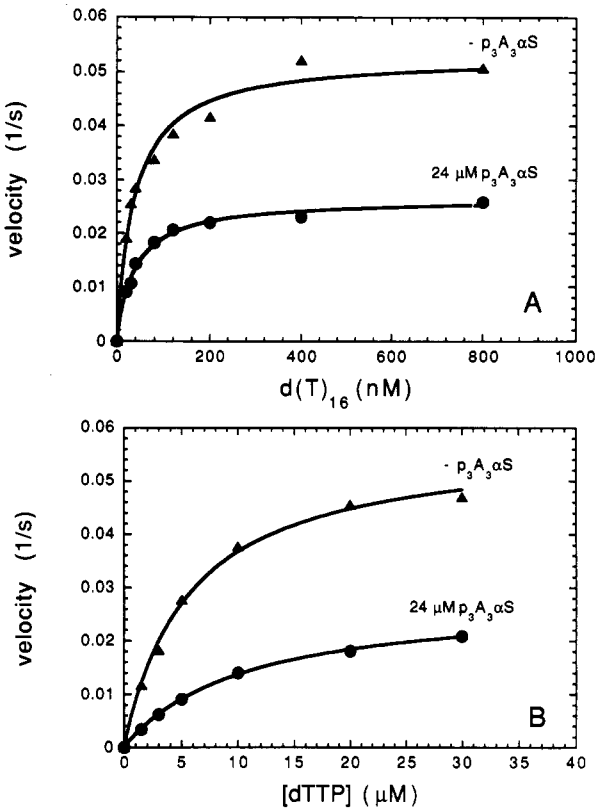

FIGURE 3: Noncompetitive inhibition of poly[d(A)-$d(T)_{16}$]/HIV-1 RT activity by $p_3A_3\alpha S$. Enzyme activity was determined as described under Materials and Methods. Apparent kinetic constants (k_{cat} and K_m) were determined from initial velocity data fitted to the Michaelis–Menten equation by nonlinear least-squares methods. (A) $d(T)_{16}$ and (B) dTTP concentrations were 0.4 and 30 μM, respectively. The K_m values were not significantly altered in the presence of the 2′,5′-$p_3A_3\alpha S$ inhibitor, whereas k_{cat} was diminished 50% in the presence of 24 μM $p_3A_3\alpha S$, indicating that inhibition was noncompetitive with respect to both substrates: (▲) minus inhibitor; (●) plus inhibitor.

Table I: Inhibition of HIV-1 RT/Primer Complex Formation by Nucleotides and Oligoadenylates

inhibitor	concn required for 50% inhbn of p66/p66-$d(T)_{16}$ complex formation[a,b] (μM)	concn required for 50% inhbn of p66/p51-$d(T)_{16}$ complex formation[a,b] (μM)
ATP	-- (200)	-- (200)
dATP	-- (200)	-- (200)
dCTP	-- (200)	-- (200)
dGTP	-- (200)	-- (200)
dTTP	-- (200)	-- (200)
A_2	-- (300)	-- (300)
A_3	-- (300)	-- (300)
A_4	-- (300)	-- (300)
pA_4	-- (100)	-- (200)
p_3A_2	328	NT[c]
p_3A_3	84	102
p_3A_4	73	55
3′,5′-A_3	-- (300)	-- (300)
3′,5′-p_3A_3	NT	-- (100)

[a] (--) = no inhibition; the number in parentheses indicates the maximum concentration tested. [b] Relationship to K_d is $[I]_{50\%} = K_i(1 + (dT_{16}/K_d)$. [c] NT = not tested.

