Cancer Chemother Pharmacol (1985) 15: 105–110

Cancer Chemotherapy and Pharmacology

© Springer-Verlag 1985

Differential calcium leucovorin protection of human lymphoid cell lines from methotrexate*

George P. Browman, Lynda Booker, and Paula Spiegl

Ontario Cancer Foundation, Hamilton Regional Center and Department of Medicine and Oncology Research Group, McMaster University, Hamilton, Ontario, Canada

Summary. Human lymphoid cell lines were studied for leucovorin requirements to protect from methotrexate (MTX)-induced growth suppression. Over a 72h continuous exposure leucovorin provided better protection to the cell lines LAZ-007 and RAJI than to the cell lines CCRF-CEM and MOLT-4. The lower leucovorin requirement for LAZ-007 protection versus CCRF-CEM was also seen over a 3h exposure period in which leucovorin protection was assessed by measuring its effect on MTX-induced suppression of ^{3}H-deoxyuridine incorporation into acid-precipitable material. Growth experiments with addition of hypoxanthine or thymidine did not abolish differential protection, suggesting that the phenomenon is not related to selective differences in the tolerance of these cells to an MTX-induced purineless or thymineless state. Preloading of cells with calcium leucovorin caused an identical shift of the CCRF-CEM and LAZ-007 MTX dose – response curves, suggesting that differential catabolism of leucovorin does not contribute to differential protection. The same degree of differential protection was observed for 5-methyltetrahydrofolate as for leucovorin, suggesting that differences in the metabolism of leucovorin do not contribute to differential protection. To elucidate the mechanism of differential protection the influence of leucovorin on [^{3}H]MTX transport and polyglutamylation were studied. Although the $Km_{(MTX)}$ influx and the $Ki_{(leucovorin)}$ for MTX uptake were lower in CCRF-CEM compared with LAZ-007 cells, the size of the difference does not seem adequate to explain differential protection. The extent of MTX polyglutamylation in CCRF-CEM and LAZ-007 cells was identical and the influence of leucovorin on MTX polyglutamylation was the same in both cell lines.

Introduction

Methotrexate (MTX) is effective against a variety of human cancers, including malignancies of lymphoid origin. The drug inhibitis the enzyme dihydrofolate reductase (DHFR), thus producing a depletion of reduced folate pools in DNA-synthesizing cells. The cytotoxic effect of MTX can be prevented or reversed in vitro and in vivo by administration of calcium leucovorin (N^5-formyl tetrahydrofolic acid), which bypasses the MTX-induced metabolic block [1]. It has been demonstrated in leukemic mice that delayed administration of leucovorin allows treatment with otherwise lethal doses of MTX by preventing toxicity without abrogating the antitumor effect [11]. The precise mechanism for this 'differential protection' is not yet known. The use of high-dose MTX (HDMTX) covered by leucovorin ('rescue') has been extensively tested in clinical trials. It has been established that leucovorin effectively minimizes the toxicity that would otherwise follow HDMTX, but there is no definite evidence from randomized trials that HDMTX with leucovorin provides a better antitumor effect than standard-dose MTX regimens [6, 24, 26]. This fact has blunted enthusiasm for this approach. However, a clinical area where HDMTX continues to receive attention is as a means of preventing central nervous system recurrence in the postinduction phase of acute lymphoblastic leukemia and in patients with B cell lymphoid malignancies (poor-prognosis lymphomas) [4, 12, 22]. In view of the continued interest in the use of HDMTX for lymphoid malignancies, we have studied the relative effects of leucovorin on MTX cytotoxicity in human lymphoid cell lines of various origins. We have found marked variability in leucovorin protection from MTX in these cells. This paper describes the results of these studies.

Materials and methods

Human lymphoid cell lines were kindly provided by Dr Herbert Lazarus (CCRF-CEM, LAZ-007) and Dr Jun Minawada (MOLT-4, RAJI) and were maintained in 5% CO_2, at 37 °C in mid-log phase growth in suspension culture in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (GIBCO), 20 m*M* Hepes buffer (Sigma Chemical Co., St. Louis, Mo), 3.6 m*M* L-glutamine (Sigma), penicillin (100 units/ml), and streptomycin 100 μg/ml (GIBCO). All cell lines were documented to be free of mycoplasma contamination [19] and had doubling times of between 24 and 30 h.

