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5′-Modified Agonist and Antagonist (2′–5′)(A)$_n$ Analogues: Synthesis and Biological Activity†

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ABSTRACT: Two 5′-modified (2′–5′)(A)$_4$ oligomers with an increased resistance to phosphatase degradation were synthesized and evaluated for their ability to develop an antiviral response when introduced into intact cells by microinjection or by chemical conjugation to poly(L-lysine). The enzymatic synthesis of 5′-γ-phosphorothioate and β,γ-difluoromethylene (2′–5′)(A)$_4$ from adenosine 5′-*O*-(3-thiotriphosphate) and adenosine β,γ-difluoromethylenetriphosphate by (2′–5′)-oligoadenylate synthetase is described. The isolation and characterization of these (2′–5′)(A)$_4$ analogues were achieved by high-performance liquid chromatography. The structures of 5′-modified tetramers were corroborated by enzyme digestion. These two 5′-modified tetramers compete as efficiently as natural (2′–5′)(A)$_4$ for the binding of a radiolabeled (2′–5′)(A)$_4$ probe to ribonuclease (RNase) L. Nevertheless, at the opposite to 5′-γ-phosphorothioate (2′–5′)(A)$_4$, β,γ-difluoromethylene (2′–5′)(A)$_4$ failed to induce an antiviral response after microinjection in HeLa cells. In addition, it behaves as an antagonist of RNase L as demonstrated by its ability to inhibit the antiviral properties of 5′-γ-phosphorothioate (2′–5′)(A)$_4$ when both are microinjected in HeLa cells. The increased metabolic stability of 5′-γ-phosphorothioate (2′–5′)(A)$_4$ as compared to that of (2′–5′)(A)$_4$ was first demonstrated in cell-free extracts and then confirmed in intact cells after introduction in the form of a conjugate to poly(L-lysine). Indeed, 5′-γ-phosphorothioate (2′–5′)(A)$_4$–poly(L-lysine) conjugate induces protein synthesis inhibition and characteristic ribosomal RNA cleavages for longer times than unmodified (2′–5′)(A)$_4$–poly(L-lysine) in the same cell system. These observations provide evidence for an intracellular activation of RNase L by this 5′-modified (2′–5′)(A)$_4$ analogue. This stabilized (2′–5′)(A)$_n$ agonist and antagonist may be of value for further studies on the biological relevance of the (2′–5′)(A)$_n$ system. Their possible utilization in chemotherapy will be severely restricted by their narrow spectrum of antiviral activity.

The (2′–5′)(A)$_n$[1] system now appears to be a part of the antiviral response of cells to interferons (IFNs) and is thought to be involved in the control of cell growth and differentiation [see Johnston and Torrence (1984) and Silverman (1984) for recent reviews]. Agonists and antagonists of RNase L, the effector enzyme in this system, thus appear as possible tools for a better understanding of the biological relevance of the (2′–5′)(A)$_n$ system as well as for antiviral and antineoplastic chemotherapy. Use for these purposes necessitates solving certain problems such as poor penetration in intact cells and low metabolic stability of (2′–5′)(A)$_n$ oligomers. A number of methods of introduction of (2′–5′)(A)$_n$ into intact cells have

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[1] Abbreviations: VSV, vesicular stomatitis virus; EMCV, encephalomyocarditis virus; m.o.i., multiplicity of infection; (2′–5′)(A)$_n$, a series of oligomers of adenylic acid with (2′–5′)-phosphate ester linkages and a triphosphate at the 5′-end, with n usually >2; "core" of (2′–5′)(A)$_n$ and its derivatives, (2′–5′)(A)$_n$ lacking the 5′-triphosphate; ATP-γ-S, adenosine 5′-*O*-(3-thiotriphosphate); ATP-β,γ-CF$_2$, adenosine β,γ-difluoromethylenetriphosphate; IFN, interferon; MEM, minimal essential medium; poly(rI)·poly(rC), poly(riboinosinic acid)·poly(ribocytidylic acid); HPLC, high-performance liquid chromatography; BAP, bacterial alkaline phosphatase; DEAE, diethylaminoethyl; RNase, ribonuclease; pfu, plaque-forming unit(s). Enzymes: bacterial alkaline phosphatase (EC 3.1.3.1); snake venom phosphodiesterase I (EC 3.1.4.1); T4 RNA ligase (EC 6.5.1.3); (2′–5′)-oligoadenylate synthetase (EC 2.7.7); (2′–5′)-(A)$_n$-dependent endoribonuclease or RNase L (EC 3.1.2.7); ribonuclease T$_2$ or T$_2$ RNase (ribonuclease 3′-oligonucleotidohydrolase from *Aspergillus oryzae*; EC 3.1.4.23).

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been found, e.g., encapsulation in liposomes (Bayard et al., 1985b) or chemical conjugation to a synthetic polypeptide such as poly(L-lysine) (Bayard et al., 1986). As far as (2′–5′)(A)$_n$ catabolism is concerned, emphasis has been put on stabilization against phosphodiesterases, essentially through modifications of (2′–5′)(A)$_n$ oligomers at their 3′-end [Bayard et al. (1984) and references cited therein]. The possible contribution of phosphatases to the catabolism of (2′–5′)(A)$_n$ oligomers has been barely studied although it deserves attention. Indeed, the affinity for RNase L of 5′-dephosphorylated or "core" (2′–5′)(A)$_n$ is about 3 orders of magnitude lower than for the corresponding di- or triphosphorylated oligomers (Knight et al., 1980). 5′-Rephosphorylation of core (2′–5′)(A)$_n$ does not seem to occur in cell extracts or in intact cells (Williams et al., 1978; Eppstein et al., 1983). Moreover, monophosphate (2′–5′)(A)$_n$ derivatives behave as competitive inhibitors of (2′–5′)(A)$_n$ in RNase L activation, at least in some cell systems (Torrence et al., 1981). Direct evidence for the dephosphorylation of (2′–5′)(A)$_n$ in cell extracts, as well as indirect arguments for the occurrence of this catabolic pathway in intact cells, has been obtained with phosphodiesterase-resistant 3′-modified (2′–5′)(A)$_n$ oligomers (Bayard et al., 1984).

