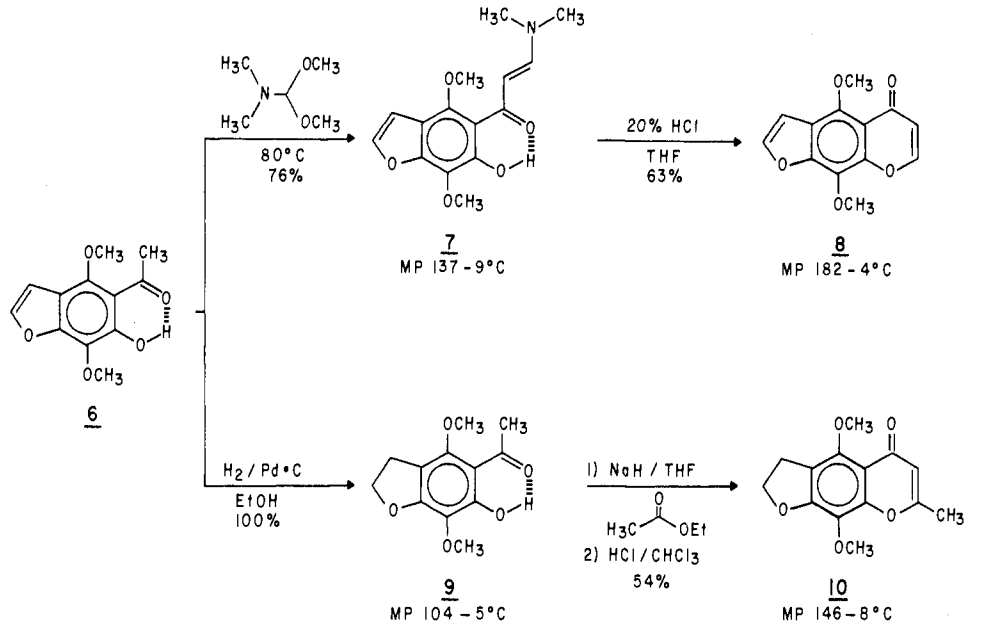
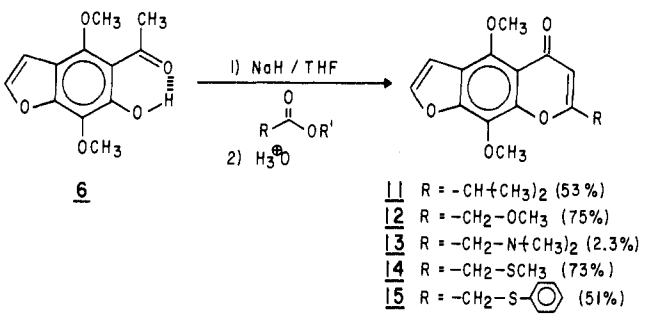
Scheme II



Scheme III



overnight with a mixture of 1.3 mL of 0.02 M Tris buffer, 0.2 mL of 0.2% heparin, and 0.2 mL of 0.4 M $CaCl_2$ at pH 7.4. After centrifugation at 4 °C, the supernate containing HDL cholesterol was poured into centrifuge tubes, 1.6 mL of absolute methanol added, the solution was mixed, let stand at 4 °C overnight, and centrifuged, and the supernate was discarded. The VLDL plus LDL precipitate was resuspended in 0.1 mL of 1 N NaOH and then extracted with 1.9 mL of 2-propanol for cholesterol determination. The HDL precipitate was extracted with 3.8 mL of 2-propanol for cholesterol determination.

Data from each study were statistically analyzed as a one-way classification[28] with values transformed to logarithms to achieve more homogeneous within-group variances. The mean response for each test compound was compared with the control mean with the LSD test.[29] Significant differences from the control mean are indicated by one asterisk for $p \leq 0.05$ and two asterisks for $p \leq 0.01$. Results are presented as treated mean/control mean ratios.

