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Inhibition of human cancer cell lines *in vitro* with mono– and polynucleotides containing 5–mercaptocytosine bases[1]

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ABSTRACT Partially thiolated polycytidylic acid (5–mercaptopolycytidylic, MPC) and its double–stranded complex with polyinosinic acid [poly (I)]·poly(I)·MPC, were assayed in both antiproliferative and cytotoxicity tests against human cell lines: lung carcinoma A549, colon carcinoma HT–29, osteosarcoma HOS, and amnion cells (WISH). Inhibitory effects of MPC were noted in the antiproliferative assay with ID_{50} of 7, 24, 33, and 35 $\mu g \cdot ml^{-1}$, and in the cytotoxicity test with ID_{50} of 164, 174, 210, and 290 $\mu g \cdot ml^{-1}$ against the HOS, A549, HT–29, and WISH cells respectively. Comparison with the corresponding partially thiolated mononucleotide (5–mercapto–CMP + CMP) and the nucleoside (5–mercapto–cytidine) demonstrated that MPC was a more potent antiproliferative agent than either of its monomeric constituents. The inhibitory effect of MPC upon the incorporation of [^{3}H]thymidine into the DNA of growing A549 cells paralleled its antiproliferative activity.

KEY WORDS Poly C; Poly I; nucleotides; 5–mercaptocytidine; cell line; cultured tumor cells; cytotoxicity tests

Partially thiolated polycytidylic acid (5–mercaptopolycytidylic acid, MPC) is a potent inhibitor of various DNA and RNA polymerases, including the reverse transcriptases of tumor viruses[1]. MPC also inhibited the colony–forming ability of murine leukemia bone marrow and spleen cells[2,3] and, upon *in vivo* administration, it showed relatively greater uptake in the liver and spleen cells of leukemic mice as compared to the same organs of normal mice[4]. Its double–stranded complex with poly(I), i.e., poly(I)·MPC was found to possess some advantageous properties over poly(I)·poly(C) as an interferon inducer and, in addition, it was capable of inhibiting human cancer cell lines[5]. In preliminary clinical trials conducted in Germany, both MPC and poly(I)·MPC showed some beneficial effects against acute lymphocytic leukemia in children[6].

In the present paper, we shall report the activities of the single–stranded MPC on several human cancer cell lines (i.e., lung carcinoma A549, colon carcinoma HT–29, osteosarcoma HOS) *in vitro*. For the understanding of the mechanism of action of MPC, its activity was compared with that of poly(I)·MPC. Moreover, in order to determine whether the direct inhibitory action is exerted by MPC itself, or by its potential metabolic degradation products, the antitumor activity of MPC against the human cancer cell lines was compared with those of the corresponding monomeric nucleotides (sh^{5}CMP + CMP) as well as 5–mercapto–cytidine.

MATERIALS AND METHODS

MPC, synthesized as described previously[7], contained 9.2% 5–mercaptocytidylate and 90.8% cytidylate units randomly

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distributed throughout the polynucleotide chain. Poly(I) and poly (C) were purchased from PL Biochemicals, Inc.

Poly(I) · MPC: annealing of poly(I) with MPC has been reported previously[5]; the product contained 5% 5–mercapto–cytidylate and had a helix–coil transition point of $T_m = 42$℃ (in phosphate buffer solution).

The "partially thiolated" cytidylic acid was prepared by identical procedure as used for the preparation of the modified polynucleotide MPC. That is, in the previously described method for the synthesis of 5–mercaptocytidylic acid[8], the modified nucleotide (sh^5CMP) was not separated from the remaining bulk of unmodified CMP. The purified reaction mixture contained about 15% sh^5CMP and 85% CMP, which is reasonably close to the ratio of bound 5–mercaptocytidylate and cytidylate units in the polymeric MPC.

5–Mercaptocytidine was also synthesized as described before[8]. The analytically pure compound was used.

[^{3}H]Thymidine was obtained from ICN Chemical and Radioisotope Division, Irvine CA, USA.

Human cell lines Lung carcinoma (A549), colon carcinoma (HT–29), osteosarcoma (HOS) and amnion cells (WISH) were obtained from the American Type Culture Collection (Rockville, MD). All the cells were maintained in MEM containing 10% fetal bovine serum (FBS).

