Biological and Clinical Effects of a Partially Thiolated Polycytidylic Acid (MPC): A Potent Inhibitor of DNA Synthesis in RNA Tumor Viruses

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Single stranded polyribonucleotides are known to act as efficient templates for the viral DNA polymerases in the presence of complementary oligo-deoxy-ribo-nucleotide primer. Chemical modification of such templates would be expected to alter the interaction between the template and the viral enzyme [1–7]. This appears to be a very useful approach for designing specific inhibitors of viral DNA polymerases, which might find application in the chemotherapy of cancer [8,9].

Our efforts to develop compounds that inhibit viral DNA polymerase by interacting directly to the enzyme led to the discovery of polycytidylic acid analog, containing 5-mercapto substituted cytosine bases (Fig. 1), a partially thiolated polycytidylic acid [10–12]. This compound, abbreviated as MPC (mercapto-polycytidylic acid) was found to inhibit the oncornaviral DNA polymerase in a very specific manner [10–15]. The mode of action of this compound as an inhibitor of viral DNA polymerase, its biological effects on viral oncogenesis, and its clinical application in the treatment of childhood leukemia will be described here.

1. Inhibition of Oncornaviral DNA Polymerase by MPC

The inhibition of DNA polymerases from RNA tumor viruses by MPC was described earlier [10–15]. Partially thiolated polycytidylic acid preparations, MPC I–III (containing 1.7%, 3.5% and 8.6% 5-mercaptocytidylate units, respectively) inhibited the DNA polymerase activity of Friend leukemia virus (FLV) in the endogenous reaction as well as in the presence of poly-rA.$(dT)_{14}$, or poly rC.$(dG)_{12\text{-}18}$; the inhibitory activities were directly proportional to the percent of thiolation. A maximum inhibition was observed with preparations containing 15–17% of the thiolated cytosine bases.

1.1 Mode of Action

The mode of action of viral DNA synthesis by MPC was investigated by product analysis of the DNA polymerase reaction in the absence or in the presence of MPC, as described elsewhere [11]. The reaction mixtures were dissolved with Na-dodecyl sulfate (1%, wt/wt, final concentration), loaded

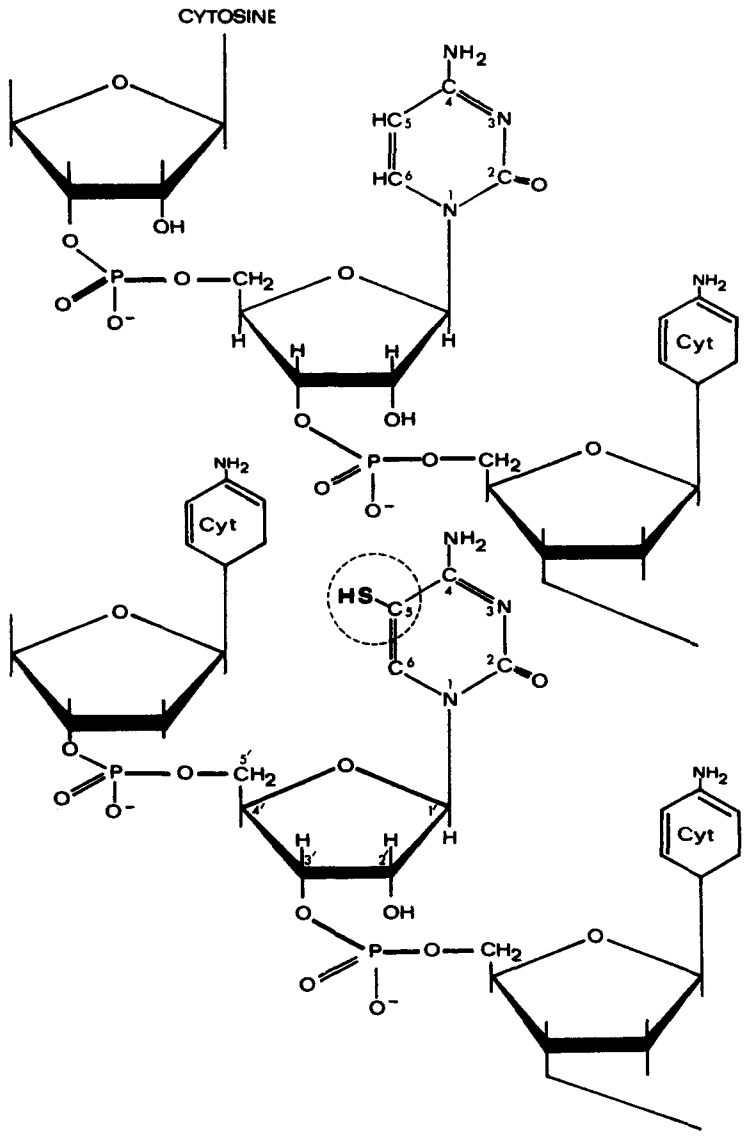

Fig. 1: Schematic presentation of the chemical structure of a partially thiolated polycytidylic acid (MPC)

