**Antiviral Properties of Polyinosinic Acids containing Thio and Methyl Substitutions**

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(Accepted 19 September 1980)

**SUMMARY**

Polyinosinic acids containing methyl and sulphur substitutions are potent inhibitors of reverse transcriptase. Substitution of sulphur for oxygen at the 6 position produces significant effects on the properties of polyinosinic acid: the kinetics of inhibition change from competitive to mixed-type and the inhibition constant falls by three orders of magnitude. In contrast, 1-methyl substitution produces no such effects. Poly(1-methyl-6-thioinosinic acid) or poly(mls6I) inhibits irreversibly, inhibiting all ten reverse transcriptases tested under a variety of assay conditions. In cell culture test systems, poly(mls6I) is capable of blocking both infection by non-transforming viruses and transformation by a sarcoma virus. The presence of poly(mls6I) in a preinfected culture results in the production of non-infectious virus particles lacking reverse transcriptase activity.

**INTRODUCTION**

Purine and pyrimidine analogues, 6-mercaptopurine, 5-fluorouracil and cytosine arabinoside are among the anti-cancer drugs currently in use (Timmis & Williams, 1967). Recently, polymeric forms of some of the substituted purines have been synthesized (Broom et al., 1976; Broom & Amarnath, 1976) and found to possess greater anti-tumour activity than the parent compound. For example, the homopolynucleotide analogue poly(thioinosinic acid) or poly(s6I), is approx. 20 times more potent than the parent 6-mercaptopurine when tested in the murine leukaemia L1210 system in vivo (Broom et al., 1978).

The mode of action of these substituted polynucleotide anti-tumour agents is unclear. Since retroviruses may be involved in murine leukaemia in general (Gilden, 1977), these compounds might act as virus inhibitors. However, virus inhibition via interferon induction is not believed to be the mechanism involved, because induction of interferon by polynucleotides requires a double-stranded secondary structure (DeClercq & Merigan, 1969), and these substitutions, especially the isosteric substitution of sulphur for oxygen, abolish complementary H-bonding (Broom & Amarnath, 1976). These single-stranded polynucleotides are, in fact, very poor inducers of interferon (Broom et al., 1978). They might, however, act as inhibitors of the retroviral enzyme, reverse transcriptase, which is essential for infectivity (for reviews, see Chandra et al., 1977; DeClercq, 1979). Various agents that inhibit this enzyme in vitro are capable of inhibiting virus infection in vivo. These include antibiotics (Green et al., 1974), homopolynucleotides (Tennant et al., 1973; Arya et al., 1975), modified polynucleotides

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(Tennant et al., 1974; DeClercq et al., 1975) and distantly related polynucleotide analogues (Pitha et al., 1973).

This report describes a comparative study of the effects of poly(s6I) and its structurally related derivatives poly(m1s6I) and poly(m1I) on the activity of reverse transcriptase in vitro and of the effects of poly(m1s6I) on virus infectivity and multiplication in cell cultures.

**METHODS**

**Polynucleotides.** Poly(m1I), poly(s6I) and poly(m1s6I) were gifts from Arthur Broom (University of Utah). Polynucleotides poly(A) and poly(I) and primer - templates for reverse transcriptase, (dG)12-18 · (rC)m and (dT)12 · (rA)n, were from P.-L. Biochemicals, Milwaukee, Wis., U.S.A.

**Viruses and polymerase.** The following viruses were obtained from the Division of Cancer Cause and Prevention, National Cancer Institute: avian myeloblastosis (AMV), Rauscher leukaemia (RLV), feline endogenous (RD114), feline exogenous (FELV), wooly monkey sarcoma (SSV), gibbon ape leukaemia (GALV), murine mammary tumour (MMTV) and rhesus monkey (MPMV) viruses. FBJ (Finkel et al., 1966) and FBR (Finkel et al., 1973) murine sarcoma viruses (MuSV) were harvested after 1 day from the media of chronically infected Balb-A31 and SC-1 roller bottle cultures respectively. Media were clarified at 10 000 g for 10 min and then centrifuged at 100 000 g for 1 h. Pellets were resuspended in 0-01 M-tris pH 8-2, 0-15 M-NaCl and 0-002 M-EDTA, layered on to a solution of 30% glycerol in the same buffer, and centrifuged at 100 000 g for 1 h. The final pellets were used for reverse transcriptase reactions. The FBR murine-associated virus (MuAV) was propagated in NIH-3T3 mouse embryo cells chronically infected with FBR virus at a post-transforming dilution and the medium collected after incubation for 24 h. This was clarified in a bench centrifuge, passed through a 0-45 μm Millipore filter and virus assayed by the XC-plaque method. Moloney-MuSV was propagated in a clone of the Moloney virus-transformed Wistar rat embryo fibroblast cell line, obtained from William Campbell (University of Tennessee). The virus was harvested as for FBR(MuAV) and assayed by focus formation. Purified reverse transcriptase of AMV was obtained from Life Sciences under a contract from the Division of Cancer Cause and Prevention, National Cancer Institute.