MTX and calcium leucovorin were purchased from Cyanamid Canada, Inc. (Willowdale, Ont). Nucleosides and bases and 5-methyltetrahydrofolate were purchased

* Supported by the National Cancer Institute of Canada

Offprint request to: G. P. Browman, Ontario Cancer Foundation, Hamilton Regional Center, 711 Concession Street, Hamilton, Ontario, L8V 1C3 Canada

Abbreviations: MTX, methotrexate; DHFR, dihydrofolate reductase; HDMTX, high-dose MTX therapy; FBS, fetal bovine serum; ^{3}H-MTX, tritiated MTX; ^{3}HdUrd, tritiated deoxyuridine; DNA, deoxyribonucleic acid; TCA, trichloroacetic acid; IC_{50}, drug concentration that inhibits cell growth to 50% of control

from Sigma. Flasks for tissue culture were purchased from Falcon Plastics (Oxnard, Calif); and Linbro multiwell plates were purchased from Flow Laboratories (Mississauga, Ont.) [^{3}H]MTX was purchased from Moravek Biochemicals, Inc. (Brea, Calif); it was purified by reverse-phase high-pressure liquid chromatography according to the method of Fry et al. [9] and adjusted to a final specific activity of 3.2 Ci/mmol. [6-^{3}H]Deoxyuridine (^{3}H-dUrd) (specific activity 20.8 Ci/mmol) and Biofluor were purchased from New England Nuclear (Boston, Mass). MTX polyglutamate standards were provided by Dr J. Jolivet. Supplies for gel filtration liquid chromatography, including Sephadex G-15, were purchased from Pharmacia (Uppsala, Sweden).

All growth experiments were performed with extensively dialyzed FBS. Dose – response curves to MTX were constructed by incubating 2×10^5 cells in 1-ml cultures in triplicate for 72h in 5% Co_2 at 37 °C with the addition of varying concentrations of MTX. Cells were counted electronically (Coulter Counter, Model ZF) and results expressed as percentages of 72-h control growth. To assess the influence of calcium leucovorin on the MTX dose-response, the cells were preloaded with 1 μM calcium leucovorin in a 24 h incubation; then the cells were washed in warm medium and the MTX dose–response curve was performed as described.

Calcium leucovorin requirements for protection from MTX-induced growth suppression were assessed by incubating 2×10^5 cells/ml with a maximum inhibitory MTX concentration (0.2 μM) and varying leucovorin concentrations for 72 h in 5% CO_2 at 37 °C. At 72 h cells were counted and growth in treated cultures was expressed as a percentage of that in control cultures. The influence of hypoxanthine (80 μM) or thymidine (20 μM) on leucovorin protection from MTX was assessed in the same way with these compounds added to cultures at the same time as MTX and leucovorin. Hypoxanthine and thymidine were also present in control cultures.

Calcium leucovorin requirements for protection from MTX-induced suppression of [^{3}H]dUrd incorporation into DNA were assessed by incubating cells suspended in fully supplemented medium containing dialyzed 10% FBS with 0.2 μM MTX and varying leucovorin concentrations for 3 h at 37 °C in 5% CO_2. At the end of the incubation cultures were pulsed with 0.5 μM [^{3}H]dUrd (5 μCi/ml) and the rate of incorporation of label into TCA-pecipitable material was measured over 40 min by adding cold TCA to give a final concentration of 5%, to 1-ml aliquots (containing 10^6 cells) at 7, 14, 21, 30, and 40 min. The precipitates were washed twice with 10% cold TCA, left at 4 °C overnight, then heated at 86 °C for 15 min in rubber-stoppered glass tubes. After heating, the precipitates were centrifuged at 1200 *g* for 12 min and the entire supernatant was collected and placed in 9 ml Biofluor for counting in a Beckman scintillation counter, model LS-3133T. The slopes of the lines representing rates of incorporation over 40 min for the different conditions were compared with those for control cultures and expressed as percentages of control values.