on a hydroxylapatit column (1 g, Bio-Rad Lab., Munich), eluted with a Na-phosphate gradient (0. 05 – 0. 4 M), collected into about 40 tubes (total vol. approx. 100 ml), and the TCA-insoluble radioactivity collected on GF/C filters (Whatman) and counted in a liquid scintillation counter.

Analysis of the endogenous products of the detergent disrupted virions exhibits 3 DNA species: single stranded DNA (ss-DNA), RNA-DNA hybrids (hy-DNA) and the double stranded DNA (ds-DNA). As follows from Fig. 2, in the presence of MPC (open circles) there is an over-all inhibition of ^{3}H-dTMP incorporation, indicating that the formation of all the 3 species is blocked. This is to be expected since the inhibitor binds to the enzyme. This has been confirmed by ultracentrifugation studies in which the binding of ^{35}S-labeled MPC to a purified FLV-DNA polymerase was investigated.

In view of the fact that all of the oncornaviral DNA polymerases examined so far do require a primer-template-like double stranded secondary structure for the initiation of DNA synthesis, it is no surprise that single

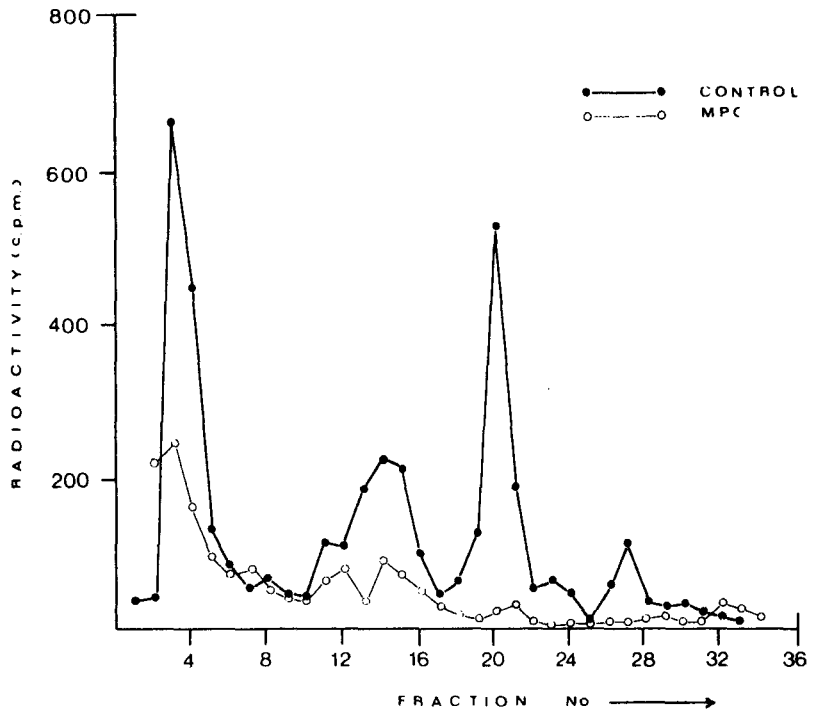

Fig. 2. Analysis of the DNA species synthesized by FLV-DNA polymerase by elution from hydroxylapatit column. Experimental details are described in the text. The first species to be eluted from the column contained ss-DNA, the second contained hy-DNA and finally, the ds-DNA, eluted in the last peak. The concentration of MPC in the reaction mixture was 20 μg/ reaction mixture

