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Combination Chemotherapy: Synergistic Inhibition of Lymphoma L5178Y Cells in Culture and *in Vivo* with 6-Mercaptopurine and 6-(Methylmercapto)purine Ribonucleoside[1,2]

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SUMMARY

The proliferation of cells of mouse lymphoma L5178Y, either in primary culture or *in vivo*, was inhibited much more effectively by combinations of 6-mercaptopurine and 6-(methylmercapto)purine ribonucleoside than would be expected from the separate effects of these agents. The existence of these synergistic inhibitory effects against the lymphoma cells in culture indicates that drug effects in the tumor cell, rather than effects at the host level, were responsible for the increase in survival time achieved by combining the drugs. In the most effective combinations of the drug pair, 6-mercaptopurine was in 5- to 10-fold molar excess, whether assayed *in vivo* or against cultured lymphoma cells. A likely biochemical mechanism for this synergism is discussed, based on the enhancement in 6-mercaptopurine anabolism caused by 6-(methylmercapto)purine ribonucleoside.

INTRODUCTION

As is well known, the therapeutic value of certain antitumor agents is enhanced when these are administered in combination (10). This study concerns the potentiation of the antitumor activities of 6-mercaptopurine (6-MP) and 6-(methylmercapto)purine ribonucleoside (Me6MPR) which results when these agents are used together against mouse lymphoma L5178Y *in vivo* or in culture.

It was reported previously from this laboratory that combinations of 6-MP and Me6MPR were much more effective than either agent used alone in the treatment of the Ehrlich ascites carcinoma (8, 12). In independent studies, Schabel *et al.* (11) found these two drugs to be "therapeutically potentiating" against mouse leukemia L1210. This drug combination has also been used with some success against human acute leukemia by Bodey *et al.* (1). In a related clinical study, Me6MPR alone was found to be inactive in the treatment of patients with 6-MP-resistant acute leukemia (6).

This report concerns our demonstration that 6-MP and Me6MPR synergize in inhibiting the proliferation of L5178Y murine leukemic lymphoblasts *in vivo* or in primary culture. The terms "synergism" and "potentiation" are used interchangeably in this report to mean that antitumor effects produced by the concurrent administration of two drugs are greater than the sum of the effects of each drug administered separately.

One of the aims of this work was to decide whether the synergism observed could be attributed to drug effects in the tumor cells or to effects at the host level (such as changes in the distribution or excretion of one drug caused by another drug). The demonstration that the lymphoma cells in culture were inhibited synergistically by combinations of the two agents, indicated that drug effects in the tumor cell were responsible for the therapeutic results.

Another aim of this work was to develop an *in vitro* assay system to detect drug combinations that would synergize *in vivo*. With this criterion in mind, the L5178Y transplantable lymphoma was chosen because these cells grow rapidly in *primary* culture.

MATERIALS AND METHODS

Me6MPR-methyl-^{14}C was the gift of Dr. I. C. Caldwell of this laboratory; 6-MP was a commercial product; other purine and purine ribonucleoside analogs were provided by the Cancer Chemotherapy National Service Center, National Cancer Institute, USPHS, Bethesda, Maryland.

The L5178Y murine lymphoma was obtained from Dr. A. C. Sartorelli of Yale University and was maintained in male BDF_1 mice by the intraperitoneal transplantation of 10^7 tumor cells each week.

Primary cultures of the lymphoma cells were employed in these studies; that is, only lymphoma cells obtained from ascitic fluids were cultured. Inocula of around 3×10^4 cells, taken 4 or 5 days after transplantation of the donor mouse, were grown in 1.0-ml volumes of Fischer's medium (5) containing 10% horse serum and antibiotics (streptomycin, 100 μg/ml; penicillin, 100 units/ml) in 15 x 85 mm test tubes. Culture tubes were kept stationary and in an upright position during incubation at 37°C; all tubes were at least in triplicate

[1]Portions of this study were reported previously (A. R. P. Paterson and A. Moriwaki, Proc. Am. Assoc. Cancer Research., *9:* 57, 1968). This is Paper No. 1 of a series on Combination Chemotherapy.

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and cell numbers were determined with an electronic particle counter (Coulter Counter, Model F).

