# EFFECT OF PYRIMIDINE AND RIBOSE MODIFICATIONS ON THE ANTIVIRAL ACTIVITY OF SYNTHETIC POLYNUCLEOTIDES

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## Summary

Homo- and copolynucleotides derived from 3-methyluridine, 5,6-dihydrouridine and 2'-azido-2'-deoxyuridine (dUz) were prepared and evaluated for antiviral activity either as single strands or as double strands when complexed with complementary homopolynucleotides. The synthetic materials were not as active as $(A)_n \cdot (U)_n$ or $(I)_n \cdot (C)_n$ in inducing the interferon system. The antiviral activity of $(A)_n \cdot (dUz)_n$ was not affected by treatment with high concentrations of pancreatic RNase A, whereas this activity was totally abolished when $(A)_n \cdot (dUz)_n$ was digested with human serum.

## Introduction

Both single- and double-stranded synthetic polynucleotides exhibit a wide variety of biological activities[1]. One such activity, the induction of interferon, has been intensively investigated as a means to circumvent the difficulties associated with the clinical use of human interferon[2-4]. Factors, currently believed to be responsible for determining the efficacy of synthetic polynucleotides as interferon inducers, are (*i*) sufficiently high molecular weight[5], (*ii*) existence as a double-stranded helix with relatively high thermal stability ($T_m$)[6], (*iii*) resistance to nucleases[7] (*e.g.*, pancreatic RNase A), and (*iv*) an intact 2'-OH group[8]. We

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have evaluated the antiviral activity of several polynucleotides, modified in both the ribose and the pyrimidine moiety, in order to gain additional information regarding the structural requirements of such interferon inducers.

## Materials and Methods

$(A)_n$ and $(U)_n$ were products of P-L Biochemicals (Milwaukee, Wisconsin). $(C,D)_n$[9] polymers and $(dUz)_n$[10] were prepared by methods previously described.* Preparation of ($m^3UMP$) was accomplished by a modification of the published procedure[11] and converted, *via* the morpholidate[12], to the 5'-diphosphate and polymerized to $(m^3U)_n$[13] using polynucleotide phosphorylase. Enzymatic copolymerization of the appropriate diphosphates gave $(m^3U,U)_n$[13], $(m^3U,A)_n$ and $(dUz,A)_n$. Where necessary, base ratios in the polynucleotides were determined by digestion of the polymer with alkaline phosphatase and venom phosphodiesterase followed by chromatography (Whatman No. 1 in *iso*-PrOH-$NH_4OH$-$H_2O$, 7/1/2), elution of the resulting nucleosides and determination of the corresponding UV absorbance. Base ratios in the $(C,D)_n$ polymers were determined as previously described[9].

Degradation of polynucleotides was monitored by one or more of the following methods: (I) removal of aliquots at various time intervals followed by paper chromatography in 95% EtOH-1.0 *M* $NH_4OAc$, pH 7.5, 7/3; (II) UV hyperchromicity at $\lambda_{max}$ of the polynucleotide; (III) $CCl_3COOH$ soluble radioactivity from labeled polynucleotides (Schwarz-Mann).

Polynucleotide complexes were prepared for antiviral assays by mixing appropriate molar quantities in 0.10 *M* NaCl, 0.01 *M* $KH_2PO_4$ (pH 7.5) buffer and incubating for at least 24 hr at 25°. Complex formation was checked by hypochromicity. General techniques used for construction of mixing curves and determination of $T_m$ will be reported elsewhere[14].

