Inhibition of oncornavirus functions by poly (2-methylthioinosinic acid)

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Received 6 November 1975

ABSTRACT

Poly (2-methylthioinosinic acid) [poly(ms²I)] was found to markedly inhibit the RNA directed DNA polymerase (reverse transcriptase) activity of murine (Moloney, Rauscher) leukemia virus and murine (Moloney) sarcoma virus, while under the same conditions the unsubstituted parent compound poly(I) showed little, if any, inhibitory effect. Copolymers of inosinic acid (I) and 2-methylthioinosinic acid₂(ms²I) showed an intermediary effect, depending on the I:ms²I ratio. Poly(ms²I) also inhibited the transformation of normal cells by murine (Moloney) sarcoma virus, as assessed by an infectious center assay.

INTRODUCTION

Homopolyribonucleotides [poly(A), poly(C), ...] and their 2'-O-methylated derivatives when complexed to a complementary oligodeoxyribonucleotide have been shown to act as templates for the RNA dependent DNA polymerase of oncornaviruses¹⁻⁴. In the absence of this oligodeoxyribonucleotide primer, homopolyribonucleotides do not serve as templates but rather inhibit the polymerization reaction⁵⁻¹³. The inhibitory effect of poly(U) and poly(C) is enhanced upon substitution of the 2'-hydroxyl by fluorine, chlorine or azido (-N=N⁺=N⁻)^{10,12}. Substitution of 2'-O-methyl or 2'-O-ethyl for 2'-OH in poly(A) increases the inhibitory potency of poly(A)¹¹, but, strangely enough, 2'-O-methylation decreases the activity of poly(I) and poly(U)¹³. Apparently, polyribonucleotides and their 2'-modified analogs compete with viral RNA (or synthetic RNA, if added exogenously to the assay mixture) for the template binding site on the DNA polymerase molecule. However, in assays in which exogenous template:primers [poly(A).oligo(dT), poly(C).oligo(dG)] were employed, poly(U), poly(A), poly(I) and their 2'-O-modified derivatives displayed inhibition kinetics for the DNA polymerase which could not be explained by simple competition with the exogenous template^{9,11,13}.

This report introduces a novel inhibitor of the reverse transcriptase, poly 2(-methylthioinosinic acid) [poly(ms²I)]. One of the major characteristics of poly(ms²I) is that it does not form a double-stranded complex with poly(C)¹⁴. Poly(ms²I), whether or not mixed with poly(C), is unable to induce interferon or resistance to (vesicular stomatitis) virus infection in cell cultures¹⁵. Poly(ms²I) has now been found effective in inhibiting the RNA directed DNA polymerase of murine leukemia and sarcoma viruses. In addition, poly(ms²I) proved effective in inhibiting the primary infection of normal cells by murine sarcoma virus. Inhibition of *in vivo* replication of oncornaviruses has previously been reported for poly(A) and O'-alkylated derivatives thereof^{7,8,16-18}.

MATERIALS AND METHODS

The synthesis and physicochemical properties of poly(ms²I), poly(I₃,ms²I₂) and poly(I₄,ms²I₁) have been described previously^{14,15}. All polymers were characterized as having chain lengths of more than 100 nucleotide units^{14,15}. Poly(I) (s_{20,w} : 9.0 S) was purchased from P-L Biochemicals, Milwaukee, Wisconsin. Poly(ms²I), poly(I₃,ms²I₂) and poly(I₄,ms²I₁) were dissolved in 0.1M Tris-HCl, 0.2M NaCl, pH 7.0 at 1 mg/ml and stored at 4°. Poly(I) was dissolved in Dulbecco's phosphate buffered saline (PBS) at 1 mg/ml and stored at -20°.

The reagents, the origin of viruses employed and the whole methodology for measuring DNA polymerase activity in oncornaviruses have been described¹². Carbopol (a carboxypolymethylene) was included in all reaction mixtures to increase the sensitivity of the assay¹⁹. The microtechnique for measuring the formation of infectious centers in MO-cells inoculated with murine (Moloney) sarcoma virus has also been described¹².