It was thus of interest to devise 5′-end modifications of (2′–5′)(A)$_n$ oligonucleotides to provide an increased resistance to phophatases while their structural characteristics that allow their binding to and/or activation of RNase L were maintained intact. This was found to be more restrictive than modifying (2′–5′)(A)$_n$ at its 3′-end since the 5′ region of the molecule is directly involved in interaction with RNase L. Some 5′-modified (2′–5′)(A)$_n$ derivatives have recently been described, including 5′-capped derivatives of (2′-5′)(A)$_n$ (Imai & Torrence, 1984), 5′-α-phosphorothioate (2′–5′)(A)$_n$ analogues (Lee & Suhadolnik, 1985), and *N*-decyl-(2′–5′)(A)$_n$ (Kariko & Ludwig, 1985). Partial characterization has revealed either increased (Lee & Suhadolnik, 1985; Kariko & Ludwig, 1985) or decreased (Imai & Torrence, 1984) activity. On the other hand, a 5′-methylphosphorothioate derivative of (2′–5′)(A)$_n$ was observed to be an interesting inhibitor of RNase L in L929 cells (Haugh et al., 1983; Watling et al., 1985).

We synthesized and briefly described two 5′-modified (2′–5′)(A)$_n$ tetramers (Bayard et al., 1985a; Bisbal et al., 1985) namely, γ-thiotriphosphate and β,γ-difluoromethylene derivatives, and report here further characterization and their biological activity as RNase L agonist or antagonist.

Materials and Methods

Materials. Media for cell cultures were obtained from Eurobio and sera from Flow Laboratories. Poly(rI)·poly(rC) and T4 RNA ligase were supplied from Pharmacia. Bacterial alkaline phosphatase type III-R, phosphodiesterase I type VII, and ribonuclease T_2 were obtained from Sigma. Adenosine triphosphate was supplied by Merck and adenosine 5′-*O*-(3-thiotriphosphate) by Boehringer. The β,γ-difluoromethylene ATP analogue was synthesized as described by Blackburn et al. (1984).

Poly(L-lysine) (M_r 14000) was from Sigma. [^{35}S]-Methionine (specific activity 1109 Ci/mmol) and adenosine 5′-*O*-[^{35}S](3-thiotriphosphate) (sp act. 800 Ci/mmol) were purchased from New England Nuclear. Human lymphoblastoid interferon (α-βHuIFN) purified to a specific activity of 10^7 IU/mg of protein was kindly provided by Dr. J. Carcagne (Institut Merieux, Lyon).

Cell Cultures and Viruses. L929 cells were grown in minimal essential medium supplemented with 5% (v/v) donor horse serum, 3 g/L bactotryptose phosphate broth, 3.4 g/L glucose, 60 IU/mL penicillin, and 50 μg/mL streptomycin. HeLa cells and L1210 cells were maintained in RPMI 1640 medium supplemented by 5% (v/v) fetal calf serum and antibiotics as above.

Vesicular stomatitis virus (VSV; Indiana strain), Mengo virus, encephalomyocarditis virus (EMCV), and vaccinia virus, were grown in L929 cell monolayers and titrated by plaque assay.

Enzymatic Synthesis, Isolation, and Characterization of (2′–5′)(A)$_n$. (2′–5′)(A)$_n$ oligonucleotides were synthesized enzymatically from ATP (or from the appropriate ATP analogue) with (2′–5′)-oligoadenylate synthetase from HeLa cell extracts as described by Minks et al. (1979). (2′–5′)(A)$_n$ oligomers were size-fractionated by HPLC chromatography on a TSK-DEAE column (LKB) eluted with a linear gradient of 125–500 mM triethylammonium bicarbonate buffer, pH 8.5 (TEAB). Fractions containing individual oligomers were pooled, concentrated under vacuum, and coevaporated 3 times with water in order to remove the TEAB. Chain lengths of purified oligomers were characterized by HPLC on a μBondapak C_{18} column in 50 mM ammonium phosphate buffer, pH 7.2, according to Brown et al. (1981).

Chemical Modifications of (2′–5′)(A)$_4$. (2′–5′)(A)$_4$ oligomers or 5′-modified (2′–5′)(A)$_4$ analogues (1 μmol) were modified at the 3′-terminal ribose residue in order to increase their stability with regard to phosphodiesterase degradation, as previously described (Bayard et al., 1984). The purity of the modified oligomers was assessed by HPLC (Brown et al., 1981). Dephosphorylated or "core" derivatives were obtained by enzymatic digestion of (2′–5′)(A)$_n$ with bacterial alkaline phosphatase.

Conjugation of (2′–5′)(A)$_4$ and γ-S-(2′–5′)(A)$_4$ to Poly(L-lysine). (2′–5′)(A)$_4$ and γ-S-(2′–5′)(A)$_4$ were coupled with poly(L-lysine) as described previously (Bayard et al., 1986). Sodium metaperiodate (0.6 μmol in 0.1 M sodium acetate buffer, pH 4.75) was added to an ice-cold solution of (2′–5′)(A)$_4$ (0.6 μmol) in 400 μL of distilled water. The reaction mixture was stirred on ice for 30 min, and 400 μL of poly(L-lysine) (0.14 μmol in 0.2 M phosphate buffer, pH 8.0) and 200 μL of sodium cyanoborohydride (20 μmol in 0.2 M phosphate buffer, pH 8.0) were added. The mixture was incubated for 2 h at room temperature and then loaded on a Sephadex G-50 column equilibrated with 0.1 M sodium acetate buffer, pH 4.75. Each fraction was assayed for its (2′–5′)-(A)$_4$–poly(L-lysine) content by the method described by Lowry et al. (1951) and by absorbance at 260 nm.

