Articles

Cordycepin Analogues of 2′,5′-Oligoadenylate Inhibit Human Immunodeficiency Virus Infection via Inhibition of Reverse Transcriptase†

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ABSTRACT: Analogues of 2′,5′-oligoadenylates (2-5A), the cordycepin (3′-deoxyadenosine) core trimer (Co_3) and its 5′-monophosphate derivative (pCo_3), were shown to display pronounced anti-human immunodeficiency virus type 1 (HIV-1) activity in vitro. Treatment of HIV-1 infected H9 cells with 1 μM Co_3 or pCo_3 resulted in an almost 100% inhibition of virus production. The compounds were encapsulated in liposomes targeted by antibodies specific for the T-cell receptor molecule CD3. Substitution of one or two cordycepin units in Co_3 or pCo_3 decreased the antiviral activity of the compounds. pCo_3 did not stimulate 2-5A-dependent ribonuclease L activity and displayed no effect on the amount of cellular RNA and protein. At a concentration of 10 μM the cellular DNA polymerases α, β, and γ were almost insensitive toward Co_3 or pCo_3. In contrast, these compounds reduced the activity of HIV-1 reverse transcriptase (RT) by 90% at a concentration of 10 μM if the viral RNA genome and the cellular $tRNA^{Lys,3}$ was used as template/primer system; if the synthetic poly(A)·$(dT)_{10}$ was used as template/primer, no marked inhibition was observed. Dot-blot, gel-retardation, and cross-linking assays showed that Co_3 or pCo_3 interfere with the binding site of $tRNA^{Lys,3}$ to RT. These results indicate that inhibition of RT at the level of initiation of the enzymic reaction is a novel approach to inhibit HIV-1 replication.

It is now widely accepted that the 2′,5′-oligoadenylate (2-5A)[1] synthetase (EC 3.1.26.–)/endoribonuclease L (RNase L) (EC 2.7.7.–) pathway is a part of the antiviral mechanism of interferon (Floyd-Smith et al., 1981) and plays an additional role in the regulation of cell growth and differentiation (Wells & Mallucci, 1985). The two double-stranded (ds) RNA dependent enzymes, the 2-5A synthetase and the protein kinase, may account for the inhibitory effects on viral RNA and viral protein synthesis. Recently, we have described that the time period after infection of cells with human immunodeficiency virus type 1 (HIV-1) is correlated with a transient activation of the double-stranded 2-5A synthetase, resulting in a strong increase in intracellular 2-5A level; this oligoadenylate in turn activates RNase L (Müller et al., 1980; Schröder et al., 1989). RNase L, which degrades HIV-1 transcripts in addition to cellular RNA (Schröder et al., 1989; Wreschner et al., 1981), was found to increase strongly after infection of cells with HIV-1, reaching maximal levels at days 2–3. Thereafter, both enzyme activities rapidly decrease simultaneously with the rise in viral protein synthesis (Schröder et al., 1989). The 2-5A-degrading 2′,3′-exoribonuclease activity (Schröder et al., 1980; Müller et al., 1980) increases only slightly during the initial stage of infection (Schröder et al., 1989). Evidence has been presented that the time period until onset of release of HIV-1 can be extended by interferon (which activates the 2-5A pathway) (Ho et al., 1985), antiviral compounds, e.g., 3′-azido-3′-deoxythymidine (which causes a delay in the decrease in intracellular level of 2-5A) (Schröder et al., 1989), or mismatched dsRNA (e.g., Ampligen) (which activates the 2-5A synthetase) (Montefiori et al., 1987). Hence our groups and others (Sawai et al., 1983) work on strategies to control HIV infection by augmentation of the intracellular 2-5A pool in the following ways: (i) by synthesis of 2-5A analogues, e.g., phosphorothioate (Montefiori et al., 1989; Suhadolnik et al., 1989) or cordycepin derivatives (Montefiori et al., 1989; Sawai et al., 1983) which are more resistant against the 2-5A-degrading 2′,3′-exoribonuclease; (ii) by development of mismatched dsRNA with low cytotoxicity (Montefiori et al., 1987), which induces 2-5A synthetase; and (iii) by an "intracellular immunization approach" (Schröder et al., 1990).

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[1] Abbreviations: AIDS, acquired immunodeficiency syndrome; 2-5A, 2′,5′-oligoadenylate; p_3A_3, trimer of adenylic acid with 2′,5′-phosphodiester linkages and a 5′-triphosphate; A_3, 5′-dephosphorylated p_3A_3; Co, cordycepin (3′-deoxyadenosine); Co_3, 2′,5′-cordycepin analogue of A_3; pCo_3, 5′-monophosphate of Co_3; DDT, dithiothreitol; HIV, human immunodeficiency virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RNase L, ribonuclease L; RT, reverse transcriptase.

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In the latter study the human 2-5A synthetase gene, under the control of a HIV 3′-LTR, is introduced into uninfected cells and is trans-activated after HIV infection by the HIV Tat protein.