RESULTS

Poly(ms²I), poly(I) and copolymers of I and ms²I were examined for their effects on the kinetics of DNA synthesis by the DNA polymerase of either MuLV (Moloney), MuLV (Rauscher) or MuSV (Moloney). No exogenous template:primer [e.g. poly(A).oligo(dT)] was added to the reaction mixtures, so that only endogenous viral RNA directed DNA synthesis was measu-

Abbreviations : MuLV (Moloney), Moloney strain of murine leukemia virus; MuLV (Rauscher), Rauscher strain of murine leukemia virus; MuSV (Moloney), Moloney strain of murine sarcoma virus; ms²I, 2-methylthioinosinic acid.

red. Poly(ms^2I) proved markedly inhibitory in all three oncornavirus DNA polymerase systems (Fig. 1). Poly(I) slightly inhibited MuLV (Moloney) DNA polymerase but did not affect MuLV (Rauscher) DNA polymerase or MuSV (Moloney) DNA polymerase. The copolymers of I and ms^2I exhibited an intermediary effect, dependent on the I: ms^2I ratio. The relative strength of inhibition was $\text{poly}(\text{ms}^2\text{I}) > \text{poly}(\text{I}_3, \text{ms}^2\text{I}_2) > \text{poly}(\text{I}_4, \text{ms}^2\text{I}_1) \approx \text{poly}(\text{I})$ for MuLV (Moloney) DNA polymerase, $\text{poly}(\text{ms}^2\text{I}) > \text{poly}(\text{I}_3, \text{ms}^2\text{I}_2) > \text{poly}(\text{I}_4, \text{ms}^2\text{I}_2) \approx \text{poly}(\text{I})$ for MuLV (Rauscher) DNA polymerase and $\text{poly}(\text{ms}^2\text{I}) > \text{poly}(\text{I}_3, \text{ms}^2\text{I}_2) \approx \text{poly}(\text{I}_4, \text{ms}^2\text{I}_1) > \text{poly}(\text{I})$ for MuSV (Moloney) DNA polymerase.

The inhibitory effect of $\text{poly}(\text{ms}^2\text{I})$ on the DNA polymerase activities of MuLV (Moloney), MuLV (Rauscher) and MuSV (Moloney) were equally dose-dependent (Fig. 2). When the concentration of $\text{poly}(\text{ms}^2\text{I})$ in the reaction mixture was increased from 5 to 80 $\mu\text{g}/\text{ml}$, inhibition of the DNA polymerase activity gradually augmented. Almost identical dose-response curves were obtained whether DNA polymerase activity was measured at 60 min or 120 min.

Figure 1. Time-dependent effect of $\text{poly}(\text{I})$, $\text{poly}(\text{I}_4, \text{ms}^2\text{I}_1)$, $\text{poly}(\text{I}_3, \text{ms}^2\text{I}_2)$ and $\text{poly}(\text{ms}^2\text{I})$ on the DNA polymerase activity of either MuLV (Moloney), MuLV (Rauscher) or MuSV (Moloney). DNA synthesis measured at different times, as indicated. Final concentration of polymers in assay mixture: 20 $\mu\text{g}/280 \mu\text{l}$. $\bullet\text{---}\bullet$: control. $\nabla\text{---}\nabla$: $\text{poly}(\text{I})$. $\square\text{---}\square$: $\text{poly}(\text{I}_4, \text{ms}^2\text{I}_1)$. $\Delta\text{---}\Delta$: $\text{poly}(\text{I}_3, \text{ms}^2\text{I}_2)$. $\bullet\text{---}\bullet$: $\text{poly}(\text{ms}^2\text{I})$.