The initial rate of entry of [^{3}H]MTX into cells was measured over 3.5 min, during which time cells were incubated at 37 °C in a shaking water bath with varying [^{3}]MTX concentrations in glucose-free Hank's balanced salt solution buffered with sodium bicarbonate and Hepes. Calcium leucovorin (5 μM) was added with [^{3}H]MTX where appropriate. Aliquots of cells were harvested at 0.5, 1, 1.5, 2, 2.5, 3, and 3.5 min, placed in 9 vol. cold PBS and then centrifuged for 30 s at 300 *g* at 4 °C. Cells were washed twice more, after which the pellets were solubilized in 1 *N* NaOH at 70 °C for 30 min and then placed in 9 ml Biofluor to be counted. Counts were adjusted for efficiency using the external standards ratio. Kinetic constants were clacu-lated from a linear regression model of double-reciprocal plots.

MTX polyglutamate formation in CCRF-CEM and LAZ-007 cells was measured by incubating cells (2×10^6/ml) in petri dishes at 37 °C in 5% CO_2–95% air in Earle's balanced salt solution (pH 7.2) containing hypoxanthine (10 μM), thymidine (10 μM), glutamine (5 μM), 10% dialyzed, heat-inactivated FBS, and purified [^{3}H]MTX (1 μM). At appropriate intervals following addition of [^{3}H]MTX, aliquots containing 4×10^6 cells were aspirated and placed into 3 ml cold phosphate-buffered saline (PBS), then centrifuged at 190 *g* for 5 min at 4 °C, the cells were then washed twice more in cold PBS. After the final wash the cells were resuspended in 100 µl PBS and sonicated. The sonicate was boiled at 100 °C for 10 min and centrifuged at 1200 *g* for 3 min at 4 °C; after this the supernatant was stored at −20 °C.

Total [^{3}H]MTX polyglutamates were separated from the monoglutamate on a 0.9×35 cm Sephadex G-15 column eluted with 25 mM sodium phosphate, 100 mM NaCl buffer, pH 7.0, at room temperature at a flow rate of 20–25 ml/h [25]. Fractions were collected every 4 min and placed in 9 ml Biofluor for counting in a Beckman scintillation counter, model LS-3133T. The proportion of ^{3}H that eluted with the polyglutamate fraction was expressed as a percentage of the total ^{3}H eluted from the MTX and MTX polyglutamate peaks. To determine the influence of leucovorin on MTX polyglutamylation, leucovorin at a final concentration of 5 μM was added to the cell incubation mixture 30 min after [^{3}H]MTX. The amount of [^{3}H]MTX polyglutamates formed over an initial 30-min incubation was measured as a control.

Results

The MTX sensitivities of the cell lines are expressed as IC_{50} values (concentration at which growth is suppressed to 50% of that in controls), shown in Table 1. The range of IC_{50} values for these cell lines is relatively narrow, the maximum difference being less than four-fold. For subsequent protection experiments an MTX concentration of 0.2 μM was chosen, since this is well beyond the maximum plateau effect for all cell lines. The inhibitory effect of this MTX concentration on all cell lines for the leucovorin protection experiments is shown in Table 1.

The dose – response relationship for calcium leucovorin protection from MTX-induced growth suppression is shown for all cell lines in Fig. 1. This can be quantitatively expressed by determining the molar concentration of leucovorin needed to protect cells to an equivalent degree (70% of control). Substantially higher concentrations of leucovorin were required for T cell protection versus B cell protection (Table 1). For the T cells CCRF-CEM and MOLT-4, 13.5 and 8.4 μM leucovorin was required, respectively, for protection against 0.2 μM MTX at the 70% level, while for the B cells the leucovorin concentrations

Table 1. MTX sensitivity and leucovorin protection in human lymphoid cell lines

	CCRF-CEM *(n)*[a]	LAZ-007 *(n)*	MOLT-4 *(n)*	RAJI *(n)*
A. *Growth Experiments*				
MTX IC_{50} (μM)	0.01 (10)	0.03 (10)	0.01 (3)	0.008 (3)
MTX inhibition in leucovorin protection experiments (% control)	19 (4)	12 (4)	19 (5)	25 (4)
Leucovorin concentration for 70% protection from MTX (μM)[c]	13.5 (4)	0.29 (4)	8.4 (5)	0.52 (4)
MTX IC_{50} in leucovorin preloaded cells (μM)	0.03 (5)	0.11 (5)	ND[b]	ND
B. ^{3}*HdUrd experiments*				
MTX suppression (% control)[c]	3.9 (4)	5.0 (4)		
Protection from MTX by 10 μM leucovorin (% control)[c]	60 (4)	96 (4)		