The first successful results showing that 2-5A analogues display anti-HIV activity in vitro were reported recently (Montefiori et al., 1989; Suhadolnik et al., 1989). The phosphorothioate and cordycepin analogues of 2-5A displayed antiviral activity in the micromolar concentration range. Surprisingly, the cordycepin analogues, which are known not to activate RNase L (Sawai et al., 1983), were reported to inhibit to some extent the HIV reverse transcriptase (RT) (Montefiori et al., 1989; Suhadolnik et al., 1989). In this paper we report that cordycepin analogues of the trimer of adenylic acid with 2′,5′-phosphodiester linkages and a 5′-monophosphate (pA_3) as well as its dephosphorylated core (A_3) are potent inhibitors of HIV replication. The compounds were delivered intracellularly by using a targeted liposome cell system (Bayard et al., 1985). Moreover, we report that this inhibition is due to an interference of these trimers with the binding of the HIV RT to the $tRNA^{Lys,3}$. This $tRNA^{Lys,3}$ has recently been demonstrated to be the primer for RT in the HIV system (Barat et al., 1989). These data demonstrate that some A_3 and pA_3 analogues are novel inhibitors of HIV RT, suggesting also a potential therapeutic value for the treatment of acquired immunodeficiency syndrome (AIDS).

Materials and Methods

Materials. The following materials were obtained: [γ-^{32}P]ATP (4000 Ci/mmol), [α-^{32}P]ATP (3000 Ci/mmol), and [^{3}H]dTTP (40 Ci/mmol) from Amersham Buchler, Buckinghamshire, England; $(A2'p)_2A$ ($=A_3$), $p_3(A2'p)_2A$ ($=p_3A_3$), and the synthetic template/primer from Pharmacia, Freiburg, Germany; T_4 polynucleotide kinase from Boehringer Mannheim, Mannheim, Germany; dipalmitoyl-L-α-phosphatidylcholine, *N*-succinimidyl 3-(2-pyridyldithio)propionate, dipalmitoyl-L-α-phosphatidylethanolamine, cholesterol, ribonuclease A, protein A (*Staphylococcus aureus*), anti-mouse IgG (peroxidase conjugated from rabbit), and anti-rabbit IgG (peroxidase conjugated from goat) from Sigma, St. Louis, MO; antibodies (polyclonal) to HIV-1 p17 and p24 from Biochrom, Berlin, Germany; monoclonal antibodies to CD3 (IOT3; isotype, IgG2a) from Dianova, Hamburg, Germany. 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2-tetrazolium hydroxide (XTT) was a gift of the National Cancer Institute (Dr. Narayanan), Bethesda, MD.

Cells and Virus Infection. H9 human T-cells (Popovic et al., 1984) were grown in RPMI 1640 medium supplemented with 15% (v/v) fetal calf serum (Müller et al., 1988a); cultures were maintained at 37 °C in a humidified 5% CO_2 in air atmosphere.

H9 cells (1×10^5) were incubated with 2 μg of anti-CD3 monoclonal antibodies (1 h; 4 °C) and then washed twice with medium. The cells were routinely seeded at a concentration of 1×10^5 cells/mL. The cells were incubated in the presence or absence of liposomes for 6 days. Routinely, 1 h after addition of the liposomes HIV-1 (HTLV-IIIB) was added to give a multiplicity of infection of 20 medium tissue culture infectious dose ($TCID_{50}$) per H9 cell. During that period of time the uninfected cells performed 2.1 doubling steps and HIV-1 infected cells 0.07 doubling steps.

Evaluation. The cell concentration was routinely monitored with the XTT colorimetric assay system (Scudiero et al., 1988) and evaluated with an ELISA reader (Bio-Rad, Model 3550 equipped with the program NCIMR IIIB). To standardize the growth curves, the cells were counted electronically (Cytocomp Counter; Model Michaelis). The effective dose, which inhibits cell proliferation by 50%, ED_{50}, as well as the number of doubling steps, was determined as described (Müller et al., 1988a).

To test for virus release, cells were removed and supernatant fluids were assayed for reverse transcriptase (RT) activity (Schröder et al., 1989). The percentage of cells expressing p24 *gag* protein of HIV-1 was determined by indirect immunofluorescence microscopy with the use of mouse monoclonal antibodies to HIV-1 p24. Positive cells were visualized by treatment with fluorescein-labeled goat anti-mouse IgG (Poiesz et al., 1980); the reactivity of the antibodies with HIV-1-infected cells was in the range of 30–40%.

Liposome Preparation. Liposomes composed of 65% (molar) dipalmitoylphosphatidylcholine, 34% cholesterol, and 1% phosphatidylethanolamine, modified with the heterobifunctional cross-linking reagent *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), were prepared as described (Hope et al., 1985; Machy et al., 1988; Leserman et al., 1981). Liposomes were mixed with *N*-succinimidyl 3-(2-pyridyldithio)propionate modified protein A (10 mol of the heterobifunctional cross-linking reagent/mol of protein A) (Carlson et al., 1978; Renneisen et al., 1990). The stoichiometric ratio of protein A molecules per liposome was calculated to be 7. The liposome preparation was termed protein A–liposomes.

To determine the encapsulation efficiency, the core trimers were 5′ end trace labeled with ^{32}P by T_4 polynucleotide kinase using [γ-^{32}P]ATP (Oshevski, 1982). Applying this approach, the encapsulation efficiency was determined to vary between 8 and 13% for the 5′-monophosphate trimers. The concentrations given for the 5′-monophosphate derivatives under Results are the final concentrations added to the cells. The concentrations for the corresponding core compounds have been estimated on an average encapsulation efficiency of 10%.

