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Synergy between 5,10-dideaza-5,6,7,8-tetrahydrofolic acid and methotrexate in mice bearing L1210 tumors

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Summary. In vivo studies with 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF), an inhibitor of glycinamide ribonucleotide transformylase, indicate that at doses ranging from 2.5 to 10 mg/kg, it prolongs the survival of mice implanted with L1210 tumors. Lower doses of this agent have no effect. Parallel studies with methotrexate indicate that DDATHF is not as potent or as efficacious as methotrexate in this animal model. Low doses of DDATHF combined with low doses of methotrexate can cause a significant increase in the survival of L1210 tumor-bearing mice, suggesting synergism between these two antifolates.

Introduction

5,10-Dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF) is a novel antifolate that inhibits the growth of a wide variety of tumors, some of which are resistant to methotrexate (MTX) [8]. Mechanistic studies with this agent have shown that it is a potent inhibitor of glycinamide ribonucleotide (GAR) transformylase, an enzyme required for purine biosynthesis [1, 6]. Thus, cells exposed to DDATHF are depleted of purines and growth is inhibited. This growth inhibition can be reversed by the addition of hypoxanthine to the culture medium.

Initial observations with cultures of *Lactobacillus casei* [5] have shown that trimethoprim (a dihydrofolate reductase inhibitor) and N^{10} propargyl-5,8-dideazafolic acid (CB 3717, a thymidylate synthetase inhibitor) were added to growth medium, the growth inhibition observed was greater than an additive response. Extending these observations, recent studies [3] have demonstrated that two antifolates with different mechanisms of action can be synergistic with each other and inhibit the growth of hepatoma cells in culture. In these studies, synergistic inhibition of growth was detected when low doses of CB3717 were added to cultures along with low doses of methotrexate, trimetrexate, or metoprine (dihydrofolate reductase inhibitors). Recent studies [4] have shown a similar synergistic effect with hepatoma cells when low doses of DDATHF were combined with low doses of dihydrofolate reductase inhibitors such as metoprine or trimetrexate. Since combination drug therapy has important clinical implications in the treatment of neoplastic diseases, we were interested in determining whether the synergism previously observed with DDATHF and methotrexate [4] could also be demonstrated in an in vivo tumor model. The results of these studies are the subject of this report.

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Materials and methods

DDATHF was synthesized [2] by a modification of a previously published procedure [8]. Male CD_2F_1 mice were implanted i.p. with L1210 tumors at a concentration of 1×10^5 cells/mouse. At 24 h after tumor implantation, mice were treated daily for 9 days with various i.p. doses of either DDATHF or methotrexate. At least eight mice were used for each dose, and they were examined every 8 h and were sacrificed on becoming moribund. The mean survival of the drug-treated mice was compared with that of controls. Statistical analysis was conducted using Student's *t*-test.

To examine the apparent supra-additive response (synergy) resulting from the combined treatment with DDATHF and methotrexate, isobolograms were constructed for the various response levels [6]. Briefly, for each of the individual treatments, the logarithm of the response (i.e., the log of increased life span) was plotted as a function of the dose. Using this plot, two curves were constructed for each response level attained by the combined treatment groups. The first of these, the "Mode I line", corresponded to the locus of individual doses whose response would sum up to the observed combination treatment response. The second curve, "Mode II line", was calculated by postulating that the effect attributable to one agent was displaced by the second agent (i.e., their actions were not independent). To facilitate the evaluation of data, an RS/1 procedure (Bolt Beranek and Newman Inc) was used to construct these isobolograms.

The subacute LD_{50} of methotrexate or DDATHF was determined as follows. Male CD_2F_1 mice (at least ten/group) were treated daily with various i.p. doses of methotrexate or DDATHF. After 9 days of daily dosing, drug treatment was suspended and the mice were monitored every 8 h, being sacrificed on becoming moribund. The number of survivors was determined on day 21. For these studies, the LD_{50} is defined as the dose of methotrexate or DDATHF that kills 50% of the mice within 21 days. The effects of combination therapy (DDATHF and methotrexate) on the mean survival of

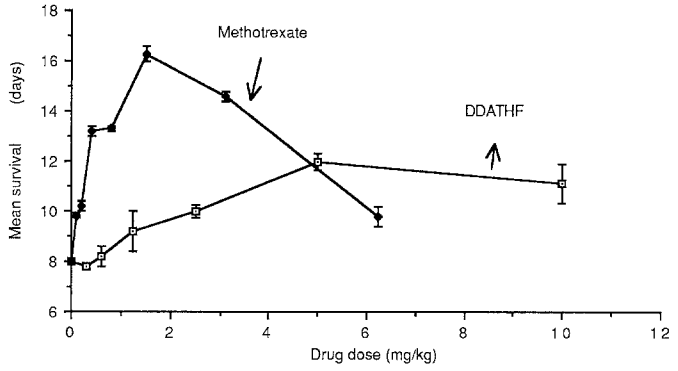

Fig. 1. Effect of various doses of DDATHF or methotrexate on the survival of mice implanted with L1210 tumor cells. *Error bars* indicate ±SEM

normal mice was also determined. In these studies, male CD_2F_1 mice (seven per group) were treated daily with various i. p. doses of DDATHF and methotrexate. After 9 days of dosing, treatment was suspended and the number of survivors was determined on day 21.