Solutions of the polynucleotides were diluted in medium containing 300 µg

* The following abbreviations are used for the non-standard bases: D, 5,6-dihydrouridine; $m^3U$, 3-methyluridine; dUz, 2'-azido-2'-deoxyuridine.

neomycin sulfate[15] per ml and incubated with primary rabbit kidney cells for 24 hr. Treated and control cultures were then challenged with vesicular stomatitus virus (VSV, MOI = 10). Eighteen to 24 hr later either viral cytopathology (CPE) was recorded or virus yield was determined by plaque assay. The biological activity was defined as the minimal effective dose (MED) or that concentration of polynucleotide which induced either a 50% reduction in the CPE or a 0.5 $\log_{10}$ reduction in yield of the challenge virus. From previous studies[16], it was concluded that the resistance to viral replication is a consequence of activation of the interferon system. The measurement of the resistance of stimulated cells to virus challenge is a more sensitive method of assay than the measurement of any interferon produced[17].

## Results and Discussion

Three modifications of uridine were examined for their effect on the antiviral activity of single- and double-stranded polynucleotides; namely, 5,6-dihydrouridine (D), 3-methyluridine ($m^3U$) and 2'-azido-2'-deoxyuridine (dUz). Introduction of these nucleosides into synthetic polynucleotides allowed examination of several of the hypotheses regarding interferon presented in the introductory paragraphs of this paper, as well as evaluation of the effect of loops on the activity of double-stranded complexes. If such alterations produced reasonable antiviral activity compared with $(A)_n \cdot (U)_n$, we intended to modify $(I)_n \cdot (C)_n$ in a similar fashion.

### Biological Evaluation of the Single-Stranded Polynucleotides

(1) Replacement of cytidine residues in $(C)_n$ by (D) (Table I) led to no significant increase in antiviral activity compared to certain commercial samples of $(C)_n$ preparations[15]. (2) Single-stranded polynucleotides (*3, 4*) which were completely resistant to pancreatic RNase A, or which (*5, 6, 7, 15*) had intermediate degrees of resistance[13] (Table I) afforded considerably less antiviral protection than the most active samples of $(I)_n$ and $(C)_n$[16]. In addition, they

showed less activity than a commercial sample of $(A)_n$, *8*, which is also resistant to RNase A at moderate enzyme concentrations. (3) There was no apparent relationship between antiviral activity of the single-stranded polynucleotides and their secondary structure as judged by $T_m$. For example, the $T_m$ of *4* is slightly higher than that of *8*, yet *4* showed no activity, whereas *8* was active at 10 μg/ml.

The finding[16,18,19] that certain commercial samples of $(I)_n$ or $(C)_n$ were able to induce the interferon system at relatively high concentrations led to the suggestion[16] that the active samples of single-stranded polynucleotides might be contaminated by a polycation or possess a conformation intermediate between a helix and a random coil which in turn would lead to increased resistance to RNase and higher antiviral activity. The results presented here show that resistance to pancreatic RNase A does not lead to antiviral activity in a single-stranded polynucleotide.

Biological Evaluation of the Double-Stranded Polynucleotides

As the results in Table I clearly show, none of the modified synthetic double-stranded polynucleotides (*12*, *13*, *14*) were as active as $(A)_n \cdot (U)_n$ or $(I)_n \cdot (C)_n$ in providing antiviral protection in rabbit kidney cells treated with VSV. The differences became even more dramatic when *10* and *16* were tested at lower concentrations under the same conditions. While the most active synthetic preparations (*12*, *13*) provided protection at 1 μg/ml, *10* and *16* (with neomycin) protected against viral infection at < 0.001 μg/ml. These results permit the following conclusions: the 2'-OH group of the ribose moiety of the $(U)_n$ strand of $(A)_n \cdot (U)_n$ is required for maximum interferon inducing ability. Replacement of the 2'-OH of $(C)_n$ by 2'-H[8], 2'-Cl[20], 2'-$OCH_3$[21], or 2'-OAc[22] also leads to dramatic decreases in the ability of the corresponding $(I)_n$ complexes to induce interferon. Since the effect of introducing such substituents (aside from H[21] and OAc[20]) into the purine member of the duplex has not yet been examined, and since the effect of more diverse substituents (F, $NH_2$, SH) at C-2' has not been determined, it is probably premature to conclude that an absolute