Antiproliferative assay (Inhibition of colony formation) Three human tumor cell lines, A549, HT–29, HOS and a nonmalignant amnion cell line were used in this study. One thousand cells of each line in a total of 5 ml of MEM (10% FBS) in 35 mm wells of cluster tissue culture trays containing 0, 10, 20, 40, 60, and 80 μg MPC · ml^{-1} respectively, were incubated in a 5% CO_2 atmosphere at 37℃ for 6–8 d. Then the medium was decanted and the cells were stained with neutral red diluted with phosphate buffered saline solution (PBS) in an incubator at 37℃ for 1 h. After aspirating the dye, the cells were washed with PBS and eluted with 50% ethanol. The eluates of each well were collected and the absorbance was determined at 540 nm in a Gilford model 2400S spectrophotometer.

Growth inhibition of HT–29 and A549 cells Both cell types were routinely grown in a modified Eagle medium[9] supplemented with 10% calf (bovine) serum (EM10C). Cells were seeded into 16 mm culture wells at 2×10^3 cells / well in 0.5 ml aliquots of EM10C. Medium was replaced with EM10C containing MPC on the following day. Cells were trypsinized and counted 3, 6, and 9 d after seeding. Rates of growth in cell doublings per day were determined from the resulting curves.

Cytotoxicity assay Suspensions of 2×10^5 cancer cells (A549, HT–29 or HOS) or amnion cells (WISH) in 1 ml of MEM (10% FBS) were pipetted into 16 mm tissue culture wells and incubated at 37℃ for several days. After the cells reached confluency, 1 ml of fresh MEM (2% FBS) containing MPC or poly (I) · MPC (0, 50, 100, 200, 300, and 400 μg) was added to the cell monolayers. Incubation was continued under the same conditions for 2–3 d. After aspiration of the medium, 1 ml of diluted neutral red solution was added and incubated at 37℃ for 1 h. After washing with PBS and eluting with 50% ethanol, the absorbance was measured at 540 nm.

Effects on DNA synthesis by MPC Suspensions of 2×10^3 lung carcinoma cells in 2.5 ml MEM (10% FBS) were added into 35 mm tissue culture dishes and incubated at 37℃ in a 5% CO_2 atmosphere. At 0, 3, and 5 d respectively, MPC 80 μg · ml^{-1} (total 200 μg / dish) was added. On d 5 from the beginning of the experiment, 4 h prior to harvest,

37 kBq · ml^{-1} of [^{3}H]thymidine was added. After harvesting, the medium was decanted and 0.2 ml of trypsin and EDTA solution was added to each dish. After 5-min incubation at 37℃, 50% TCA 0.5 ml was added and this was followed by 2 ml of 5% TCA. The mixture was filtered and washed with 5% TCA twice, followed by 95% ethanol, then the filter with the precipitate was dried under an infrared lamp. Radioactivity was measured in 5 ml of spectrofluoroscintillator using a Packard Tri-Carb Model 3320 scintillation counter.

RESULTS

Antiproliferative effects Tab 2 shows that MPC inhibited the growth of human lung carcinoma, colon carcinoma, osteosarcoma cells, and also of amnion cells, *in vitro*. All these inhibitions were dose-related as exemplified by inhibition of the rate of growth of A549 and HT-29 cells (Tab 1). These cell lines exhibited similar sensitivities to MPC in both assay systems described in METHODS. This result suggests that MPC is cytotoxic to broad cell lines including malignant and nonmalignant cells (WISH).

Tab 1. Inhibition on growth of HT-29 and A549 cells by MPC. 2×10^3 Cells in 0.5 ml aliquots of EM10C were seeded into 16-mm tissue culture wells. The following day, medium was replaced with EM10C containing the indicated concentrations of MPC. Cells were trypsinized and counted 3, 6, and 9 d after seeding ($10^3\times$ Cells / ml).

Cell lines	Days after cell seeded	MPC (μg · ml^{-1}) 0	25	50	100
HT-29	3	3.4			
	6	7.6		6.1	
	9	45	34	9.6	34
A549	3	1.7			
	6	10	7.0	1.5	
	9	160	55	24	0.98

The results obtained in parallel assays using poly(I) · MPC were similar to those previously reported for this polynucleotide complex[5]. Compared on a weight basis the inhibitory activities of MPC on the above cell lines were generally somewhat more potent than those of poly(I) · MPC. However, the latter contained only one-half as many 5-mercapto-nucleotides per molecule than did MPC, and neither poly(I) nor poly(C) showed any significant activity in the antiproliferative assay. Thus, using the A549 cell line, at drug concentration of 20, 40, and 80 μg · ml^{-1} poly(I) showed 10.8%, 12.3%, and 3.8% and poly(C) showed −3.7%, 1.9%, and 22.5% inhibition respectively.

