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Modulation of Methotrexate Toxicity by Thymidine: Sequence-Dependent Biochemical Effects

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SUMMARY

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Biochemical consequences of treatment with combinations of methotrexate (MTX) and thymidine (dThd) were examined in four mammalian cell lines *in vitro*. When 40 μM dThd was added to medium, 1 h after a lethal dose of MTX, fibroblasts and lymphoblasts were partially protected, but N_1S_1 tumor cells were not. Cytotoxicity of this combination correlated with cellular thymidylate synthetase activity, and in the presence of MTX, followed by dThd, N_1S_1 tumor cells accumulated dihydrofolate, whereas dihydrofolate accumulation was less extensive in the other cell lines. In fibroblasts, lymphoblasts, and N_1S_1 tumor cells, MTX caused a marked accumulation of cellular deoxyuridylate. This effect was decreased, but not eliminated, by administration of dThd 1 h after MTX. The effects of administration of dThd (40 μM) 2 h before MTX were markedly different from the effects of dThd after MTX; no accumulation of deoxyuridylate or dihydrofolate was observed, and the dThd pretreatment conferred a greater degree of protection from MTX toxicity than dThd "rescue" in fibroblasts, lymphoblasts, and N_1S_1 tumor cells. In N_1S_1 cells MTX had a pronounced antipurine effect, as measured by the decrease in dGTP pools; this effect was largely prevented by dThd pretreatment, but dThd administration after MTX restored the dTTP pool without abolishing the antipurine effect of the MTX. The abolition of the MTX antipurine effect by dThd pretreatment is attributed to decreased cellular thymidylate synthetase activity consequent upon the reduction in deoxyuridylate caused by dThd pretreatment. In cells of the well-differentiated Morris hepatoma 8999S, pretreatment with 40 μM dThd gave no protection from MTX, though partial protection was obtained at 100 μM dThd.

INTRODUCTION

It was shown many years ago that mammalian cells could be protected from the toxic effects of the antifolate drugs methotrexate (MTX)[1] or aminopterin, if thymidine and a purine (such as hypoxanthine) were supplied in the growth medium (1). The ability of cells to survive under these conditions depended upon the integrity of the salvage pathways, an observation that provided the basis for the HAT selective medium (2). In the presence of an otherwise lethal MTX concentration thymidine appears to be an absolute requirement for survival in all mammalian cells. However, the antipurine effect of MTX showed wide variation among cell lines (3). Borsa and Whitmore showed that MTX toxicity toward mouse L cells *in vitro* was enhanced by simultaneous addition of a purine (4). This suggested that in L cells MTX caused a state of unbalanced growth resembling that which causes thymineless death in bacteria (5); i.e., MTX led to depletion of both dTTP and purines, and addition of purine resulted in a more lethal selective depletion of dTTP. In contrast with those results, Hryniuk *et al.*, working with the L5178Y mouse lymphoma, found that MTX induced a lethal purineless state (6); the antipurine effect of MTX in this cell line was greatest in cells of rapid growth rate (7). Tattersall *et al.* (3) also found a correlation between growth rate and the antipurine effect of MTX, and attributed this correlation to the higher activity of thymidylate synthetase in the rapidly growing cells, which would result in faster depletion of the tetrahydrofolate cofactor pools necessary for *de novo* purine biosynthesis. A similar situation was seen in a series of four cultured rat hepatoma lines (8), though the effect of the MTX/dThd combination was complicated by the rapid catabolism of dThd in some hepatomas.

In such *in vitro* studies it was possible to maximize the antipurine effect of MTX+dThd combinations by using culture medium devoid of purines so that all cellular purines must be formed by the *de novo* biosynthetic

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[1] Abbreviations used: MTX, methotrexate (amethopterin); HAT, hypoxanthine + aminopterin + thymidine; dThd, thymidine.

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pathway. The situation *in vivo* is complicated by the presence of purine bases and nucleosides which may be utilized by the MTX-insensitive salvage pathways. There have been several *in vivo* studies of the MTX+dThd combination (9–12) and the results suggest that thymidine may increase MTX selectivity, perhaps because tumors primarily utilize purines synthesized by the *de novo* pathway, while bone marrow and intestinal epithelium rely mainly upon salvage of circulating preformed purines.