The inhibitory effect of poly(ms²I) on the in vitro reverse transcriptase activity of MuSV (Moloney) was paralleled by a similar inhibitory effect on the in vivo transformation of normal cells by MuSV (Moloney). As shown in Table 1, poly(ms²I) inhibited the development of infectious centers in MO-cells infected with MuSV (Moloney). This inhibitory effect was almost

Figure 2. Dose-dependent effect of poly(ms²I) on the DNA polymerase activity of either MuLV (Moloney), MuLV (Rauscher) or MuSV (Moloney). DNA synthesis measured at 60 min (●—●) or 120 min (○—○). Final concentrations of polymer in assay mixtures, as indicated.

TABLE 1. EFFECT OF POLY(I), POLY(I₄,ms²I₁), POLY(I₃,ms²I₂)
AND POLY(ms²I) ON THE DEVELOPMENT OF MuSV (MOLONEY)
INFECTIOUS CENTERS IN MO-CELLS

Infectious Centers (% of control)					
Polymer	<u>Polymer concentration (µg/ml)</u>				
	5	10	20	40	80
Poly(I)	-	-	-	-	77
Poly(I ₄ ,ms ² I ₁)	-	-	-	-	54
Poly(I ₃ ,ms ² I ₂)	-	-	-	-	57
Poly(ms ² I)	74	67	64	39	31

Average values for two observations.

equally dose-dependent as the inhibition of MuSV (Moloney) DNA polymerase activity by poly(ms²I), and, as may have been anticipated from the DNA polymerase data (Fig. 1), poly(I) and I:ms²I copolymers were less active than poly(ms²I) in inhibiting cell transformation by MuSV (Table 1).

When administered intraperitoneally to newborn mice (1.3-1.9 g) at 10 or 50 µg per mouse 4 h prior to MuSV (Moloney) challenge, poly(ms²I) failed to inhibit tumor development and death (data not shown). In similar conditions, poly(2'-O-methyladenylic acid), also an inhibitor of reverse transcriptase^{7,11} and oncornavirus replication in cell culture^{7,16,18}, suppressed sarcoma development and death¹⁷. The latter effect was ascribed to an enhancement of the hosts immune response¹⁷. Whether poly(ms²I), unlike poly-(2'-O-methyladenylic acid), is unable to stimulate the immune responsiveness of newborn mice and, therefore, fails to curtail MuSV oncogenesis, is a possibility worth exploring.

DISCUSSION

There are both similarities and dissimilarities in the structural parameters underlying the interferon inducing capacity of synthetic polynucleotides and their inhibitory effect on the reverse transcriptase activity of oncornaviruses.

-1°. A sufficiently high molecular size ($s_{20,w} \geq 4$ S) appears to be required for both interferon induction²⁰ and inhibition of reverse trans-

criptase⁹ as well as for several other biological activities of synthetic polynucleotides such as inhibition of complement activity²¹ and release of DNA template restrictions²².

-2°. To induce interferon, the polynucleotide should be double-stranded, not single- or triple-stranded^{20,23}. To inhibit the reverse transcriptase, the polynucleotide should be single-stranded (as concluded from references 5 through 13).

-3°. Whereas modifications in the purine ring, e.g. substitution of CH for N⁷ in poly(A) or poly(I), weaken or abolish the interferon inducing capacity, partly because of a reduction of the thermal stability of the corresponding complexes with poly(U) or poly(C)^{23,24}, such modifications do not impair or rather increase the inhibitory effects of these purine homopolyribonucleotides on reverse transcriptase activity²⁵. Similarly, introduction of a 2-methylthio substituent in poly(I) prevents the polymer from forming an interferon inducing complex with poly(C)¹⁵. Yet, poly(I) becomes more effective as reverse transcriptase inhibitor upon 2-methylthio substitution (this report). Other purine or pyrimidine modifications which have been shown to enhance the inhibitory effects of polynucleotides on oncornavirus DNA polymerases are the introduction of a 1,N⁶-ethenobridge in poly(A)²⁶ and the introduction of 5-mercapto groups in poly(C)^{27,28}. In the latter experiments^{27,28}, a partially thiolated poly(C) preparation was employed that contained 9.5 % 5-mercaptocytidylate units. The same C: 5-mercapto C copolymer, when annealed to poly(I), proved significantly less active in inducing interferon than the unsubstituted poly(C).poly(I) duplex²⁹.

