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Comparison of leucovorin protection from variety of antifolates in human lymphoid cell lines*

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Summary. Leucovorin requirements for protection of the T cell line CCRF-CEM and the B cell line LAZ-007 against the cytotoxic effects of a variety of antifolates were studied. Differential leucovorin protection for DDMP-induced growth suppression occurred in the opposite direction to that for MTX, with CCRF-CEM requiring less leucovorin than LAZ-007 for equivalent protection. A pattern of differential protection from DDMP different from that of protection from MTX was also seen for the cell lines RAJI and MOLT-4. Differential leucovorin protection was observed for the chain-extended MTX analogue PT441. The degree of differential protection was similar to that seen for MTX, and transport studies showed that PT441 was a weak inhibitor of tritiated MTX uptake into CCRF-CEM cells. Differential leucovorin protection was observed for the lipophilic antifolate trimetrexate glucoronate (TMQ) but the degree of differential protection was smaller than that seen for PT441 or for MTX. Since TMQ is not transported into cells by the reduced folate system, while PT441 is a weak competitive inhibitor of [^{3}H]MTX transport, and since neither is polyglutamylated, these results support the conclusion reached in previous experiments that differential leucovorin protection of MTX is unlikely to be a transport-related phenomenon and is not due to an effect on polyglutamylation. In addition, the different patterns of relative leucovorin requirements for DDMP and MTX protection suggest that differential metabolism or catabolism of leucovorin does not account for differential protection.

Introduction

The folate analogue methotrexate (MTX) is widely used to treat a variety of human malignancies, including those of lymphoid origin. A major problem in its clinical use is the rapid development of tumor resistance. Mechanisms of acquired MTX resistance have been well characterized in experimental tumors and more recently in human tumors [6]. Two major strategies have been explored in attempts to overcome the known and hypothesized mechanisms of resistance: (a) the use of high-dose MTX (HDMTX) with leucovorin cover to prevent toxicity (for review see [1]); and (b) the development of nonclassic antifolate compounds with transport and other characteristics different from those of MTX. HDMTX with leucovorin, when administered to tumor-bearing animals, results in an antitumor effect without excessive toxicity [11]. In humans leucovorin provides protection from the severe toxicity of HDMTX, but does not apparently abrogate the antitumor effect, although superiority of this regimen over standard-dose MTX has not been proven [7, 17, 18]. The mechanism by which leucovorin preferentially protects normal tissues from HDMTX is not known, although in vivo animal studies suggest a role for MTX polyglutamylation which influences the retention time of MTX within the cells [10, 15, 16].

In an in vitro model system we have demonstrated that leucovorin differentially protects human lymphoid cell lines from MTX cytotoxicity [2, 5]. This system may provide a useful model to increase our understanding of differential leucovorin protection of tumor and normal tissues in vivo. In this paper we describe investigations of leucovorin protection from a chain-extended and from nonclassic antifolates compared with MTX, performed to attempt to determine the mechanism of differential leucovorin protection from MTX.

Materials and methods

Human lymphoid cell lines were obtained from Dr Herbert Lazarus (CCRF-CEM, LAZ-007) and from Dr Jun Minawada (MOLT-4, RAJI) and were maintained as previously described [5].

MTX and calcium leucovorin were purchased from Cyanamid Canada, Inc. (Willowdale, Ont.). Nucleosides and bases were purchased from Sigma Chemical Co. (St. Louis, Mo), and all tissue culture supplies were purchased as previously described [5]. [^{3}H]MTX was purchased from Moravek Biochemicals, Inc. (Brea, Calif), purified by HPLC [9], then adjusted to a final specific activity of 3.2 Ci/mmol. [6-^{3}H]Deoxyuridine (^{3}HdUrd) (specific activity 20.8 Ci/mmol) and Biofluor were purchased from New England Nuclear (Boston, Mass). DDMP was provided by Dr Charles Nichol, Burroughs-Wellcome Co (Research

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Abbreviations: MTX, methotrexate; HDMTX, high dose MTX; HPLC, high pressure liquid chromatography; [^{3}H]dUrd, tritiated deoxyuridine; DDMP, 2,4-diamino-5-(3', 4' dichlorophenyl)-6-methylpyrimidine; PT44], N-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-α-aminoadipate; TMO, 2,4-diamino-5-methyl-6-((3,4,5-trimethoxy-anilino)methyl) quinazoline

Triangle Park, NC). PT441 was provided by Dr André Rosowsky. Trimetrexate glucuronate (TMQ) was provided by Dr J. R. Bertino. DDMP was dissolved in lactate and the pH adjusted with NaOH, while the other antifolates were dissolved in 1 *N* HCl and the pH adjusted with NaOH.

