The Antileukemic Effectiveness of 5-Fluorouracil and Methotrexate in the Combination Chemotherapy of Advanced Leukemia L1210 in Mice[1]

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Summary

Enhanced therapeutic effectiveness has been demonstrated by combining methotrexate and 5-fluorouracil in the treatment of murine leukemia L1210. Optimal combinations of the 2 drugs consistently showed greater antileukemic activity than optimal treatment with each drug by itself. The treatment schedules resulting in this enhanced activity were characterized by relatively low and therapeutically ineffective doses of 5-fluorouracil, whereas the associated level of methotrexate ranged from slightly less than optimal to optimal. The therapeutic activity of the combination of the drugs was limited by the development of host toxicity similar to that observed when the drugs were used by themselves.

Introduction

The fluorinated pyrimidine, 5-fluorouracil (FU)[2] has been shown (4, 15, 16, 22) to have antitumor activity against several experimental animal neoplasms. However, in both laboratory and clinical situations where antitumor effects have been realized by the use of this antimetabolite, it has been only at the expense of well-defined toxicity (1, 3, 6, 23).

Biochemical studies (19, 20) have shown that the activation of FU proceeds through 5-fluorouridine-5'-monophosphate (FURP) to 5-fluoro-2'-deoxyuridine-5'-monophosphate (FUdRP). It is as the deoxyribonucleotide that FU inhibits thymidylate synthetase, thus preventing the methylation of deoxyuridylic acid to thymidylic acid (2, 5). Inhibition of this reaction is considered likely to lead to the observed tumor inhibition by FU since it results in a reduced synthesis of DNA. FU may also act through 2 additional mechanisms. These are (a) inhibition of the incorporation of uridine monophosphate into RNA, and (b)

incorporation of FU or 5-fluorouridine (FUR), presumably as nucleotides, directly into RNA to give a fraudulent RNA. However, concentrations of FU which markedly inhibit DNA synthesis and cellular proliferation have no effect on RNA or protein synthesis (7).

The studies in this report were designed to investigate the concerted action of the fluorinated pyrimidine (FU) and the folic acid antagonist methotrexate (MTX, amethopterin) on the survival of mice inoculated with leukemia L1210 (21). The rationale for using this particular combination was as follows: MTX, as an inhibitor of dihydrofolate reductase, reduces the availability of tetrahydrofolate coenzymes involved in thymidylate and inosinate biosynthesis (16). This inhibition in itself reduces considerably the rate of DNA synthesis. Addition of a fluorinated pyrimidine to this system results in the direct inhibition of the enzyme, thymidylate synthetase (14). Thus, a combination of MTX and FU would be expected, as a form of sequential blockade, to achieve almost total inhibition of thymidylate synthesis. Additionally, since MTX also inhibits purine biosynthesis *de novo*, this would constitute concurrent (parallel) blockade, the net effect being a dramatic reduction in DNA synthesis which should certainly exceed that achieved with either agent used alone. It might be anticipated, therefore, that any therapeutic effect that these drugs exert individually might be enhanced when they are used together. As reported preliminarily, it does appear possible to obtain an enhanced therapeutic effect, in mice, when a combination of the agents is used.

Materials and Methods

The materials and methods are essentially the same as those described in previous reports from this laboratory (11, 12). A saline suspension of cells prepared from leukemia (L1210)-infiltrated spleens of CDBA stock mice was inoculated (0.2 ml/mouse) s.c. into the right hind leg of male or female CDBA or BDF₁ hybrid mice. All the animals used in these experiments weighed 18–26 gm at the beginning of the experimental period.

Treatment was initiated (day noted in the individual tables) when the local tumor at the site of s.c. leukemic inoculation was estimated by manual palpation to be in the range of 7–12 mm in diameter. In each experiment, on the day of treatment initiation, 4–6 mice selected at random from the inoculated population were killed and splenic cell suspensions were prepared and implanted s.c. into normal mice of the same strain. The resulting tumor-

induced death of the recipients was taken to indicate that the disease was systemic in the donor mice.

MTX was obtained from the Lederle Laboratories Division, American Cyanamid Co., and FU from Hoffmann-LaRoche, Inc.

The vehicles employed for the administration of the drugs were 2% $NaHCO_3$ solution for MTX, and physiologic saline for FU (except in Experiment 1, for which FU was prepared as a suspension in 0.5% methylcellulose).