Syntheses of 2-5A Analogues. The chemical syntheses of the 2′,5′-cordycepin analogues have been described (Charubala et al., 1980; Montefiori et al., 1989). The following cores were prepared: A-A-Co [A2′p5′A2′p5′Co], A-Co-Co, Co-Co-A, Co-A-Co, and Co-Co-Co (Charubala et al., 1980; Montefiori et al., 1989). A-A-A ($=A_3$) and p_3A_3 were purchased. The corresponding 5′-phosphorylated compounds were obtained by phosphorylation using T_4 polynucleotide kinase and either unlabeled ATP or [γ-^{32}P]ATP as substrate (Oshevski, 1982).

Isolation and Labeling of $tRNA^{Lys,3}$. $tRNA^{Lys,3}$ was isolated from beef liver by chromatographic methods (Fournier et al., 1976) and subsequently purified (Raba et al., 1979). Subsequently, the $tRNA^{Lys,3}$ was 5′-labeled by use of T4 polynucleotide kinase and [γ-^{32}P]ATP (Ausubel et al., 1987). The ^{32}P-$tRNA^{Lys,3}$ was purified from 1% low melting temperature agarose gels. The specific activity was 5×10^6 cpm/μg of tRNA.

Gel-Retardation Assay. ^{32}P-$tRNA^{Lys,3}$ (0.3 or 0.6 pmol) was added to 0.3–2.0 pmol of the p66 recombinant RT (Larder et al., 1987) in the RT assay (with the exception of the dNTPs) (see below) in the absence or presence of the trimer analogues in a final volume of 20 μL. After incubation at 30 °C for 30 min the reaction product was analyzed by 0.8% agarose gel electrophoresis (Müller et al., 1990).

Protein–RNA Dot-Blot Analysis. One hundred nanograms (=1.5 pmol) of recombinant RT (Larder et al., 1987) was spotted onto nitrocellulose filters and probed in the RT assay (with the exception of the dNTPs) (see below) with 0.5 nmol of ^{32}P-$tRNA^{Lys,3}$ in the absence or presence of the trimer analogues as described (Barat et al., 1989); the final volume was 200 μL.

Ribosomal RNA Cleavage Assay. This assay system, essentially as described previously (Wreschner et al., 1981) was used to estimate the function of RNase L. In brief, extracts from L5178y mouse lymphoma cells were prepared (Kariko & Ludwig, 1985), incubated for 1 h at 30 °C (Wreschner et al., 1981), and supplemented with the A_3, p_3A_3, or Co_3. Ribosomal RNA was extracted and analyzed by agarose gel electrophoresis (Wreschner et al., 1981). RNA bands were analyzed after staining with ethidium bromide.

tRNA–RT Cross-Linking. Cross-linking of ^{32}P-tRNA$^{Lys.3}$ with RT was performed according to the procedure described by Tukalo et al. (1987). The 10-μL RT reaction mixture was composed of 2.0 pmol of recombinant RT [p66 enzyme (Larder et al., 1987)], 0.6 pmol of ^{32}P-tRNA$^{Lys.3}$, and the indicated amount of 2-5A analogues. After an incubation period of 20 min at 37 °C *trans*-diaminedichloroplatinum(II) (final concentration 0.2 mM) was added, and incubation proceeded for an additional 60 min at 20 °C in the dark. The RNA was partially digested by T1 RNase and then labeled again with T_4 polynucleotide kinase in the presence of [γ-^{32}P]ATP essentially as described (Barat et al., 1989). Reaction product was purified from the free RNA fragments by gel filtration over Sephadex G-50 (Barat et al., 1989). The tRNA–RT complex which eluted in the void volume was collected and analyzed by 10% polyacrylamide gel electrophoresis in the presence of SDS (Aldovini et al., 1986). Prior to electrophoresis the sample was heated at 70 °C with 1% SDS but in the absence of β-mercaptoethanol. Finally, the gel was autoradiographed.

Polymerases and Enzyme Assays. DNA polymerase α was isolated and partially purified from calf thymus as described by Bollum et al. (1974). The DNA polymerase α pool obtained from the DE-11 cellulose column was further fractionated by ammonium sulfate precipitation and Sephadex G-200 gel filtration (Chang & Bollum, 1981). The specific activity of the final preparation was 2340 units/mg. DNA polymerase β was purified from calf thymus by phosphocellulose column chromatography, gel filtration, and affinity on DNA cellulose (Chang, 1973). The specific activity was 54 500 units/mg. DNA polymerase γ was isolated and purified from rat liver mitochondria by DEAE-cellulose and phosphocellulose column chromatography (Vogel & Chandra, 1981). The specific activity was determined to be 12.1 units/mg. HIV-1 RT was isolated from HTLV-IIIB-infected H9 cells by successive chromatography on DEAE-cellulose and phosphocellulose columns (Vogel & Chandra, 1981). Where indicated, endogenous RT from disrupted HIV-1 virions was used. The virions were purified by density gradient centrifugation (Popovic et al., 1984); prior to RT testing, the virions were disrupted (Prince et al., 1985). The specific activity was 13.2 units/mg [using poly(A)·$(dT)_{10}$ as template/primer]. One unit of enzyme is determined as 1 nmol of total nucleotide polymerized/h.