Tab 2. Antiproliferative effect of MPC and poly(I) · MPC against various human cancer lines. $\bar{x}\pm$SD.

Cell line	Expts	IC_{50} (μg · ml^{-1}) MPC	Poly(I) · MPC
A549	6	24 ± 12	33 ± 5
HT-29	5	33 ± 11	56 ± 25
HOS	4	7 ± 8	15 ± 14
WISH	4	35 ± 28	39 ± 20

Cytotoxicity test Tab 3 shows that MPC exerted some cytotoxic effect on A549, HOS, HT-29, and WISH cell lines, but it was not as potent under these assay conditions as it was in the antiproliferative assay. The results obtained for poly(I) · MPC in the cytotoxicity test against the various cell lines

Tab 3. Cytotoxicity test of MPC and poly(I) · MPC against various human cancer lines. $\bar{x}\pm$SD.

Cell line	Expts	IC_{50} (μg · ml^{-1}) MPC	Poly(I) · MPC
A549	6	174 ± 20	192 ± 7
HT-29	5	210 ± 124	283 ± 66
HOS	4	174 ± 17	249 ± 35
WISH	4	290 ± 36	213 ± 14

were similar to those obtained with MPC

Comparison of the antiproliferative effects of MPC with those of the partially thiolated CMP and pure 5–mercaptocytidine In comparison to the significant inhibitory effect of MPC in the antiproliferative assay using the A549 cell line, the corresponding modified mononucleotide (5–mercapto–CMP + CMP) was seven times less active. For example, the LD_{50} of MPC for the A549 cell line was 25, $\mu g \cdot ml^{-1}$; while the LD_{50} of the mononucleotides was estimated to be 153 $\mu g \cdot ml^{-1}$. In the case of the pure nucleotide 5–mercaptocytidine, the antiproliferative effect was even less, the ID_{50} being 218 $\mu g \cdot ml^{-1}$ (Tab 4).

Tab 4. Antiproliferative effects of MPC, partially thiolated CMP and 5–mercaptocytidine on A549 cells in culture. $\bar{x} \pm SD$.

Drug $\mu g \cdot ml^{-1}$	Growth, % of control		
	MPC	5–SH–CMP	5–SH–cytidine
0	100	100	100
10	72 ± 12	–	100
20	53 ± 13	82	97
40	32 ± 14	73	87
80	22 ± 12	66	79
IC_{50}	26 ± 7	153	218

All groups are different from each other at $P < 0.05$ by weighted probit analysis

Tab 5. Effects of MPC (80 $\mu g \cdot ml^{-1}$) on incorporation of [^{3}H]thymidine into DNA of lung carcinoma (A549) cells. $\bar{x} \pm SD$.

Time at which MPC was added	Incorporation of [^{3}H]thymidine into DNA	
	Total cpm	% inhibition
Control	1654 ± 310	–
6 h before harvest	1537 ± 240	7
3 d before harvest	977 ± 208	41
5 d before harvest	145 ± 26	91

Effect on DNA synthesis The effects of MPC on DNA synthesis of lung carcinoma cells was demonstrated by the experiment summarized in Tab 5. It is seen that 80 $\mu g \cdot ml^{-1}$ of MPC inhibited DNA synthesis even if the drug was added on d 5, 6 h before harvest. If MPC was in contact with the cancer cells for a longer time, the inhibitory potency increased.