[a] *n*, number of experiments; all values represent means of *n* experiments. Standard error $\leq 20\%$ for all experiments

[b] ND, not done

[c] For leucovorin protection experiments, values were read from the composite curves shown in the figures

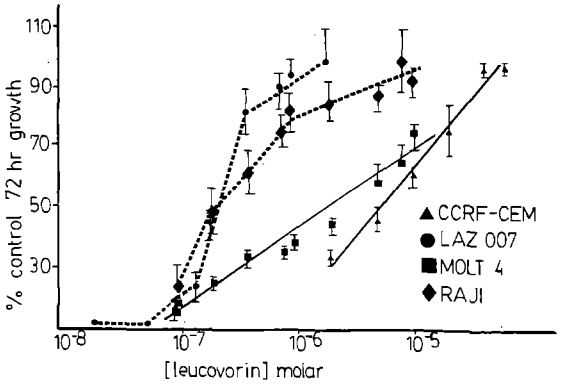

Fig. 1. Dose–response curves of leucovorin protection. Cells were incubated for 72 h in 0.2 μM MTX and varying leucovorin concentrations. Results are expressed as percentages of control growth. *Bars* = ±SE

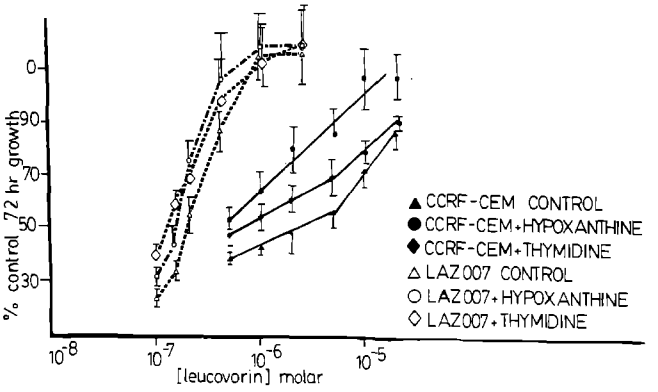

Fig. 2. Effect of hypoxanthine and thymidine on leucovorin protection in CCRF-CEM and LAZ-007. Cells were incubated for 72 h in 0.2 μM MTX, either hypoxanthine (80 μM) or thymidine (20 μM), and varying concentrations of leucovorin. Results are expressed as percentages of control cultures with hypoxanthine or thymidine. *Bars* = ±SE

required for the same degree of protection were only 0.29 μM for LAZ-007 and 0.52 μM for RAJI. This represents a 16-to-46-fold leucovorin concentration range for equivalent protection from the same concentration of MTX in these cell lines.

Since it has been reported that cells may differentially catabolize reduced folates [5, 13, 23], we investigated whether this mechanism might account for differential leucovorin protection by examining the influence of a 24h preincubation with leucovorin (leucovorin preloading) on the shift in the MTX dose – response curves for CCRF-CEM and LAZ-007. As expected (results in Table 1), there was a shift of the MTX dose – response curves to the right in both cell lines, which is reflected in the increased MTX IC_{50} values; however, the degree of shift was similar for the two cell lines. In addition, we compared the requirements for leucovorin and 5-methyltetrahydrofolate protection from MTX in the two cell lines. The molar concentration of 5-methyltetrahydrofolate required was slightly greater than the concentration of leucovorin required for equivalent protection; but the increased requirement was identical for both cell lines (data not shown). To determine whether differential leucovorin protection could be related to relative differences in the sensitivities of these cells to MTX-induced selective de novo purine or thymidylate synthesis, we performed leucovorin protection experiments in the presence of either hypoxanthine (80 μM) or thymidine (20 μM). Figure 2 shows that exposure to thymidine did not alter the relative difference in leucovorin requirements between CCRF-CEM and LAZ-007 cells. Exposure to hypoxanthine shifted the CCRF-CEM leucovorin protection curve to the left and narrowed the concentra-