DNA polymerase activities were determined essentially as described (Wondrak et al., 1988). The standard reaction system (final volume 100 μL) contained the following components: for DNA polymerase α, 50 mM Tris-HCl, pH 8.2, 1 mM DDT, 0.01% NP 40, 25 mM KCl, and 5 mM $MgCl_2$; for DNA polymerase β, 50 mM Tris-HCl, pH 8.2, 1 mM DDT, 0.01% NP 40, 100 mM KCl, and 0.5 mM $MnCl_2$; for DNA polymerase γ, 50 mM Tris-HCl, pH 8.2, 1 mM DDT, 0.01% NP 40, 100 mM KCl, and 0.5 mM $MnCl_2$; and for HIV-1 RT, 50 mM Tris-HCl, pH 8.2, 1 mM DDT, 0.01% NP 40, 100 mM KCl, and 5 mM $MgCl_2$. To the assays for DNA polymerases α, β, and γ, as well as HIV-RT, was added 1.0 A_{260} unit of activated herring sperm DNA. In one series of experiments DNA was replaced by 0.2 A_{260} unit of poly(A)·$oligo(dT)_{10}$. In a third approach endogenous RT from disrupted virions (20 μg with respect to protein) was added to the RT assay, which contained the endogenous viral RNA and 0.1 μg of exogenously added purified tRNA$^{Lys.3}$ but no additional template/primer. The radioactively labeled precursor in all assays was 16 μM [^{3}H]dTTP (400 dpm/pmol). Incubations were performed at 37 °C for 1 h; acid-insoluble radioactivity was then determined (Arya et al., 1985). During that period of time the reaction kinetics of the different enzyme systems used were linear. The inhibition studies were performed as described earlier (Müller et al., 1988b).

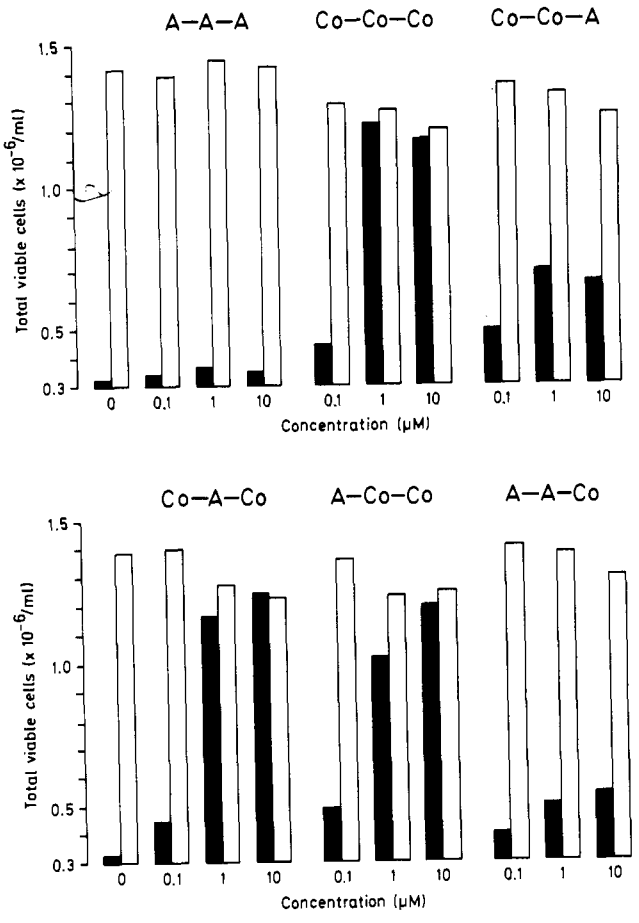

FIGURE 1: Influence of 2-5A and selected 2-5A analogues on cell growth of HIV-infected and uninfected H9 cells in vitro. The number of viable cells was determined; the final concentrations of the compounds are given on the abscissa. Solid bars, virus-treated, and open bars, uninfected control cells. The means of six parallel experiments are shown; the SD does not exceed 10%.

Results

Anti-HIV Activity of Cordycepin Analogues in Intact Cell System. In a previous study we showed that H9 cells, treated with anti-CD3 antibodies, very efficiently bind protein A–liposomes at the cell surface (Renneisen et al., 1990); under the conditions used earlier and also in the present study, approximately 3500 of the 85-nm liposomes bound per cell.