DISCUSSION

Nucleoside analogs and compounds with no structural relationship to dNTP have been reported to inhibit the HIV-1 life cycle [for review, see Schinazi et al. (1992)]. Some of these inhibitors affect either the N-terminal portion of HIV-1 RT where DNA polymerase activity is located, or they affect

Table II: Inhibition Primer/HIV-1 RT Complex Formation by 3′-Deoxyadenosine Derivatives of 2-5A

inhibitor[a]	concn required for 50% inhbn of p66/p66-d(T)$_{16}$ complex formation[a] (μM)	concn required for 50% inhbn of p66/p51-d(T)$_{16}$ complex formation[a] (μM)
(3′-dA)$_3$	– – (300)	– – (300)
(3′-dA)$_3$-RpRp	– – (200)	– – (200)
(3′-dA)$_3$-SpRp	– – (200)	– – (200)
(3′-dA)$_3$-RpSp	– – (200)	– – (200)
(3′-dA)$_3$-SpSp	– – (200)	– – (200)
p(3′-dA)$_2$	100	NT[b]
p(3′-dA)$_3$	NT	200
p$_3$(3′-dA)$_2$	110	82
p$_3$(3′-dA)$_3$	60	62

[a] (– –) = no inhibition; the number in parentheses indicates the maximum concentration tested. [b] NT = not tested.

Table III: Inhibition Primer/HIV-1 RT Complex Formation by Phosphorothioate Derivatives of Oligoadenylates

inhibitor	concn required for 50% inhbn of p66/p66-d(T)$_{16}$ complex formation[a] (μM)	concn required for 50% inhbn of p66/p51-d(T)$_{16}$ complex formation[a] (μM)
pRpRp	(~15%, 200)	NT[b]
pSpRp	NT	NT
pRpSp	NT	NT
pSpSp	NT	NT
pRpRpRp	– – (1)	– – (1)
pSpRpRp	– – (1)	– – (1)
pRpSpSp	– – (1)	– – (1)
pSpSpSp	– – (1)	– – (1)
p$_3$A$_2$$\alpha$S	10	NT
p$_3$A$_3$$\alpha$S	NT	13
p$_3$A$_4$$\alpha$S	7	7
3′,5′-RpRp	– – (200)	– – (200)
3′,5′-SpRp	– – (200)	– – (200)
3′,5′-RpSp	– – (200)	– – (200)
3′,5′-SpSp	– – (200)	– – (200)

[a] (– –) = no inhibition; the number in parentheses indicates the maximum concentration tested. [b] NT = not tested.

the C-terminal portion which possesses RNase H activity. These functions are independent of each other (Wilson & Abbotts, 1992). Recently, Suhadolnik, Wilson, Steitz, and their co-workers have used several approaches to define subdomains of HIV-1 RT (Sobol et al., 1991; Kohlstaedt et al., 1992; Kumar et al., 1993). Sobol et al. (1991) identified a polynucleotide binding domain involved in primer binding by V8 proteolysis of HIV-1 RT/primer complexes. Kohlstaedt et al. (1992) used the crystalline structure of HIV-1 RT and reported that the p66 RT subunit is folded into five subdomains. Kumar et al. (1993) used carefully controlled V8 protease and tryptic digestions of three forms of HIV-1 RT (p66, p66/p51 heterodimer, and p51) to generate domain fragments and thereby map the N-terminal, middle, and C-terminal regions of HIV-1 RT, and they identified the HIV-1 RT peptide fragments that bind nucleic acids. Recently, we reported on a new antiretroviral activity of the 2-5A molecule, that is, inhibition of HIV-1 RT, in lysates of HIV-1-infected MT-2 and H9 cells in culture (Montefiori et al., 1989; Sobol et al., 1990; Müller et al., 1991). Evidence was presented which clearly demonstrated that 2-5A and 2-5A derivatives inhibit primer/HIV-1 RT complex formation (Sobol et al., 1990; Müller et al., 1991).