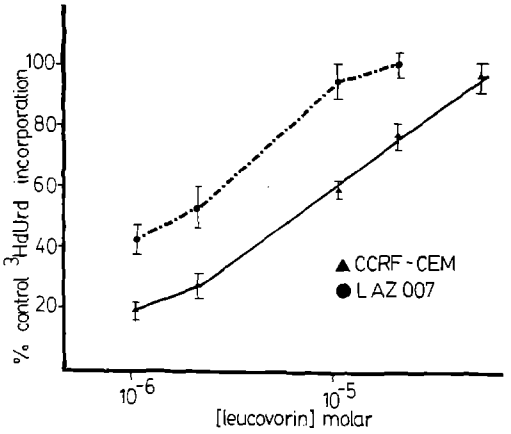

Fig. 3. Dose–response curves for leucovorin protection of MTX-induced suppresion of [^{3}H]dUrd incorporation into acid-precipitable material. Cells were incubated for 3 h in 0.2 μ*M* MTX and varying leucovorin concentrations. Each *point* represents the mean ± SE of the rate of incorporation measured over 40 min

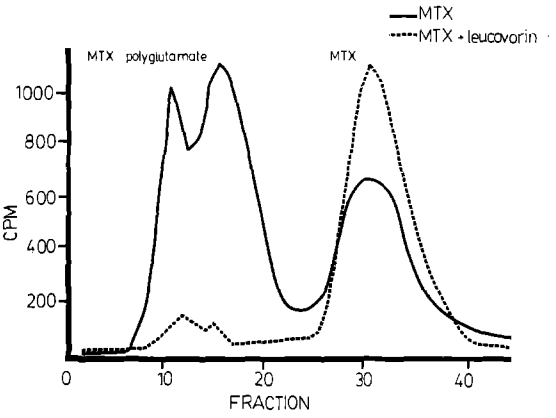

Fig. 4. MTX polyglutamylation and influence of calcium leucovorin in CCRF-CEM cells. Cells were incubated for 4.5 h with 1 μ*M* [^{3}H]MTX, 10 μ*M* thymidine, 10 μ*M* hypoxanthine with or without 5 μ*M* leucovorin added at 30 min. MTX polyglutamates were separated by Sephadex G-15 gel filtration [25]

tion ratio for 70% protection in CCRF-CEM relative to LAZ-007 from 34-fold to 7.5-fold. This suggests that relative differences in the purineless state induced by MTX are partly responsible for differential protection; nonetheless, even in the presence of hypoxanthine there was still a substantial difference in the degree of protection between the two cell lines.

We were concerned that the differential leucovorin protection phenomenon which we observed might be an artifact of the growth system we were using. Therefore, we investigated whether this occurred with short-term exposure to MTX. Leucovorin protection from MTX was compared in CCRF-CEM and LAZ-007 cells over a 3h exposure period in which protection from MTX-induced suppression of [^{3}H]dUrd incorporation into acid-precipitable material was assessed. Figure 3 shows that even over this brief exposure period differential protection is observed. Despite the similar effect of 0.2 μ*M* MTX on [^{3}H]dUrd incorporation in the two cell lines (Table 1), 10 μ*M* leucovorin produced 96% incorporation in LAZ-007 as against only 60% in CCRF-CEM cells.

To determine whether differences in the folate transport system of these cells might account for differential protection we adopted two approaches; we examined the leucovorin requirements for protection from other antifolates not transported by the reduced folate carrier. These results are presented in the companion paper [3]. In addition, we measured the initial rates of [^{3}H]MTX influx and the influence of leucovorin on [^{3}H]MTX transport in CCRF-CEM and LAZ-007 cells. As expected, we demonstrated a competitive interaction between MTX and leucovorin for cell uptake for both cell lines. The Km for [^{3}H]MTX uptake was lower for CCRF-CEM (2.1 ± 0.6 μ*M*) than for LAZ-007 (4.9 ± 1.6 μ*M*), as was the Ki of leucovorin for [^{3}H]MTX uptake (2.9 ± 1.8 μ*M* for CCRF-CEM and 3.6 ± 1.7 μ*M* for LAZ-007). The V_{max} was similar (CCRF-CEM 1.5 ± 0.4, LAZ-007 2.1 ± 0.6 pm/min/10 cells). The magnitude of these differences could explain the differential sensitivities of these cell lines to MTX, but seems unlikely to account for the degree of differential leucovorin protection observed. Efflux studies with [^{3}H]MTX in the presence and absence of leucovorin were also performed, and the results do not explain differential protection.