Applying the same approach, selected cordycepin analogues of A_3 (trimer of adenylic acid with 2′,5′-phosphodiester linkages) were first studied for their cytostatic activity. The experiments revealed (data not presented in a table) that A-A-A, Co-Co-A, Co-A-Co, A-A-Co, pA-A-A, and pA-A-Co displayed no cytostatic activity below 30 μM, while Co-Co-Co, A-Co-Co, pCo-Co-A, and pCo-A-Co reduced cell proliferation

Table I: Effect of A_3, Co_3, and Their 5′-Phosphorylated Analogues Encapsulated into Targeted Liposomes on Release of HIV-1 from Infected H9 Cells[a]

encapsulated trimers	concn (μM)	% inhibition of RT	% inhibition of expression of p17	% inhibition of expression of p24
none		0	0	0
A-A-A	1.0	5	10	10
	10.0	9	5	15
Co-Co-Co	1.0	85	65	80
	10.0	90	85	90
Co-Co-A	1.0	25	15	5
	10.0	35	25	15
Co-A-Co	1.0	60	80	75
	10.0	65	85	85
A-Co-Co	1.0	55	75	65
	10.0	60	85	70
A-A-Co	1.0	10	15	5
	10.0	20	25	15
pA-A-A	1.0	10	5	5
	10.0	10	5	5
pCo-Co-Co	1.0	95	90	90
	10.0	100	95	95
pCo-Co-A	1.0	15	5	10
	10.0	25	10	10
pCo-A-Co	1.0	85	75	75
	10.0	90	90	95
pA-Co-Co	1.0	45	50	55
	10.0	55	65	60
pA-A-Co	1.0	5	5	10
	10.0	15	15	10

[a] Liposomes were added 1 h prior to virus infection. The RT activity was determined in the supernatants 6 days post infection; the data are given as percent of inhibition of RT activity, compared to the infected controls. In addition, the values for the inhibition of HIV-1 protein p17 and p24 expression are shown. As a control, the growth rate of uninfected cells is set at 100%; the cell growth of the HIV-infected nontreated cultures was 3%. The means of five parallel experiments are given. The SD does not exceed 15%.

by 50% at concentrations between 20 and 30 μM. The highest cytostatic activities were determined for pCo-Co-Co (ED_{50} concentration of 17.5 μM) and pA-Co-Co (ED_{50} 19.9 μM).

Testing for anti-HIV activity among the core structures revealed that the A-A-A and A-A-Co trimers were without considerable cytoprotective effect up to concentrations of 10 μM, while Co-Co-Co, Co-A-Co, and A-Co-Co at 1 and 10 μM caused an almost total inhibition of viral cytopathic effect (Figure 1). Substitution of the Co trimer by A at the 3′-terminus (Co-Co-A) strongly reduced the cytoprotective effect. The 5′-monophosphate analogues of A-A-A, Co-Co-A, and A-A-Co displayed no impressive cytoprotection while pCo-Co-Co, pCo-A-Co, and pA-Co-Co showed a more than 50% anti-HIV activity at a concentration as low as 1 μM (data not shown).

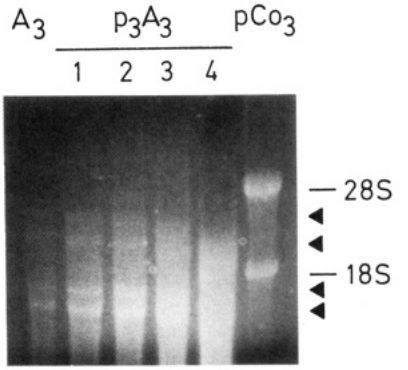

FIGURE 2: Effect of A_3, p_3A_3, and pCo_3 on the RNase L activity, according to the ribosomal RNA cleavage assay. L5178y cell extract was supplemented with A_3 (0.2 μM), p_3A_3 [0.03 μM (track 1); 0.1 μM (track 2); 0.3 μM (track 3); 1 μM (track 4)], or 20 μM pCo_3. The positions of 28S and 18S rRNA as well as the positions of the specific cleavage products (SCP) (arrow-heads) are given.

In order to demonstrate that the observed cytoprotective effect is indeed due to an inhibition of virus production, the following additional parameters were determined: (i) the amount of HIV-1 released from the cells on the basis of the RT activity in the medium and (ii) the percentage of HIV-p17 and p24 positive cells. The results confirm the cytoprotection data (Table I); the strongest anti-HIV activity was measured with Co_3, pCo_3, Co-A-Co, and pCo-A-Co.

Effect on Ribonuclease L. Wreschner et al. (1981) described a sensitive assay for the determination of RNase L activity. This assay is based on the observation that rRNA is cleaved by the enzyme into discrete products in the presence of p_3A_3. As shown in Figure 2, addition of 0.2 μM A_3 or 0.03–1.0 μM p_3A_3 resulted in the appearance of two discrete bands which were termed specific cleavage products; these characteristic cleavage products could not be detected any more at higher p_3A_3 concentrations. In contrast, 20 μM pCo_3 was without any effect on RNase L activity; only the 28S and 18S rRNA bands were visible. These data confirm earlier observations (Sawai et al., 1983) which have been made by

Table II: Effect of 2-5A and 2-5A Analogues and Their 5′-Monophosphates (10 μM) on the Activity of Different Purified Cellular DNA Polymerases and on Purified HIV Reverse Transcriptase[a]

compound	inhibition (%) caused by 10 μM compound polymerase α (act. DNA)	β (act. DNA)	γ (act. DNA)	HIV RT (act. DNA)	HIV RT [poly(A)·(dT)$_{10}$]	disr HIV (endog RNA)
A-A-A	17	5	12	18	12	69
Co-Co-Co	12	7	10	22	15	89
Co-Co-A	6	11	14	16	8	84
Co-A-Co	7	11	8	16	14	87
A-Co-Co	13	9	14	19	13	78
A-A-Co	8	12	9	16	18	74
pA-A-A	12	7	8	18	22	81
pCo-Co-Co	18	9	13	21	28	92
pCo-Co-A	9	11	9	13	18	85
pCo-A-Co	14	8	16	20	21	88
pA-Co-Co	15	16	9	17	20	93
pA-A-Co	9	11	16	19	25	80