-4°. One of the most stringent requirements for the induction of interferon by double-stranded RNAs is the presence of intact 2'-hydroxyl groups^{20,30}. Substitutions of 2'-F³¹, 2'-Cl³², 2'-O-CH₃^{33,34}, 2'-O-CH₂-CH₃³⁵ or 2'-N₃³⁶ for 2'-OH in the purine or pyrimidine strand of poly(I).poly(C) or poly(A).poly(U) invariably led to dramatic decrease in the interferon inducing properties of the corresponding duplexes. Yet, the same modifications [2'-F¹⁰, 2'-Cl¹⁰, 2'-O-CH₃^{7,11}, 2'-O-CH₂-CH₃¹¹, 2'-N₃¹²] potentiated the inhibitory effects of either poly(U), poly(C) or poly(A) on oncornavirus DNA polymerase. That 2'-O-methylation actually decreased the inhibitory potency of poly(I) and poly(U) for MLV (Moloney) DNA polymerase¹³, is a dissonant.

In their study on the inhibition of MuLV (Moloney) DNA polymerase by 2'-O-alkylated polyadenylic acids, Arya *et al.*¹¹ found that poly(I) was

even more effective in inhibiting the poly(A).oligo(dT) directed polymerization than either poly(2'-O-methyl A) or poly(2'-O-ethyl A). In further studies, Arya *et al.*¹³ found poly(I) also more effective than either poly(2'-O-methyl I), poly(2'-O-methyl U), poly(2'-O-methyl C), poly(U) or poly(C). In our assay, in which no exogenous template:primer was used, 2-methylthio substituted poly(I) proved clearly more effective than the unsubstituted poly(I) (Fig. 1). Furthermore, the extent of activity depended on the $ms^2I:I$ ratio in the substituted molecule.

Poly(ms^2I) may owe its inhibitory potential for oncornavirus DNA polymerase to its inability to base pair with primer oligonucleotides. Unlike poly(I), poly(ms^2I) does not form a double-stranded complex with poly(C)¹⁴. The bulky, non-hydrogen bonding substituent in C-2 may hamper complex formation with oligonucleotides as well. Hence, poly(ms^2I) may remain unpaired and free to compete with viral RNA for the template site on the DNA polymerase molecule. Inability to base pair with primer oligonucleotides has been envisaged previously to explain the inhibitory effect of poly(1,N⁶-ethenoadenylic acid) on AMV (Avian myeloblastosis virus) DNA polymerase²⁶.

As demonstrated before with poly(2'-azido U), poly(2'-O-methyl A) and other polynucleotides^{7,8,12,16,18}, the inhibitory effect of poly(ms^2I) on the *in vitro* reverse transcriptase activity was matched by an inhibition of *in vivo* oncornavirus replication (Table 1). This correlation suggests but, obviously, does not prove that poly(ms^2I) inhibits those steps in virus replication that are governed by the reverse transcriptase of the virion. In this sense, our data provide additional evidence for the role of the reverse transcriptase in the early stages of oncornavirus replication^{37,38}.

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ACKNOWLEDGMENTS

This investigation was supported by grants from the Belgian F.G.W.O. (Fonds voor Geneeskundig Wetenschappelijk Onderzoek), A.S.L.K. (Algemene Spaar- en Lijfrentekas) and the Katholieke Universiteit Leuven (Fonds Derde Cyclus). The technical assistance of Miette Stuyck and Chris Dillen and the editorial help of Janine Putzeys are deeply appreciated.

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