TABLE I

Antiviral Activity of Modified Synthetic Polynucleotides[a]

| Compound No. | Polynucleotide | $s_{20}$[b] | Base ratio | $T_m$[c] | Behavior toward pancreatic RNase A[d] | MED[e] | Effect of human serum on activity | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | No serum MED | Serum[f] MED |
| 1 | $(D,C)_n$ | -- | $\frac{D}{C} = 0.05$ | -- | --- | > 10 | -- | -- |
| 2 | $(D,C)_n$ | -- | $\frac{D}{C} = 0.50$ | -- | --- | 7[g] | -- | -- |
| 3 | $(dUz)_n$ | 8 | -- | 12° | completely resistant (I,II) | > 10 | -- | -- |
| 4 | $(dUz,A)_n$ | 4.5 | $\frac{dUz}{A} = 0.26$ | 43°(br) | completely resistant (I) | > 10 | -- | -- |
| 5 | $(m^3U)_n$ | 6 | -- | < 0° | slower than poly(U) (I) | > 10 | -- | -- |
| 6 | $(m^3U,U)_n$ | 6 | $\frac{m^3U}{U} = 0.46$ | < 0° | slower than poly(U) (I) | > 10 | -- | -- |
| 7 | $(m^3U,A)_n$ | 4.5 | $\frac{m^3U}{A} = 0.33$ | 32°(br) | degraded slowly to oligo (I) | > 10 | -- | -- |
| 8 | $(A)_n$ | 9.8 | -- | 38°(br) | completely resistant at 10 µg/ml RNase (I,II,III) | 10 | (10)[h] | 10 |
| 9 | $(U)_n$ | 8 | -- | 6° | degraded quickly even at 0.01 µg/ml (I,II) | > 10 | -- | -- |
| 10 | $(A)_n \cdot (U)_n$ | 9.8,8 | -- | 57° | degraded slowly only at 10 µg/ml (II) | < 0.1[i] | < 0.1 | < 0.1 |
| 11 | $(A)_n \cdot 2(U)_n$ | 9.8,8,8 | -- | 45°,57° | --- | 0.3 | 0.3 | 0.07 |
| 12 | $(A)_n \cdot (dUz)_n$ | 9.8,8 | -- | 59° | not degraded even at 10 µg/ml (II) | 1.0 | (1.0)[h] | 10 |
| 13 | $(A)_n \cdot (m^3U,U)_n$ | 9.8,6 | $\frac{m^3U}{U} = 0.46$ | 32°(br) | --- | 0.7 | (0.7)[h] | > 10 |
| 14 | $(U)_n \cdot (m^3U,A)_n$ | 8,4.5 | $\frac{m^3U}{A} = 0.33$ | 38°(br) | --- | > 10 | -- | -- |
| 15 | $(A,U)_n$ | 10.9 | $\frac{A}{U} = 1.1$ | -- | slowly degraded to oligo (I) | > 10 | -- | -- |
| 16 | $(I)_n \cdot (C)_n$ | -- | -- | 60° | degraded slowly at 10 µg/ml RNase | < 0.1[i] | (< 0.1)[h] | < 0.1 |

[a] All assays were conducted in the presence of 300 μg/ml neomycin sulfate. The antiviral activities of compounds *1* and *2* were determined using rat cells, whereas the remaining compounds (*3–16*) were assayed using rabbit kidney cells.

[b] $S_{20}$ values were determined with a Beckman Model E analytical ultracentrifuge using an AN-G titanium six-hole rotor and 0.01 *M* NaCl, 0.001 *M* $NaH_2PO_4$, 0.001 *M* EDTA (pH 6.5) buffer. For the doubly-stranded polynucleotides, the $s_{20}$ values represent the constants determined for the constituent chains in the order named in column 2.

