3′-*O*-Methylated analogs of 2–5A as inhibitors of virus replication

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We have explored analogs of 2′–5′-linked andeylic acid trimer (2–5A): 3′-O-methylated 2–5A, 2′-end modified adenylate trimer with deoxyadenosine or araadenine, methyl phosphonate and methyl phosphorotriester analogs as potential antiviral agents. For the treatment of virus infections, 2–5A and its analogs may serve in lieu of interferon, however, the use of 2–5A has two serious limitations: it is presumed to be impermeable to most cells, and moreover, cellular enzymes rapidly degrade it. Methylated analogs of 2–5A core strongly inhibited virus growth when added directly to cells in culture. 2′-End modified adenylate trimer with araadenine also inhibited virus growth, however, neither 2–5A nor other analogs showed any significant antiviral activity. The inhibition of virus growth was not due to the toxic effect of these compounds on cell growth as they had no inhibitory effect on the growth of uninfected cells

Interferon *2–5A* *Inhibition, of virus growth*

1. INTRODUCTION

Treatment of cells with interferon induces the synthesis of an enzyme 2–5A synthetase which, when activated by certain double-stranded RNAs, converts ATP into a series of 5′-triphosphorylated 2′–5′-linked oligo riboadenylates [1,2]. The major active component is a trimer, 5′-triphosphate, $pppA^{2'}p^{5'}A^{2'}p^{5'}A$ [3]. It activates an endonuclease at nM levels. This endonuclease-mediated degradation of RNA has been suggested as a mechanism by which interferon exerts its anticellular and antiviral effects [1,2]. However, a direct evaluation of anticellular or antiviral effect of 2–5A has not been possible because of its polar nature which makes it impermeable to most cells. Attempts have been made to introduce 2–5A and its analogs into cells made permeable by hypertonic shock followed by resealing [4], or by increasing cell permeability by treatment with lysolecithin [5] or by the calcium phosphate co-precipitation technique [5–8]. Although these studies revealed inhibition of both cellular and viral protein synthesis as well as RNA synthesis and enhanced degradation of RNA, and in some cases inhibition of virus growth in cells in culture [5,6,8,9], they do not point to possible clinical use. Moreover, a cellular phosphodiesterase rapidly degrades 2–5A so that its biological effects are transient.

Removal of the 5′-triphosphate of 2–5A results in drastic reduction of the polar nature of this molecule so that it becomes permeable to cells in culture. Although the 2–5A core is inactive in vitro [1,2,10,11], in intact cells, it produces inhibition of protein synthesis, although at much higher concentrations. This is presumably because in intact cells, kinases are thought to exist that introduce the 5′-triphosphate group in the 2–5A core molecule, although this has never been demonstrated. Moreover, addition of 2–5A core or other analogs directly to cells in culture inhibits concanavalin A-induced mitogenesis of mouse spleen lymphocytes [12], growth of 3T3 cells [13,14], growth of Daudi

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cells [15] and Epstein-Barr virus-induced transformation of human lymphocytes [16].

2. MATERIALS AND METHODS

3′-*O*-methylated 2–5A core, 2′-end modified adenylate trimer core with deoxyadenosine, araadenine and methyl phosphonate and methyl phosphorotriester analogs [18,19] were synthesized as described earlier. 2–5A core compound was procured from P-L Biochemicals.

Mouse L929 and African green monkey kidney (Vero) cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% fetal calf serum. Vaccinia virus was grown in roller bottles and purified [10]. Inhibition of virus growth was assayed by the inhibition of plaque formation in L929 cells. Mouse L-cells (0.25×10^6) were seeded in 35-mm petri dishes with 2-mm grids and incubated at 37°C for 72 h to obtain a confluent monolayer. The monolayer was washed once with 2 ml MEM and the cells were exposed to 2–5A analogs in MEM. After 1 h of incubation at 37°C, the medium was removed, 0.5 ml of MEM containing vaccinia virus was added, and the dishes were incubated for 30 min at 37°C; the dishes were rocked gently every 10 min. These experiments were done in triplicate: control dishes received MEM and no virus. After virus adsorption, the medium was aspirated, the cells were washed with 2 ml MEM, and 1 ml agar overlay (0.28% agarose in MEM, without phenol red but containing 2% fetal calf serum and 0.175% $NaHCO_3$) was added. To the petri dishes in which the effect of 2–5A analogs on virus replication was studied, the compounds were also added in the agar overlay. On top of the agar overlay, 0.1 ml of MEM without phenol red but containing 2% fetal calf serum and 0.175% $NaHCO_3$ was added.