Growth experiments, including dose–response curves and leucovorin protection curves, investigations of leucovorin protection from DDMP-induced suppression of [^{3}H]dUrd into DNA, and [^{3}H]MTX cellular transport studies, were performed as previously described [5].

Results

The relative cytotoxic potencies of the antifolates DDMP, PT441, and TMQ were assessed by dose–response experiments and compared with MTX. The IC_{50} values (drug concentration which inhibits growth by 50%) are shown in Table 1. The cells were most sensitive to TMQ and least sensitive to DDMP, as would be expected from previous reports [3, 8]. Sensitivity to PT441 was intermediate between that for TMQ and that for DDMP in both cell lines.

For leucovorin protection experiments equivalent maximum inhibitory concentrations of the drugs were used. The results in Table 1 verify that the drug concentrations chosen to test protection produced similar cytotoxic effects.

Leucovorin protection curves for DDMP (0.8 μM), PT441, (0.2 μM), and TMQ (0.2 μM) for the CCRF-CEM and LAZ-007 cell lines are shown in Fig. 1 and can be compared with the curves generated for MTX in the companion paper [5].

In contrast to the differential protection between the cell lines observed for MTX, the leucovorin requirement for DDMP protection is slightly greater for LAZ-007 than for CCRF-CEM. On the other hand, the difference in leucovorin requirement for protection from PT441 is similar to that seen for MTX. Differential protection is not observed to any significant degree for TMQ.

The data in Table 1 quantitatively express the leucovorin requirement for protection from these antifolates in CCRF-CEM and LAZ-007 cells as the concentration of leucovorin needed to protect to 70% of the control growth. The leucovorin concentration ratio required to achieve 70% protection in CCRF-CEM relative to LAZ-007 is 47-fold for 0.2 μM MTX (13.5 : 0.29), 0.4-fold for 0.8 μM DDMP (0.03 : 0.07), 66-fold for 0.2 μM PT441 (5.9 : 0.09), and 4-fold for 0.2 μM TMQ (2.0 : 0.5). Differential leucovorin protection is maintained at a similar level when antifolate concentrations are increased (see Table 1). This demonstrates that the phenomenon is not merely an artifact of carefully chosen drug concentrations.

To determine whether the difference in differential leucovorin protection between MTX and DDMP also applied to other cells, we measured leucovorin protection from these drugs in MOLT-4 and RAJI cell lines, which had previously been shown to display differential leucovorin protection to MTX [5]. Figure 2 shows that in these two cell lines there is only a small degree of differential protection from DDMP, which is consistent with observations for CCRF-CEM and LAZ-007 (Fig. 1 a).

We had previously shown that differential MTX protection could also be demonstrated in a short-term expo-

Table 1. Cytotoxic effects and leucovorin protection from antifolates in CCRF-CEM and LAZ-007 cell lines

			CCRF-CEM	(n)[a]	LAZ-007	(n)	Differential protection ratio [leucovorin] CEM/007
IC_{50} (μM)	MTX[c]		0.01[b]	(10)	0.03	(10)	
	DDMP		0.13	(7)	0.13	(6)	
	PT441		0.05	(6)	0.07	(4)	
	TMQ		0.007	(1)	0.006	(1)	
Growth inhibition for leucovorin protection experiments (% control)							
	MTX	0.2 μM[c]	19[b]	(4)	12	(4)	
	DDMP	0.8 μM	16	(5)	13	(4)	
	PT441	0.2 μM	16	(3)	13	(3)	
	TMQ	0.2 μM	22	(7)	14	(7)	
Leucovorin conc. for 70% protection (μM)[d]							
	MTX	0.2 μM[c]	13.5	(4)	0.29	(4)	47
		2.0 μM	75	(5)	1.7	(5)	44
	DDMP	0.8 μM	0.03	(5)	0.07	(4)	0.43
	PT441	0.2 μM	5.9	(3)	0.09	(3)	66
		2.0 μM	69	(5)	3.3	(5)	21
	TMQ	0.1 μM	0.4	(4)	0.1	(4)	4
		0.2 μM	2.0	(7)	0.5	(7)	4

[a] n = number of separate experiments performed
[b] Values are means with SE $\leq$ 20%
[c] Results for MTX are as previously reported [5], and are included here for comparative purposes
[d] Values were read off leucovorin protection curves (see Fig. 1)

