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Effect of increasing thiolation of the polycytidylic acid strand of poly I:poly C on the α, β and γ interferon-inducing properties, antiviral and antiproliferative activities

Kailash C. Chadha[a], Wlodzimierz E. Dembinski[b], Christopher B. Dunn[c], Janos Aradi[d], Thomas J. Bardos[d], Joseph A. Dunn[c], Julian L. Ambrus Sr.[b,*]

[a] *Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, New York 14263, USA*
[b] *Department of Medicine, SUNYAB-Buffalo General Hospital/Kaleida Health System, State University of New York at Buffalo, 100 High Street, Bldg. E, Room 320, Buffalo, New York 14203, USA*
[c] *OmniPharm Research International, 255 Great Arrow Ave., Buffalo, New York 14210, USA*
[d] *Department of Medicinal Chemistry, State University of New York at Buffalo, Buffalo, New York 14214, USA*

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Abstract

Double-stranded RNAs induce interferons and cause the development of antiviral and antiproliferative activities. Antiviral activity is related to the production of interferons and other proteins that stimulate various immunologic activities, which appear to contribute to their overall antiproliferative activity. The most active double-stranded RNA, polyI:polyC, was shown to be too toxic for therapeutic use. We conducted selective thiolation of the polyC strand at the five position of the cytosine bases, generating a partially thiolated polyC (MPC) which after annealing with a complimentary unmodified polyI, gave the thiolated double-stranded RNA, pI:MPC. We have explored antiviral and antiproliferative activities at various levels of thiolation and found that optimal responses can be obtained at 7.4% level of thiolation. This compound deserves further study of antiviral and antiproliferative responses in vivo, and eventually clinical exploration. Earlier studies have shown that this and related compounds are active against HIV-1, in human cells, and against DNA polymerases of DNA and RNA tumor viruses.

Keywords: Interferon(s)-α, -β, -γ; Antiviral agents; Thiolated polyI:polyC; dsRNA; Vesicular stomatitis virus (VSV); Cellular proliferation; HIV-1 infection; AIDS

1. Introduction

Double-stranded RNAs (dsRNAs) have the capacity to induce interferons in animal and human cells and thus increase their resistance to viral infections (Haines et al., 1991; Torrence and DeClercq, 1981; DeClercq, 1974; Sen et al., 1978). The multiple mechanisms of signal transduction by interferons involve dsRNA at several points as activator, co-factor, or indirectly as inhibitor (Kalvakolanu and Bordon, 1996). The prototype dsRNA, polyI:polyC (pI:pC), has been reported also to inhibit the growth of some tumor cell lines as well as the rate of growth of human tumor xenografts in mice (Levy et al.,1969; Levy, 1970). However, the antiproliferative effects of pI:pC do not correlate with its interferon inducing activity (Fisher et al., 1972) and may involve other mechanisms as well, such as (1) activation of interferon-induced dsRNA dependent protein kinase which inhibits protein synthesis; (2) activation of 2′,5′-oligoadenylate synthetase which in turn activates a latent endoribonuclease (Rnase L); and (3) activation of adenylate cyclase, thus increasing the concentration of cAMP (Ruffner et al., 1990; Hovanessian, 1989; Li and Petryshyn, 1991; Wathelet et al., 1989; Chapekar et al., 1988; Hubbel et al., 1991). The immunologic activities of

* Corresponding author. Tel.: +1 716 859 1399; fax: +1 716 859 3659.
E-mail address: jlambrus@netscape.net (J.L. Ambrus Sr.).

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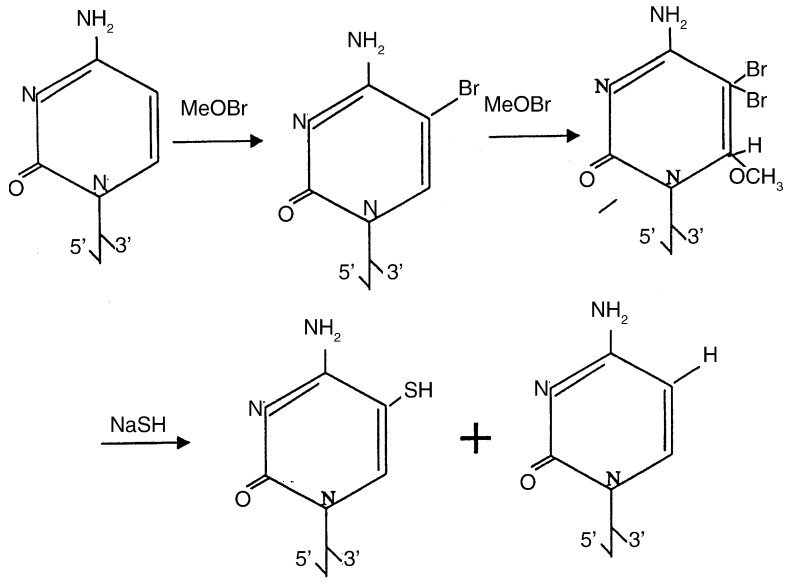