For *in vivo* assays of therapeutic activity, male BDF_1 mice (A. R. Schmidt Co. Madison, Wisconsin, or Microbiological Associates Inc., Bethesda, Maryland) were implanted intraperitoneally with 10^6 lymphoma cells obtained from ascitic fluid 4 or 5 days after implantation. Therapeutic agents were administered by intraperitoneal injection in a series of 8 doses at 12- or 24-hour intervals, as noted, starting 24 hours after implantation. 6-MP, ground to a very fine powder, was suspended in physiologic saline, and Me6MPR was dissolved; injection volumes were 0.2 ml for the agents singly or in combination. Treatment and control groups contained 10 and 20 mice each respectively.

RESULTS

To determine whether two drugs had synergistic or additive effects when used together, the following procedure was used. The first step was to determine concentrations (7) (or doses) of the individual drugs that would inhibit cell proliferation (or increase survival time) to the same extent. Next, fractions of these concentrations were tested in combination, as in Table 1. It is seen in Table 1 that 6-MP and Me6MPR together had only additive inhibitory effects in 48- and 72-hour cultures of the lymphoma cells when the medium was not changed. However, when the culture medium was replaced daily (Expt. III), the agents in combination had inhibitory effects that were greater than additive.

Chart 1 represents the data of Table 1 in the graphic form used by Hitchings (3). Additive effects, by definition, give points on the diagonal line joining the extreme points which represent the effects of the separate treatments, A and B. Synergistic effects resulting from fractional combinations of A and B give points which tend toward the origin. It is apparent in Chart 1 that 6-MP and Me6MPR in combination synergized when the culture medium was replaced daily.

Table 2 shows that combinations of 6-MP and Me6MPR were distinctly synergistic when cells were counted after 96 hours

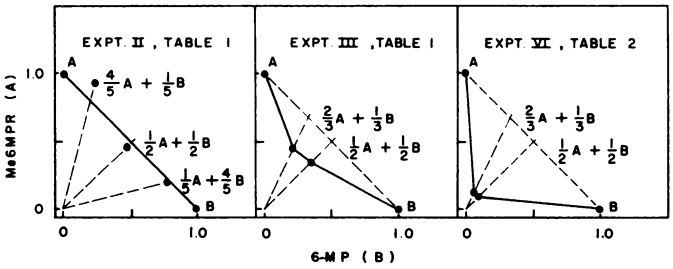

Chart 1. Graphic representation of data from Tables 1 and 2 showing additive and synergistic effects. Unity on the graph axes represents concentration *A* of Me6MPR, or concentration *B* of 6-MP, which individually inhibited cell proliferation by about 30% in Expt. II (Table 1), by about 50% in Expt. III (Table 1), and by 75% in Expt. VI (Table 2). 6-MP, 6-mercaptopurine; Me6MPR, 6-(methylmercapto)purine ribonucleoside.

Table 1

Experiment	Growth period (hours)	Medium changed daily	Inhibitors	Final cell number × 10^{-4} (cells/tube)	Growth rate (%)
I	48	No	*A*: Me6MPR 1×10^{-7} M	27.2 ± 0.8[a]	77.9 ± 2.3[a]
			B: 6-MP 5×10^{-6} M	24.9 ± 0.3	71.3 ± 0.9
			$\frac{1}{2}A + \frac{1}{2}B$	22.9 ± 1.2	65.6 ± 3.4
			Control	34.9 ± 0.3	100
II	72	No	*A*: Me6MPR 9×10^{-8} M	70.2 ± 1.9	73.4 ± 2.0
			B: 6-MP 1.5×10^{-6} M	66.9 ± 2.1	70.0 ± 2.2
			$\frac{1}{2}A + \frac{1}{2}B$	62.3 ± 1.1	65.2 ± 1.2
			$\frac{1}{5}A + \frac{4}{5}B$	65.5 ± 2.2	68.5 ± 2.3
			$\frac{4}{5}A + \frac{1}{5}B$	78.3 ± 2.0	81.9 ± 2.1
			Control	95.6 ± 3.5	100
III	72	Yes	*A*: Me6MPR 1.2×10^{-7} M	40.1 ± 2.4	60.0 ± 3.6
			B: 6-MP 5×10^{-6} M	30.3 ± 1.7	45.3 ± 2.5
			$\frac{1}{2}A + \frac{1}{2}B$	23.0 ± 1.1	34.5 ± 1.6
			$\frac{2}{3}A + \frac{1}{3}B$	22.0 ± 2.8	32.9 ± 4.2
			Control	66.9 ± 2.0	100

Additive inhibitory effects against L5178Y lymphoma cells in culture with combinations of 6-MP and Me6MPR. Me6MPR, 6-(methylmercapto)purine ribonucleoside; 6-MP, 6-mercaptopurine.