Stability of (2′–5′)(A)$_4$ Analogues. The stability of (2′–5′)(A)$_4$ analogues was estimated in HeLa cell extracts by measuring the disappearance of oligonucleotides. (2′–5′)(A)$_4$ (5 μL) was incubated at a final concentration of 0.02 mM with 5 μL HeLa cell extract (22 mg of protein/mL). The reaction was stopped by heating at 100 °C for 2 min and was centrifuged at 10000*g* for 10 min. (2′–5′)(A)$_3$ (1 nM) was added to the supernatant as an internal standard, and residual products were quantified by HPLC as described above.

Radiobinding Assay for (2′–5′)(A)$_4$ and (2′–5′)(A)$_4$–Poly(L-lysine) Conjugates. Radiobinding assays were performed according to Knight et al. (1980), using an S30 unfractionated bovine spleen extract as source of (2′–5′)(A)$_n$–dependent endoribonuclease (RNase L). The labeled probe (2′–5′)ApCp was synthesized by ligation of [^{32}P]pCp (sp act. 3000 Ci/mmol) on (2′–5′)(A)$_4$ with T4 RNA ligase. (Silverman et al., 1981).

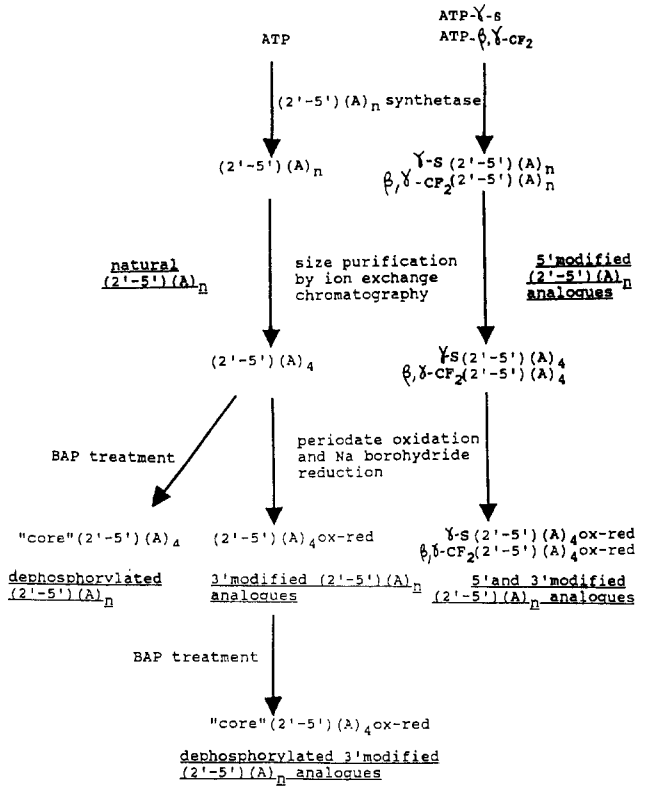


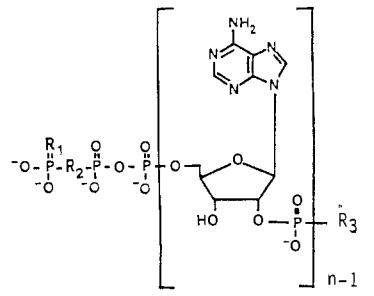
FIGURE 1: Synthesis and chemical modification of $(2'–5')(A)_n$ oligomers.

Cell Microinjection. HeLa cells were grown on small pieces of glass (2 mm^2) at densities that allowed about 200 cells to become attached to each glass fragment as previously described (Graessman & Graessman, 1983). An average volume of 0.5 pL (i.e., approximately one-tenth of the cell volume) was injected in the cytoplasm of each cell with 0.5-μm diameter glass micropipets. Injections were monitored under a Leitz Diavert phase-contrast microscope with a magnification of 320.

Protein Synthesis Assay. L1210 cell cultures (2.5×10^5 cells) were incubated with $(2'–5')(A)_4$–poly(L-lysine) conjugates as specified in the legends of the figures, washed in methionine-free RPMI 1640 medium, and labeled for 30 min at 37 °C with 10 μCi of [^{35}S]methionine (sp act. 1109 Ci/mmol) in 0.25 mL of methionine-free medium supplemented by 5% (v/v) fetal calf serum at the times indicated in the individual experiments. Cells were then processed for protein synthesis analysis. In some experiments ^{35}S-labeled proteins were analyzed by 10% (w/v) polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970). Labeled proteins were located by fluorography after exposure to 10% (v/v) 2,5-diphenyloxazole in dimethyl sulfoxide (Laskey & Hills, 1975).

Assay for Ribosomal RNA Cleavage in Intact Cells. A total of 5×10^6 L1210 cells was incubated with $(2'–5')(A)_4$–poly(L-lysine) conjugate as indicated in the legends of the figures. Denatured cytoplasmic RNAs were isolated and analyzed by agarose gel electrophoresis as described previously (Hugues & Robins, 1983). After electrophoresis at 50 mA for 2 h, the gels were stained with ethidium bromide and the RNA bands were examined in an UV light box.

Assay of Antiviral Activity. Cells were infected at the times indicated (usually 1 h after microinjection) with VSV, Mengo virus, EMCV, or vaccinia virus at a multiplicity of 10 for 1 h at 37 °C in RPMI medium supplemented with 5% (v/v) fetal calf serum. Unadsorbed viruses were carefully removed by three washings with RPMI containing 10% (v/v) fetal calf serum. The viruses produced were titrated 18 h later by an end-point assay as described previously (Milhaud et al., 1983).



with R_1 representing an O or an S atom

R_2 representing an O atom or a CF_2 group

R_3 representing:

a) an adenosine residue

b) an adenosine residue with an open residue ring

c) an adenosine residue conjugated to ε-amino group of poly(L-lysine)