The drugs were administered s.c. in the scapular region over a wide range of logarithmically spaced dose levels in the constant volume of 0.01 ml/gm of body weight. The various treatment schedules employed for the drugs when given singly or in combination are noted in the tables.

The antileukemic effect of FU used alone or when administered in combination with MTX was rated relative to the efficacy of daily s.c. administered MTX in increasing the survival time of the leukemic mice.

Results

The therapeutic effectiveness, against advanced leukemia L1210, of FU in combination with MTX was investigated, 3 schedules of treatment being used: (a) FU given on a daily schedule in combination with daily MTX treatment, (b) FU given as a single injection followed by MTX administered daily, and (c) FU given as a single injection followed by MTX administered every 4 days.

The results in Table 1 are from an experiment in which FU and MTX were administered, alone or in combination. The dose levels of FU were 40, 80, 160, and 320 mg/kg when given alone as a single dose, and 40, 80, and 160 mg/kg when given as a single dose in combination with MTX. The dose levels of MTX were 0.25, 0.50, 1.0, 2.0, and 4.0 mg/kg on a daily treatment schedule, 5, 10, 20, 40, and 80 mg/kg on the intermittent schedule, and 5, 10, 20, and 40 mg/kg on the intermittent schedule in combination with a single dose of FU.

The most effective combination, 80 mg FU/kg (single) plus 10 mg MTX/kg (intermittent) was more effective than an optimal single treatment with FU (320 mg/kg) or optimal intermittent therapy with MTX alone (80 mg/kg). For this experi-

ment the combination was also more effective than treatment with daily MTX alone. It is of interest that the optimal combination treatment was achieved with doses of FU and MTX substantially below the optimal individual doses of FU or MTX. The necessity for reduction in dosage of both drugs suggests that with this schedule of therapy the enhanced therapeutic effect was accompanied by substantial additive host toxicity.

In a 2nd experiment FU was given alone or in combination with MTX administered daily (Table 2). Both drugs were given at logarithmically spaced dose levels so that each dose was 0.6 of the next highest level. The dose levels for FU were 65–500 mg/kg for single dose administration alone, 23–180 mg/kg for daily administration alone, and 23–65 mg/kg for single dose administration in combination with MTX. MTX was given alone and in combination with FU on a daily schedule of 0.39–3.0 mg/kg. For each schedule of treatment, only the optimal dose of the chemical agents is reported in Table 2. In this experiment a single treatment with 23 mg FU/kg plus daily treatment with 1.08 mg MTX/kg constituted the most effective therapy, giving a 78% increase in survival time as compared with daily treatment with MTX alone. It may be noted that the enhanced therapeutic effect of the combination was obtained with an ordinarily optimal daily dose of MTX when employed alone, indicating that, for the combination, the single dose of FU (23 mg/kg) did not contribute appreciably to toxicity.

The series of experiments summarized in Table 3 shows that concomitant treatment with daily FU in combination with daily MTX was more effective in increasing the survival time of the mice than daily treatment with either drug alone. In all of the 5 experiments each drug alone was given as a wide range of daily doses. Also in each experiment the drugs were combined in a number of FU to MTX dosage ratios and a wide range of daily treatment levels was used in each combination ratio. Daily MTX alone was consistently more effective than daily FU alone, and optimal combination treatment was 11–57% more effective than the optimal daily dose of MTX alone. Maximal effectiveness was achieved when the ratio of the drugs, on a mg/kg basis, ranged from 4 to 16 parts of FU to 1 part MTX. Of interest was the observation that in each experiment, an improvement in therapeutic usefulness was attained when a relatively low daily dose of FU

TABLE 1

INFLUENCE OF TREATMENT SCHEDULE ON THE EFFECTIVENESS OF METHOTREXATE (MTX) PLUS
5-FLUOROURACIL (FU) AGAINST ADVANCED LEUKEMIA L1210 IN MICE[a]

Treatment schedule	Optimal treatment levels (mg/kg)	Range of individual survival times (days)	MST[b] (days)	% Increase over controls	Relative increase in MST over controls[c]
MTX, daily from Day 6	1.00	11–30	18	100	100
MTX, every 4 days from Day 6	80.0	10–15	13.5	50	50
FU, Day 6 only	320	13–17	14	56	56
FU, Day 6 only	80.0	13–27	23	156	156
+MTX, every 4 days from Day 6	+10.0				
Controls		9–10	9		

[a] Experiment 1: s.c. treatment was begun 6 days after s.c. inoculation of tumor; BDF_1 female mice (19–24 gm) were employed; 8 mice in treated groups and 16 mice in untreated group.
[b] MST, median survival time.
[c] MTX alone daily = 100.

was combined with a dose of MTX which was optimal for daily treatment with MTX when used alone (Experiments 5 and 6) or actually higher than the optimal daily dose of MTX alone (Experiments 3, 4, and 7). These observations suggest that on a daily schedule, FU contributed to the enhanced therapeutic effect without contributing significant toxicity for the host.