Fig. 1. Chemical structure and partial "5-thiolation" of cytosine residues of polynucleotides. MeOBr: methyl hypobromide; NaSH: sodium sulfhydride.

dsRNAs also include, in addition to interferon induction, the activation of macrophages and augmentation of natural killer cell activity (Djeu et al., 1978; Heider and Bardos, 1996). While pI:pC is the most potent among the dsRNAs tested, it has proved to be too toxic for therapeutic application (Stebbing and Grantham, 1976; Bacur et al., 1998).

Selective thiolation at the 5 position of some of the cytosine bases in pI:pC resulted in a partially thiolated dsRNA, poly I:MPC (pI:MPC) containing typically 9% 5-mercaptocytosine bases randomly distributed throughout the poly C strand; however the percentage of 5-mercaptocytosines could be varied with the amounts of the reagents, MeOBr and NaSH (Bardos et al., 1975), (Fig. 1) (Milkulski et al., 1973). Previous studies have demonstrated that pI:MPC is a potent inducer of interferons α, β and γ (O'Malley et al., 1975; Vastola et al., 1984) and that it possesses potent cytotoxic and antiproliferative activities against a variety of human solid tumor cell lines that are either unaffected or, to a much lesser degree, inhibited by the unmodified pI:pC (Vastola et al., 1984; Geng et al., 1991). Moreover, pI:MPC was found to be significantly less toxic than pI:pC in vivo in mice, rabbits and guinea pigs (Vastola et al., 1984). Partial thiolation enhanced the resistance of poly C to degradation by plasma ribonucleases (Ho et al., 1979), and increased its uptake into cells in vitro and in vivo (Paffenholz et al., 1976; Kung et al., 1984). In rats, plasma clearance rates of single-stranded MPC decreased with dose escalation suggesting that clearance of the polynucleotide is preceded by nuclease hydrolysis (Kung et al., 1984). MPC, in its single-stranded form, has been found to possess potent "antitemplate" activity against DNA and RNA polymerases, including reverse transcriptase (Chandra and Bardos, 1972; Milkulski et al., 1973; Kung et al., 1976; Cavanaugh et al., 1982; Bardos et al., 1992). Organ distribution studies using ^{35}S labeled pI:MPC demonstrated significantly greater tissue accumulation of radioactivity in the livers and spleens in leukemic versus normal mice at all time points examined (Kung et al., 1984). Comparing the radioactivity in suspended and washed spleen cells harvested at 60 min post-injection indicated that 4–10 times more pI:MPC and/or ^{35}S labeled oligonucleotides were localized and bound to cells in the spleens of leukemic mice than to those of normal mice (Kung et al., 1984). In addition, pI:MPC and other partially 5-thiolated oligo and polynucleotides particularly in the deoxyriboside series have been demonstrated to potently inhibit HIV-replication in primary human lymphocytes in culture (Bardos et al., 1992).

In our attempt to improve the biological properties of pI:MPC, we have conducted a new study to establish the relative effect of the percent of thiolation on the interferon inducing activities of pI:MPC. In this paper we report the results of that study.

2. Materials and methods

2.1. Preparation of pI:MPC

Polycytidylic acid (pC), polyinosinic acid (pI) and poly I:poly C (pI:pC) used in this study were obtained from Amersham Biosciences (Piscataway, NJ). The details of structural modifications of polycytidylic acid by partial 5-thiolation resulting in the formation of mercaptopolycytidylate (MPC) (Fig. 1) has been described previously (Milkulski et al., 1973; Bardos et al., 1978). Several preparations of MPC varying in percentage of thiolated bases were prepared. The value of percent thiolation was determined by ultraviolet spectra and by neutron activation analysis. Three different preparations of MPC having 1.1%, 7.4% and 14.3% thiolation were used in

the current studies. Annealing of MPC with pI was carried out by mixing them in equimolar concentrations of buffer containing 10 mM phosphate, 150 mM NaCl, and 1 mM MgCl at pH 7.2 for 3 h at room temperature. T_m values were recorded using a standard spectrophotometer (Bardos et al., 1975).