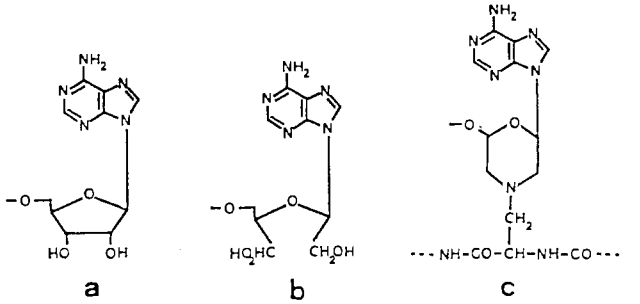


FIGURE 2: Structure of $(2'–5')(A)_n$ and its analogues. Nomenclature adopted throughout was as follows: For $(2'–5')(A)_4$, R_1 = O, R_2 = O, R_3 = a, n = 4; for $(2'–5')(A)_4$(oxred), R_1 = O, R_2 = O, R_3 = b, n = 4; for $(2'–5')(A)_4$–poly(L-lysine), R_1 = O, R_2 = O, R_3 = c, n = 4; for γ-S-$(2'–5')(A)_4$, R_1 = S, R_2 = O, R_3 = a, n = 4; for γ-S-$(2'–5')(A)_4$(oxred), R_1 = S, R_2 = O, R_3 = b, n = 4; for γ-S-$(2'–5')(A)_4$–poly(L-lysine), R_1 = S, R_2 = O, R_3 = c, n = 4; for β,γ-CF_2-$(2'–5')(A)_4$, R_1 = O, R_2 = CF_2, R_3 = a, n = 4; for β,γ-CF_2-$(2'–5')(A)_4$(oxred), R_1 = O, R_2 = CF_2, R_3 = b, n = 4; for β,γ-CF_2-$(2'–5')(A)_4$–poly(L-lysine), R_1 = O, R_2 = CF_2, R_3 = c, n = 4.

RESULTS

Synthesis and Characterization of 3'- and 5'-Modified $(2'–5')(A)_4$ Analogues. The method of synthesizing $(2'–5')(A)_n$ analogues outlined in Figure 1 was used. It led to $(2'–5')(A)_4$ oligomers and to 3'-, 5'-, or 3'–5'-modified analogues (Figure 2). Most of the studies described here were carried out with $(2'–5')(A)_4$ and its analogues after purification by ion-exchange chromatography. Tetramers were chosen because 3' modification of $(2'–5')(A)_4$ oligomers (see Figures 1 and 2) opens the 3'-terminal ribose ring and leaves unchanged the trinucleotide moiety known to be important for RNase L binding and activation (Johnston & Torrence, 1984). $(2'–5')(A)_n$ synthetase converted ATP, ATP-γ-S and ATP-β,γ-CF_2 to the corresponding $(2'–5')(A)_n$ oligomers after 4-h incubation; yields were approximately 80%, 60% and 15% respectively. The disgestion of oligomers by snake venom phosphodiesterase I but not by T_2 RNase demonstrated the presence of the 2',5'linkage.

Stability and Activity of $(2'–5')(A)_4$ and Its Analogues in Cell-Free Extracts. The stability of $(2'–5')(A)_4$ and its analogues was determined by using HPLC to monitor the disappearance of these substances after incubation in HeLa cell extracts (Figure 3). No increase in stability was observed

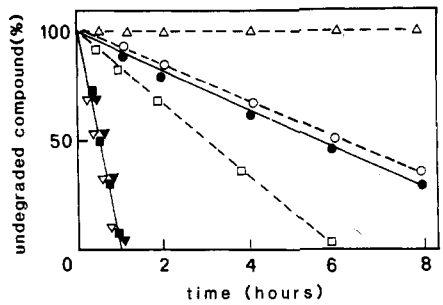


FIGURE 3: Stability of (2′–5′)(A)$_4$ and analogues in HeLa cell extracts. (2′–5′)(A)$_4$ (■), (2′–5′)(A)$_4$(oxred) (□), γ-S-(2′–5′)(A)$_4$(oxred) (●), β,γ-CF$_2$-(2′–5′)(A)$_4$(oxred) (○), core (2′–5′)(A)$_4$(oxred) (△), γ-S-(2′–5′)(A)$_4$ (▼), and β,γ-CF$_2$-(2′–5′)(A)$_4$ (▽) were incubated in HeLa cell extracts. Incubation was stopped by boiling at the times indicated, and denatured proteins were removed by centrifugation. Residual oligomers in the supernatant were analyzed by HPLC as described under Materials and Methods.

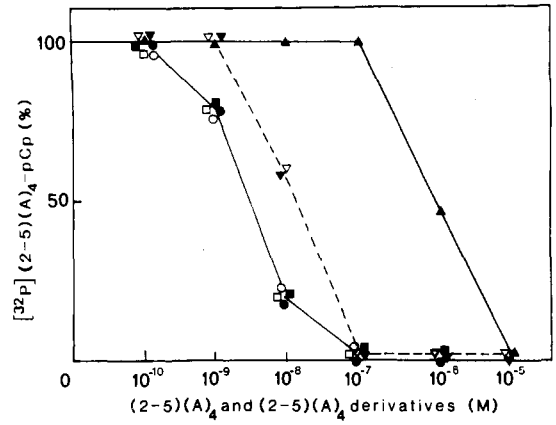


FIGURE 4: Binding of (2′–5′)(A)$_4$, (2′–5′)(A)$_4$ analogues, and (2′–5′)(A)$_4$–poly(L-lysine) conjugates to RNase L. (2′–5′)(A)$_4$ (■) (2′–5′)(A)$_4$(oxred) (□), γ-S-(2′–5′)(A)$_4$(oxred) (●), β,γ-CF$_2$-(2′–5′)(A)$_4$(oxred) (○), core (2′–5′)(A)$_4$ (▲), (2′–5′)(A)$_4$–poly(L-lysine) (▽), and γ-S-(2′–5′)(A)$_4$–poly(L-lysine) (▼) were compared for their ability to inhibit the binding of the radioactive [^{32}P](2′–5′)(A)$_4$pCp probe to RNase L in the radiobinding assay.