[c] The buffers used for determination of $T_m$ are as follows: *3*, *5*, *6* and *9*, 0.01 *M* $MgCl_2$ (pH 7.6); *4*, 0.1 *M* Na citrate, 0.01 *M* $MgCl_2$ (pH 7.5); *7*, 0.15 *M* KCl, 0.05 *M* KOAc (pH 4.6); *8*, 0.15 *M* NaCl, 5 mM $NaH_2PO_4$ (pH 7.2); *10*, *11*, *13*, *14* and *16*, 0.1 *M* NaCl, 0.01 *M* $NaH_2PO_4$ (pH 7.5); *12*, 0.2 *M* NaCl, 0.01 *M* Na cacodylate (pH 7.0). The double inflection in the melting curve of *15* represents a 3 → 2 transition ($T_m$ = 45°) followed 2 → 1 transition ($T_m$ = 57°). Broad or non-cooperative $T_m$'s (> 5°) are indicated as (br).

[d] Determined by methods I, II or III (see Materials and Methods).

[e] Minimal effective dose (μg/ml) = lowest concentration of compound which resulted in 0.5 $\log_{10}$ reduction in HA yield of VSV (for rat cells, reduction in Sindbis virus HA yield).

[f] Polynucleotides were incubated for 4 hr at 37° in the presence of 13% human serum. Neomycin was added *after* this digestion.

[g] 0.9 $\log_{10}$ reduction in Sindbis virus HA yield at 10 μg/ml, the highest concentration tested.

[h] Results taken from previous assay.

[i] Greater than 3.0 $\log_{10}$ inhibition of virus at lowest concentration (0.1 μg/ml) tested.

requirement for the 2'-OH exists. (2) A highly ordered complementary base-paired complex is a necessary but not sufficient condition for maximum interferon stimulating capacity. One example of this is compound *11*, the triply-stranded complex, which is about a thousandfold less active than *10*, the double-stranded complex (also see ref 6). The most striking example is *12* which has been shown[9,14] to possess a double-stranded structure with a $T_m$ at least equal to *10*. Yet *12* is a thousandfold less effective as an interferon inducer. (3) Resistance of the double-stranded complex to pancreatic RNase A is not a good guide for predicting antiviral activity. Compound *12* is totally resistant to high concentrations of RNase A, yet is much less active than *10* which is degraded at the same RNase A concentration. (4) Although compounds *13* and *14* have similar molecular weights and $T_m$'s, *13* has significant antiviral activity, whereas *14* is completely inactive. At least two interpretations are possible, (a) the presence of $m^3U$ in *13* renders the $(A)_n$ more susceptible to degradation; (b) the purine member of the polynucleotide duplex may be more important than the pyrimidine strand in the induction process. Such a possibility has been indicated in other experiments[24-26]. (5) The reduction of activity brought about by the nonbase pairing nucleotide $m^3U$ is probably due to formation of loops in the polynucleotide duplex[25].

Effect of Human Serum on Biological Activity

Since it has been shown that human serum contains an activity which degrades double-stranded polynucleotides[27,28], and since *12* is totally resistant to high concentrations of pancreatic RNase A, we evaluated the effect of preincubating several polynucleotide duplexes with serum on their ability to provide antiviral protection in the hope that the resistance of *12* to degradation would lead to an effective increase in its activity in comparison to *16* or *10*. The results (Table I) were surprising in that *12*, completely resistant to RNase A by chemical criteria, showed complete loss of antiviral activity after incubation with human serum.

In a separate experiment, we compared the effect of RNase A and human

serum. Using experimental conditions described in Table I, we found that serum (13%) caused a tenfold drop in the activity of $(I)_n \cdot (C)_n$, while RNase A (10 μg/ml) caused a > hundredfold drop. $(A)_n \cdot (dUz)_n$ (*12*), however, showed no decrease in activity upon prior incubation with RNase A (10 μg/ml), but lost all activity when preincubated with human serum (13%). These results indicate that the human serum nuclease(s) which degrade $(I)_n \cdot (C)_n$ may have a different substrate specificity from pancreatic RNase A. Because of the importance of such a possibility with regard to the design of polynucleotide interferon inducers for human usage, we are presently engaged in further studies to elucidate the nature of this serum activity.