We hypothesized that competition between MTX and leucovorin for polyglutamylation might explain differential protection. We therefore measured the effect of leucovorin on the formation of MTX polyglutamates in CCRF-CEM and LAZ-007 cells. For these experiments, 5 μ*M* leucovorin was added 30 min after the addition of 1 μ*M* [^{3}H]MTX to cells to ensure that MTX entry was not a factor in determining the rate of polyglutamylation.

Figure 4 shows total MTX polyglutamates and MTX monoglutamate in CCRF-CEM cells after a 4.5h incubation with [^{3}H]MTX and the influence of 5 μ*M* leucovorin on MTX polyglutamate formation. The data in Table 2 compare the extent of MTX polyglutmylation over a 6.5h time period and the influence of leucovorin in LAZ-007 and CCRF-CEM cell lines. The extent of MTX polyglutamate formation is almost identical in the two cell lines and is consistent with results reported by Mini et al. for CCRF-CEM [15]. Leucovorin added at 30 min completely prevented further MTX polyglutamate formation in both cells up to 6.5 h of incubation.

Discussion

We have previously shown that exposure of CCRF-CEM and LAZ-007 cells to a single fixed concentration of leucovorin added 12 h after MTX results in differential rescue of these cells [2]. The results reported here quantifiy the effect more precisely and in more detail, with simultaneous exposure of cells to MTX and leucovorin. Our results are consistent with those of Dudman et al., who demonstrated, under different conditions, that rescue of MTX-treated (50 μ*M*) LAZ-007 cells with 5 μ*M* 5-methyltetrahydrofolate or leucovorin was superior to rescue of MTX-treated CCRF-CEM cells [7].

This paper describes experiments designed to examine an in vitro model system for differential leucovorin protection. Although it has not been possible to elucidate the precise mechanism(s) of the differential protection observed, important progress has been made. Results obtained by direct measurement of [^{3}H]MTX transport kinetic experi-

Table 2. MTX polyglutamylation and influence of leucovorin in CCRF-CEM and LAZ-007 cell lines

Incubation time (h)	Condition	Percent MTX polyglutamate	
		LAZ-007	CCRF-CEM
0.5	MTX 1 μ*M*	11 ± 1[a]	12 ± 1
2.5	MTX 1 μ*M*	48 ± 4	43 ± 6
	MTX 1 μ*M* + leucovorin 5 μ*M*	11 (14,8)[b]	16 (20, 12)
4.5	MTX 1 μ*M*	59 ± 7	55 ± 5
	MTX 1 μ*M* + leucovorin 5 μ*M*	9 (11, 7)	11 (12, 9)
6.5	MTX 1 μ*M*	73 ± 1	66 ± 4
	MTX 1 μ*M* + leucovorin 5 μ*M*	(11)	(9.3)

[a] All values are means of at least three separate experiments ± SE
[b] Values in parentheses indicate all data. Leucovorin was added 30 min after addition of ^{3}HMTX

ments do not support a role of differential competition between MTX and leucovorin for uptake in these cells as a mechanism for differential protection. Results from experiments with leucovorin preloading and with N^5-methyl-tetrahydrofolate protection suggest that leucovorin conversion to more readily usable forms and leucovorin catabolism by cells are not likely to account for differential protection. The similar sensitivities of these cell lines to MTX and the results of experiments with leucovorin preloaded cells also make it unlikely that differential protection is related to differences in the rate of utilization of intracellular reduced folates during thymidylate synthesis [16]. The inclusion of thymidine in protection experiments did not significantly reduce the degree of differential leucovorin protection between cells. This suggests that differential protection is not related to selective differences between cells in sensitivity to MTX-induced thymidylate deficiency or to differences in the ability of cells to utilize reduced folate for thymidylate synthesis in the presence of MTX. The inclusion of hypoxanthine in protection experiments, however, substantially reduced the degree of differential protection although a 7.5-fold difference in leucovorin requirements for 70% protection persisted. This suggests that differential protection may be partially explained by differential sensitivity of these cell lines to a MTX-induced purineless state, an interpretation supported by previous work which demonstrated that the concentration of purine required to reverse MTX cytotoxicity in the presence of optimal thymidine concentrations was less in LAZ-007 than in CCRF-CEM [2]. If the purineless state is the major factor in explaining differential leucovorin protection, one would expect all antifolates to produce the observed differential effect. This is not the case as shown by results in the companion paper [3].