stranded synthetic polynucleotides (unprimed templates) can act as inhibitors of the polymerization reaction. This, presumably, is due to hydrogen bonding of the base sequences between the added polymer and the functional template. Thus, the specificity of inhibition by such polymers is not limited to the viral enzyme system only. On the other hand, minor modifications in the chemical structure of synthetic polynucleotides might be useful to develop inhibitors that interact directly with the enzyme but fail to be transcribed, i.e. they function as a "dead template" for the enzyme. The data from our laboratory have shown that the partially thiolated polycytidylic acid is functioning as a "dead template" in the DNA polymerase system of FLV [11]. The results of these studies can be summarized as follows: 1. The incorporation of ^{3}H-dGMP into DNA by the viral enzyme is stimulated to about 9-fold (compared to the endogenous value) in the presence of Poly rC.$(dG)_{12-18}$. However, under similar conditions a hybrid of MPC.$(dG)_{12-18}$ failed to stimulate the incorporation of ^{3}H-dGMP into DNA; 2. In the presence of MPC.$(dG)_{12-18}$, the increasing concentrations of poly rC.$(dG)_{12}$ in the reaction mixture have no effect on the activity of the enzyme; however, at higher enzyme concentrations the stimulatory effect of poly rC.$(dG)_{12-18}$ gradually reappears. These data indicate that the viral enzyme has higher binding affinity towards MPC than to its optimal template poly rC. The presence of zinc in reverse transcriptase makes it attractive to suggest that the mercapto group may undergo an interaction with zinc to form a stable complex.

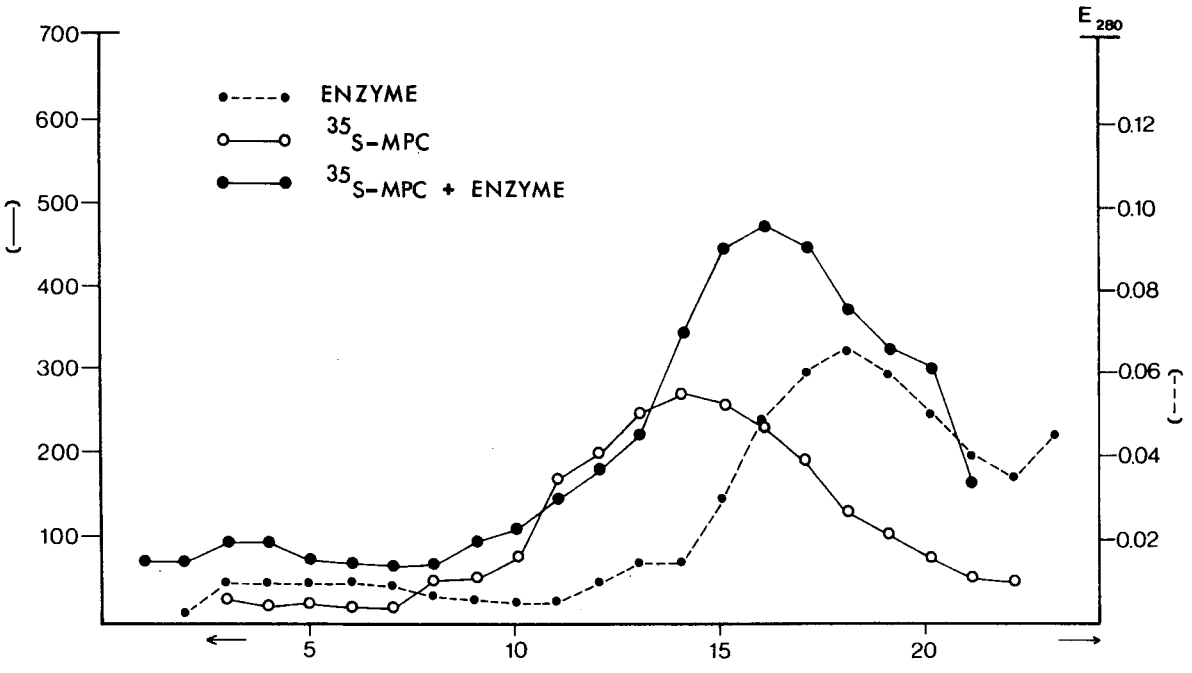

Fig. 3. Binding of ^{35}S-labeled MPC to a purified FLV-DNA polymerase. The specimen in 0.1 M Tris/HCl buffer (pH 7.4) were layered on linear gradients of 10–40% sucrose (RNase-free) in the same buffer and spun at 35,000 rpm (swingout rotor) at 4 °C for 20 hr. The gradients were dripped from below, fractions collected and after dilution, were analyzed for their radioactivity or absorbtion at 280 nm. The MPC preparation contained 10.1% of thiolated cytosine bases (sp. Act. 141 c. p. m./μg MPC)

1.2 Selectivity of MPC Action

In order to determine the selectivity of MPC action, further studies were conducted using DNA polymerases from different sources. As follows from Table 1, the viral DNA polymerases are most sensitive towards inhibition by MPC, whereas MPC is completely unable to inhibit the bacterial DNA poly-