[a]Mean ± S.E.

Table 2

Experiment	Medium changed daily	Horse serum (%)	Inhibitors	Final cell number × 10^{-4} (cells/tube)	Growth rate (%)
IV	Yes	10	*A*: Me6MPR 1.5×10^{-7} M	114.8 ± 3.7[a]	58.0 ± 1.9[a]
			B: 6-MP 5×10^{-6} M	88.6 ± 1.4	44.8 ± 0.7
			$\frac{1}{2}A + \frac{1}{2}B$	28.5 ± 0.8	14.4 ± 0.4
			$\frac{2}{3}A + \frac{1}{3}B$	23.7 ± 1.0	12.0 ± 0.5
			Control	197.9 ± 6.2	100
V	No	10	*A*	47.3 ± 2.6	48.1 ± 2.6
			B	39.8 ± 1.6	40.4 ± 1.6
			$\frac{1}{2}A + \frac{1}{2}B$	19.7 ± 1.1	20.0 ± 1.1
			$\frac{2}{3}A + \frac{1}{3}B$	19.5 ± 1.4	19.8 ± 1.4
			Control	98.3 ± 1.4	100
VI	Yes	20	*A*	100.4 ± 10.6	33.3 ± 3.5
			B	64.3 ± 2.9	21.3 ± 1.0
			$\frac{1}{2}A + \frac{1}{2}B$	13.8 ± 0.4	4.6 ± 0.1
			$\frac{2}{3}A + \frac{1}{3}B$	13.8 ± 0.5	4.6 ± 0.1
			Control	301.8 ± 2.3	100
VII	No	20	*A*	61.7 ± 5.0	54.9 ± 4.5
			B	43.4 ± 2.0	38.6 ± 1.8
			$\frac{1}{2}A + \frac{1}{2}B$	21.0 ± 1.2	18.7 ± 1.1
			$\frac{2}{3}A + \frac{1}{3}B$	19.1 ± 1.7	17.0 ± 1.5
			Control	112.3 ± 3.8	100

Synergistic inhibitory effects against L5178Y lymphoma cells in culture with combinations of 6-MP and Me6MPR. A 96-hour growth period was used in all experiments. Me6MPR, 6-(methylmercapto)purine ribonucleoside; 6-MP, 6-mercaptopurine.
[a]Mean ± S.E.

of culture. Synergistic effects were evident whether or not the medium was replaced daily, although they were more pronounced when this was done. Thus, for the synergistic effect to be clearly seen, cell numbers in control cultures had to increase by about 50-fold; the effect was greater still when larger increases in cell number resulted from improved nutrition (replacement of medium) and an adequate growth period.

Daily replacement of the culture medium also served to maintain drug concentrations. That disappearance of Me6MPR from the medium was a factor of importance is evident in Table 3, which shows that (*a*) the uptake of Me6MPR-methyl-^{14}C was related to the concentration of cells and (*b*) when the lymphoma cells numbered about 3 × 10^5 per ml, roughly half of an initial concentration of 1.5 × 10^{-7} M Me6MPR was taken up during a 24-hour period of culture. Thus, in order to keep the concentration of Me6MPR reasonably close to the initial value, cultures were subdivided daily (with replacement of medium as a necessary part of this procedure); as cell numbers were kept below 10^5 per ml, concentrations of Me6MPR probably did not fall below 80–85% of the initial value, judging from Table 3.

Table 3

Initial number of cells × 10^{-4}	Number of cells after 24 hrs of incubation × 10^{-4}	Percentage loss of Me6MPR-methyl-^{14}C during 24 hours of incubation
1.5	1.8	4
4	5.1	7
10	12	17
30	57	46
100	50	43

Disappearance from the culture medium of Me6MPR-methyl-^{14}C during culture of L5178Y cells. Cultures of L5178Y cells were set up, with initial cell concentrations as noted, in media containing 1.5 × 10^{-7} M Me6MPR-methyl-^{14}C. After incubation for 24 hours, the loss of ^{14}C from the medium was measured. Me6MPR, 6-(methylmercapto)purine ribonucleoside.