Results and Discussion

Removal of both the C-4 and C-9 methoxyl groups, compound **2** (see Table I), resulted in complete loss of lipid-altering activity. The 9-methoxyfurochromone **4** (C-4 methoxyl absent) exhibited marginal activity, while visnagin (**3**) was found to be equal in potency to khellin. The 5,6-dihydrofurochromone **5** was quite active, while the 2,3-dihydrofurochromone **10** was devoid of activity at the screening dose. Removal or replacement of the C-7 methyl group in khellin with alkyl, heteroalkyl, or heteroalkylaryl groups was not detrimental to the lipid-altering activity of the molecule.

These results indicate that within this series, the C-4 methoxyl group is required for lipid-altering activity, while the C-9 methoxyl is neither required nor significantly contributes to the observed lipidemic activity in this test system. Interestingly, the planar furan ring is critical to lipid-altering activity, while absence of planarity in the C-ring, as well as a C-7 substituent, is well tolerated. It is important to point out that the mechanism by which these furochromones affect lipid levels is unknown. Our data have provided insight into the initial questions raised concerning the influence of planarity and various subfunctional units and functionalities on activity. However, the lack of activity of the 2,3-dihydro analogue, which represents a change in both planarity and subfunctional units, raises an interesting question regarding the relationships between molecular modifications of the furochromone nucleus, metabolic biotransformations, absorption, distribution, clearance, and the observed lipid-altering activity.

Registry No. 1, 82-02-0; 2, 7674-96-6; 3, 82-57-5; 4, 87249-41-0; 5, 3380-63-0; 8, 49572-91-0; 10, 26239-04-3; 11, 76301-21-8; 12, 76301-18-3; 13, 76301-38-7; 14, 76301-19-4; 15, 76301-20-7.

Supplementary Material Available: Physical and analytical properties of compounds 5, 7, and 11-15 (3 pages). Ordering information is given on any current masthead page.

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Structure-Activity Relationships for and Potentiation of the Antimitogenic Activity of 2-5A Core Derived from 2-5A, a Mediator of Interferon Action

Sir:

One mechanism by which interferon exerts its antiviral effect is through the 2-5A system.[1] Double-stranded RNA,

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a product of virus replication, activates the interferon-induced enzyme 2-5A synthetase, which then generates 2-5A[2] [ppp5′A2′(p5′A2′)$_n$5′pA][3] from ATP. The 2-5A thus synthesized then activates a latent cellular endoribonuclease (RNase L or RNase F), which subsequently degrades viral RNA,[1] possibly selectively.[4] This results in an inhibition of translation and blockade of virus growth. The involvement of the 2-5A system with the antiviral properties of interferon and its possible additional roles[1,5,6] in the regulation of growth, development, and differentiation of normal cells have spurred an interest in the exploitation of the 2-5A system as a novel approach to antiviral and/or antitumor agents.

Possibly due to its highly ionic nature, 2-5A does not show significant translational inhibitory or antiviral properties in the intact cell unless rather drastic treatments, such as microinjection,[7] calcium phosphate coprecipitation,[8,9] or hypertonic salt[10,11] or lysolecithin[12] treatments, are employed. Of course, such procedures are totally impractical from a chemotherapeutic vantage point. Therefore, especially in view of the antiproliferative and antitumor effects of interferon,[13,14] considerable interest greeted the reports that 2-5A core (A2′p5′A2′p5′A or 5′-dephosphorylated 2-5A trimer) exhibited antimitogenic activity in mouse splenic leukocytes,[15,16] Balb/c 3T3 cells,[17] lymphoblastoid cells,[18] and Swiss 3T3 cells,[19] just as did interferon itself.[20] Although never rigorously established, the suggestion was advanced that such activity was due to conversion of the 2-5A core to 2-5A itself by phosphorylation in the intact cell.[17] 2-5A core analogues based on xylofuranosyladenine[19,21] or cordycepin[22] (3′-deoxy-