Results

The effects of various doses of methotrexate or DDATHF on the mean survival of mice implanted with L1210 tumors was examined. Results shown in Fig. 1 indicate that DDATHF treatment at doses ranging from 2.5 to 10 mg/kg (optimal dose, 5 mg/kg) resulted in a significant increase in the life span of tumor-bearing mice. At lower doses the drug was ineffective. With methotrexate, the survival was significantly prolonged at doses ranging from 0.4 to 3 mg/kg (optimal dose, 1.25 mg/kg). At methotrexate doses of <0.4 mg/kg, the drug did not prolong the survival of tumor-bearing mice. From the data shown in Fig. 1, it is apparent that in this animal model methotrexate was more potent and more efficacious than DDATHF. In addition, the survival of tumor-bearing mice treated with the optimal dose of methotrexate was considerably higher than that in animals treated with the optimal dose of DDATHF.

The subacute LD_{50} of the two agents was determined in normal mice using the dosing schedule described in Materials and methods. The LD50 for methotrexate was 10 mg/kg and that for DDATHF was 125 mg/kg. Since the toxicity of DDATHF is dependent on the plasma or intracellular concentration of hypoxanthine [1] and because DDATHF has no effect on the enzymes of the purine salvage pathway, a high LD_{50} was expected for DDATHF.

Since DDATHF was not as potent or efficacious as methotrexate (Fig. 1), we were interested in determining whether its efficacy could be improved if it were combined with low doses of methotrexate. To conduct these combination therapy studies, we chose methotrexate doses that were ineffective in prolonging the survival of mice bearing L1210 tumors. For example, the mean survival of mice treated with methotrexate at 0.1 mg/kg was 7.9 days; those treated with 0.2 mg/kg survived for 8.3 days and control mice, for 8 days. In these combination therapy studies, mice implanted with L1210 tumor cells (eight mice/group) were treated with various doses of DDATHF combined with either 0.1 or 0.2 mg/kg methotrexate. Results of a typical experiment are shown in Fig. 2.

When tumor-bearing mice were treated with methotrexate (0.1 mg/kg) and DDATHF (at doses of up to 2.5 mg/kg), a significant enhancement in survival was observed (as compared with the individual treatment groups). At a higher dose of DDATHF (5.0 mg/kg), a significant increase in survival was not observed; at 10 mg/kg DDATHF, drug toxicity may have been responsible for the reduction in mean survival. When tumor-bearing mice were treated with methotrexate (0.2 mg/kg)