for 5′-modified (i.e., phosphatase-protected) (2′–5′)(A)$_4$ analogues. 3′-Modified (i.e., phosphodiesterase-protected) (2′–5′)(A)$_4$ "cores" were almost completely stable while unprotected (2′–5′)(A)$_4$ "cores" were degraded as rapidly ($t_{1/2}$ = 30 min) as is (2′–5′)(A)$_4$ (data not shown). Phosphorylated 3′-modified (2′–5′)(A)$_4$, i.e., (2′–5′)(A)$_4$(oxred), was still degraded at an appreciable rate ($t_{1/2}$ = 2.5 h) essentially through dephosphorylation (Bayard et al., 1984). Both the 5′- and 3′-modified (2′–5′)(A)$_4$ analogues (i.e., protected against phosphatases and phosphodiesterases) described in Figure 2 displayed increased stability ($t_{1/2}$ = 8 h). Both phosphodiesterases and phosphatases thus contribute to (2′–5′)(A)$_n$ catabolism, at least in cell-free extracts.

Binding of (2′–5′)(A)$_4$ and Its Analogues to RNase L. The affinities of (2′–5′)(A)$_4$ and its analogues for RNase L were compared by using the radiobinding assay described by Knight et al. (1980). This procedure is based on the ability of (2′–5′)(A)$_4$ analogues to compete with a ^{32}P-labeled (2′–5′)(A)$_4$ probe for specific binding to RNase L. The displacement curves illustrated in Figure 4 reveal no significant differences in the affinity of (2′–5′)(A)$_4$ and its 5′- or 3′-modified analogues for RNase L. Dephosphorylation of the 5′-terminal triphosphate moiety resulted in a dramatic decrease in the ability to bind RNase L. Conjugation of both (2′–5′)(A)$_4$ and γ-S-(2′–5′)(A)$_4$ to poly(L-lysine) (vide infra) resulted in a significant decrease in affinity for RNase L.

Table I: Antiviral Activity of Microinjected (2′–5′)(A)$_4$ and Analogues against VSV[a]

treatment	oligomer concn	virus yield (pfu/200 cells)	% of control
H_2O		3.3×10^5	100
(2′–5′)(A)$_4$(oxred)	10 μM	$<10^2$	<0.01
(2′–5′)(A)$_4$(oxred)	100 nM	2.6×10^2	0.08
(2′–5′)(A)$_4$(oxred)	10 nM	1.1×10^3	0.33
H_2O		4.0×10^5	100
γ-S-(2′–5′)(A)$_4$(oxred)	10 μM	$<10^2$	<0.01
γ-S-(2′–5′)(A)$_4$(oxred)	100 nM	$<10^2$	<0.01
γ-S-(2′–5′)(A)$_4$(oxred)	10 nM	$<10^2$	<0.01
γ-S-(2′–5′)(A)$_4$(oxred)	1 nM	$<10^2$	<0.01
H_2O		2.2×10^4	100
ATP-γ-S	10 μM	1.3×10^4	59
H_2O		1.3×10^4	100
β,γ-CF$_2$-(2′–5′)(A)$_4$(oxred)	10 μM	6.1×10^3	47

[a] Two hundred HeLa cells were microinjected with 0.5 pL each of (2′–5′)(A)$_4$ or analogues at the indicated concentration. One hour after, cells were challenged with VSV at a m.o.i. of 10, and the virus titers were determined 18 h later by an end-point assay in L929 cells. Since 0.5 pL represents approximately one-tenth of the cell volume, final intracytoplasmic concentrations of the oligomers are one-tenth of the values listed in this table and Tables II and III.

Biological Activity of (2′–5′)(A)$_4$ and Its Analogues in Intact Cells. Microinjection of (2′–5′)(A)$_4$ and its analogues into intact cells with micropipets (Bayard et al., 1984; Defilippi et al., 1986) enabled direct comparison of their antiviral and growth-inhibitory activities.

3′-Modified (2′-5′)(A)$_4$ derivatives were compared in dose–response experiments to 5′- and 3′- modified (2′–5′)(A)$_4$ analogues for their capacity to inhibit the multiplication of vesicular stomatitis virus (VSV) inoculated at a m.o.i. of 10 into HeLa cells 1 h after microinfection. As illustrated in Table I, 3′-modified (2′–5′)(A)$_4$, i.e., (2′–5′)(A)$_4$(oxred), causes complete inhibition of VSV growth when microinjected at 10 μM and still reduces virus output by more than 2 log units at 10 nM (e.g., at an approximate concentration of 1 nM in cell cytoplasm).

The 3′- and 5′-modified tetramer, i.e., γ-S-(2′–5′)(A)$_4$(oxred), is even more potent in reducing VSV growth since inhibition was still complete at 1 nM. A lower limit has not been given in Table I since it varies from one experiment to another in the 100 pM–1 nM range. β,γ-CF$_2$-(2′–5′)(A)$_4$(oxred) had no significant antiviral activity whether VSV (Table I) or EMCV (data not shown) was used as virus challenge.

The growth properties of uninfected cells microinjected with (2′–5′)(A)$_4$ or its stabilized analogues were not significantly affected over a 3-day period at doses (10 nM) promoting pronounced antiviral activity (data not shown).

To examine whether these (2′-5′)(A)$_4$ analogues exhibit a broad spectrum of antiviral activity as IFNs themselves, two commonly used and unrelated laboratory virus species were compared to VSV for their sensitivity to (2′–5′)(A)$_4$ and its analogues. Neither (2′–5′)(A)$_4$(oxred) nor γ-S-(2′–5′)(A)$_4$-(oxred) significantly affected the multiplication of EMCV, vaccinia virus (Table II), or Mengo virus (data not shown) when microinjected at doses up to 1 μM into HeLa cell cytoplasm.