The summary data for Experiment 6, Table 3, are shown in Chart 1. Included are the dosages employed and median survival times for the leukemic mice and for treated nonleukemic control mice. As observed previously (22) the toxicity of FU severely limited its therapeutic usefulness as indicated by the toxicity of therapeutic dose levels to normal mice. With MTX, toxicity also limited therapeutic effectiveness, but this is partially attributable to reduced tolerance of leukemic mice to the drug (13). The

improved therapy with an optimal dose level of MTX plus a relatively low, nontoxic, and not detectably effective dose of FU is apparently attributable to improved tolerance of the mice accompanying the more successful therapy. With the combination, the limitation to therapy was the toxicity as observed for normal mice treated with MTX plus FU.

Discussion

The present studies show that therapeutic synergism (10) was obtained when FU was administered in combination with MTX. That is, a therapeutic response was observed with combination therapy which was greater than that achieved when either of the chemical agents was administered alone.

TABLE 2
EFFECTIVENESS OF METHOTREXATE (MTX) PLUS 5-FLUOROURACIL (FU) AGAINST ADVANCED LEUKEMIA L1210 IN MICE[a]

Treatment schedule	Optimal treatment levels (mg/kg)	Range of individual survival times (days)	MST[b] (days)	% Increase over controls	Relative increase in MST over controls[c]
MTX, daily from Day 6	1.08	8–26	17	112	100
FU, daily from Day 6	39.0	10–16	14.5	81	72
FU, Day 6 only	108	12–16	15	88	78
FU, Day 6 only +MTX, daily from Day 6	23.0 +1.08	8–28	24	200	178
Controls		8–10	8		

[a] Experiment 2: s.c. treatment was begun 6 days after s.c. inoculation of tumor: CDBA female mice (19–25 gm) were employed; 10 mice in treated groups and 20 mice in untreated group.

[b] MST, median survival time.

[c] MTX alone daily = 100.

TABLE 3
COMBINATION TREATMENT OF ADVANCED LEUKEMIA L1210 WITH METHOTREXATE (MTX) PLUS 5-FLUOROURACIL (FU) IN MICE[a]

EXPERIMENT No.	AGENT	OPTIMAL TREATMENT LEVELS (mg/kg) Separately	Combined	RATIO FU TO MTX	TEST AGENT Separately Range of individual survival times (days)	MST (days)	Combined Range of individual survival times (days)	MST (days)	CONTROLS Range of individual survival times (days)	Median survival time (days)	RELATIVE INCREASE IN MST[b] AT OPTIMAL DOSE OVER CONTROLS[c] Separately	Combined
3	MTX	0.8	1.6	4:1	9–23	17	15–26	21	8–12	10	100	157
	FU	51.2	+6.4		13–16	14					57	
4	MTX	0.5	1.0	8:1	11–28	24.5	27–35	31.5	9–11	10	100	148
	FU	64.0	+8.0		14–15	14					28	
5	MTX	1.08	1.08	10:1	17–22	20	19–30	24	8–9	8	100	133
	FU	50.0	+10.8		13–17	16					67	
6	MTX	0.8	0.8	16:1	10–29	22	18–30	26	8–10	9	100	131
	FU	51.2	+12.8		13–15	14					38	
7	MTX	0.8	1.6	4:1	16–27	23.5	24–26	25	9–14	10	100	111
	FU	51.2	+6.4		14–16	15					37	

[a] Daily s.c. treatment was begun 6 or 7 days after s.c. tumor inoculation; CDBA and BDF$_1$ male and female mice (18–27 gm) were employed; 10 mice in treated groups and 20 mice in untreated group.