Table 1. Evaluation of the inhibitory response of partially thiolated (SH = 13%). Polycytidylic acid on DNA polymerase from various sources

Source of DNA polymerase	Type of DNA polymerase	Template used	Compound required to inhibit 50% of the Reaction (μg/ml)
Human lymphocytes[a] (1788)	I	Poly(dA)·$(dT)_{12-18}$	30
	II	Poly(dA)·$(dT)_{12-18}$	38
Regenerating rat liver	I	CT-DNA	>100 (38% inhibition at 78 μg/ml)
E. coli K_{12}	I	Poly(dA-dT)	No inhibition >100
RMuLV[a]	Reverse transcriptase	Poly(rA)·$(dT)_{12-18}$	20
FLV	Reverse transcriptase	Poly(rA)·$(dT)_{12-18}$	19.2

[a] These studies were done by Dr. R. Graham Smith at the Laboratory of Tumor Cell Biology of the National Cancer Institute, Bethesda, Md.

merase. Enzymes from human lymphocytes are more sensitive towards MPC inhibition than the DNA polymerase I of regenerating rat liver. In spite of the fact that the experiments were carried out in different laboratories, a comparative evaluation shows that the viral enzymes are at least twice as sensitive as DNA polymerase from another source.

The studies on the selectivity of MPC action were substantiated using the cellular DNA polymerases (α, β and γ) and a reverse transcriptase from human spleen. We have recently discovered a reverse transcriptase in the spleen of patient with myelofibrosis [16,17], a preleukemic disease. This reverse transcriptase is antigenically related to DNA polymerases of primate RNA tumor viruses [16,17], the Simian sarcoma virus (SiSV) and Baboon leukemia virus (BaLV). This observation implicates the viral origin of the reverse transcriptase in myelofibrotic spleen (Fig. 4).

The effect of MCP (SH = 15%) on the activity of cellular DNA polymerases, and on the reverse transcriptase activity from human spleen is shown in Fig. 4. At a concentration of 1 μg/reaction mixture [cf ref. 16], none of the cellular enzymes was inhibited. In contrast, the reverse transcriptase activity was inhibited to approx 20%. At a concentration of 16 μg, the reverse transcriptase activity was inhibited to 80%, whereas the cellular enzymes lost only 25–40% of their activities. Kinetic studies on the DNA-polymerase reaction, catalyzed endogenously by SiSV, revealed that MPC inhibition is of a non-competitive nature (Fig. 5).

2. Effects of MPC on Oncogenesis by RNA Tumor Viruses

To measure the effect of MPC (SH = 8.6%) on the production of splenomegaly by Friend leukemia virus (FLV), we first incubated the cell-free extracts of spleen from mice (Groppel strain) with 100 μg/ml of MPC at 37 °C for 1 hr. In the control group, where no compound was used, the cell-free spleen suspension was preincubated with Tris/HCl buffer, pH 7.4, the solvent for MPC. The aliquots of this suspension were injected (0.2 ml, LD_{90}) into each group, consisting of 10 animals. The spleen weights were analyzed on the 8th or the 12th day after infection [8]. There was a 60% reduction of spleen weights (arithmatic mean of five individual values) in the MPC-treated group, measured on the 8th day after FLV-infection. However, no differences were observed on the 12th day. This is probably due to fact that at this MPC concentration the whole of virus is not inactivated, so that the residual active virus particles lead to potentiation of leukemogenesis.

The studies reported above were extended using MPC in-vitro and in-vivo. The animals were divided into four groups of five each (donors): 1. Group 1 was injected with a viral suspension (citrate plasma from FLV-infected animals, dose LD_{90}) preincubated with Tris/HCl buffer, pH 7.6 for 30 min. at 37 °C; 2. Group 2 was injected with the viral suspension, as in 1, but preincubated with MPC (200 μg per 0.2 ml of suspension) at 37 °C for 30 min. These animals received in addition, on day 5 and day 9 (post infection) 50 μg of MPC, injected intraperitoneally; 3. Group 3 was treated similar to group