DISCUSSION

It has been reported that various double–stranded RNAs have antitumor activities. Thus, poly(I)· poly(C), poly(A· poly(U), poly(I)· MPC[5], and poly(I)· poly(C_{12}, U) were reported to inhibit the growth of tumor cells *in vitro* and / or *in vivo*. Since double–stranded RNAs can induce interferons and enhance the activities of natural killer cells, it may not be justified to ascribe their antitumor effect solely to their direct action on the tumor cells, i.e., arresting their proliferation or killing the cells. However, the single–stranded thiolated polynucleotide, MPC has been shown to have no significant immunomodulatory activity *in vivo*[10] and to have no interferon inducing activity *in vitro*[5]. Thus, both the antiproliferative and cytotoxic effects of MPC observed in the present study should be attributed to its direct antitumor activity. In view of the known susceptibility of polyribonucleotides to hydrolysis, one might question whether MPC enters the cells as a polymer, or whether it is hydrolyzed first by the ubiquitous ribonucleases to the monomeric nucleoside and nucleotide analogs. The much less potent activities of the 5–mercapto analogs of CMP and of cytidine against these cell lines appear to argue against the latter possibility. In addition, our previous studies have indicated that the enzymatic digestion of MPC with nucleases yielded a mononucleotide fraction almost void of SH groups; that is, essentially only unmodified CMP–residues were released in the mononucleotide form.

while the 5–mercapto CMP residues remained bound in the oligonucleotide fractions[11]. Thus, it is the polymeric (or, at least, oligomeric) form of MPC which appears to be responsible for the antitumor action.

As to its mechanism of action, MPC may act intracellularly in several ways. In our previous studies, we have shown conclusively that 1) MPC and other thiolated polynucleotides bind more strongly to the template sites of certain DNA and RNA polymerases than do the functional templates; 2) they do not function as templates; and 3) they are potent inhibitors of these polymerases, reversible with the functional templates[1,12,13], therefore, they were termed " antitemplates" [12]. An additional mechanism may also be considered since MPC should be capable of binding to poly(dC) · poly(dG) tracts within the double–stranded DNA by triple helix formation[14] or alternately, to poly(G) tracts of the mRNAs by "antisense–type" sequential base– matching, causing translation arrest[15]. Notwithstanding its sensitivity to hydrolysis by ribonucleases, the inactivity of poly(C), however, tends to negate the involvement of this additional mechanism for which so far in this case no evidence exists.

On the other hand, the greater activity of the " partially thiolated" mononucleotide ($sh^5CMP+CMP$) as compared to the pure 5–mercaptocytidine may indicate that the latter is such a poor substrate for the kinases that even the extra–cellularly administered 5–mercapto–CMP, despite its presumably unfavorable membrane transport, is a more active inhibitor than the nucleoside. Therefore, the possibility cannot be excluded that MPC may act by releasing the 5–mercapto–nucleotide intracellularly in an activated form.

The cell lines used in the present study are relatively insensitive to inhibition by the unmodified poly(I) · poly(C)[6]. The fact that they are significantly inhibited by poly(I) · MPC implies that MPC retains its direct antitumor action after annealing to poly(I). Poly(I) · MPC is more stable to blood nucleases than is MPC[4]. Therefore even disregarding its previously demonstrated immunomodulatory activities[5,10], it could be used at least as a more effective form for the administration of MPC. The action of MPC inside the cells is relatively fast since 7 % inhibition of DNA synthesis is observed between 2 and 6 h after addition of MPC to tumor cells. The inhibition increases to 91 % when MPC is allowed to act in the cells for 5 d. Whether the inhibition of the cellular incorporation of [^{3}H]thymidine into DNA is the cause or a consequence of the cytotoxicity of MPC could not be determined in this study. More detailed investigations of the modes of action of this class of mercaptopolynucleotides is continuing in our laboratories.

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含巯胞嘧啶的单和聚核苷酸体外抑制人癌细胞株[1]

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提要 观察巯聚胞 MPC 对人癌细胞株：肺癌 A549、大肠癌 HT-29、骨肉瘤 HOS 和人羊膜细胞株 WISH 的抗增殖和细胞毒作用，并与巯聚肌胞(Poly I·MPC)作比较，MPC 明显抑制以上细胞增殖，其 IC_{50} 分别为 7, 24, 33 和 35 $\mu g \cdot ml^{-1}$，细胞毒的 IC_{50} 为 164, 174, 210 和 290 $\mu g \cdot ml^{-1}$，以含量相比，MPC 作用较 Poly I·MPC 为强，与巯单核苷酸(5-巯 CMP+CMP)及巯核苷(5-巯胞苷)相比则更强. MPC 抑制 A549 细胞[^{3}H]TdR 掺入 DNA 的结果与其抗增殖活性相一致.

关键词 多聚胞苷酸类；多聚肌苷酸类；核苷酸类；5-巯胞苷；细胞株；培养肿瘤细胞；细胞毒性试验