**Polymerase assays.** Standard exogenous template reactions were conducted essentially as described by Spiegelman et al. (1970). Fifty μl preincubation buffer (0-01 M-tris-HCl pH 8-2, 0-01 mM-dithiothreitol and 0-3% NP40) containing either virus pellets (50 to 100 μg) or purified reverse transcriptase of AMV (2 ng) were incubated with an equal volume of reaction mixture containing, in the final concentration, 0-05 M-tris-HCl pH 8-2, 0-01 M-NaCl, 2 mM-dithiothreitol, 10 mM-MgCl2, 50 μM-8-3H-dGTP at 1-2 × 106 ct/min/pmol (ICN, Irvine, Calif., U.S.A.) and 25 μg/ml (dG)8-12 · (rC)m. When other templates were used, the reactions were carried out at the same template concentration and conditions as above. In the case of (dT)12 · (rA)n, the substrate was replaced with methyl-3H-TTP at the same concentration and specific activity. With (dG)12-18 · (rC)m as template (Gerard et al., 1974; Gerard, 1975), the cation was replaced by MnCl2 at 0-01 mM. After incubation at 37 °C for the times indicated, the reaction mixtures or aliquots thereof were assayed for acid-precipitable radioactivity.

Endogenous reactions (Baltimore, 1970; Temin & Mizutani, 1970) were conducted with 50 μl detergent-disrupted virus (50 to 200 μg) and 50 μl of the reaction mixture, containing in final concentrations: 60 mM-tris-HCl pH 8-2, 1 mM each of dATP, dGTP, dCTP, 0-1 mM-methyl-3H-TTP at 86 Ci/mmmol (ICN), 8 mM-MgCl2, 5 mM-dithiothreitol, 100 μg/ml actinomycin D and 0-15 μg/ml (dT)12.

**Cell cultures.** D55, A31 and SC-1 cell cultures were obtained and propagated as previously reported (Lee et al., 1979).
Virus inhibition by substituted poly(I)

Fig. 1. Inhibition of exogenous reaction. The three substituted poly(I)'s were added at the concentrations (μg/ml) indicated to standard reactions with detergent-disrupted AMV. The kinetics were monitored by assaying 15 μl samples at the times indicated. (a) Poly(m'l): O--O, none; △--△, 0.36; ×--×, 1.82; □--□, 3.6; ●--●, 5.46; ▲--▲, 7.3. (b) Poly(s'q): O--O, 0; △--△, 0.025; ×--×, 0.05; □--□, 0.075; ●--●, 0.1; ▲--▲, 0.125; ■--■, 0.15; ▽--▽, 0.2. (c) Poly(m'sq'I): O--O, 0; △--△, 0.012; ×--×, 0.025; □--□, 0.037; ●--●, 0.05; ▲--▲, 0.062; ■--■, 0.075; ▽--▽, 0.1.

Infectivity assays. XC-plaque and focus assays for non-transforming and transforming viruses were conducted on D55 indicator cells as described by Lee et al. (1979). Concentrations of poly(m'sq'I) up to 50 μg/ml were non-toxic to D55 cells, as indicated by the absence of microscopically visible cytopathic effects on the monolayer cultures during the 2 week test period.

Ultrastructural assay. Viral pellets and infected cell preparations were processed for electron microscopic examination by thin-section methods.