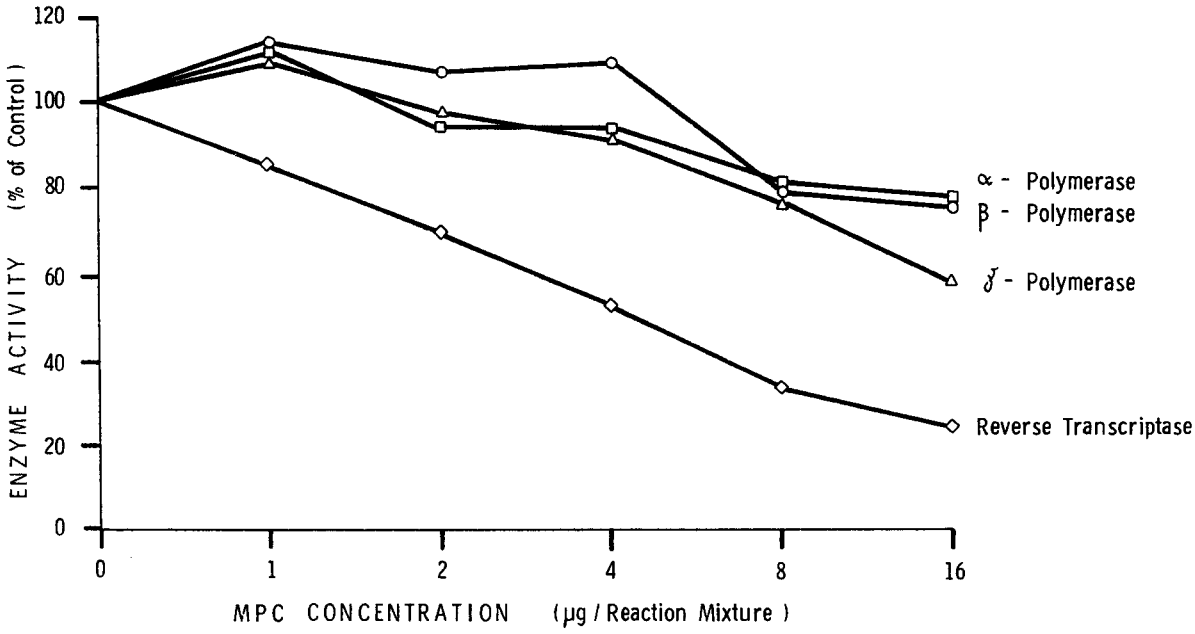

Fig. 4. Effect of MPC (SH = 15%) on the activity of cellular DNA polymerases (α, β and γ), and on the reverse transcriptase activity from human spleen of a patient with myelofibrosis. Bivalent cation and template specificities for cellular DNA polymerases and reverse transcriptase from human myelofibrotic spleen are described elsewhere [16]