In Chart 2 cumulative cell numbers are plotted; that is, cell numbers have been multiplied by the dilution factors involved in each culture subdivision. Under these circumstances, growth rates for control cultures and for cells in the presence of Me6MPR were constant; when the concentration of Me6MPR was 1.5 × 10^{-7} M, the growth rate was reduced by about 70%.

Chart 2 shows that the growth curve obtained in the presence of Me6MPR differed distinctly from that with 6-MP; in the latter the growth rate decreased progressively after 48 hours. Thus, the two agents have quite different effects on the lymphoma cells. 6-MP appears to have a delayed effect, that is, after several divisions lethal damage appears to accumulate. When both 6-MP and Me6MPR were present in the culture, the trend apparent in the 6-MP growth curve was exaggerated.

Table 4 summarizes experiments which show that the 6-MP-Me6MPR pair is therapeutically potentiating in the treatment of lymphoma L5178Y *in vivo*. The increase in survival time of animals treated with the drug pair was much larger than would be expected from the sum of the separate drug effects. It is apparent that the biochemical events responsible for the *in vivo* potentiation are taking place in the tumor cells and not at the host level.

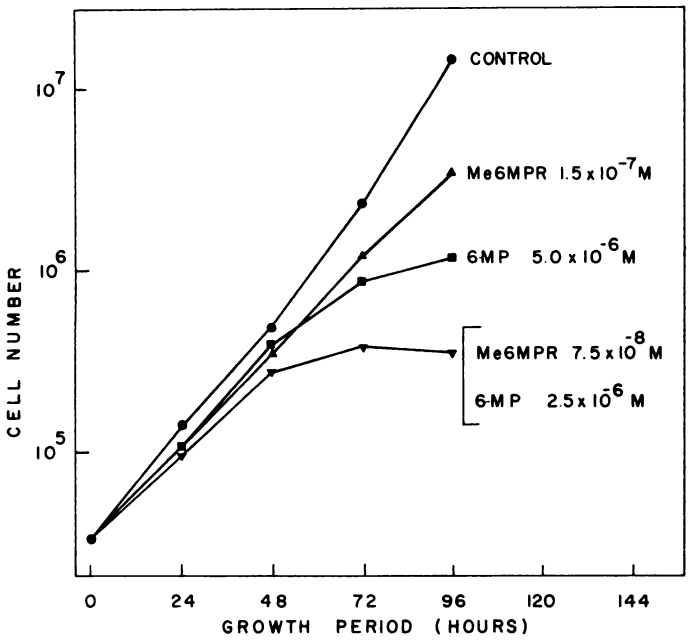

Chart 2. Influence of 6-MP and Me6MPR on the growth rate of L5178Y lymphoma cells cultured with daily subdivision of cultures and daily replacement of medium. Cell numbers were kept below 10^5 cells per ml. Cumulative cell numbers are plotted. 6-MP, 6-mercaptopurine; Me6MPR, 6-(methylmercapto)purine ribonucleoside.

Table 4

Dosage 6-MP	Dosage Me6MPR	Median survival time (days)	Mean survival time (days)
8 treatments at 24-hr intervals			
Saline		9.5	11.3 ± 2.3[a]
	15	12.0	12.0 ± 0.8
30		13.0	13.5 ± 0.8
15	7.5	16.0	18.2 ± 3.9 (1)[b]
10	10	19.0	20.4 ± 4.8 (2)
20	5	16.5	20.1 ± 5.5 (1)
8 treatments at 12-hr intervals			
Saline		10.0	11.1 ± 0.9
	15	12.5	12.7 ± 0.8
30		14.0	15.1 ± 2.7
15	7.5	16.0	15.8 ± 2.5
7.5	3.8	18.0	22.2 ± 0.9 (4)

Therapeutic potentiation resulting from the combined use of 6-MP and Me6MPR in the treatment of lymphoma L5178Y in BDF_1 mice. The experiment was terminated at 30 days and any surviving mice were counted as 30-day survivors in calculation of mean survival time. 6-MP, 6-mercaptopurine; Me6MPR, 6-(methylmercapto)purine ribonucleoside.

[a] Mean deviation.

[b] The number of mice surviving 30 days is given in parentheses.