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References

1. Beers, R. F. and Braun, W., Biological Effects of Polynucleotides, Springer-Verlag, New York, 1971.
2. Colby, C., Progr. Nucl. Acid Res. Mol. Biol., 11, 1 (1971).
3. Kleinschmidt, W. J., Annu. Rev. Biochem., 41, 517 (1972).
4. Grossberg, S. E., New Engl. J. Med., 287, 13 (1972).
5. Niblack, J. F. and McCreary, M. B., Nature New Biol., 233, 52 (1971).
6. DeClercq, E. and Merigan, T. C., Nature, 222, 1148 (1969).
7. DeClercq, E., Eckstein, F., Sternbach, H. and Merigan, T. C., Virology, 42, 421 (1970).
8. Colby, C. and Chamberlin, J. J., Proc. Nat. Acad. Sci., U.S.A., 63, 160 (1969).
9. Torrence, P. F. and Witkop, B., Biochemistry, 11, 1737 (1972).
10. Torrence, P. F., Waters, J. A. and Witkop, B., J. Amer. Chem. Soc., 94, 3638 (1972).
11. Kochetkov, N. K., Budowsky, E. I., Shibaev, V. N., Eliseeva, G. I., Grachev, M. A. and Demushkin, V. P., Tetrahedron, 19, 1207 (1963).
12. Moffatt, J. G. and Khorana, H. G., J. Amer. Chem. Soc., 83, 649 (1961).
13. Szer, W. and Shugar, D., Acta Biochem. Polon., 8, 235 (1961).
14. Torrence, P. F., Bobst, A. M., Waters, J. A. and Witkop, B., in preparation.
15. Billiau, A., Buckler, C. E., Dianzani, F., Uhlendorf, C., Baron, S., Proc. Soc. Exp. Biol. Med., 132, 790 (1969).
16. Baron, S., Bogomolova, N. N., Billiau, A., Levy, H. B., Buckler, C. E., Stern, R., Naylor, R., Proc. Nat. Acad. Sci., U.S.A., 64, 67 (1969).
17. Buckler, C. E., Wong, K. T., Baron, S., Proc. Soc. Exp. Biol. Med., 127, 1258 (1968).
18. Finkelstein, M. S., Bausek, G. H. and Merigan, T. C., Science, 161, 465 (1968).
19. Fukada, T., Kawade, Y. and Vjihara, M., Jap. J. Microbiol., 12, 329 (1968).
20. Black, D. R., Eckstein, F., Hobbs, J. B., Sternbach, H. and Merigan, T. C., Virology, 48, 537 (1972).
21. DeClercq, E., Zmudzka, B. and Shugar, D., FEBS Letters, 24, 137 (1972).
22. Steward, D. L., Hernden, W. C., Jr., Schell, K. R., Biochim. Biophys. Acta, 262, 227 (1972).

23. Vilček, J., Ng, Mun H., Friedman-kien, A. E. and Krawciw, T., J. Virol., 2, 648 (1968).
24. DeClercq, E. and DeSomer, P., J. Virol., 9, 721 (1972).
25. Carter, W. A., Pitha, P. M., Marshall, L. W., Tazawa, I., Tazawa, S. and Ts'o, P. O. P., J. Mol. Biol., 70, 567 (1972).
26. Mohr, S. J., Brown, D. G. and Coffey, D. S., Nature New Biol., 240, 250 (1972).
27. Norlund, J. J., Wolff, S. M. and Levy, H. B., Proc. Soc. Exp. Biol. Med., 133, 439 (1970).
28. Stern, R., Biochem. Biophys. Res. Commun., 41, 608 (1970).