[a] The assay conditions are described under Materials and Methods; the standard assay systems were supplemented with 5–10 μg of enzyme protein with the exception of the experiments with disrupted ("disr") virions, for which 20 μg of viral protein was added; the incubation period was 60 min. The nature of template/primer activated ("act.") DNA, endogeneous ("endog") RNA (together with $tRNA^{Lys,3}$), or synthetic poly/oligonucleotides is given in parentheses. The values are means of 5 parallel experiments; the standard deviation did not exceed 14%; the values are given as percent of inhibition (total inhibition, 100%).

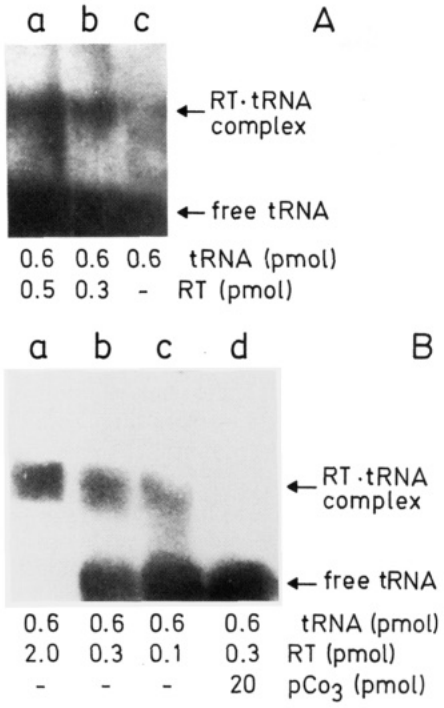

FIGURE 3: Effect of pCo_3 on binding of RT to ^{32}P-$tRNA^{Lys.3}$. The standard gel-retardation assay was composed of 0.6 pmol of ^{32}P-$tRNA^{Lys.3}$ (panel A, lanes a–c; panel B, lanes a–d) and of RT as indicated. One assay was also supplemented with 20 pmol of pCo_3. After incubation the samples were analyzed by electrophoresis as described under Materials and Methods.

using a different assay system for the detection of RNase L activity.

Influence on Activities of Cellular Polymerases and HIV-1 Reverse Transcriptase. In a first set of experiments a constant concentration (10 μM) of trimer cores and 5′-monophosphate derivatives was added to assays containing cellular DNA polymerases (α, β, or γ) or HIV-1 RT. As summarized in Table II, all purified cellular polymerases and also the viral enzyme, under the assay conditions used with activated DNA as template/primer, were inhibited only by 5–22%. Also, with the 5′-monophosphate derivatives the inhibition never exceeded 22%. If the DNA in the RT assays was replaced by the synthetic primer [poly(A)·$(dT)_{10}$], the degree of inhibition was not markedly changed. However, if the nonpurified RT preparation (disrupted HIV virion) was used, together with the endogenous RNA and exogenously added $tRNA^{Lys.3}$ as template/primer, the percent inhibition reached values of greater than 70%; the inhibition was pronounced especially for the cordycepin analogues (Co_3 inhibition by 89%; pCo_3 inhibition by 92%). In control experiments its was established that the reaction measured in the assays with disrupted virions is catalyzed by reverse transcriptase; the template was identified as RNA (digestion with ribonuclease A) and the product as DNA (deoxyribonuclease I digestion) (Müller et al., 1971).

Inhibition of Binding of Reverse Transcriptase onto $tRNA^{Lys.3}$. As described recently (Barat et al., 1989), HIV-1 RT binds to the anticodon domain of $tRNA^{Lys.3}$ which comprises the sequence 5′-A-C-U-S(modified uridine ester)-U-U-R(modified adenosine)-A-Ψ-C-U-G-3′ (the anticodon S-U-U is underlined). We were interested to learn whether this complex formation can be weakened by pCo_3. Consequently, we performed (i) gel-retardation, (ii) dot-blot, and (iii) cross-linking analyses to study the influence of pCo_3 on the interaction between HIV-1 RT and purified ^{32}P-labeled $tRNA^{Lys.3}$

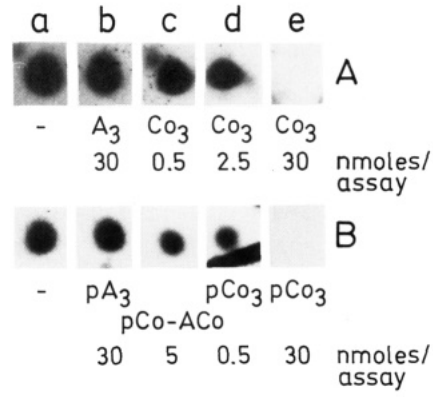

FIGURE 4: Influence of A_3 and Co_3 analogues on binding of ^{32}P-$tRNA^{Lys.3}$ to RT. Recombinant RT [100 ng (=1.5 pmol of RT)] was spotted onto nitrocellulose and subsequently incubated with 1 nmol of ^{32}P-$tRNA^{Lys.3}$ in a final volume of 200 μL either in the absence of a trimer (lanes A-a and B-a) or in the presence of 30 nmol of A_3 (A-b) or pA_3 (B-b), of 0.5–30 nmol of Co_3 (A-c–e), of 5 nmol of pCo-A-Co (B-c), and of 0.5 and 30 nmol of pCo_3 (B-d and -e). The filters were then autoradiographed.