Selection of the most effective molar ratio of the two drugs was attempted with cultured cells in the experiments of Chart 3. On the basis of preliminary experiments, concentrations of Me6MPR and of 6-MP were chosen which separately inhibited cell proliferation by about 50%. However, as the response to a given drug concentration varied somewhat in repeated experiments, equal inhibitions were not achieved in the experiments of Table 3 with the chosen drug concentrations. When both drugs were present together in the culture medium in fractions of the chosen concentrations, growth inhibitory effects were greater than additive, and the degree of this potentiation varied with the relative molar proportions of the two drugs, whether or not the medium was replaced daily. The maximum inhibitory effect was achieved when 6-MP was present in about 10-fold molar excess over Me6MPR. Schabel *et al.* (11) tested against leukemia L1210 *in vivo* 3 different molar ratios of 6-MP to Me6MPR in combinations of equal host toxicity and found that ratios of 5 and 1.25 were more effective than 20.

A similar type of experiment was performed to determine the most effective composition of 6-MP and Me6MPR mixtures in the treatment of animals implanted with the L5178Y lymphoma. The results, shown in Chart 4, indicate that the molar ratio of 6-MP to Me6MPR was about 10 to 1 in the most effective combinations of the two agents.

The selection of effective drug ratios for therapy was explored further, as described in Table 5. In this experiment, combination treatments with 6-MP to Me6MPR ratios of 5 and 10 were compared, using schedules in which drugs were injected at intervals of 12 and 24 hours. Table 5 shows that when treatments were given at 24-hour intervals, the 10-fold molar excess of 6-MP over Me6MPR was the more effective, although a difference between the two treatments was not apparent in the 12-hour schedule. Treatment at 12-hour intervals was as effective as treatment at twice the drug dosage level given at 24-hour intervals.

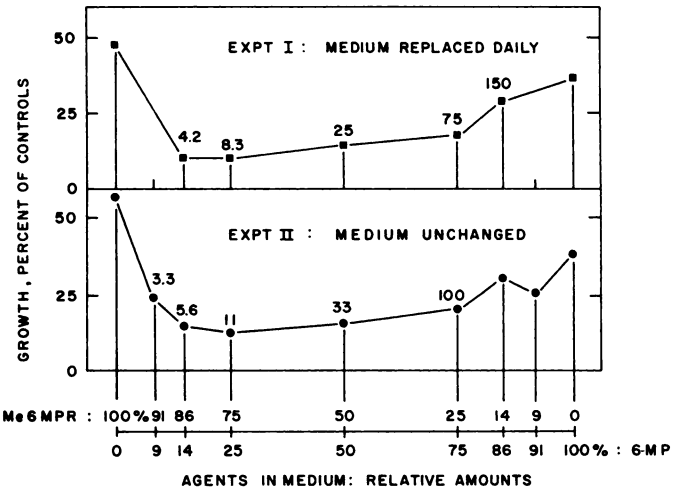

Chart 3. Combinations of 6-MP and Me6MPR: selection of molar ratio most inhibitory to L5178Y cells in culture. Expt. I. (■) Culture medium was not changed during the 96-hour growth period. Drug concentrations (100%): Me6MPR, 1.5×10^{-7} M; 6-MP, 5×10^{-6} M. Expt. II. (●) Culture medium was changed daily. Drug concentrations (100%): Me6MPR, 2×10^{-7} M; 6-MP, 5×10^{-6}M. Values above the plotted points represent the molar ratio of 6-MP to Me6MPR. 6-MP, 6-mercaptopurine; Me6MPR, 6-(methylmercapto)purine ribonucleoside.