2.2. Cells

Primary human foreskin fibroblast (BG-9) cells were isolated, characterized and made available to us by Horoszewicz et al. (1978). Human amnionic epithelium (WISH, CCL-25) cells (with HeLa markers), human lung carcinoma (A-549, CCL-185) cells and human cervical epitheloid carcinoma (HeLa, CCL-2) cells were obtained from the American Type Culture Collection (Bethesda, MD). All cells were maintained in Minimal Essential Medium (MEM) containing non-essential amino acids and 10–15% fetal bovine serum (FBS). Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from normal, healthy volunteers, using Ficoll-Hypaque density gradient centrifugation method of Boyum (1968). BG-9 cells were used for interferon antiviral assays and also for interferon-β induction. PBMCs at a density of 1–3 × 10^6 cells/mL were used for interferon-α and -γ production.

2.3. Interferon production and antiviral activity

Confluent monolayers of BG-9 cells (IFN-β study) in 24-well tissue culture trays or PBMCs at a density of 1–3 × 10^6 cells/mL (IFN-α and IFN-γ study, respectively) were exposed at 37 °C to pI:pC or to different preparations of pI:MPC having variable levels of thiolation for 20 h for IFN-α and IFN-β, or 72 h for IFN-γ production. Each inducer with varying level of thiolation was used at five different concentrations (0, 1.0, 10, 100 and 1000 μg/mL) and each concentration was added to triplicate wells. The interferon released into the culture medium was collected after low-speed centrifugation, dialyzed overnight against PBS and stored at −70 °C until assayed. Interferon activity was measured as total antiviral activity on BG-9 cells and as individual interferons (α, β and γ) using commercially available ELISA assay kits (PBL Biomedical Laboratories, New Brunswick, NJ. For total antiviral activity, confluent monolayers of BG-9 cells in 24-well tissue culture plates were exposed to a serial two-fold dilutions of interferon preparations. Each plate had its own cell and viral controls. After 24 h, monolayers were washed with warm medium and challenged with Vesicular Stomatitis Virus (VSV). When cells in "virus control" wells were lysed (36 h), the level of protection was quantitated by the dye up-take method of Finter (1969). Monolayers were exposed to the vital dye, neutral red and the amount of dye taken up by the living cells was extracted and measured spectrophotometrically at 540 nm. Each plate contained the reference standard and results are expressed as International Units (IU). Antiviral activity was measured by ELISA assay, following the instructions provided by the manufacturer. The results are expressed as pg/mL.

2.4. Cytotoxicity of polynucleotides

Normal human fibroblasts (BG-9) cells were grown to confluency in 24-well tissue culture trays in MEM containing 10% FBS. A range of concentrations of pI:pC and pI:MPC (0, 10, 100 and 1000 μg/ml) in maintenance medium was added in triplicate to confluent cell monolayers and incubated at 37 °C for up to 3 days. At the end of each 24 h time period, a set of monolayers was stained with neutral red dye (Finter, 1969). The dye taken up by the living cells was eluted and quantitated spectrophotometrically at 540 nm and compared to control cells.

2.5. Antiproliferative activity

Cells were seeded in 96-well tissue culture trays at a concentration of 3 × 10^4 cells/mL in the growth medium (0.1 mL/well). After 48 h of seeding the trays, the old medium was removed and replaced with new medium containing a range of pI:pC or partially thiolated pI:MPC (1, 10, 100 and 1000 μg/mL). Cells were allowed to grow for 7 days and at this point, cell growth was measured with Cell Titer 96^R AquousOne Solution Cell Proliferation Assay (Promega, Madison, WI). The quantity of dye (a formazan product) taken up by the cells (490 μm absorbence) is directly proportional to the number of living cells in culture.