Stability of (2′–5′)(A)$_4$ and Its Analogues in Intact Cells. (2′–5′)(A)$_4$ and analogues were coupled with poly(L-lysine) to gain further information about their activity. Such conjugates constitute an efficient tool for the introduction of (2′–5′)(A)$_n$ into intact cells (Bayard et al., 1986), thus allowing evaluation of biochemical parameters linked to RNase L activation, such as protein synthesis inhibition or the characteristic cleavage pattern of ribosomal RNAs (rRNAs) in

Table II: Spectrum of Antiviral Activity of Microinjected $(2'-5')(A)_4$ and Analogues[a]

treatment	oligomer concn	virus	virus yield (pfu/200 cells)	% of control
H_2O		EMCV	5.5×10^6	100
$(2'-5')(A)_4$(oxred)	1 μM	EMCV	1.8×10^6	33
$(2'-5')(A)_4$(oxred)	10 nM	EMCV	1.5×10^6	27
H_2O			1.2×10^6	100
γ-S-$(2'-5')(A)_4$(oxred)	1 μM	EMCV	3.3×10^6	>100
γ-S-$(2'-5')(A)_4$(oxred)	10 nM	EMCV	3.1×10^6	>100
H_2O		vaccinia	2.3×10^6	100
$(2'-5')(A)_4$(oxred)	1 μM		8.9×10^6	>100
$(2'-5')(A)_4$(oxred)	10 nM	vaccinia	8.2×10^5	36
H_2O		vaccinia	2.3×10^6	100
γ-S-$(2'-5')(A)_4$(oxred)	1 μM	vaccinia	6.0×10^6	>100
γ-S-$(2'-5')(A)_4$(oxred)	10 nM	vaccinia	1.8×10^6	81

[a] Two hundred HeLa cells were microinjected with 0.5 pL each of $(2'-5')(A)_4$ or analogue at the concentration indicated and challenged 1 h later with EMCV or vaccinia virus at a m.o.i. = 10. Virus yields were determined 18 h later by an end-point assay.

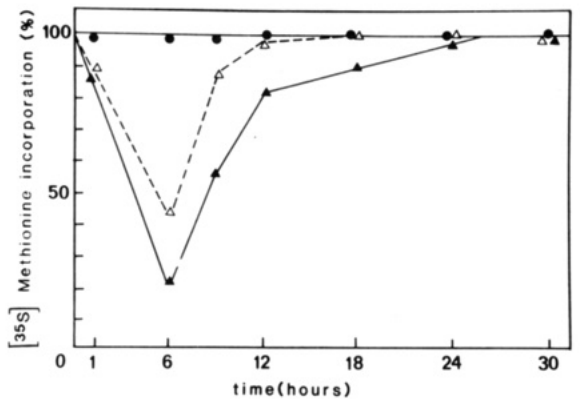


FIGURE 5: Kinetics of inhibition of protein synthesis by $(2'-5')(A)_4$–poly(L-lysine) or γ-S-$(2'-5')(A)_4$–poly(L-lysine). L1210 cells were treated with acetate buffer (●), 9×10^{-7} M $(2'-5')(A)_4$–poly(L-lysine) (△), or 9×10^{-7} M γ-S-$(2'-5')(A)_4$–poly(L-lysine) (▲) [expressed in $(2'-5')(A)_4$ concentration]. The molar ratio of $(2'-5')(A)_4$ to poly(L-lysine) was 2:1. Protein synthesis was monitored at the times shown by pulsing with [^{35}S]methionine for 30 min, and the amount of acid-precipitable radioactivity was determined as described under Materials and Methods.

uninfected cells (Wreschner et al., 1981).

The kinetics of protein synthesis inhibition in cells incubated with 5′-unprotected or 5′-modified $(2'-5')(A)_4$–poly(L-lysine) conjugates are shown in Figure 5. A slight but significant inhibitory effect was observed 1 h after the start of incubation, and maximum inhibition was reached about 6 h after exposure of cells to the conjugate. γ-S-$(2'-5')(A)_4$–poly(L-lysine) inhibits rather more protein synthesis than does $(2'-5')(A)_4$–poly(L-lysine) (Figure 5). β,γ-CF_2-$(2'-5')(A)_4$–poly(L-lysine) or poly(L-lysine) alone did not reduce protein synthesis (data not shown). The most striking difference between the two active conjugates resides however in the increased length of time during which γ-S-$(2'-5')(A)_4$–poly(L-lysine) inhibits protein synthesis as compared to $(2'-5')(A)_4$–poly(L-lysine) at the same extracellular concentration. Although the actual intracellular concentrations of $(2'-5')(A)_4$ or $(2'-5')(A)_4$ analogues obtained by this procedure have not been determined, the differences observed between these conjugates are probably not caused by the efficiency with which they are internalized in intact cells. Indeed, a more sustained inhibition of cellular protein synthesis has also been observed when γ-S-$(2'-5')(A)_4$(oxred) is microinjected directly into HeLa cells, as compared to $(2'-5')(A)_4$(oxred). Likewise, β,γ-CF_2-$(2'-5')(A)_4$(oxred) did not significantly inhibit cellular