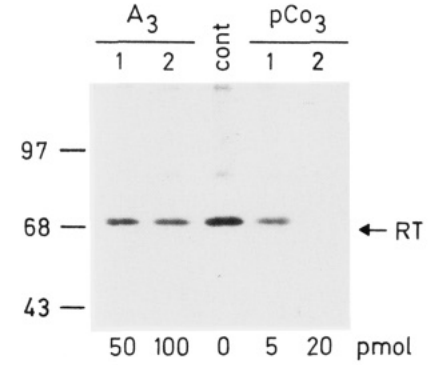

FIGURE 5: Effect of A_3 and Co_3 on complex formation between recombinant RT and ^{32}P-labeled $tRNA^{Lys.3}$. RT (2.0 pmol) was incubated together with tRNA (0.6 pmol) in the absence ("cont") or presence of (i) A_3 (lane 1, 50 pmol; lane 2, 100 pmol) or (ii) Co_3 (lane 1, 5 pmol; lane 2, 20 pmol). Then the reaction product was cross-linked, labeled with ^{32}P, and purified by gel filtration essentially as described under Materials and Methods. The material eluting in the void volume was analyzed by polyacrylamide gel electrophoresis followed by autoradiography. The migration position of HIV RT (not cross-linked to tRNA) was determined in a parallel gel (marked RT). The molecular weight size markers are given at the margin.

The gel-retardation experiments revealed that if given in a molar excess of RT over tRNA (2.0 pmol of RT and 0.6 pmol of ^{32}P-labeled $tRNA^{Lys.3}$), all labeled tRNA was complexed with RT and no free tRNA could be detected (Figure 3B, lane a). In a reversed ratio (0.6 pmol of tRNA and 0.1–0.5 pmol of RT) only a portion of the tRNA could be recovered in the retarded band (Figure 3A, lanes a and b; Figure 3B, lanes b and c). In the absence of RT only free tRNA could be identified (Figure 3A, lane c). Addition of 20 pmol of pCo_3 to the retardation assay completely abolished the binding of 0.6 pmol of tRNA to 0.3 pmol of RT (Figure 3B, lane d). This finding indicated that binding of $tRNA^{Lys.3}$ to RT can be abolished by a 30-fold molar excess of pCo_3 with respect to $tRNA^{Lys.3}$.

In a second approach the influence of Co_3 and its analogues on binding of $tRNA^{Lys.3}$ onto RT was studied by dot-blot analysis. RT, spotted onto nitrocellulose, bound ^{32}P-$tRNA^{Lys.3}$ as already described (Barat et al., 1989) (Figure 4). Addition of A_3 or pA_3 in a 30-fold higher molar ratio with respect to $tRNA^{Lys.3}$ caused only a moderate decrease of the binding efficiency (Figure 4, lane A, part b, and lane B, part b). In contrast, at a 2.5-fold higher ratio Co_3 and pCo_3 displayed an

almost 50% reduction of the binding; at a 30-fold higher ratio the binding of tRNA was negligible (lane A, part e, and lane B, part e). At a 5-fold higher ratio the pCo-A-Co analogue displayed a reduction of the binding to 50% (lane B, part c). This finding indicates that the Co_3 compounds but not A_3 or pA_3 compete with $tRNA^{Lys,3}$ for the binding site at the RT.

In a last approach cross-linking experiments were performed to demonstrate the inhibition of binding of RT (p66 protein) to $tRNA^{Lys,3}$ in the presence of Co_3. As shown in Figure 5, this trimer at an amount of 20 pmol totally prevented the binding of RT to purified ^{32}P-labeled $tRNA^{Lys,3}$ (Figure 5; pCo_3, lane 2). In comparison, at a concentration of 50 pmol of A_3 the suppression of the binding was only approximately 50% and at 100 pmol 60% (Figure 5; A_3 lanes 1 and 2) (based on the intensity of the radioactively labeled band). At a concentration of 5 pmol of pCo_3 the amount of $tRNA^{Lys,3}$ cross-linked to RT was reduced by 80%. The RT–tRNA oligoribonucleotide complex was shifted by 5 kDa relative to the nontreated RT (Figure 5).

Discussion

To date, inhibitors of HIV RT have proven to be the most successful antiviral agents in HIV infection; of particular value has been a class of nucleoside analogues, the dideoxynucleosides, with the prominent members 3′-azido-3′-deoxythymidine and 2,3-dideoxycytidine (Mitsuya & Broder, 1987). These compounds, in their triphosphate stage, inhibit the RT in a competitive manner with respect to their normal substrates, the dNTPs. One disadvantage of compounds like this is their mutagenic potential both for the virus and for the host cell (Cohen, 1987). RT enzyme poisons, e.g., phosphonoacetic acid (Wondrak et al., 1988) or modified DNAs (Müller et al., 1988b), which are devoid of direct mutagenic effects, are apparently less powerful.