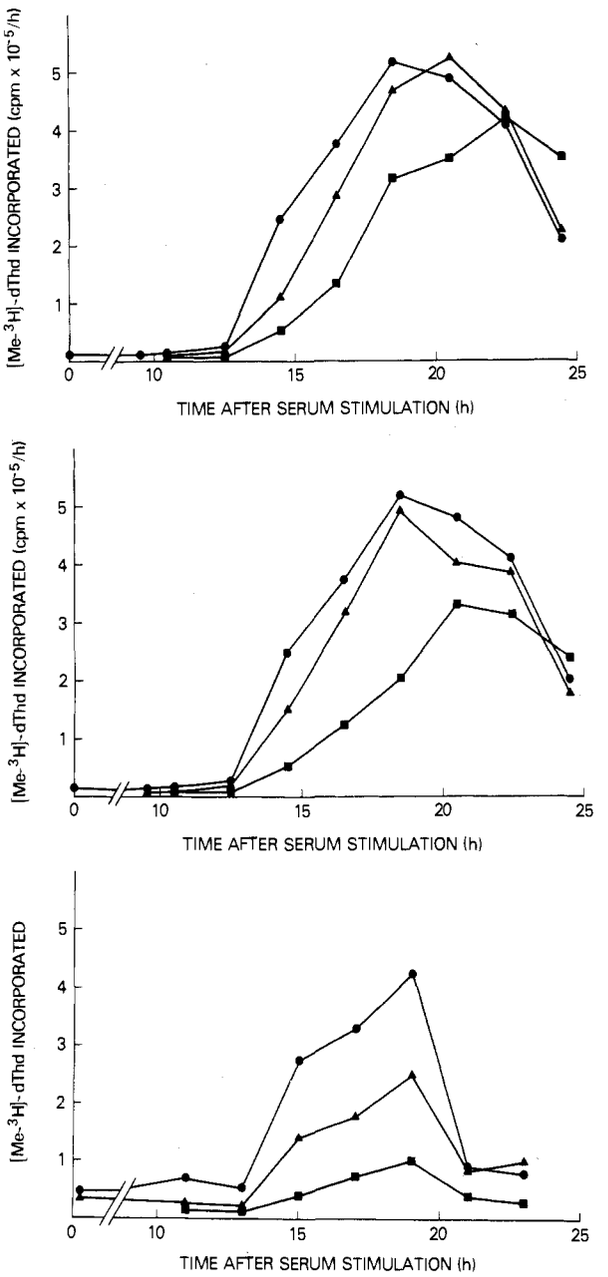

Figure 1. Effect of A2′p5′A2′p5′A (top), p5′A2′p5′A2′p5′A (middle), and p5′A2′p5′A2′p5′A2′p5′A (botton) on incorporation of [*Me*^{3}H]thymidine into DNA of serum-stimulated Balb/c 3T3 cells. As explained in the text, cells were grown to confluency and synchronized by 24-h incubation in medium without fetal calf serum but containing 10% spent medium from confluent cultures. Such synchronized cells were then stimulated with fresh medium plus 10% fetal calf serum with no further additions (●) or with 5 μM (▲) or 50 μM (■) oligoadenylate. At the indicated time intervals, duplicate cultures were pulse labeled for 1 h with [*Me*-^{3}H]thymidine to measure incorporation into DNA.

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(3) Standard abbreviations are used for oligonucleotides. For instance 5′-monophosphoryladenylyl(2′→5′)adenylyl(2′→5′)-adenosine is written p5′A2′p5′A2′p5′A. A shortened version is used for the P^2-phenyl-substituted adenosine diphosphate: Phpp5′A stands for P^2-phenyl P^1-adenosine 5′-diphosphate.

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(20) Leanderson, et al. have argued[18] that interferon and core 2′5′-oligoadenylates act by different mechanisms, since their flow cytometric analysis showed that interferon increased the ratio of cells in G_0/G_1 (resting phase), but core oligoadenylate increased the number of cells in the S (growth) phase.

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adenosine) have been reported to possess antimitogenic, antiviral, or antitumor properties, but it is possible that those activities are mediated by degradation of the trinucleotides to the parent cytotoxic nucleoside or nucleotide,[23] especially since the corresponding 5′-triphosphates of these analogues are not efficient activators of the 2-5A-dependent endonuclease.[24,25]

We have prepared and evaluated for antimitogenic activity a series of 2-5A core congeners that differ in oligonucleotide chain length and the nature of the substituent at the 5′-terminus. A significant potentiation of antimitogenic activity has been achieved by such structural modification.