2.6. Statistical analysis

To analyze our data, we used means with standard error of means (S.E.M. ±) and one-way ANOVA with Turkey's multiple comparison post-test using Graph Pad Prism version 3.02 for windows, Graph Pad Software (San Diego, CA, USA, www.graphpad.com). All error bars in the figures refer to S.E.M. Wherever required, *P*-values are indicated in the text.

3. Results

3.1. Cytotoxicity of pI:MPC

Cytotoxicity of pI:MPC compounds (Fig. 1) was tested in primary human fibroblasts (BG-9) cells. No cytotoxicity was seen at any of the concentrations (1.0, 10, 100 and 1000 μg/mL) of all three pI:MPCs tested. These results are similar to earlier reports that thiolation of pI:pC reduced its cytotoxicity (Vastola et al., 1984).

3.2. Influence of level of thiolation on total antiviral activity

Partially thiolated pI:pC (pI:MPC) significantly modifies the response of PBMCs to interferon production. Antiviral

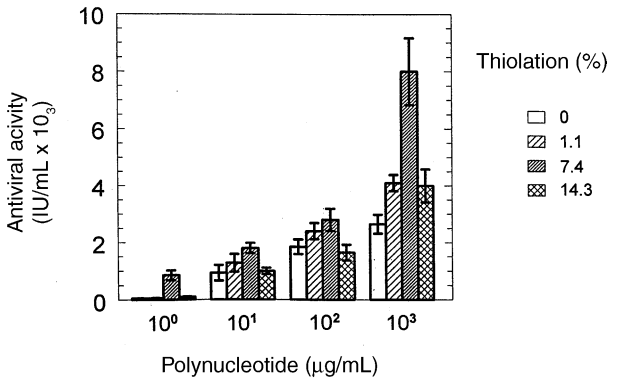

Fig. 2. Levels of total antiviral activity ± S.E.M. induced by pI:MPC in PBMC. Freshly isolated PBMC were exposed to indicated range of pI:MPC or pI:pC for 6 h. Inducer was removed and cells washed three times with warm medium and incubated for an additional 18 h. The total antiviral activity was measured on confluent monolayers of BG-9 cells as described in Section 2.

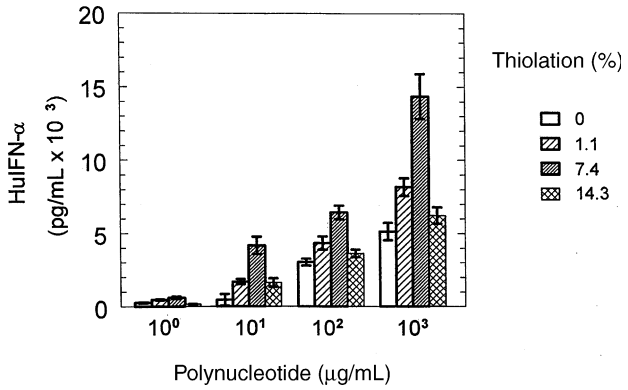

Fig. 3. Production of HuIFN-α by pI:MPC in PBMC ± S.E.M. Freshly isolated PBMC were exposed to indicated levels of pI:MPC or pI:pC for 6 h. Inducer was removed and cells were washed with warm medium and incubated for an additional 18 h. HuIFN-α antiviral activity released in the culture medium was measured by multi-species ELISA kit (#41105, Lot 1188) from PBL Biomedical Laboratories (Piscataway, NJ). This kit is specific for HuIFN-α and has no cross reactivity to HuIFN-β or HuIFN-γ.

activity of the interferon produced by the pI:MPC treated PBMC was measured on BG-9 cells. The results are shown in Fig. 2. At 1000 μg/mL and at 7.4% levels of thiolation, the total antiviral activity produced by pI:MPC treated PBMC was significantly higher than pI:pC treated PBMC ($P < 0.01$). Also, it is important to mention here that at 1 μg/mL, a significant amount of interferon antiviral activity (1000 IU) was seen at 7.4% thiolation when no detectable level of antiviral activity was seen in case of pI:pC or other two levels (1.1% and 14.3%) of thiolation (Fig. 2). At all concentrations of pI:MPC, 7.4% thiolation gave maximal antiviral activity. At all concentrations of pI:MPC, a definite decline in antiviral activity was seen at 14.3% thiolation as compared to 7.4% thiolation.