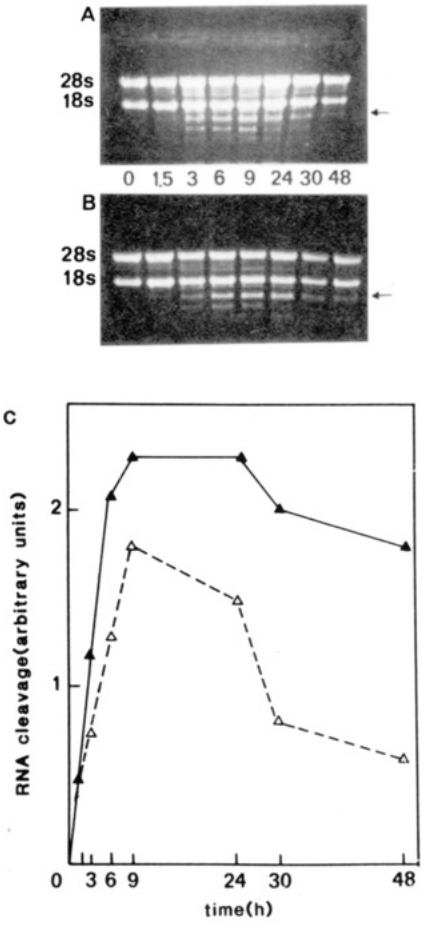


FIGURE 6: Ribosomal RNA cleavage assay by $(2'-5')(A)_4$–poly(L-lysine) or γ-S-$(2'-5')(A)_4$–poly(L-lysine) in L1210 cells. (A) Cells were treated for 0, 1.5, 3, 6, 9, 24, 30, and 48 h with 500 nM $(2'-5')(A)_4$–poly(L-lysine) conjugate. RNAs were extracted, denatured, and analyzed by 1.2% (v/v) agarose gel electrophoresis as described under Materials and Methods. The agarose gel was stained with ethidium bromide for 1 h and photographed under UV illumination. Positions of the 28S and 18S rRNAs as well as locations of the major specific cleavage products are shown by arrows. (B) Cells were treated as described with 500 nM γ-S-$(2'-5')(A)_4$–poly(L-lysine). All conditions were the same. (C) Densitometric analysis of tracks from (A) (△) or (B) (▲). The cleavage band marked with an arrow in panels A and B was quantified with a Vernon densitometer. The amount of cleavage product is represented in arbitrary units.

protein synthesis upon microinjection (data not shown).

Activated RNase L degrades rRNAs into highly characteristic cleavage products when activated by $(2'-5')(A)_n$ or $(2'-5')(A)_n$ analogues that have been introduced into intact cells either by calcium phosphate coprecipitation (Wreschner et al., 1981) or by poly(L-lysine) conjugation (Bayard et al., 1986). This provides another way of comparing the biological activities and functional stabilities of $(2'-5')(A)_n$ and its 5′-modified analogues in intact cells. Both $(2'-5')(A)_4$–poly(L-lysine) (Figure 6A) and γ-S-$(2'-5')(A)_4$–poly(L-lysine) (Figure 6B) gave rise to the characteristic cleavage patterns of rRNAs (Wreschner et al., 1981). Once again, γ-S-$(2'-5')(A)_4$–poly(L-lysine) mainly differs from $(2'-5')(A)_4$–poly(L-lysine) by its prolonged activity in intact cells. This is seen more clearly in Figure 6C where the intensity of one of the cleavage bands (indicated by an arrow) was recorded by densitometric scanning of the autoradiographs shown in Figure 6A,B. Poly(L-lysine) alone or β,γ-CF_2-$(2'-5')(A)_4$–poly(L-lysine) conjugates do not significantly degrade rRNAs (data not shown).

β,γ-CF_2-$(2'-5')(A)_4$(oxred) as an Antagonist of RNase L Activation. The biological and metabolic properties of β,γ-

Table III: Microinjected β,γ-CF_2-(2′–5′)(A)$_4$(oxred) Inhibits the Antiviral Activity of (2′–5′)(A)$_4$(oxred)[a]

first injection	second injection	VSV yield (pfu/200 cells)
H_2O	H_2O	5.3×10^5
1 μM (2′–5′)(A)$_4$(oxred)	H_2O	1.8×10^2
1 μM β,γ-CF_2-(2′–5′)(A)$_4$(oxred)	1 μM (2′–5′)(A)$_4$(oxred)	2.6×10^5
H_2O	H_2O	1.7×10^5
H_2O	1 μM (2′–5′)(A)$_4$(oxred)	$<10^2$
100 nM β,γ-CF_2-(2′–5′)(A)$_4$(oxred)	H_2O	1.4×10^5
100 nM β,γ-CF_2-(2′–5′)(A)$_4$(oxred)	1 μM (2′–5′)(A)$_4$(oxred)	3.8×10^3
H_2O	H_2O	9.7×10^5
H_2O	1 μM (2′–5′)(A)$_4$(oxred)	1.7×10^2
1 μM β,γ-CF_2-(2′–5′)(A)$_4$(oxred)	1 μM (2′–5′)(A)$_4$(oxred)	1.6×10^4
100 nM β,γ-CF_2-(2′–5′)(A)$_4$(oxred)	1 μM (2′–5′)(A)$_4$(oxred)	1.2×10^4
10 nM β,γ-CF_2-(2′–5′)(A)$_4$(oxred)	1 μM (2′–5′)(A)$_4$(oxred)	$<10^2$

[a] Two hundred HeLa cells were microinjected at an interval of 1 h with 0.5 pL each of the compounds as indicated. Cells were challenged with VSV 1 h after the last injection at a m.o.i. = 10. Virus yields were determined 18 h later by an end-point assay.

CF_2-(2′–5′)(A)$_4$(oxred) described in previous sections led us to expect it to be another analogue inhibitor of RNase L.

The experiments described in Table III strongly suggest that β,γ-CF_2-(2′–5′)(A)$_4$(oxred) effectively behaves as an analogue inhibitor of RNase L in human cells. In these experiments, β,γ-CF_2-(2′–5′)(A)$_4$ was microinjected in HeLa cells 1 h before (2′–5′)(A)$_4$(oxred) and shown to display dose–dependent inhibition of the antiviral activity of the latter compound.

Discussion

We have described the synthesis and characterization in terms of stability and biological activity of a series of 5′-, 3′-, or 5′- and 3′-modified (2′–5′)(A)$_4$ oligonucleotides.

Discrete modifications of the terminal ribose residue of (2′–5′)(A)$_4$ oligomers give them almost complete resistance to phosphodiesterases and a concomitant substantial increase in biological activity. In these experiments, (2′–5′)(A)$_4$(oxred) inhibited VSV multiplication to the same extent as *N*-morpholino-(2′–5′)(A)$_n$, another 3′-stabilized (2′–5′)(A)$_n$ analogue (Defilippi et al., 1986), when introduced into HeLa cells by microinjection. 5′-γ-Phosphorothioate (2′–5′)(A)$_n$ analogues display increased stability toward bacterial alkaline phosphatase (data not shown) or to phosphatases in cell–free extracts. The increased antiviral activity of such 5′- and 3′-modified (2′–5′)(A)$_4$ analogues when microinjected in HeLa cells, or their prolonged biological activity (protein synthesis inhibition, rRNA cleavages) when introduced in L1210 cells in the form of poly(L-lysine) conjugates, is consistent with enhanced resistance to phosphatase degradation as well. Seen as a whole, these and previous data (Bayard et al., 1984) support contribution of phosphatase to (2′–5′)(A)$_n$ catabolism in addition to the well-established role of phosphodiesterases (Schmidt et al., 1979).