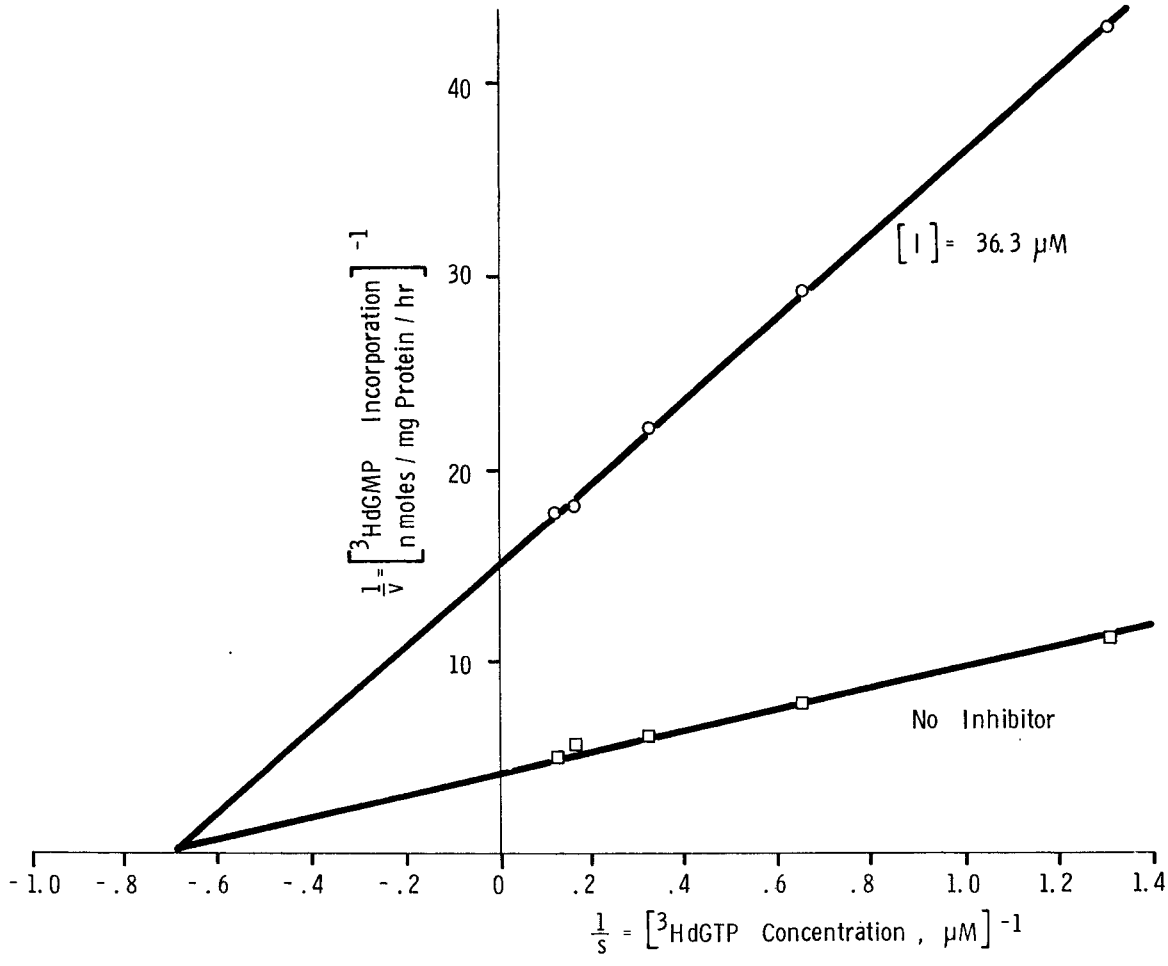

Fig. 5. Lineweaver-Burk plot the kinetics of DNA polymerase reaction, catalyzed endogenously by Simian Sarcoma Virus (SiSV). The reaction conditions have been described by Chandra and Steel [16]

1, except that the viral suspensions were preincubated for 2 hrs.; 4. Group 4 was treated in a similar manner as group 2, except that the viral suspensions were preincubated for 2 hrs at 37°C. On the 10th day, animals were sacrificed and spleen extracts were prepared, as described elsewhere [8]. The spleen extract from each mouse was then analyzed individually, with respect to their leukemogenic potentiality. Each "donor" spleen specimen was reinjected to a different "receipient" mouse (20 in total), and the leukemogenesis was followed, as shown in Table 2.

Table 2. Assay for leukemogenic potential of spleen extracts from FLV-infected mice after their in-vitro/vivo treatment with MPC

Treatment of donor mice	Leukemogenesis in recepient mice after infection with spleen extract[a]		
	No. of positive Total no. of mice	Mean survival Time (days)	Mean spleen Weight (g)
Virus Suspension[b] (0.2 ml) + Tris buffer (37°C, 30 min)	5/5	47	2.41
Virus Suspension + 200 μg of MPC (37°C, 30 min) + 50 μg MPC, i.p. (day 5 & 9)	2/5	123[c]	1.05 (1.78, 2.10, 0.52, 0.41, 0.44)
Virus Suspension + Tris buffer (37°C, 2 hr)	5/5	52.2	1.80
Virus Suspension + 200 μg of MPC (37°C, 2 hr) + 50 μg MPC, i.p. (day 5 & 9)	1/5	110 4 (123)[c] 1 (97)	0.38 (0.74, 0.29, 0.34, 0.22, 0.31)

[a] Cell-free spleen extracts were prepared (see reference 8) from spleens of individual donors on the 10th day after being challenged with the virus, or other treatments as shown.
[b] Citrate plasma from FLV-infected mice was used as the source of visus (LD_{90}).
[c] The experiment was terminated on day 123 and all animals were sacrified on this day. Therefore, the term "mean survival period" does not apply to these animals.

All animals in groups 1 and 3 developed splenomegaly and died between 40–60 days; whereas, in the MPC treated groups, of the 10 animals only 3 showed signs of splenomegaly. In group 2, 2 animals had splenomegaly but, in spite of that, all animals survived till the 123rd day, at which time our experiment was terminated. Similarly, in the last group 4 animals survived till the 123rd day; one died on the 97th day. The spleen weights, shown in the last column, also exhibit large differences between the MPC-treated group, and the control group. In another study we have analyzed the effect of MPC on normal mice of the same strain. We failed to observe any effect of MPC on the spleen weights of non-infected mice.