3.3. pI:MPC and human interferon-α (HuIFN-α) production

PBMC cells, freshly isolated (3×10^6 mL) from normal, healthy donors, were induced in triplicate with pI:MPC having different levels of thiolation and at four different concentrations (1.0, 10, 100 and 1000 μg/mL). In all cases pI:pC was used as control. HuIFN-α antiviral activity was measured by ELISA assays using commercially available kits that have no cross-reactivity to human β and γ interferons (PBL Biomedical Laboratories, New Brunswick, NJ). As shown in Fig. 3, the maximal HuIFN-α production was seen at 7.4% thiolation, and at all concentrations of pI:MPC used ($P < 0.001$). At 1000 μg/mL and at 7.4% thiolation, HuIFN-α production was maximal and nearly four-fold higher than that of pI:pC. Also, at 14.3% thiolation, no significant differences were seen in HuIFN-α production at all concentrations of pI:MPC used as compared to pI:pC (Fig. 3).

3.4. pI:MPC and HuIFN-β production

Confluent monolayers of normal human fibroblast (BG-9) cells were exposed to pI:pC or pI:MPC for 24 h for HuIFN-β production. Thiolation levels of pI:MPC and their concentrations used remained the same as for HuIFN-α. HuIFN-β levels were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN) that has no cross reactivity to human interferon α or γ. As shown in Fig. 4, 10 μg/mL and 7.4% thiolation produced significantly higher amounts of HuIFN-β as compared to pI:pC ($P < 0.05$). The maximal HuIFN-β was produced at 100 μg/mL and at 7.4% thiolation. The HuIFN-β yield declined at higher concentrations of the inducers and at higher level of thiolation. There was nearly 50% decline in HuIFN-β antiviral activity at 14.3% thiolation as compared to 7.4% thiolation at 100 μg/mL concentration (Fig. 4). The degree of thiolation appears to result in bell-shaped activity curves. Higher thiolation level usually results in lower HuIFN-β production (Fig. 4).

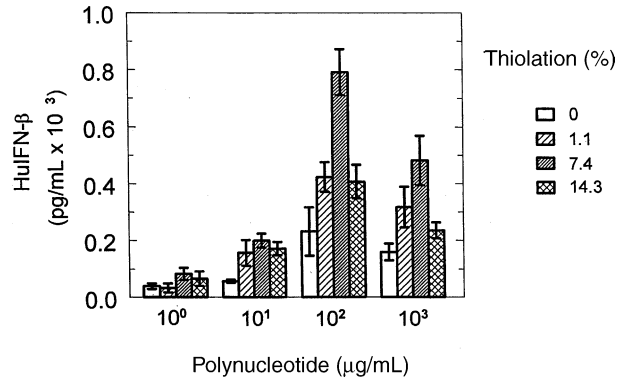

Fig. 4. Production of HuIFN-β by pI:MPC in BG-9 cells ± S.E.M. Confluent monolayers of BG-9 cells were exposed to indicated levels of pI:MPC or pI:pC for 6 h. Inducer was removed and monolayers washed with warm medium and incubated for an additional 18 h. HuIFN-β antiviral activity released in the culture medium was measured by an ELISA kit (#41400, Lot 1228) from PBL Biomedical Laboratories (Piscataway, NJ). This kit is specific for HuIFN-β and has no cross reactivity to HuIFN-α or HuIFN-γ.

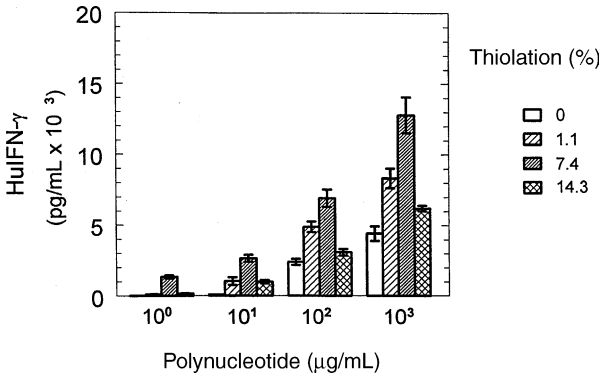

Fig. 5. Production of HuIFN-γ by pI:MPC in PBMC ± S.E.M. Freshly isolated PBMC were exposed to indicated levels of PI:MPC or pI:pC for 6 h. Inducer was removed by low speed centrifugation and cells incubated in culture medium for an additional 18 h. HuIFN-γ antiviral activity was measured in the culture fluid by an ELSIA kit (#41500, Lot 1222) from PBL Biomedical Laboratories (Piscataway, NJ). This kit is specific for HuIFN-γ and has no cross-reactivity to HuIFN-α or HuIFN-β.