Studies by Sirotnak et al. and by Fry et al. have demonstrated a possible role for MTX polyglutamylation as a mechanism of differential leucovorin rescue from MTX in normal and tumor tissues in vivo in mice [10, 20, 21]. Along these lines, Sato et al. recently presented evidence in a cell-free system that MTX and reduced folates may compete for polyglutamylation [18], and Rosenblatt et al. have shown that leucovorin will inhibit MTX polyglutamylation in human fibrobalsts [17]. The results reported here also show inhibition of MTX polyglutamylation in the presence of leucovorin. This effect was identical in the two cell lines, as was the extent of MTX polglutamylation in the absence of leucovorin. These results provide indirect evidence that competition between MTX and leucovorin for polyglutamylation may not explain differential protection. Furthermore, differential polyglutamylation of reduced folates in the presence of MTX would be expected to substantially influence thymidylate and de novo purine synthesis, since polyglutamylation markedly influences the efficiency of folate cofactors in the reactions catalyzed by thymidylate snythase and AICAR transformylase [14]. In this context the failure of hypoxanthine or thymidine to completely abolish differential protection therefore argues against polyglutamylation as an important mechanism for differential protection. Evidence provided in the companion paper is consistent with this conclusion [3].

In the companion paper to this we present further evidence, based on studies with 'nonclassic' antifolates, that MTX transport, folate catabolism, and competition between MTX and leucovorin for polyglutamylation are unlikely to account for differential leucovorin protection [3].

References

1. Bertino JR (1977) "Rescue" techniques in cancer chemotherapy: use of leucovorin and other rescue agents after methotrexate treatment. Semin Oncol 4: 203–216
2. Browman GP (1982) Comparison of thymidine and folinic acid protection from methotrexate toxicity in human lymphoid cell lines. Cancer Treat Rep 66: 2051–2059
3. Browman GP, Spiegl P, Booker L, Rosowsky A (1985) Comparison of leucovorin protection from a variety of antifolates in human lymphoid cell lines. Cancer Chemother Pharmacol 15: 111–114
4. Cadman E, Farber L, Berd D, Bertino J (1977) Combination chemotherapy for diffuse histiocytic lymphoma that includes antimetabolites. Cancer Treat Rep 61: 1109–1116
5. Clynes MM, O'Neill C (1980) Folic acid degradation is not a property specific to cancer cells in culture. Cancer Lett 10: 133–140
6. DeConti RC, Schoenfeld D (1981) A randomized prospective comparison of intermittent methotrexate, methotrexate with leucovorin, and a methotrexate combination in head and neck cancer. Cancer 48: 1061–1072