RESULTS

Template activity

The substituted poly(m'l), poly(s'q) and poly(m'sq'I) and the unsubstituted poly(I) were tested for their ability to function as templates in a reverse transcriptase reaction with detergent-disrupted AMV virion enzyme. Except for poly(I), none of the polynucleotides showed appreciable template activity, even at the higher concentration (100 μg/ml) tested (data not shown).

Inhibition of exogenous reaction

The addition of the poly(I) analogues to exogenous template reactions consisting of (dG)_{12-18}·(rC)_n and purified AMV reverse transcriptase resulted in strong inhibitions of the enzyme (Fig. 1). This was not caused by nuclease contamination as each of the analogues was shown to be nuclease-free by incubation with 3H-cellular RNA, or by a shift in pH, since the analogues are themselves neutral and the reaction mixtures remain at approximately the optimum pH.

The poly(I) analogues equally inhibited reactions directed by other templates, such as (dT)_{12}·(rA)_n or (dG)_{12-18}·(rCm)_n (data not shown). Moreover, poly(m'sq'I) strongly inhibited the reverse transcriptase activities of all 10 viruses tested, including examples of types B, C, and D viruses (Table 1).
**Table 1. Inhibition of reverse transcriptase by poly(m's6I)**

<table>
<thead>
<tr>
<th>Retrovirus</th>
<th>Incorporation (ct/min × 10^-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No virus</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>Type C</td>
<td>AMV</td>
</tr>
<tr>
<td></td>
<td>RLV</td>
</tr>
<tr>
<td></td>
<td>FBJ</td>
</tr>
<tr>
<td></td>
<td>FBR</td>
</tr>
<tr>
<td></td>
<td>FELV</td>
</tr>
<tr>
<td></td>
<td>RD114</td>
</tr>
<tr>
<td></td>
<td>SSV</td>
</tr>
<tr>
<td></td>
<td>GALV</td>
</tr>
<tr>
<td>Type B</td>
<td>MMTV</td>
</tr>
<tr>
<td>Type D</td>
<td>MPMV</td>
</tr>
</tbody>
</table>

* Exogenous reactions were performed for 1 h with the appropriate amounts of various viruses and 25 μg/ml (dG)_{12-18}·(rC)_n in the absence and presence of 10 μg/ml poly(m's6I).

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Poly(m'sI) was a competitive inhibitor, like poly(A) and poly(I) (Fig. 2a). In contrast, poly(s6I) and poly(m'sq) showed mixed-type inhibition kinetics (Fig. 2b, c) and a decrease of three orders of magnitude in inhibition constants ($K_i$) (Table 2).

The effects of varying the time and order of addition of poly(m's6I) to a standard exogenous reaction mixture were studied. Results in Fig. 3(a) showed that the addition of the inhibitor at various times resulted in immediate and total inhibition. If the inhibitor was added at zero time, the subsequent addition of excess template at various times would not start the reaction (Fig. 3b).

**Inhibition of endogenous reaction**

Poly(m's6I) inhibited an endogenous reaction of the reverse transcriptase of detergent-disrupted SSV (Fig. 4a). When the deproteinized reaction mixture of an inhibited endogenous reaction was analysed by velocity-gradient centrifugation, there was a total absence of $^3$H-DNA products, free or complexed with template 60 to 70S virus RNA (Fig. 4b).
Virus inhibition by substituted poly(I)

Table 2. Inhibitor constants (Ki)*

<table>
<thead>
<tr>
<th>Polynucleotide</th>
<th>Type of inhibition</th>
<th>Ki (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td>Competitive</td>
<td>2.1 x 10^{-6}</td>
</tr>
<tr>
<td>(I)</td>
<td>Competitive</td>
<td>5.1 x 10^{-6}</td>
</tr>
<tr>
<td>(m1I)</td>
<td>Competitive</td>
<td>1.0 x 10^{-6}</td>
</tr>
<tr>
<td>(m1s6I)</td>
<td>Mixed</td>
<td>14.0 x 10^{-9}</td>
</tr>
<tr>
<td>(s6I)</td>
<td>Mixed</td>
<td>7.1 x 10^{-9}</td>
</tr>
</tbody>
</table>

* From data in Fig. 2, Ki's of competitive inhibitors were calculated by standard formula (Lineweaver & Burk, 1934). Ki's for the mixed-types were determined by the graphical method of Hunter & Downs (1945), plotting \( \frac{1}{v} \) versus \( s \).