In another biological study we have analyzed the in-vivo effect of MPC on leucocytes of mice infected with the active Friend virus (Fig. 6). Within 12–24 hrs. after MPC injection (50 μg/mouse) a dramatic fall in the leucocyte count of animals infected with FLV was observed; MPC failed to reduce the leucocyte number in mice not-infected with the virus. It is interesting to note

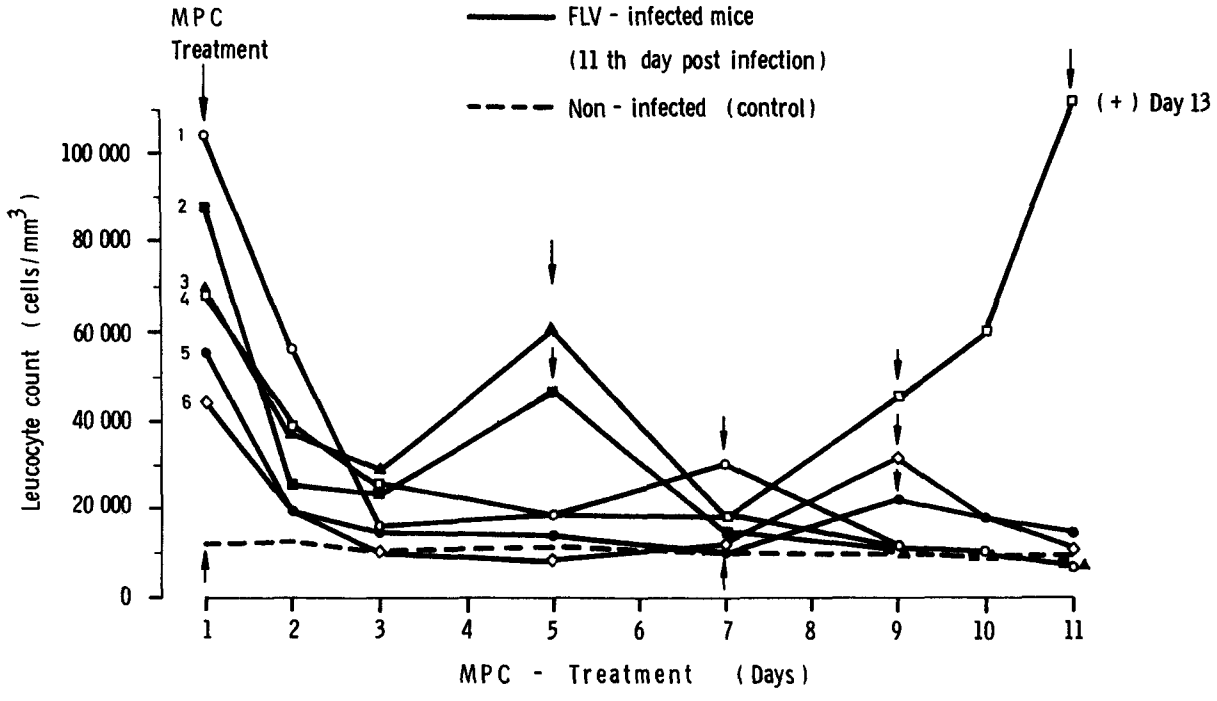

Fig. 6. In-vivo effect of MPC (SH = 15%) on the leucocyte number of mice infected with the active Friend Leukemia Virus (FLV). Arrows indicate the day of MPC treatment. MPC was injected intraperitoneally (50 μg per mouse). The MPC treatment was started on the 11th day postinfection with FLV. The hatched line indicates the leucocyte number of a control mouse treated with MPC. The control group (non-infected) had 5 animals, but the effect of MPC was similar to the one represented by the hatched line

that in one of the infected animals MPC failed to suppress the leucocyte number;on the contrary, there was a gradual increase in leucocyte number. This animals died on the 13th day of MPC treatment. Unfortunatly, we were not able to analyze the spleen of this animal. It is therefore difficult to interpret the reasons for failure of MPC effect in this animal.

3. Clinical Trails with MPC in the Treatment of Childhood Leukemia

Clinical data of patients submitted to MPC trials are shown in Table 3. Of the 18 cases treated with MPC, were in the terminal phase of the disease. These patients were resistent to all previous chemotherapeutic regimes which involved drugs, such as prednisone, vincristin, daunorubicin, L-asparaginase, Ara-C, 6-mercaptopurine, methotrexate, cyclophosphamide and actinomycin D.

MPC used in our clinical trials contained 15% of thiolated cytosine bases. The lyophilized product (MPC) was dissolved in 0.1 M Tris/HCl buffer, pH 7.6, and diluted with 0.9% NaCl before use. This solution was sterilized by passing through a membrane filter (Millipore GmbH, Neu Isenburg, Germany). It was kept at 4 °C and used immediately, or within the next five days; solutions older than 5 days were reprecipitated, purified on the column and resterilized. In our clinical trials, MPC (sterile) was given intravenously at a dose 0.5 mg/kg body weight. The injections were given once a week.