After incubation for 72 h at 37°C, the plaques were counted in a plaque viewer (Bellco Glass) after staining with 0.20% crystal violet [20]. The amount of virus was adjusted to give 80–100 plaques/petri dish in the controls.

The effect of 2–5A analogs on cellular growth was measured as follows: Mouse L-cells (0.25×10^6) were seeded in 35-mm petri dishes and incubated at 37°C. After 24 h the monolayer was washed with 2 ml MEM. Fresh medium (with or without 2–5A methylated core analogs) was added to growing L-cells and incubation continued at 37°C. After 24–48 h, the medium was aspirated, the cells were removed by trypsin and counted for viability by trypan blue exclusion. All determinations for cell viability were made in triplicate cultures.

3. RESULTS AND DISCUSSION

Table 1 shows that non-phosphorylated 3′-*O*-methylated analogs of 2–5A, methylated at the terminal 3′-OH or methylated at all 3 hydroxyl groups, when added directly to the culture medium, strongly inhibited replication of vaccinia virus, as measured by inhibition of plaque formation. The analog methylated only at the 3′-terminal 3′-hydroxyl group was more active than the compound methylated at all 3′-hydroxyl groups. The inhibition of virus growth was not due to their toxic effect on the cells as these compounds had no effect on the growth of uninfected L-cells (table 2). Besides the methylated 2–5A analogs, only the 2′-end modified 2–5A adenylate trimer with araadenine-inhibited virus growth. However, neither the unmodified 2–5A core nor its 5′-phosphorylated analog, 2′-end modified 2–5A adenylate trimer with 2′- or 3′-deoxyadenosine, methyl phosphonate nor methyl phosphorotriester analogs inhibited virus growth. Several analogs of

Table 1

Effect of different 2′–5′-linked oligoadenylic acids on vaccinia virus growth in L-cells

Additions	Conc. (μM)	% Inhibition of virus growth
AAA	50	0
pAAA	20	0
pppAAA	20	0
AAAm	1	37
	5	50
	10	60
	25	83
AmAmAm	25	55
AA2′dA	50	20
AA3′da	50	10
AAaraA	50	50
3′dACH$_3$dACH$_3$dA	50	11
3′dAOCH$_3$dAOCH$_3$dA	50	6

Table 2
Effect of 2′–5′-linked oligoandeylic acid on the growth of L-cells

Additions	Viable cells ($\times 10^6$) after	
	24 h	48 h
None	1.56	2.94
AAAm (10 μM)	1.68	2.84
AmAmAm (25 μM)	1.83	3.47

2–5A have been recently synthesized [17–19, 21–26] to increase the biological stability of 2–5A, as it is rapidly degraded by cellular phosphodiesterase [1,2], thus limiting its efficacy. Both the xylo [14] and the cordycepin 2–5A analogs [14,16,21] show increased resistance to hydrolysis and consequently increased efficacy in inhibiting cell growth. The analog of 2–5A methylated at the 3′-terminal 3′-hydroxyl is also more resistant to degradation and causes more prolonged inhibition of protein synthesis when introduced into cells by the calcium phosphate coprecipitation technique [7]. The 2–5A analog methylated at all 3 hydroxyl groups was inactive under these conditions [7]. Since both the mono- and trimethylated 2–5A core analogs inhibited virus growth as shown in table 1, these compounds may be inhibiting virus growth by a mechanism different from the 2–5A activated endonuclease. The inhibition of DNA synthesis by the nonphosphorylated 2–5A, its xylo and cordycepin analogs lend support to this hypothesis [12,14].

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