These (2′–5′)(A)$_n$ analogues have a narrow spectrum of antiviral activity. Indeed, neither picornaviruses [Defilippi et al. (1986) and data presented here] nor vaccinia virus (our data) is sensitive to exogenous (2′–5′)(A)$_n$ at least in amounts that do not inhibit cell growth itself. A possible explanation has been proposed for picornaviruses (Defilippi et al., 1986). These viruses indeed inactivate RNase L and hence switch off the (2′–5′)(A)$_n$ pathway unless cells are pretreated with IFNs (Cayley et al., 1982). The accumulation of (2′–5′)(A)$_n$ and related substances in the absence of antiviral activity in some IFN-treated vaccinia-infected cell lines possibly reflects vaccinia virus induced inactivation of the (2′–5′)(A)$_n$ pathway as well (Rice et al., 1984). In any case, these data illustrate differences in the way in which IFNs and exogenous (2′–5′)(A)$_n$ affect virus multiplication. Such problems will severely limit attempts to utilize (2′–5′)(A)$_n$ and their analogues in antiviral chemotherapy.

Stable and specific inhibitors of RNase L should prove valuable for investigations of the role of the (2′–5′)(A)$_n$ pathway in the control of virus multiplication by IFNs as well as in other situations (i.e., cell growth and differentiation) where its involvement has been postulated (Johnston & Torrence, 1984). A single (2′–5′)(A)$_n$ analogue [i.e., CH_3-p-(A2′p)$_2$A2′pp3′-OCH_3] (Watling et al., 1985) has proved to work as an antagonist of RNase L in intact cells but, surprisingly, behaves as expected in murine cells only (Watling et al., 1985).

The β,γ-difluoromethylene (2′–5′)(A)$_4$(oxred) derivative described here binds to RNase L with the same affinity as natural (2′–5′)(A)$_4$ without activating it. It therefore behaves as an antagonist of RNase L when introduced into HeLa cells together with RNase L agonist. It is fairly stable toward phosphodiesterases and phosphatases; this is essential in order to avoid intracellular conversion into RNase L agonists or inactive products. It could thus become a valuable tool for studies on the biological role(s) of the (2′–5′)(A)$_n$ system. Since however the yield with which (2′–5′)(A)$_n$ synthetase converts β,γ-difluoromethylene ATP into the corresponding (2′–5′)(A)$_n$ analogue is low, chemical synthesis would be more appropriate for future use on a larger scale.

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Registry No. RNase L, 76774-39-5; (2′–5′)(A)$_4$, 65954-95-2; (2′–5′)(A)$_4$(oxred), 92510-53-7; γ-S-(2′–5′)(A)$_4$, 108969-90-0; γ-S-(2′–5′)(A)$_4$(oxred), 104453-86-3; β,γ-CF_2-(2′–5′)(A)$_4$, 108969-91-1; β,γ-CF_2-(2′–5′)(A)$_4$(oxred), 108969-92-2; phosphatase, 9013-05-2; phosphodiesterase, 9025-82-5.

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Sequence Dependence of the B to Z Transition in Crystals and Aqueous NaCl Solutions for Deoxyoligonucleotides Containing All Four Canonical DNA Bases†

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ABSTRACT: A laser Raman study has been made on the conformation of a series of self-complementary octameric deoxynucleotides that contain all four canonical deoxynucleotide bases [guanine (G), cytosine (C), adenine (A), and thymine (T)] in order to determine which sequences will crystallize in the Z form and which sequences will go into the Z form in aqueous solution at high salt concentrations (4–6 M NaCl). All four octadeoxynucleotides, d(TGCGCGCA) (I), d(CACGCGTG) (II), d(CGTGCACG) (III), and d(CGCATGCG) (IV), have been crystallized from low-salt solutions. The Raman spectra of microcrystals show that I, II, and IV crystallize in a rigorous Z form while III crystallizes in the B form. Sequences I and II go into a Z form in 4–6 M NaCl solution at 0 °C while sequences III and IV remain in the B form in 6 M salt. There are substantial differences in the Raman spectra of oligonucleotides in the Z form found in the crystal and in high-salt solutions. The Raman spectra of the Z forms in 6 M NaCl solution at 0 °C are not linear combinations of the Raman spectra of the complete Z form in the crystal and the complete B form in low-salt solutions. The terminal residues of these oligomers do not appear to be in a strict Z form. A detailed analysis of the ring puckers and syn/anti conformation for all of the residues both in solution and in the crystal has been made. From these data together with data found in recent literature, some simple rules are suggested that may prove useful for predicting which DNA sequences containing all four canonical bases will go into the Z form in aqueous solution under high-salt conditions. The tendency of these four sequences to go into the Z form may be ranked I > II > IV > III.

Although the discovery of Z DNA has led to a great deal of discussion concerning its possible biological significance (Rich et al., 1983; Jovin et al., 1983; Rich et al., 1984), it is not clear at the present time which base sequences in DNA will support the left-handed conformation and which environmental conditions are necessary to induce the Z form for a given base sequence. The initial discovery of the left-handed conformation of poly(dG-dC) in 4 M aqueous salt solutions (Pohl & Jovin, 1972) has been followed by several investigations of alternating pyrimidine–purine sequences (Jovin et al., 1983; Rich et al., 1984). There has been an increasing number of reported examples of both polydeoxynucleotides and oligonucleotides that will support this left-handed conformation. With the exception of $d(CG)_n$ where $n \geq 2$, either most of the sequences that may be induced into the Z form

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