Antimitogenic activity[26] was explored by using the synchronized (serum-starved) Balb/c 3T3 cell system previously employed by Kimchi et al. in their initial studies on 2-5A core antimitogenicity.[17] In this system, Balb/c 3T3 cells were grown to confluence in Eagle's minimal essential medium with 10% fetal calf serum and then synchronized by incubation for 25 h in the same media without fetal calf serum but containing 10% spent medium from the confluent cell cultures. For the [^{35}S]methionine incorporation studies, methionine-free medium was used during the synchronization period. Synchronized cells were then stimulated to grow with fresh medium plus 10% fetal calf serum plus or minus the agent to be tested for antimitogenic behavior. Then, at the time intervals indicated in Figure 1, duplicate cultures were pulse labeled (1 h) with [*Me*-^{3}H]thymidine (40 Ci/mmol) or [^{35}S]methionine (147 mCi/mmol) to measure incorporation into DNA or protein, respectively.

Oligoadenylate 5′-triphosphates were prepared as described previously.[27] All 2′,5′-oligoadenylate 5′-monophosphates were synthesized by lead-ion-catalyzed polymerization of adenosine 5′-phosphorimidazolidates according to the method of Sawai et al.[28] The oligoadenylates thus prepared were obtained as homogeneous preparations by a combination of nuclease P_1 digestion of the crude reaction mixture, followed by reverse-phase

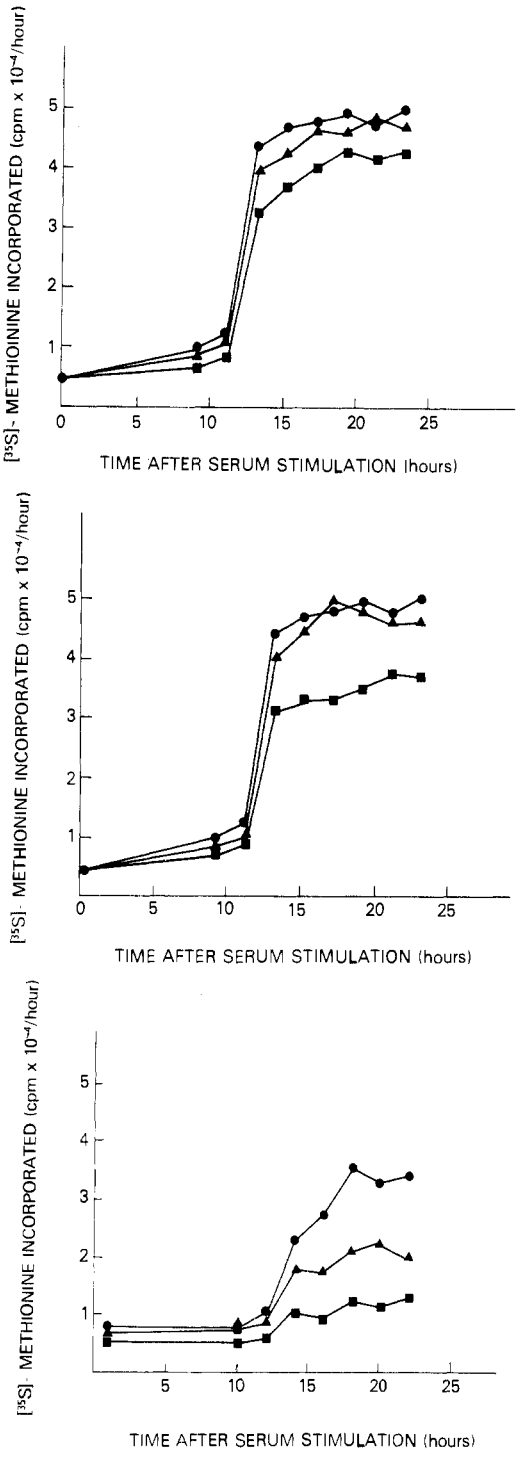

Figure 2. Effect of A2′p5′A2′p5′A (top), p5′A2′p5′A2′p5′A (middle), and p5′A2′p5′A2′p5′A2′p5′A (bottom) on incorporation of [^{35}S]methionine into protein of serum-stimulated Balb/c 3T3 cells. Details of cell growth and synchronization are given in the text and in the legend to Figure 1. Synchronized cells were stimulated with fresh medium (without methionine) plus 10% fetal calf serum with no additions (●) or with 5 μM (▲) or 50 μM (■) oligoadenylate. At the indicated time intervals, duplicate cultures were pulse labeled for 1 h with [^{35}S]methionine.