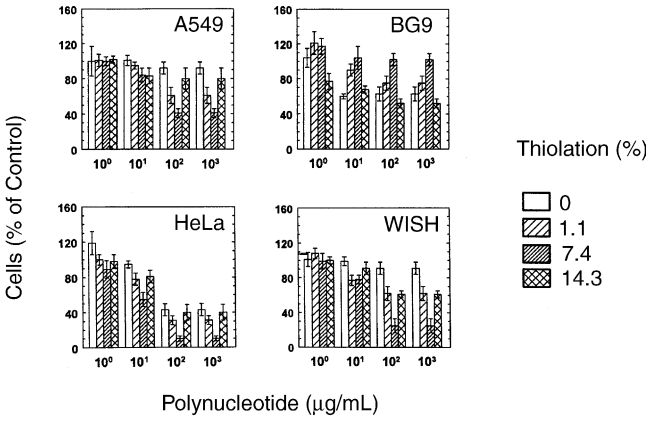

Fig. 6. Antiproliferative activity of pI:MPC ± S.E.M. in normal and neoplastic human cells: Cells were seeded in 96-well tissue culture plates at density of 3×10^4 cells/mL. After 48 h, the growth medium was removed and replaced with indicated levels of pI:MPC or pI:pC in growth medium to triplicate wells. Cells were allowed to grow for 7 days. Cell growth was measured with a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit from Promega (Madison, WI). Error bars: S.E.M.

3.5. pI:MPC and HuIFN-γ production

Freshly prepared PBMCs from normal, healthy blood donors (1×10^6 mL^{-1}) were induced with a range of pI:pC and pI:MPC concentrations and at comparable levels of thiolation as carried out for HuIFN-α and -β. As shown in Fig. 5, at 1.0 and 10 μg/mL, pI:pC did not induce any detectable amounts of HuIFN-γ. At 10 μg/mL, all three pI:MPC induced detectable levels of HuIFN-γ. The maximal HuIFN-γ antiviral activity was seen at 7.4% thiolation and at all concentrations of this inducer and in a dose-dependent manner ($P < 0.001$). HuIFN-γ was maximally induced at a concentration 1000 μg/mL of pI:MPC and at 7.4% thiolation (Fig. 5). This concentration is 10-fold higher than needed for maximal HuIFN-β induction at 7.4% thiolation. However, this concentration is comparable to that needed for maximal induction of HuIFN-γ.

3.6. pI:MPC and antiproliferative activity

Human amnionic cell-derived cultured epithelial cells (WISH), human cervical epitheloid carcinoma (HeLa) cells, human lung carcinoma (A-549) cells and normal human fibroblast (BG-9) cells were used in evaluating the antiproliferative activity of various pI:MPC analogs. The results are shown in Fig. 6. Both the concentration of pI:MPC and the level of thiolation have distinct effect on its antiproliferative activity. Normal human fibroblast (BG-9) cells show qualitatively different sensitivity to pI:MPC from the neoplastic cell lines. There is a bell shaped dose response curve with the least toxicity at the 7.4% thiolation level. In BG-9 cells the maximal antiproliferative activity was seen at 100 μg/mL and at 14.3% thiolation. Among neoplastic cells, the maximal antiproliferative effect was seen at 100 μg/mL and at 7.4% thiolation. The fact that pI:MPC at 7.4% thiolation is essentially non-toxic to normal human cells and is toxic to neoplastic cells, in a dose dependent manner, is potentially an important observation. This suggests that normal cells at 7.4% thiolation are spared and cancer cells at the same concentration are being hit the hardest. It is understood that other factors, particularly the "antitemplate" activity (inhibition of DNA polymerases) of thiolated polynucleotides (see Section 4), may also contribute toward the total antiproliferative activity of these compounds.