Table 3. Clinical data of patients with ALL and AML submitted to MPC trials

No.	Init.	Age	Sex	Diag.	Stage	Results
1	D.M.	8	♀	ALL	3rd rel.	PR, WBC ↓
2	T.I.	6 6/12	♀	ALL	2nd rel.	?
3	B.M.	6 7/12	♂	ALL	2nd rel.	?
4	M.M.	8 4/12	♂	ALL	2nd rel.	CR
5	J.O.	3 6/12	♂	ALL	2nd rel.	?
6	N.A.	10	♀	ALL	2nd rel.	CR
7	B.C.	5 8/12	♀	ALL	3rd rel.	CR
8	N.N.	7 11/12	♂	ALL	3rd rel.	?
9	M.A.	12	♂	ALL	1st rel.	?
10	M.I.	8 6/12	♂	ALL	1st rel.	?
11	K.C.	11 3/12	♀	ALL	4th rel.	?
12	K.K.	10 9/12	♀	ALL	5th rel.	PR
13	L.J.	7	♂	ALL	3rd rel.	WBC ↓
14	F.D.	2 3/12	♂	ALL	init. ph.	WBC ↓
15	S.B.	7 11/12	♀	ALL	init. ph.	WBC ↓
16	H.B.	12 5/12	♀	ALL	init. ph.	WBC ↓
17	S.N.	4	♀	AML	init. ph.	WBC ↓
18	W.H.	5 6/12	♂	AML	init. ph.	WBC ↓

Of the 13 terminal cases, complete remission was achieved in 3, and a partial remission achieved in 2 other cases. Fever, occasionally accompanied by shivering, was frequently observed under MPC treatment in the first hour after injection. However, these symptoms never lasted more than the first hour, and no other sideeffects could be observed.

On the basis of our experience with MPC on terminal cases, we were motivated to give MPC a clinical trial in the beginning of leukemia. A monotherapy with MPC, as devised for terminal cases is, however, not possible. We therefore decided to introduce MPC (0.5 mg/kg body weight) therapy in the beginning of treatment of cases which at the time of diagnosis had leucocytosis. This initial treatment, a single injection of MPC, was then followed up by polychemotherapeutic protocol, adopted by the university hospitals in Berlin, Frankfurt and Münster [see ref. 18]. As shown in Fig. 7, 24 hrs. after MPC injection, there was a significant reduction leukemic cells in all the cases. Five of these seven children had ALL, and two AML.

The status of this drug in the chemotherapy of fresh leukemic cases is not known, since monotherapy with MPC in such cases has not been done. The fact that under the pressent polychemotherapeutic protocols one can frequently achieve longterm remissions, hinders one ethically to use MPC as a monotherapeuticum in fresh cases. However, its use in the initial phase of the acute disease, and its use as a monotherapeutic agent in terminal cases are quite encouraging. On the basis of our to-date experience with MPC we could summarize by saying: a) MPC is useful to initiate the therapy in freshly diagnosed acute leukemic cases, b) it has shown promise as an effective drug in the treatment of leukemic cases in the terminal phase, and c) it could

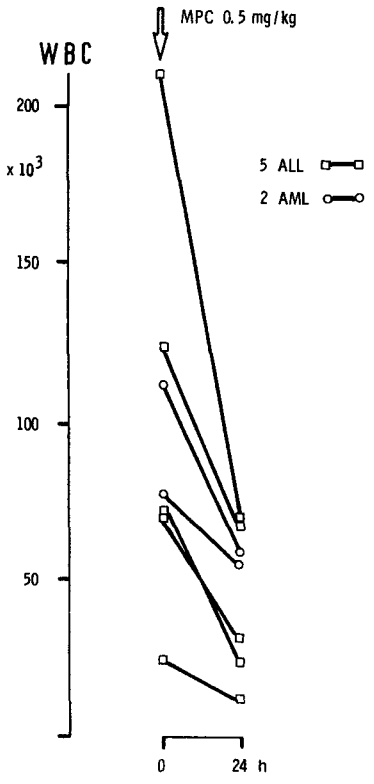

Fig. 7. MPC trials in the initial treatment of freshly diagnosed leukemic cases. Details are given in the text

be used in the remission maintenance therapy. This aspect is yet to be investigated. This, as a matter of fact, is the rationale for its therapeutic application, since it is a potent inhibitor of reverse transcriptase.

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