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(26) Antimitogenic activity was assessed by measurement of both [^{3}H]thymidine and [^{35}S]methionine incorporation according to the precedents set by earlier publications. In most instances, there was a close correlation between the results of [^{3}H]thymidine uptake and [^{35}S]methionine uptake, making it unlikely that an effect on transport could be involved. Separate experiments showed that at 0.8 μM, the oligomer p5′A2′p5′A2′p5′A2′p5′A had no effect on the uptake of labeled thymidine or methionine or on the TCA-soluble radioactivity associated with the cells. At higher concentrations (5 and 50 μM), p5′A2′p5′A2′p5′A2′p5′A resulted in an inhibition of the quantity of label appearing in the TCA-insoluble fraction and diminished the clearance of [^{3}H]thymidine or [^{35}S]methionine from the cell culture medium but had no effect on the TCA-soluble pools. However, if the primary effect of p5′A2′p5′A2′p5′A2′p5′A is on protein synthesis and/or DNA synthesis, then diminution of label uptake would occur as a result. In concert with this hypothesis, it was demonstrated that p5′A2′p5′A2′p5′A2′p5′A and other oligomers had a demonstrable effect on cell growth that paralleled their potency as antimitogenic agents measured by the isotope method (Figure 2).

(27) Imai, J.; Torrence, P. F. *J. Org. Chem.* **216**, 4015–4021. All compounds employed in this study were homogeneous by both HPLC and TLC. The identity of each compound was assured by enzymatic digestion results, UV spectra, and high-resolution NMR. Details concerning the preparation of the P^2-phenyl-substituted derivatives will be published soon (K. Lesiak and P. F. Torrence, in preparation).

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high-performance liquid chromatography (HPLC) (Zorbax ODS column, 9.6 × 25 cm, linear gradient of 0–35% B in 35 min; buffer A: 50 mM ammonium phosphate, pH 7; buffer B: MeOH–H_2O, 1:1), and final chromatography on DEAE-Sephadex (triethylammonium bicarbonate buffer gradient) to remove HPLC buffer salts. The deoxy oligomer, p5′(2′dA)3′p5′(3′dA)3′p5′(2′dA), was a product of PL Biochemicals (Milwaukee, WI) and was used without additional purification. The dinucleotides, A2′p5′A and A3′p5′A, and the trinucleotide, A3′p5′A3′p5′A, were obtained from Sigma Chemical Co. (St. Louis, MO) and used as such. 2-5A trimer core was either prepared as previously described[29] or obtained from Calbiochem (La Jolla, CA). Higher core oligoadenylates were purchased from Calbiochem and further purified by reverse-phase HPLC using an Accupak C_{18} column (4.0 mm × 10 cm) and linear gradient of 0–100% B (where buffers A and B were as previously defined), followed by DEAE-Sephadex chromatography. The P^2-phenyl diphosphate derivatives of adenosine, A2′p5′A and A2′p5′A2′p5′A, were prepared by using modifications of the phosphorimidazolidate method.[30]