The 2-5A synthetase/RNase L system is critical in the antiviral mechanism of mammalian cells (Lengyel, 1982). 2-5A, a mediator of the intracellular action of interferons, is synthesized by 2-5A synthetase and exerts its biological effect

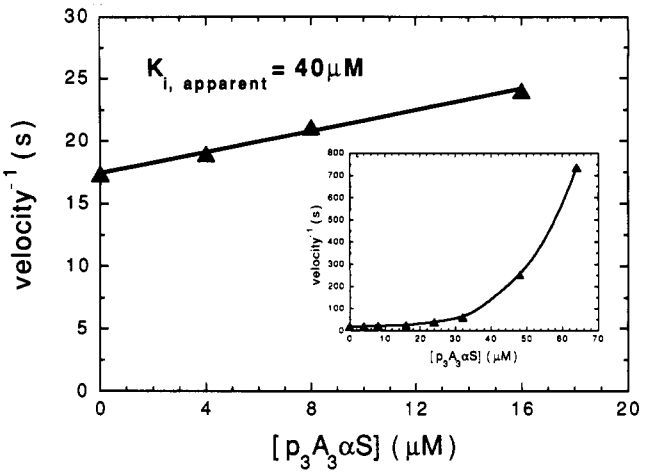

FIGURE 4: Dixon plot of p$_3$A$_3$$\alpha$S inhibition of dTTP incorporation into poly[d(A)-d(T)$_{16}$]. Concentrations of dTTP and poly[d(A)-d(T)$_{16}$] were 50 and 0.4 μM (expressed as 3′-primer termini), respectively. From the low inhibitor concentration range (0–16 μM), $K_{i,apparent}$ is 40 μM. The inset illustrates that, at high inhibitor concentrations, inhibition is more complex as illustrated by the parabolic character of the plot.

by binding to and activating the unique endoribonuclease, RNase L, at subnanomolar concentrations. The preferential degradation of viral RNA associated with the 2-5A synthetase/RNase L system has been shown in retrovirus- and picornavirus-infected cells. However, roles for 2-5A in cellular processes other than RNase L activation have been reported, including inhibition of viral mRNA capping (Kimchi et al., 1979; Sharma & Goswami, 1981; Leanderson et al., 1982; Schmidt et al., 1984), DNA topoisomerase I (Castora et al., 1991; H. C. Schröder, M. Kelve, H. Schäcke, W. Pfleiderer, R. Charubala, R. J. Suhadolnik, and W. E. G. Müller, manuscript in preparation), VSV RNA polymerase (Subramanian et al., 1990), and poly(ADP–ribose) polymerase (Leone et al., 1985).

Previous studies have shown that the template/primer recognition process of HIV-1 RT initiates with primer binding to the free enzyme (Majumdar et al., 1989). We have utilized oligo d(T)$_n$ as model primer probes to characterize the nature of primer/HIV-1 RT complex formation, to differentiate the specificity of primer recognition, and to localize the primer binding site on HIV-1 RT (Sobol et al., 1991). It has been established that oligo d(T)$_8$ and oligo d(T)$_{16}$, specifically bound to the HIV-1 RT p66 homodimer at the primer binding site and in UV cross-linking assays, are ideal for the investigation of primer/HIV-1 RT complex formation (Sobol et al., 1991). A comparison of the binding and UV cross-linking of pd(T)$_n$ to p66/p66 and p66/p51 suggests that the primer binding site is specific for the 66-kDa subunit of the heterodimer and probably only one of the two identical subunits in the p66/p66 homodimer. Furman and co-workers reported dTTP or template/primer cross-links only to the p66 subunit (Cheng et al., 1991; Furman et al., 1991). LeGrice et al. (1991) reported that only mutations in putative active-site residues of the p66 subunit affect function. Barat et al. (1989) chemically cross-linked tRNA^{Lys-3} to both p66 and p51. Although the use of different primers and different cross-linking techniques preclude direct comparison between the results of Barat et al. and our results, on a speculative level, it would not be surprising that p51 plays a role in tRNA recognition to initiate DNA synthesis but has only minor interactions during cDNA synthesis. Finally, V8 proteolysis of the [^{32}P]pd(T)$_{16}$-p66/p51 complex yields identical proteolysis patterns to that of the [^{32}P]pd(T)$_{16}$-p66/p66 complex,

lending further support to the supposition that $pd(T)_n$ binds and cross-links to the same site on both the p66/p66 and p66/p51 forms of HIV-1 RT.