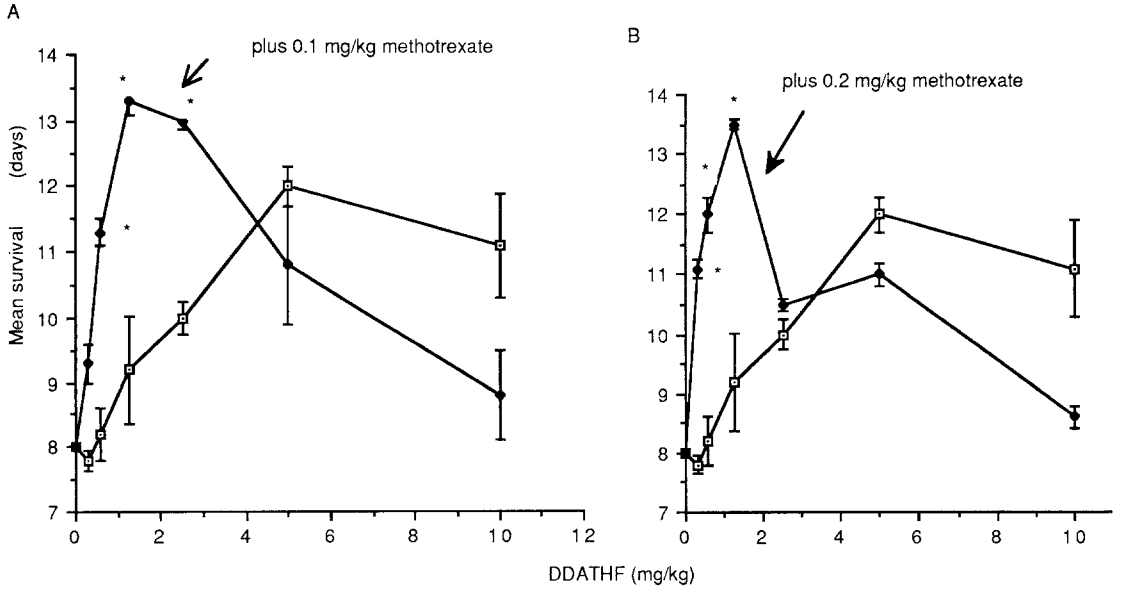

Fig. 2. Effect of **A** 0.1 mg/kg methotrexate and **B** 0.2 mg/kg methotrexate along with various doses of DDATHF on the survival of mice bearing L1210 tumor cells. At least eight mice were used in each group. $*P<0.05$ as compared with controls (i. e., animals receiving DDATHF alone)

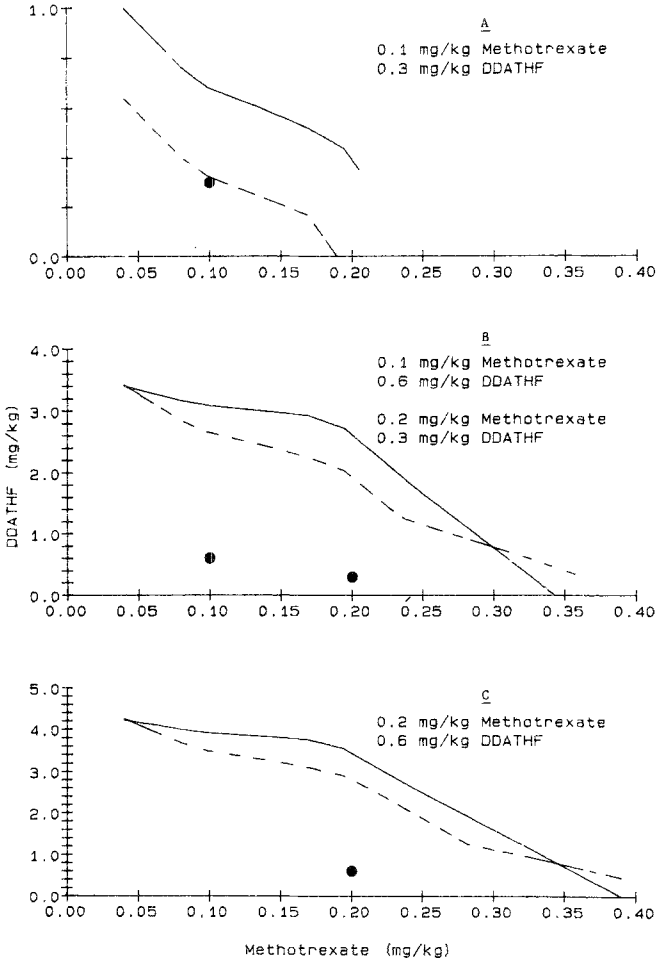

Fig. 3. Isobologram analysis of mice treated with **A** DDATHF (0.3 mg/kg) and methotrexate (0.1 mg/kg) **B** DDATHF (0.6 or 0.3 mg/kg) and methotrexate 0.1 or 0.2 mg/kg), and **C** DDATHF (0.6 mg/kg) and methotrexate (0.2 mg/kg). ———, Mode I line; – – – –, Mode II line. ● indicates values obtained for combination therapy

and various doses of DDATHF (up to 1.25 mg/kg), survival was significantly enhanced. At 2.5 mg/kg DDATHF and 0.2 mg/kg methotrexate, the survival was not significantly different from that observed in controls. At higher doses of DDATHF (5 and 10 mg/kg), the mean survival was lower, suggesting that these doses were toxic. It should be noted that the mean survival of mice treated with a combination of DDATHF and methotrexate was always lower (Fig. 2) than the survival of tumor-bearing mice treated with the optimal dose of methotrexate (Fig. 1).