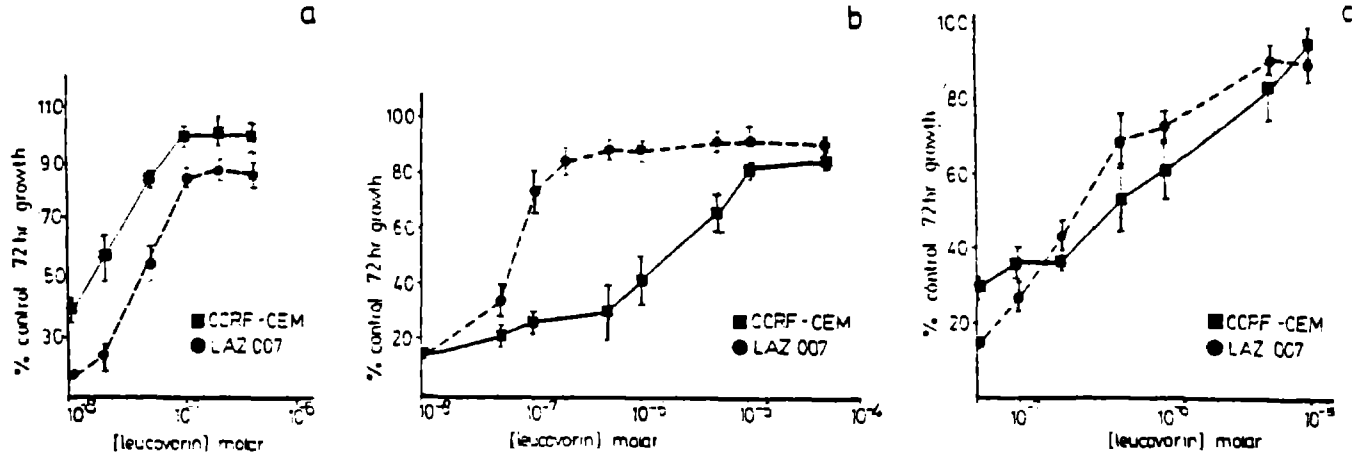

Fig. 1a–c. Dose–response curves of leucovorin protection from **a** DDMP (0.8 μ*M*), **b** PT441 (0.2 μ*M*), and **c** TMQ (0.2 μ*M*) in CCRF-CEM and LAZ-007. Cells were incubated for 72 h in antifolate and varying leucovorin concentrations. Results are expressed as percentages of 72 h control growth. *Error bars* = ±1 SE

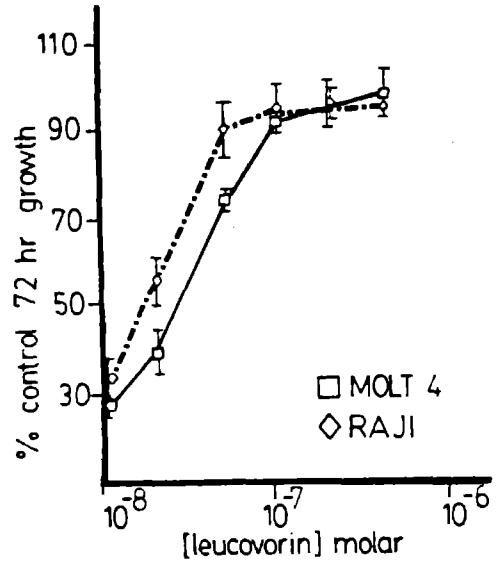

Fig. 2. Dose–response curves of leucovorin protection from DDMP (0.8 μ*M*) in RAJI and MOLT-4 cell lines. Cells were incubated for 72 h in DDMP and varying leucovorin concentrations. Results are expressed as percentages of 72 h control growth. *Error bars* = ± SE

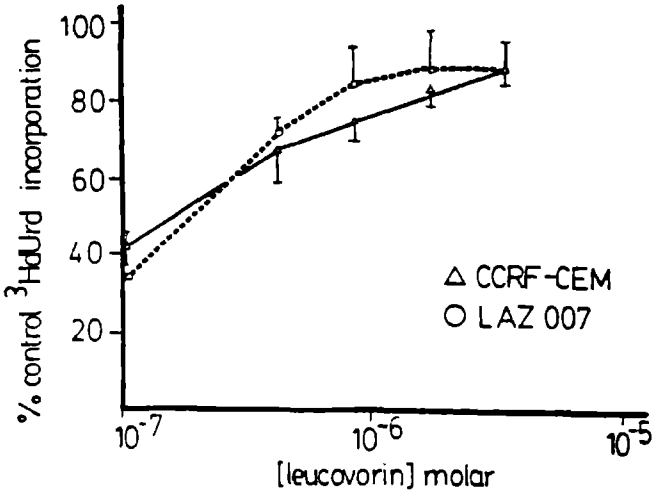

Fig. 3. Dose–response curves for leucovorin protection from DDMP (0.8 μM)-induced suppression of [^{3}H]dUrd incorporation into TCA-precipitable material. Cells were incubated for 3 h in DDMP and varying leucovorin concentrations. Each *point* represents the mean ±SE of the rate of incorporation measured over 40 min

sure over 3 h by measuring the effect of leucovorin on drug-induced suppression of ^{3}HdUrd incorporation into trichloroacetic acid-precipitable material [5]. The effect of leucovorin on DDMP-induced ^{3}HdUrd suppression was compared with that of MTX to determine whether the observations in the growth experiments were consistent over a shorter incubation period. Figure 3 shows that as in the 72-h growth experiments, the differential leucovorin protection observed for MTX is not observed for DDMP in CCRF-CEM and LAZ-007 cells.