7. Dudman NPB, Slowiaczek P, Tattersall MHN (1982) Methotrexate rescue by 5-methyl-tetrahydrofolate or 5-formyltetrahydrofolate in lymphoblast cell lines. Cancer Res 42: 502–507
8. Fisher RI, DeVita VT, Hubbard SM, Longo DL, Wesley R, Chabner BA, Young RC (1983) Diffuse aggressive lymphomas: increased survival after alternating flexible sequences of ProMACE and MOPP chemotherapy. Ann Intern Med 98: 304–309
9. Fry DW, Yalowich JC, Goldman ID (1982) Rapid formation of polyglutamyl derivatives of methotrexate and their association with dihydrofolate reductase as assessed by high-pressure liquid chromatography in the Ehrlich ascites tumor cell in vitro. J Biol Chem 257: 1890–1896
10. Fry DW, Anderson LA, Borst M, Goldman ID (1983) Analysis of the role of membrane transport and polyglutamation of methotrexate in gut and the Ehrlich tumor in vivo as factors in drug sensitivity and selectivity. Cancer Res 443: 1087–1092
11. Goldin A, Venditti JM, Kline I, Mantel N (1966) Eradication of leukemic cells (L1210) by methotrexate and methotrexate plus citrovorum factor. Nature 212: 1548–1550
12. Green DM, Brecher ML, Blumenson LE, Grossi M, Freeman AI (1982) The use of intermediate dose methotrexate in increased risk childhood acute lymphoblastic leukemia. A comparison of three versus six courses. Cancer 50: 2722–2727
13. Halpern R, Halpern BC, Stea B, Dunlap A, Conklin K, Clark B, Ashe H, Sperling L, Halpern JA, Hardy D, Smith RA (1977) Pterin-6-aldehyde, a cancer cell catabolite: identification and application in diagnosis and treatment of human cancer. Proc Natl Acad Sci USA 74: 587–591
14. McGuire JJ, Bertino JR (1981) Enzymatic synthesis and function of folylpolyglutamates. Mol Cell Biochem 38: 19–48
15. Mini E, McGuire JJ, Moroson BA, Bertino JR (1983) Correlation of methotrexate polyglutamate formation and cell kill in the human lymphoblast leukemic cell line CCRF-CEM. Proc Am Assoc Cancer Res 24: 275
16. Moran RG, Mulkins M, Heidelberger C (1979) Role of thymidylate synthetase activity in development of methotrexate cytotoxicity. Proc Natl Acad Sci USA 76: 5924–5928
17. Rosenblatt DS, Whitehead VM, Matiaszuk NV, Pottier A, Vuchich MJ, Beaulieu D (1982) Differential effects of folinic acid and glycine, adenosine, and thymidine as rescue agents in methotrexate-treated human cells in relation to the accumulation of methotrexate polyglutamates. Mol Pharmacol 21: 718–722
18. Sato JK, Moran RG (1984) Interaction of methotrexate and citrovorum factor at folyl polyglutamate synthetase. Proc Am Assoc Cancer Res 25: 312
19. Schneider EI, Stanbridge EJ, Epstein CJ (1974) Incorporation of ^{3}H-uridine and ^{3}H-uracil into RNA. A simple technique for the detection of mycoplasma contamination of cultured cells. Exp Cell Res 84: 311–318
20. Sirotnak FM, Donsbach RC, Dorick DM, Moccio DM (1976a) Tissue pharmacokinetics, inhibition of DNA synthesis, and tumor cell kill after high-dose methotrexate in murine tumor models. Cancer Res 36: 3672–4678
21. Sirotnak FM, Donsbach RC, Moccio DM, Dorick DM (1976b) Biochemical and pharmacokinetic effects of leucovorin after high-dose methotrexate in a murine leukemia model. Cancer Res 36: 22.Skarin A, Canellos G, Rosenthal D, Case D, Maloney W, Frei E (1980) Therapy of diffuse histiocytic and undifferentiated lymphoma with high-dose methotrexate and citrovorum factor rescue, bleomycin, adriamycin, cyclophosphamide, oncovin and decadron. Proc Am Soc Clin Oncol 21: 163
23. Stea B, Backlune PS Jr, Berkey PB, Cho AK, Halpern BC, Halpern RM, Smith RA (1978) Folate and pterin metabolism by cancer cells in culture. Cancer Res 38: 2378–2384
24. Vogler WR, Jacobs J, Moffitt S, Velez-Garcia E, Goldsmith A, Johnson L, MacKay S (1979) Methotrexate therapy with or without citrovorum factor in carcinoma of the head and neck, breast, and colon. Cancer Clin Trials 2: 227–236
25. Whitehead VM, Perrault MM, Stelcner S (1975) Tissue-specific synthesis of methotrexate polyglutamates in the rat. Cancer Res 35: 2985–2990
26. Woods RL, Fox RM, Tattersall MHN (1981) Methotrexate treatment of advanced head and neck cancers: a dose-response evaluation. Br Med J 282: 600–602

Received December 10, 1984/Accepted January 20, 1985