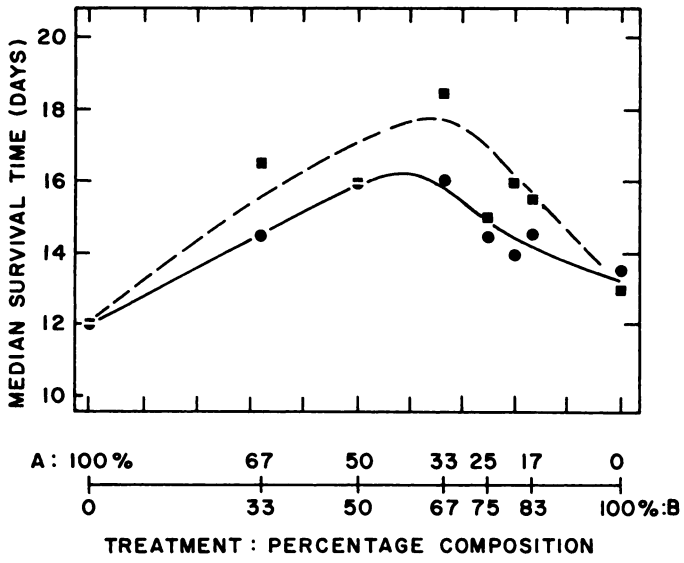

Chart 4. Combination chemotherapy of lymphoma L5178Y: selection of the most effective molar ratio of 6-MP to Me6MPR. Shown are data from 2 separate experiments which employed mice from different suppliers: --■-- A. R. Schmidt Co. (median survival time of saline-treated control mice was 9.5 days). —●— Microbiological Associates Inc. (median survival time of saline-treated control mice was 11.0 days). The relative molar proportions of the two drugs in each combination treatment were as follows: Treatment *A*: 6-(methylmercapto) purine ribonucleoside (Me6MPR), 15 mg/kg; treatment *B*: 6-mercaptopurine (6-MP), 30 mg/kg.

Treatment:	% *A*	67	50	33	25	20	17
	% *B*	33	50	67	75	80	83
Molar ratio	(*B*/*A*)	1.8	3.5	7	10.5	14	18

Table 5

Molar ratio: 6-MP/ Me6MPR	Dosage (mg/kg)		Median survival time (days) when treatments given at intervals of:	
	6-MP	Me6MPR	12 hr	24 hr
	Saline		11.0	
5	15	5.0	18.0	15.5
5	7.5	2.5	18.0	13.0
10	15	2.5	19.0	22.0
10	7.5	1.3	18.0	13.0

Chemotherapy of lymphoma L5178Y with combinations of 6-MP and Me6MPR. Starting 24 hours after implantation, 8 treatments were given at 12- or 24-hour intervals, as indicated; control mice received 8 injections of saline at 12-hour intervals. 6-MP, 6-mercaptopurine; Me6MPR, 6-(methylmercapto)purine ribonucleoside.

DISCUSSION

Therapeutic potentiation resulting from the combination of antitumor agents is a very important extention of their usefulness. In these experiments, the events responsible for therapeutic potentiation appear to be taking place in the tumor cells, because the two agents combined, 6-MP and Me6MPR, were clearly synergistic in inhibiting the proliferation of the lymphoma cells in culture.

In respect to the optimal relative proportions of the two drugs, the correspondence between the *in vivo* and the *in vitro* results may be related to the ability of the L5178Y cells to proliferate immediately upon explanation. Whether this correspondence will be found with other drug combinations, and the more basic question of whether synergism *in vitro* with other drug combinations will predict similar *in vivo* effects, are matters presently under study.

Other studies in this laboratory have suggested biochemical events in the tumor cells which might account for the synergism between 6-MP and Me6MPR. We have found that treatment of Ehrlich ascites cells *in vivo* with a therapeutic dose of 6-MP and Me6MPR increases several-fold the rate at which 6-MP in a following dose is converted into nucleotides. This enhancement of 6-MP anabolism also occurs on prior treatment of Ehrlich ascites tumor cells (9) or lymphoma L5178Y cells (M. C. Wang and A. R. P. Paterson, unpublished data) with Me6MPR alone and appears due to a diversion of phosphoribosyl pyrophosphate (PRPP) into the phosphoribosyl transferase reaction, by which 6-MP is converted into its nucleotide derivative. [Ehrlich ascites cells synthesize and retain Me6MPR 5'-monophosphate (2), which, as a potent inhibitor of PRPP-amidotransferase, blocks the utilization of PRPP by way of the *de novo* pathway of purine nucleotide synthesis.] Since the formation of 6-MP nucleotide is an obligatory process in the mechanisms of action of 6-MP (4), it is reasonable to speculate that the enhancement of 6-MP anabolism in tumor cell resulting from exposure to Me6MPR may be the mechanism of the synergism of 6-MP and Me6MPR. These observations show that the enhancement in 6-MP anabolism and the synergistic effects have a circumstantial relationship, but they do not prove that this enhancement is the mechanism of the synergism.

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