[b] MST, median survival time.
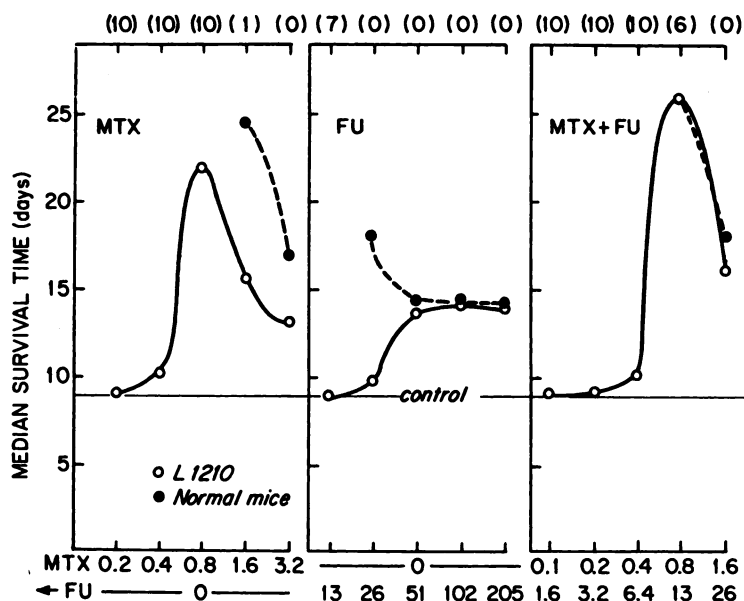
[c] MTX alone daily = 100.

CHART 1.—Dose response of normal and leukemic (L1210) mice to treatment with 5-fluorouracil (FU) and methotrexate (MTX), alone and in combination. The doses of the drugs are given in mg of drug/kg of body weight and were administered, s.c., from the 6th day after leukemic inoculation. Treatment was given daily until death or until the 70th day. Figures in parentheses represent the number of normal animals alive on the 70th day.

The enhanced antileukemic activity shown by the combination treatments was characterized by relatively low doses of FU, whereas the associated level of MTX was either a suboptimal one or an effective one for treatment with MTX alone. When FU was given as a single treatment with MTX on a 4-day schedule, there was an appreciable reduction in both the optimal dosage of FU and of the folic acid antagonist relative to the optimal dosage when the drugs were employed individually. This finding indicates that on this schedule the drugs showed increased toxicity for the host. However, the increased therapeutic effect appeared to be proportionately more extensive than the increased toxicity for the host as evidenced by the increased survival time of the leukemic animals. When FU and MTX were administered on a daily schedule, a therapeutic advantage was obtained with a substantially decreased level of the fluorinated pyrimidine, but the dose level of MTX remained unchanged or was even higher than when employed alone. Thus, with this regimen of therapy the therapeutic advantage of combination therapy was achieved without appreciable increased toxicity for the host. Undesirable toxicity of FU was avoided by the use of a nontoxic dose level, which by itself exerted no observable therapeutic effect. Yet this low dosage contributed markedly to the chemotherapy and even appeared to permit greater tolerance of the host to MTX. The relative success of the combination therapy was limited only by the ultimate toxicity of the combination to the host.

Although the current experiments were undertaken with the biochemical rationale that therapeutic synergism might be produced by combination treatment with FU and MTX as the result of a sequential blockade in thymidylate biosynthesis (9), or as a form of concurrent blockade involving purine and thymidylate biosynthesis (9) the precise nature of the enhanced therapeutic response remains to be determined. It would, how-

ever, be of interest to determine whether there is an enhanced biochemical effect on thymidylate synthesis as a result of treatment with a combination of FU and MTX.

A number of combinations of drugs have now been demonstrated to provide a therapeutic advantage both in animal and clinical leukemia (8). Detailed investigations of the mechanism of action of combinations of drugs will undoubtedly, as with individual drugs, provide additional increments of control of the leukemic process.

References

1. Berlin, N. L., Rall, D. P., Mead, J. A. R., Freireich, E. J., Van Scott, E., Hertz, R., and Lipsett, M. B. Folic Acid Antagonists. Effects on the Cell and the Patient. Ann. Internal Med., *59:* 931–56, 1963.
2. Bosch, L., Harbers, E., and Heidelberger, C. Studies on Fluorinated Pyrimidines. V. Effects on Nucleic Acid Metabolism *in vitro*. Cancer Res., *18:* 335–43, 1958.
3. Brennan, M. J., and Vaitkouicius, V. K. 5-Fluorouracil in Clinical Cancer. Experience with 155 Patients. Cancer Chemotherapy Rept., *6:* 8–11, 1960.
4. Calabresi, P., and Welch, A. D. Chemotherapy of Neoplastic Diseases. Ann. Rev. Med., *13:* 147–202, 1962.
5. Cohen, S. S., Flaks, J. G., Barner, H. D., Loeb, M. R., and Lichtenstein, J. The Mode of Action of 5-Fluorouracil and Its Derivatives. Proc. Natl. Acad. Sci. U. S., *44:* 1004–12, 1958.
6. Curreri, A. R., Ansfield, F. J., McIver, F. A., Waisman, H. A., and Heidelberger, C. Clinical Studies with 5-Fluourouracil. Cancer Res., *18:* 478–84, 1958.
7. Danneberg, P. B., Montag, B. J., and Heidelberger, C. Studies on Fluorinated Pyrimidines. IV. Effects on Nucleic Acid Metabolism *in vivo*. Ibid., *18:* 329–34, 1958.
8. Frei, E., III, and Freireich, E. J. Progress and Perspectives in the Chemotherapy of Acute Leukemia. *In:* A. Goldin and F.