Fig. 3. Effect of (a) time and (b) order of addition of poly(m1s6I). Poly(m1s6I) was tested in standard exogenous reactions with disrupted AMV and 10 µl amounts of each reaction mixture were sampled to follow reaction kinetics. (a) With template (25 µg/ml) present at zero time, inhibitor (10 µg/ml) was added at the times indicated: ■■■, 50 min; ▼▼▼, 40 min; ●●●, 30 min; □□□, 20 min; △△△, 10 min; ×××, zero time. (b) With inhibitor (10 µg/ml) present at zero time, template (50 µg/ml) was added at 0, 15 and 30 min (●●●●). Control reactions, without inhibitor, also received template at 0 (××××), 15 (●●●) and 30 (△△△) min.

Fig. 4. Inhibition of endogenous reaction. Endogenous reactions were carried out in standard 100 µl mixture with and without poly(m1s6I). (a) Reaction kinetics: 15 µl amounts of the reaction mixtures consisting of 0.4 mg SSV and various concentrations of the inhibitor were monitored at the times indicated. Inhibitor concentrations were as follows: ○○○, none; ×××, 0.01 µg/ml; △△△, 0.1 µg/ml; □□□□, 0.1 µg/ml; ▼▼▼▼, 0.5 µg/ml; ●●●●, 10 and 50 µg/ml. (b) Reaction products: endogenous reactions of FBR(MuSV), freshly harvested from 30 ml medium, were carried out for 30 min in the absence (○○○) and presence (××××) of 100 µg/ml poly(m1s6I). After deproteinization, reaction mixtures were analysed in glycerol density gradients (Schlom & Spiegelman, 1971).
Table 3. Inhibition of virus infection

<table>
<thead>
<tr>
<th>Virus dilution*</th>
<th>Poly(m^1s^6I) (μg/ml)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>FBR(MuAV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^{-4.9}</td>
<td>&gt;400</td>
<td>60</td>
</tr>
<tr>
<td>10^{-4.5}</td>
<td>110</td>
<td>23</td>
</tr>
<tr>
<td>10^{-5.0}</td>
<td>47</td>
<td>8</td>
</tr>
<tr>
<td>Moloney MuSV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^{-3}</td>
<td>85</td>
<td>18</td>
</tr>
<tr>
<td>10^{-4}</td>
<td>17</td>
<td>3</td>
</tr>
</tbody>
</table>

* Dilutions of FBR(MuAV) were inoculated in duplicate on to overnight cultures of D55 indicator cells maintained in the absence or presence of 30 μg/ml of poly(m^1s^6I). Standard XC-plaque assays were performed. Similarly, two dilutions of Moloney MuSV were tested.
† Average of two plates.

Table 4. Effects of poly(m^1s^6I) on virus replication*

<table>
<thead>
<tr>
<th>Passage</th>
<th>Day</th>
<th>Polymerase (ct/min × 10^{-5}) at inhibitor concn. (μg/ml) of</th>
<th>Plaques (no./plate) at inhibitor concn. (μg/ml) of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>2.6</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>2.6</td>
<td>0</td>
</tr>
</tbody>
</table>

* Balb-A31 cells chronically infected with FBJ-MuSV(MuAV) were maintained in the presence of 0, 20 and 40 μg/ml poly(m^1s^6I). Virus replication was monitored by polymerase (exogenous template) and plaque assays on 1-day spent media at days and passages indicated.
† Not assayed.
‡ The culture exposed to 40 μg/ml was split into two matched flasks at the second passage; one was maintained at 40 μg/ml, the other received no inhibitor. Both cultures were assayed for polymerase 6 days later.