Experiments defining the antimitogenic behavior are illustrated in Figures 1 and 2 for three representative oligomers, and the results obtained with each of the oligoadenylates are presented in Table I. In general, there was good correlation between antimitogenic activity, as monitored by inhibition of [*Me*-^{3}H]thymidine incorporation into DNA and that monitored by inhibition of [^{35}S]-methionine into protein. The following generalizations could be made from the data of Table I. (1) Antimitogenic activity was strongest among, but not limited to, the 2′,5′-phosphodiester-linked oligoadenylates. Although, in contrast to the findings of Eppstein et al.,[19] A3′p5′A3′p5′A was devoid of antimitogenic activity, some activity was shown by the 3′,5′-linked dimer core, A3′p5′A, and the 3′,5′-deoxyadenylate trimer core monophosphate, p5′-(2′dA)3′p5′(2′dA)3′p5′(2′dA). The ability of A3′p5′A to block [*Me*-^{3}H]thymidine, however, was not matched by the corresponding ability to inhibit [^{35}S]methionine incorporation, implying that this was not a true antimitogenic effect. (2) In the class of 2′,5′-linked core oligoadenylates (Table I), the most potent antimitogenic was 2-5A trimer core, A2′p5′A2′p5′A. Oligomers of shorter or longer chain length were less active. The oligomers could be listed in the following order of (descending) activity: A2′p5′A2′p5′A > A2′(p5′A2′)$_2$p5′A > A2′(p5′A2′)$_3$p5′A > A2′p5′A ≥ A2′(p5′A2′)$_4$p5′A. (3) 5′-Monophosphorylated 2′,5′-core oligoadenylates also were potent antimitogenic agents; however, in this case the most potent of the series was the tetramer, p5′A2′(p5′A2′)$_2$p5′A. The 5′-phosphorylated oligoadenylates could be arranged in the following order of (descending) activity: p5′A2′(p5′A2′)$_2$p5′A > p5′A2′-(p5′A2′)$_3$p5′A ≥ p5′A2′p5′A2′p5′A > p5′A2′(p5′A2′)$_4$ p5′A > p5′A2′p5′A. (4) Surprisingly, 5′-triphosphorylated 2′,5′-core oligomers, including 2-5A trimer itself, possessed some antimitogenic properties. In this case, the dimer triphosphate, ppp5′A2′p5′A, was the most potent agent, followed by the trimer triphosphate, ppp5′A2′p5′A2′p5′A. The tetramer triphosphate, ppp5′A2′(p5′A2′)$_2$5′pA was nearly without activity. (5) P^2-Phenyl-substituted 2′,5′-linked core 5′-diphosphates showed antimitogenic behavior, which was maximal with the trimer derivative, Phpp5′A2′p5′A2′p5′A. The dimer, Phpp5′A2′p5′A, expressed some activity, but the derivative of adenosine itself, i.e., PhppA, was totally devoid of biological activity in this system.

Several compounds emerged from this study as either equipotent with or even more potent than 2-5A core, A2′p5′A2′p5′A, as antimitogens. These included p5′A2′p5′A2′p5′A, p5′A2′(p5′A2′)$_2$p5′A, p5′A2′-(p5′A2′)$_3$p5′A, ppp5′A2′p5′A, and Phpp5′A2′p5′A2′p5′A. Outstanding among these was the tetramer 5′-monophosphate, p5′A2′(p5′A2′)$_2$p5′A, which was definitely more active than 2-5A trimer or tetramer core. To ensure that these results were not due to some effect of the oligomer on uptake or pool size of the thymidine or methionine, the ability of several of the most active oligonucleotides to block the actual increase in cell number was determined. As is apparent from Figure 3, the oligoadenylates A2′p5′A2′p5′A2′p5′A, p5′A2′(p5′A2′)$_2$p5′A, and Phpp5′A2′p5′A2′p5′A, representative of three different classes of oligonucleotides, all inhibited cell proliferation

Table I. Antimitogenic Activity as Monitored by Inhibition of DNA and Protein Synthesis

compound	% inhibn of DNA synthesis at the following concns[a] 5 μM	50 μM	% inhibn of protein synthesis at the following concns[a] 5 μM	50 μM
3′,5′ Core Oligomers				
A3′p5′A	13	30	0	0
A3′p5′A3′p5′A	0	0	0	0
2′,5′ Core Oligomers				
A2′p5′A	0	6	0	0
A2′p5′A2′p5′A	14	48	0	14
A2′(p5′A2′)$_2$p5′A	13	21	19	24
A2′(p5′A2′)$_3$p5′A	2	20	0	20
A2′(p5′A2′)$_4$p5′A	0	0	0	0
5′-Monophosphorylated 2′,5′-Core Oligomers				
p5′A2′p5′A	0	0	0	0
p5′A2′p5′A2′p5′A	15	54	0	33
p5′A2′(p5′A2′)$_2$p5′A	46	79	42	66
p5′A2′(p5′A2′)$_3$p5′A	35	48	24	42
p5′A2′(p5′A2′)$_4$p5′A	10	23	0	16
5′-Triphosphorylated 2′,5′-Core Oligomers				
ppp5′A2′p5′A	35	45	23	38
ppp5′A2′p5′A2′p5′A	11	27	11	21
ppp5′A2′(p5′A2′)$_2$p5′A	0	10	0	15
P^2-Phenyl-Substituted 2′,5′-Core 5′-Diphosphates				
Phpp5′A	0	0	0	0
Phpp5′A2′p5′A	14		0	20
Phpp5′A2′p5′A2′p5′A	18	45	21	37
5′-Monophosphorylated 3′,5′-Core of 2′-Deoxyadenylate				
p5′(2′dA)3′p5′(2′dA)-3′p5′(2′dA)	3	18		26