As seen in Fig. 1 and quantitatively expressed in Table 1, the degree of differential protection observed for PT441 was similar to that observed for MTX. To determine the transport characteristics of PT441 we compared the influence of 5 μ*M* PT441 and 5 μ*M* leucovorin on the initial rate of [^{3}H]MTX uptake in CCRF-CEM cells. The results, illustrated as double-reciprocal plots in Fig. 4, demonstrate that PT441 is not a potent competitive inhibitor of MTX influx but may be transported by the reduced folate carrier system. Since the magnitude of the PT441 effect on [^{3}H]MTX influx is so small and the degree of differential leucovorin protection from this compound is at least as great as that seen for MTX, this suggests that differential leucovorin protection is unlikely to be a transport-related phenomenon.

Discussion

This report describes the characteristics of leucovorin protection against a variety of antifolates in human lymphoid cell lines. Whereas we had previously shown that human lymphoid cell lines differed markedly in the amount of leucovorin required for protection from MTX [5], no such differential protection was seen with the lipophilic antifo-

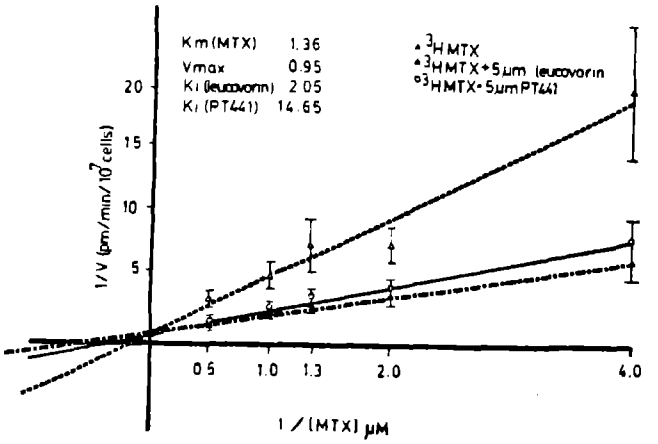

Fig. 4. Double reciprocal plots of [^{3}H]MTX influx into CCRF-CEM cells and the influence of leucovorin and PT441. Initial rate of [^{3}H]MTX uptake at varying MTX concentrations was measured over 3.5 min in the absence and presence of 5 μ*M* PT441 or 5 μM leucovorin. *Error bars* = ±SE

lates DDMP and TMQ. DDMP binds to dihydrofolate reductase, and detailed experiments indicate that its mode of action is identical with that of MTX [3]. However, the compound is not transported by the reduced folate carrier system, since MTX-transport-resistant cells are not cross-resistant to DDMP [8, 13]. Furthermore, DDMP would not be expected to be polyglutamated. Therefore, our initial hypothesis was that failure to observe differential leucovorin protection from DDMP could be due to a transport-related phenomenon or to competition between MTX and leucovorin metabolites for polyglutamylation [4]. However, the results of transport experiments and direct measurements of MTX polyglutamylation in CCRF-CEM and LAZ-007 cells presented in the companion paper make it unlikely that these mechanisms explain differential leucovorin protection [5]. This conclusion is supported by results reported in this paper for leucovorin protection from PT441. This compound is a chain-extended analogue of MTX, in which an extra $-CH_2$ group is added to the glutamate protion of the molecule to form an aminoadipate [14]. Experiments reported in this paper indicate that it is a relatively weak competitive inhibitor of MTX transport, and Moran et al. have previously shown that it is a poor substrate for folate polyglutamate synthetase derived from mouse liver [12]. Nevertheless, the degree of differential leucovorin protection observed in CCRF-CEM and LAZ-007 cells from PT441 was at least as marked as that seen for MTX.

The 2,4-diaminoquinazoline, TMQ, is another compound which is not cross-resistant to MTX in MTX-transport-resistant cells and is not polyglutamylated [8]. Differential protection is consistently observed with this compound although the small magnitude of this difference requires more experiments for statistical confirmation of this observation.

Although the precise mechanism of differential protection has not been elucidated, these studies have helped to narrow the focus of enquiry and have helped to eliminate a number of suggested mechanisms.

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