Hawking (eds.). Advances in Chemotherapy, Vol. II, pp. 269–98. New York: Academic Press, Inc., 1965.

9. Goldin, A. The Employment of Methods of Inhibition Analysis in the Normal and Tumor-Bearing Mammalian Organism. Advan. Cancer Res., *4:* 113–48, 1956.

10. Goldin, A., and Mantel, N. The Employment of Combinations of Drugs in the Chemotherapy of Neoplasia: A Review. *Ibid., 17:* 635–54, 1957.

11. Goldin, A., Venditti, J. M., Humphreys, S. R., and Mantel, N. Modification of Treatment Schedules in the Management of Advanced Mouse Leukemia with Amethopterin. J. Natl. Cancer Inst., *17:* 203–12, 1956.

12. ———. Qualitative Evaluation of Chemotherapeutic Agents Against Advanced Leukemia in Mice. *Ibid., 21:* 495–511, 1958.

13. Goldin, A., Venditti, J. M., and Mantel, N. Preclinical Screening and Evaluation of Agents for the Chemotherapy of Cancer: A Review. Cancer Res., *21:* 1334–51, 1961.

14. Hartmann, K. U., and Heidelberger, C. Studies on Fluorinated Pyrimidines. XIII. Inhibition of Thymidylate Synthetase. J. Biol. Chem., *236:* 3006, 1961.

15. Heidelberger, C., and Ansfield, F. J. Experimental and Clinical Use of Fluorinated Pyrimidines in Cancer Chemotherapy. Cancer Res., *23:* 1226–43, 1963.

16. Heidelberger, C., Chaudhuri, N. K., Danneberg, P., Mooren, D., Griesbach, L., Duschinsky, R., Schnitzer, R. J., Pleven, E., and Scheiner, J. Fluorinated Pyrimidines, a New Class of Tumor-Inhibiting Compounds. Nature, *179:* 663–66, 1957.

17. Holland, J. F. Symposium on the Experimental Pharmacology and Clinical Use of Antimetabolites. VIII. Folic Acid Antagonists. Clin. Pharmacol. Therap., *2:* 374–409, 1961.

18. Kline, I., Venditti, J. M., and Goldin, A. 5-Fluorouracil (FU) and Methotrexate (MTX) in the Combination Chemotherapy of Advanced Leukemia L1210. Proc. Am. Assoc. Cancer Res., *5:* 35, 1964.

19. Mukherjee, K. L., Boohar, J., Wentland, D., Ansfield, F. J., and Heidelberger, C. Studies on Fluorinated Pyrimidines. XVI. Metabolism of 5-Fluorouracil-2-C^{14} and 5-Fluoro-2'-deoxyuridine-2-C^{14} in Cancer Patients. Cancer Res., *23:* 49–66, 1963.

20. Mukherjee, K. L., and Heidelberger, C. Studies on Fluorinated Pyrimidines. XV. Inhibition of the Incorporation of Formate-C^{14} into DNA Thymidine of Ehrlich Ascites Carcinoma Cells by 5-Fluoro-2'-deoxyuridine-5'-monophosphate and Related Compounds. *Ibid., 22:* 815–22, 1962.

21. Venditti, J. M., and Goldin, A. Drug Synergism in Antineoplastic Chemotherapy. Advan. Chemotherapy, *1:* 397–448, 1964.

22. Venditti, J. M., Kline, I., and Goldin, A. Evaluation of Antileukemic Agents Employing Advanced Leukemia L1210 in Mice. VIII. Cancer Res. (Suppl.), *24:* 827–79, 1964.

23. Welch, A. Some Metabolic Approaches to Cancer Chemotherapy. Cancer Res., *21:* 1475–90, 1961.