[a] Determined by pulse (1 h) incorporation of [Me-^{3}H]-thymidine or [^{35}S]methionine as described in the text in the absence or presence of the indicated oligonucleotide at a final concentration of either 5 or 50 μM. Percent inhibition = 100[1 – (TCA insoluble cpm in cultures with oligonucleotide/TCA insoluble cpm in cultures without oligonucleotide)]. For purposes of this calculation, the summation of thymidine incorporation during the period of peak control DNA synthesis (e.g. 15–16, 17–18, 19–20 h) was compared to the corresponding times in the cultures that received oligonucleotide. Neither adenosine nor phenylphosphoric acid gave any inhibition even at 50 μM concentration. Figures 1 and 2 represent typical experiments from which these data were compiled.

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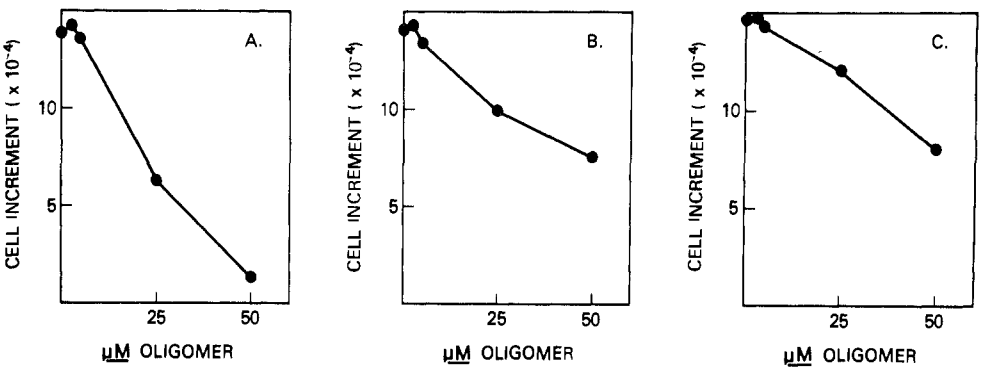

Figure 3. Effect of p5′A2′p5′A2′p5′A2′p5′A (panel A), A2′p5′A2′p5′A2′p5′A (panel B), and Phpp5′A2′p5′A2′p5′A (panel C) on the growth of Balb/c 3T3 cells. The cells were seeded at 12 800 cells/well and allowed to proliferate for 91 h in Eagle's minimal essential medium with 10% fetal calf serum and varying concentrations of oligonucleotide. At the end of this incubation period, the cell layers (in duplicate) were trypsinized and enumerated with a Coulter counter. The increment of cells is graphed as a function of inhibitor concentration. Increment is defined as number of cells at 91 h − number of cells at 0 h.

to an extent generally consistent with the results of the [*Me*-^{3}H]thymidine and [^{35}S]methionine labeling experiments. The exception was A2′p5′A2′p5′A2′p5′A, which displayed a somewhat more effective inhibition of proliferation than the labeling data would have suggested. A comparison of Figure 3 (panels A and B) and the data of Table I reveals that 5′-phosphorylation of 2-5A core oligonucleotide did not diminish antimitogenic activity but, in fact, potentiated it. For instance, 5′-phosphorylation of 2-5A trimer core, A2′p5′A2′p5′A, gave p5′A2′p5′A2′p5′A, which was equipotent with 2-5A trimer core, while 5′-phosphorylation of tetramer, pentamer, or hexamer core gave 5′-monophosphates with significantly enhanced antimitogenic activity.