4. Discussion

The results show that partial thiolation of pI:pC has a biphasic effect upon its interferon inducing activity. There is an increase in interferon inducing ability of all three types of human interferons when 5-mercaptocytosine concentration in the polyC strand of pI:pC increases from 0% to 7.4%. This effect generally decreases at higher levels of thiolation. Previous studies have shown that some of the immunologic activities of these thiolated dsRNAs, for example macrophage and natural killer cell activation, increase with the length of unmodified inosinate–cytidylate region between two neighboring 5-mercaptocytidylate units (Cavanaugh et al., 1996), which in the case of 14.3%, 7.4% and 1.1% thiolated MPC would correspond to 6, 12.5 and 90 uninterrupted base pairs, or approximately between 0.5 and 8 full turns of the double helix (Lodish et al., Molecular Cell Biology, 4th ed., 1999), while the induction of interferons in the present study appears to show an optimal value. It appears that across the range of % thiolations studied, immunologic activities vary.

The antiproliferative activity is the result of a combination of at least two different activities of thiolated polynucleotides, one is its antitemplate effect causing inhibition of DNA polymerases and the other is its interferon inducing effects as described above. In a previous study (Geng

et al., 1991), we compared the antiproliferative activities of pI:MPC with those of the single-stranded pI:MPC, which has no interferon inducing effects. The results showed the same dose–activity relationships on a weight basis for the double-stranded pI:MPC as for its single-stranded MPC component, although the former contained only one-half per weight of the thiolated polynucleotide. This leaves room for a possible enhancement of the antiproliferative activity by the interferon inducing effect in the case of pI:MPC, although some other factors may also explain its greater antiproliferative activity compared to MPC. The present results show that 7.4% thiolation of pI:MPC has the peak activity in inhibiting cell proliferation. This is true of neoplastic cells (A-549, HeLa and WISH) but not of normal (BG-9) cells which are least inhibited by the 7.4% thiolated pI:MPC. Previous studies on leukemic cells have shown that pI:MPC is taken up and accumulated in neoplastic cells to a greater degree than in normal leukocytes (Kung et al., 1976). Previous studies have also shown that the in vivo toxicity and pyrogenecity of pI:MPC in both guinea pigs and mice is significantly lower than that of pI:pC (Bardos et al., 1975). This observation is essentially similar to "mismatched" dsRNA such as Ampligen (Ts'O et al., 1976; Carter et al., 1991). Although introduction of thiol groups into the 5-position of cytidine does not change its base-pairing preference for complimentary inosine (since the 2, 3 and 4 positions involved in the H-bonding remain unchanged), the ionized 5-SH groups cause hydration and loop formation, disrupting the double-strandedness at the site of the 5-mercaptocytidylate units (Cavanaugh et al., 1996). An important difference is that the 5-SH groups in the "loop" decrease the sensitivity of pI:MPC toward enzymatic degradation by nucleases (increased stability), while the "loop" in the non-thiolated "mismatched" dsRNAs, in contrast, increase the latter's sensitivity to nucleases. Another important advantage of pI:MPC is the established antitemplate activity of thiolated polynucleotides (Chandra and Bardos, 1972; Milkulski et al., 1973; Kung et al., 1976; Cavanaugh et al., 1982; Bardos et al., 1992) which in itself, without interferon induction and other immunologic activities, is capable of directly inhibiting tumor cell growth via inhibition of DNA and RNA polymerases (Bardos and Ho, 1982).

The present study was undertaken due to renewed interest in the therapeutic potential of these thiolated, double-stranded RNA polynucleotides having immunological, antiviral and chemotherapeutic antitumor activities, with increased stability and decreased toxicity. Experimental clinical studies conducted with our collaborators at the University of Frankfurt (Germany) Medical School evaluating the effects of MPC and pI:MPC against acute childhood leukemia indicated that a single intravenous dose of either the single-stranded or the double-stranded thiolated polynucleotide produced a marked reduction in the number of leukemic cells in the blood of treated patients without significant toxicity (Chandra et al., 1977; Kornhuber et al., 1979a; Kornhuber et al., 1979b). The present experimental data as well as those of the earlier studies referred to in this paper appear to justify further pre-clinical and clinical evaluation of pI:MPC as an antiviral and an antineoplastic agent. The spectrum of antiviral sensitivities have to be studied further as well. We have shown earlier that related single-stranded thiolated ribo- and deoxyribo oligo and polynucleotides are effective against HIV-1 proliferation in human cells (Bardos et al., 1992), and against DNA polymerases of RNA and DNA tumor viruses (Chandra and Bardos, 1972; Tokes and Aradi, 1996).

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