In the present study a new strategy to inhibit HIV RT via inhibition of binding of the RT to $tRNA^{Lys,3}$ via the anticodon region (Barat et al., 1989) is outlined. Hitherto, oligonucleotide analogues complementary to a segment of the viral genome, e.g., to the *tat*-III gene splice acceptor and donor sites (Zamecnik et al., 1986), were found to display specific inhibitory effects on expression of HIV-1 in cultured cells. Now we demonstrate that the cordycepin analogues of the physiological products of 2-5A synthetase, pA_3 and A_3, inhibited HIV production in vitro. The compounds were delivered into the cells in a way similar to that described earlier for both DNA (Leonetti et al., 1990) and RNA (Milhaud et al., 1989) encapsulated into liposomes. For the studies reported here the in vitro synthesized Co_3 and pCo_3 analogues were encapsulated in protein A bearing liposomes which were directed to CD3 molecules at the surface of the cells by an anti-CD3 antibody bound to target H9 cells.

The Co_3 and pCo_3 analogues inhibited cell proliferation at concentrations between 20 and 30 μM and acted in an antiviral manner at concentrations around 1 μM. The parent compounds A_3 and pA_3, delivered to the cells in the same way, were without any antiviral effect up to concentrations of 10 μM. A substitution of the 3′-terminal cordycepin by adenosine abolished the antiviral activity of Co_3 and pCo_3 more strongly than the same replacement at the 5′-terminus. This finding is another indication that the intracellular stability of these trimers is controlled by the 2-5A-degrading 2′,3′-exoribonuclease activity which starts hydrolysis from the 5′-terminus (Schröder et al., 1980).

In contrast to A_3 and pA_3 and some of their analogues which activate RNase L and in consequence cause (i) degradation of viral and cellular mRNA and (ii) reduction of viral and cellular protein synthesis (Suhadolnik et al., 1989), our preliminary data (not shown) indicate that the cordycepin analogues did not display such effects. Northern blot hybridization studies revealed that the level amount of cellular GAPDH transcripts remained almost unchanged under conditions that were found to be inhibitory for HIV production. In contrast, the level of HIV-*pol* transcripts was found to be reduced by 40% or by 95% if the HIV-infected cultures were treated with 0.1 or 1 μM pCo_3, respectively. Moreover, our preliminary Western blotting studies suggest that after incubation of the cells for 5 days in the presence of 0.1 μM pCo_3 an 80% decrease in the amount of viral Tat could be measured; at 1 μM no Tat could be determined. In contrast, the level of the cellular protein, tubulin, was not changed under otherwise identical conditions used. These semiquantitative results are a first indication that pCo_3 at concentrations which displayed antiviral activity had no drastic influence on the level of cellular proteins. These data are in accordance with data obtained in a different assay system, using the rRNA cleavage assay (Wreschner et al., 1981). Previous data and the presented results demonstrate that the cordycepin analogues bind to RNase L but do not activate that enzyme (Sawai et al., 1983).

The likely target of the anti-HIV compounds, Co_3 and pCo_3, which had been elucidated in the present study is the inhibition of RT. By use of the "natural" RT assay system, disrupted HIV particles, which contain besides the RT and genomic viral RNA also the $tRNA^{Lys,3}$ primer for the RT reaction [reviewed in Barat et al. (1989)], a 90% inhibition of enzyme activity was detectable. Dot-blot, gel-retardation, and cross-linking experiments revealed that Co_3 and pCo_3 (at concentrations below 5 pmol/10 μL) but not the adenosine analogues (at concentrations below 30 pmol/10 μL) competed with the binding site of the $tRNA^{Lys,3}$ [around the anticodon region which is comprised of four uridines (one of them a modified one) in a row] for the RT. In a different approach, by use of a competition assay, the binding of RT to $oligo(dT)_8$ was inhibited also by tRNAs others than $tRNA^{Lys,3}$ (Sobol et al., submitted for publication). In contrast, in the studies summarized here, we measured directly the effect of 2-5A and p2-5A analogues on the natural primer/template system. At present, we have no experimental data from which an explanation for the failure of the adenosine analogues to bind to the RT can be deduced. In contrast, applying the artificial template/primer system $poly(A)\cdot(dT)_{10}$ instead of viral RNA and $tRNA^{Lys}$ primer, only a 30% inhibition of RT reaction by the cordycepin analogues could be detected. This finding is plausible because no competition of Co_3 or pCo_3 with $(dT)_{10}$ for the primer binding site at the RT can be expected. The other cellular DNA polymerases (α, β, and γ) were insensitive toward A_3, pA_3, and their cordycepin analogues.

From the data presented we can conclude that the Co_3 and pCo_3 analogues of the naturally occurring adenosine analogues do not markedly interfere with the 2′,5′-oligoadenylate synthetase/endoribonuclease L system but display their antiviral activity in vitro via strong inhibition of HIV RT. Moreover, these compounds were found to be metabolically stable (Doetsch et al., 1981); no mutagenic potential of these compounds has been detected in the Ames test (to be published). Therefore, we conclude that Co_3 and pCo_3 represent a novel class of anti-HIV compounds acting at the RT level in a highly specific way.

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