In the present study, structural and stereochemical changes have been made in the 2-5A molecule, and we evaluated these changes on the inhibition of primer/HIV-1 RT complex formation and RT-mediated DNA-directed DNA synthesis. The inhibition of primer/HIV-1 RT complex formation and enzyme activity by 2′,5′- or 3′,5′-adenylate trimer 5′-triphosphates were evaluated using purified recombinant HIV-1 RT (p66/p66 and p66/p51), providing an opportunity to determine and compare the K_d's obtained as a function of (i) ribosyl moiety, (ii) 2′,5′-internucleotide linkage, (iii) chain length, and (iv) degree of phosphorylation at the 5′-terminus of the 2-5A molecule.

Authentic 2-5A exhibits marked inhibition of primer binding to purified, recombinant HIV-1 RT p66/p66 homodimer and p66/p51 heterodimer. This inhibition is dependent on the degree of 5′-phosphorylation, since the corresponding cores or 5′-monophosphates exhibit no inhibition at concentrations up to 200 μM for either the homodimer or heterodimer forms. Authentic 2′,5′-oligoadenylate dimer, trimer, and tetramer 5′-triphosphates exhibit K_d values in the range of 55–328 μM. Further, this inhibition is specific for the 2′,5′-internucleotide linkage in that the corresponding 3′,5′-isomers exhibit little or no inhibition. 2-5A derivatives with increased hydrophobicity exhibit an even greater inhibition of primer binding to RT and inhibition of enzyme activity, as demonstrated by the increased inhibition by the 3′-deoxyadenosine- or phosphorothioate-substituted analogs of 2-5A. From these studies, a new antiretroviral activity for the 2-5A molecule has been demonstrated, i.e., inhibition of HIV-1 RT/primer complex formation. Recent studies by Basu et al. (1992) and Mitchell and Cooperman (1992) using a tryptic digest of $[^{32}P]pd(T)_{15}$ covalently cross-linked HIV-1 RT identified Leu^{289}-Thr^{290}-Leu^{295}-Thr^{296} as the probable sites of UV cross-linking. When the active site of HIV-1 RT was covalently linked with phenylglyoxal, Mitchell and Cooperman (1992) provided data that Arg^{277} was involved in primer/template binding.

The noncompetitive nature of the inhibition of the DNA-dependent DNA polymerase activity of the RT was unexpected, since 2-5A and its derivatives inhibit UV cross-linking of $[^{32}P]d(T)_{16}$. The noncompetitive nature, however, was not simple as illustrated by the nonlinear Dixon plot (Figure 4, inset). This indicates that the inhibitor can bind to both free enzyme and enzyme/substrate complexes. How the inhibitor may effect the binding affinity of substrates in these complexes as well as if more than one inhibitor can bind per RT dimer requires a rigorous kinetic characterization. Several new classes of potent inhibitors of RT have recently been described (Merluzzi et al., 1990; Pauwels et al., 1990; Goldman et al., 1991). They appear to bind to a distinct site on the RT as they can compete with one another for this site, but dNTPs or template/primer afford no protection to binding (Goldman, et al., 1991; Wu et al., 1991; Dueweke et al., 1992). Inhibition by one of these non-nucleoside inhibitors, nevirapine (BI-RG-587) has been shown to be noncompetitive with respect to template/primer as well as dNTPs (Merluzzi et al., 1990). It binds at a site near both the 'palm' and the 'thumb' subdomains (Kohlstaedt et al., 1992), and an azido analog has been cross-linked to tyrosines at positions 181 and 188 (Cohen et al., 1991). It remains to be seen if 2-5A or its derivatives may also bind to this site. The studies described here suggest that 2-5A and metabolically stable 2-5A derivatives are an interesting class of inhibitors of the HIV-1 life cycle. The 2′,5′-phosphorothioates in particular may be potentially useful in therapy of HIV-1 infection in that they can activate the natural cellular antiretroviral defense system and simultaneously inhibit HIV-1 replication.

ACKNOWLEDGMENT

The authors thank Nikolaos Tairis, Temple University School of Medicine, for the chemical synthesis of 3′,5′-p_3A_3.

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