Effects on virus infection and replication

In cell culture test systems at a subtoxic concentration of 30 μg/ml, poly(m^1s^6I) inhibited the infectivity of FBR(MuAV), as monitored by XC-plaque assays. Similarly, focus formation assays indicated that the transforming activity of Moloney MuSV was also inhibited (Table 3). The effects of poly(m^1s^6I) on virus replication in a chronically infected, high virus-producing culture were tested (Table 4). At the subtoxic concentrations of 20 and 40 μg/ml poly(m^1s^6I), virus polymerase and XC-plaque assays showed that virus production was inhibited. It was resumed when poly(m^1s^6I) was omitted from the culture medium on the second subculture, whereas the matched culture, maintained in the presence of 40 μg/ml poly(m^1s^6I), continued to be negative. However, other detection procedures that do not depend on a functional reverse transcriptase indicated that virions were indeed being produced at the same level or at an even higher level in the presence of the polynucleotide analogue. Fig. 5 shows that more labelled virus particles of density 1.16 g/ml were produced in the presence of 30 μg/ml of the analogue. In addition, electron microscopy revealed budding and mature type C virus particles in the same relative abundance in the presence or absence of 30 μg/ml poly(m^1s^6I) (data not shown).
**Virus inhibition by substituted poly(I)**

**DISCUSSION**

The data indicate that the single-stranded polyinosinic acids containing thio and methyl substitutions are potent inhibitors of reverse transcriptase although they have no template functions. These substituted polynucleotides inhibit reverse transcription under a variety of assay conditions that involve different templates and enzymes.

Methyl substitutions at the 1 position did not significantly alter the effect of polyinosinic acid on reverse transcriptase. Both poly(m1sI) and poly(I) showed comparable Ki's and competitive inhibition kinetics, suggesting that they compete for the same active site. The isosteric substitution of sulphur for oxygen at the 6 position, however, resulted in polynucleotides with different characteristics. Poly(s6I) and poly(m1s6I) showed mixed-type inhibition kinetics suggesting that they bind to additional sites. Moreover, Ki measurements, which differed by three orders of magnitude, indicated that they bind to reverse transcriptase more strongly than poly(m1I) or poly(I) and poly(A). The unusually high affinity of the two polynucleotide analogues for reverse transcriptase suggests that they would be superior materials for affinity chromatography of the enzyme. The binding was characteristically tight and essentially irreversible under reaction conditions. Furthermore, poly(m1s6I) is effective whether added before or after the initiation of a reaction.

Since endogenous virus RNA transcription is an essential step for successful infection, the fact that poly(m1s6I) also inhibits the endogenous reaction suggests that this substituted polynucleotide may have antiviral activity *in vivo*. Indeed, poly(m1s6I) prevented the induction of XC plaques by murine non-transforming virus and the induction of morphological transformation by a murine sarcoma virus. However, addition of poly(m1s6I) after infection resulted in the production of non-infectious virus particles lacking reverse transcriptase activity. Since the function of reverse transcriptase is to catalyse virus RNA transcription, these effects of poly(m1s6I) on acute and chronic infections indicate that poly(m1s6I) specifically inhibits the transcription step in the infective phase and none of the subsequent steps in the productive phase of the replicative cycle.
The above results could be explained by the strong affinity and binding of reverse transcriptase to the polynucleotide. Presumably poly(MLS6I) and the enzyme form stable inactive complexes. In acute infections, such binding would inhibit RNA transcription, resulting in an abortive infection. In chronic infections, the polynucleotide may complex with the enzyme molecules as soon as they are synthesized, thus resulting in the absence of functional reverse transcriptase in the progeny virus particles. Whether or not the inactive complexes are encapsulated in the defective particles remains to be established.

The mechanism(s) of action of these anti-tumour polynucleotide analogues in the L1210 murine leukaemia test system is unknown. The present studies indicate that poly(MLS6I) is a potent antiviral agent \textit{in vitro} by virtue of its ability to inhibit reverse transcriptase. In animal species, such as chickens, mice, cats (Gilden, 1977) and cows (Ferrer \textit{et al.}, 1974; Abt \textit{et al.}, 1976), these polynucleotide analogues could be effective in preventing naturally occurring retrovirus-induced malignancies.

We are indebted to Dr Arthur Broom for his generous gift of the substituted polynucleotides. We thank M. Williams for the propagation of FBJ and FBR viruses. This research was jointly supported by the U.S. Department of Energy under contract No. W-31-109-ENG-38 and by the Division of Cancer Cause and Prevention, National Cancer Institute, through an Interagency Agreement, contract No. YO1 CP 7-0504.

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Virus inhibition by substituted poly(I)


(Received 14 May 1980)