Within any given series (i.e., cores, 5′-monophosphates, or P_2-phenyl-substituted diphosphates), the antimitogenic activity depended upon the chain length or the charge of the oligomer, but this optimal chain length or charge differed from one class of oligonucleotides to another. For the core series, activity peaked at the trimer (−2 nominal charge). In the case of the 5′-monophosphates, activity was greatest with tetramer (−5 nominal charge), whereas for the triphosphates, the dimer (−5 nominal charge) was of greatest activity. In the P^2-phenyl-substituted diphosphate series, the trimer (−4 nominal charge) achieved the greatest antimitogenic effect.

What is the mechanism by which such oligoadenylates operate to effect an antimitogenic response? On the one hand, the 2-5A-dependent ribonuclease from a number of sources (mouse, rabbit, and human) is not activated by dimeric 2-5A, ppp5′A2′p5′A,[2,8,10,31,32] and in accord with this, neither A2′p5′A nor p5′A2′p5′A showed significant antimitogenic behavior. On the other hand, both ppp5′A2′p5′A and Phpp5′A2′p5′A possessed substantial activity. 2-5A trimer, tetramer, pentamer, and hexamer have been shown to be as active as RNase L activators,[2,8,10,31] although the heptamer and hexamer may be slightly less efficient.[31] In contrast with this latter result from cell-free systems, core pentamer and hexamer were much less effective than core trimer or tetramer as antimitogens. A significant, albeit not as drastic, decrease in activity also was seen with the pentamer and hexamer 5′-monophosphates. Finally, Phpp5′A2′p5′A2′p5′A has been found not to activate the 2-5A-dependent endonuclease in an in vitro L cell systems.[30] Viewed as a whole, these results would not seem consistent with any proposed role for the 2-5A-dependent endonuclease in the antimitogenic effects of 2′,5′-linked oligoadenylates. A similar conclusion was reached by Eppstein et al.,[33] who could find no evidence of 2-5A-characteristics ribosomal RNA cleavages in rRNA extracted from Swiss 3T3 cells treated with 2-5A trimer core under conditions that gave rise to an antimitogenic effect. On the other hand, Kimchi et al.[17] reported that NIH 3T3 cells, deficient in the 2-5A-dependent endonuclease, were insensitive to the antimitogenic effects of 2-5A trimer core.

In summary, the results presented here demonstrate that chemical modification of the 5′-hydroxyl of 2-5A cores does not mitigate but rather potentiates the antimitogenic action of 2′,5′-linked oligoadenylates. Whether the mechanism behind this phenomena shares a common molecular basis with the antiviral action of either interferon or 2-5A and whether these obervations can be further developed into useful antimitogenic agents for use in the whole animal remain to be established.

Registry No. A3′p5′A, 2391-46-0; A3′p5′A3′p5′A, 917-44-2; A2′p5′A, 2273-76-9; A2′p5′A2′p5′A, 70062-83-8; A2′(p5′A2′)$_2$p5′A, 73853-00-6; A2′(p5′A2′)$_3$p5′A, 73853-01-7; A2′(p5′A2′)$_4$p5′A, 86416-36-6; p5′A2′p5′A, 20307-28-2; p5′A2′p5′A2′p5′A, 61172-40-5; p5′A2′(p5′A2′)$_2$p5′A, 66048-58-6; p5′A2′(p5′A2′)$_3$p5′A, 66048-59-7; p5′A2′(p5′A2′)$_4$p5′A, 87413-35-2; ppp5′A2′p5′A, 65954-94-1; ppp5′A2′p5′A2′p5′A, 65954-93-0; ppp5′A2′(p5′A2′)$_2$p5′A, 65954-95-2; Phpp5′A, 2464-80-4; Phpp5′A2′p5′A, 87413-36-3; Phpp5′A2′p5′A2′p5′A, 87413-37-4; p5′(2′dA)3′p5′(2′dA)3′p5′(2′dA), 14258-27-6.

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