The data shown in Fig. 2 suggest that at low doses of DDATHF and methotrexate (0.1 and 0.2 mg/kg), a synergistic effect between these agents may have been responsible for the prolonged survival. To prove that the results of combination therapy indicated synergism between DDATHF and methotrexate rather than an additive response, isobolograms [6] were constructed for each treatment group; the results indicate that synergy between DDATHF and methotrexate was observed at doses of 1.25, 0.6, or 0.3 mg/kg DDATHF and 0.1 and 0.2 mg/kg methotrexate. Three of these isobolograms are shown in Fig. 3. In the three cases illustrated (*panels A–C*), the response resulting from treatment with methotrexate and DDATHF fall to the left of the envelope of additivity [6] delineated by the mode I and II lines. When a measured response between two agents is synergistic rather than additive, results such as those shown in Fig. 3 are typical [6]. In panel A, the doses of DDATHF and methotrexate were 0.3 and 0.1 mg/kg, respectively. In panel B, the doses of DDATHF were 0.6 or 0.3 mg/kg and the methotrexate doses were 0.1 or 0.2 mg/kg. In panel C, the doses of DDATHF and methotrexate were 0.6 and 0.2 mg/kg, respectively. Synergy between DDATHF (1.25 mg/kg) and methotrexate (0.1 or 0.2 mg/kg) was also demonstrated using isobolograms, but these are not shown.

The subacute toxicity of combination therapy (DDATHF and methotrexate) was ascertained in normal

Table 1. The effect of combination therapy (DDATHF and methotrexate) on the mean survival (MS) of normal CD_2F_1 mice

Group	DDATHF (mg/kg)	Methotrexate (mg/kg)	Weights (±SEM)	Dead/treated on day 21	MS (days ± SEM)
Normal	–	–	19.5±0.6	0/7	NA
1	5.0	0.5			9.7±0.4
2		0.2			10.2±0.3
3		0.1			10.4±0.2
4	2.5	0.5			9.7±0.2
5		0.2			11.0±0.4
6		0.1			12.1±0.4
7	1.25	0.5	15.8±0.3[a]	3/7	NA
8		0.2	18.6±0.7	0/7	NA
9		0.1	18.3±0.5	0/7	NA
10	0.6	0.5	19.2±0.3	0/7	NA
11		0.2	18.7±0.6	0/7	NA
12		0.1	19.1±0.5	0/7	NA
13	0.3	0.5	19.0±0.1	0/7	NA
14		0.2	20.0±0.4	0/7	NA
15		0.1	21.2±0.3	0/7	NA

NA, not applicable
[a] Significantly different from normal values

mice. The results of these studies are shown in Table 1. No toxicity was observed when methotrexate (0.1 and 0.2 mg/kg) was combined with DDATHF (0.3–1.25 mg/kg), suggesting that these doses were safe. In addition, the weight of mice treated at these doses was not significantly different from that of control mice.

Discussion

The present studies examined the effects of DDATHF and methotrexate in a murine tumor model. The results of these studies indicate that DDATHF is not as potent or as efficacious as methotrexate in prolonging the survival of mice bearing L1210 tumors. Moreover, at optimal doses, the survival of tumor-bearing mice treated with methotrexate was considerably higher than that of those treated with DDATHF. The therapeutic index for DDATHF (LD_{50}/optimal effective dose) is 25, which is considerably higher than that for methotrexate (therapeutic index of 8), suggesting that a greater margin of safety exists when DDATHF is used in therapy. A highlight of these studies is that low and ineffective doses of DDATHF (0.3–1.25 mg/kg) can be synergistic with low and ineffective doses of methotrexate (0.1 and 0.2 mg/kg), prolonging the survival of tumor-bearing mice. Subacute toxicity studies have shown that these doses of DDATHF and methotrexate are safe, resulting in no overt toxicity. However, the mean survival of mice undergoing synergistic therapy was still lower than that observed with the optimal dose of methotrexate.

At low doses, synergistic activity in combination drug therapy is of great clinical interest because of its potential to (a) significantly increase the therapeutic index of the treatment regimen and (b) avoid drug resistance. In the present studies, we demonstrated that low doses of DDATHF can be synergistic with low doses of methotrexate, increasing the life span of mice implanted with L1210 tumors. Since these two antifolates have differing mechanisms of action, combination of the two agents may have increased the susceptibility of the tumor cells to cell death and reduced host toxicity. For example, it is known that low levels of methotrexate can deplete the cells of reduced folates including N^{10} formyl tetrahydrofolate. Since this reduced folate is a cofactor for glycinamide (GAR) transformylase, limiting the intracellular concentrations of N^{10} formyl tetrahydrofolate may have made the enzyme susceptible to inhibition by low concentrations of DDATHF. If this were the case, synergy between DDATHF and methotrexate would be expected to occur, as demonstrated in these studies. In spite of this synergistic effect, the low efficacy of DDATHF (when used as a single agent or in combination with low doses of methotrexate) will be a prime consideration when clinical studies are conducted with this agent.

Previous studies [1, 2, 4] have shown that methotrexate and DDATHF are transported into the cell by the use of a common transport protein. Both of these antifolates can be converted intracellularly to their respective polyglutamates. It is possible that at high concentrations of DDATHF, cellular transport or glutamylation of methotrexate was impaired and synergy was therefore not observed.

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