The possible therapeutic value of the MTX+dThd combination makes it desirable to have a deeper understanding of the reasons for the selectivity of the combination. In the present study it has been shown that the protective effect given by dThd against antifolate toxicity is sequence dependent. In the subsequent discussion, the term "protection" is used to describe pretreatment of cells with dThd before addition of MTX, whereas "rescue" implies addition of dThd to cultures after an otherwise lethal MTX administration. Thymidine not only overcame the antithymidylate effect of MTX, but also antagonized the antipurine effect. The biochemical basis for these observations has been established.

MATERIALS AND METHODS

Nucleosides and nucleotides, DNA (calf thymus) and oligonucleotides, dihydrofolate, tetrahydrofolate, and MTX were purchased from Sigma Chemical Company, St. Louis, Missouri. DNA polymerase from *Micrococcus luteus* was a product of Miles Laboratories, Elkhart, Indiana. Radioactive thymidine and deoxyuridylic acid were from Amersham Corporation (Arlington Heights, Ill.). Reagent chemicals were obtained from Fisher Scientific Company, Fair Lawn, New Jersey, or from J. T. Baker Chemical Company Phillipsburg, New Jersey, and analytical grades were used where obtainable. Tissue culture supplies were purchased from Grand Island Biological Company, Grand Island, New York, or from Flow Laboratories, Rockville, Maryland.

Cell culture. All cell lines were maintained in McCoy's medium 5A, supplemented with 10% fetal calf serum, penicillin (100 u/ml), and streptomycin (100 μg/ml). For growth inhibition experiments 10-ml cultures were initiated in plastic flasks (Falcon Plastics, Oxnard, Calif.) at a cell density of 50,000/ml. Novikoff hepatoma cells of the subline N_1S_1-67 (13) were grown in stationary suspension culture; their mean log-phase doubling time under these conditions was 11 h. Morris hepatoma cells of the line 8999S (14) were grown in monolayer culture, with a mean log-phase doubling time of 49 h. BF5 is a rat fibroblast line initiated in this laboratory from skin of the BUF rat. It grew in monolayers with a mean doubling time of 28 h. W1-L2 lymphoblasts (15) were grown in stationary suspension culture and had a doubling time of 19 h. The cultures reached limiting densities of 1.8×10^7 (N_1S_1), 1.4×10^7 (W1-L2), 3.5×10^6 (BF5), and 2.0 and 10^6 (8999S) cells per flask. Cultures were tested and found to be free of mycoplasma contamination using a test kit obtained from Flow Laboratories.

Deoxyribonucleotide assays. Deoxyribonucleoside triphosphates were measured in methanol extracts prepared by the method of Tyrsted (16) as modified by Harrap and Paine (17). dGTP and dCTP were measured by the method of Solter and Handschumacher (18), using calf thymus DNA as template/primer. dTTP and dATP were assayed according to Lindberg and Skoog (19), using poly(dA–dT) as template/primer. dUMP was measured enzymatically with *Lactobacillus casei* thymidylate synthetase, as previously described (20, 21).

Dihydrofolate assays. Dihydrofolate was assayed enzymatically (22) using dihydrofolate reductase from L1210/R71 mouse leukemia cells (23) purified by affinity chromatography (24).

Enzyme assays. Cells (5×10^7) were harvested in mid-log phase, washed by centrifugation at 400*g* for 7 min in phosphate-buffered saline, resuspended in 2 ml 0.05 M Tris–chloride buffer, pH 7.2, and lysed by three freeze–thaw cycles in which the cell suspension was placed alternately in liquid nitrogen and a 37°C water bath. After centrifugation of the lysate at 105,000*g* for 30 min, the supernatant fraction was used for enzyme assays. Dihydrofolate reductase (EC 1.5.1.3) was measured by the spectrophotometric method of Mathews and Huennekens (25) modified as previously described (22). Thymidylate synthetase (EC 2.1.1.45) was measured in the same extract by the tritium release procedure of Lomax and Greenberg (26).

RESULTS

The effect of dThd on the growth rates of the four cell lines used in this study is shown in Fig. 1. Growth of Morris hepatoma 8999S was not inhibited by dThd at concentrations up to 100 μM. The other cell lines were all slightly inhibited by 40 μM dThd and extensively inhibited at higher concentrations. Preliminary experiments that examined the possibility of reversing MTX cytotoxicity with dThd are illustrated in Fig. 2. When 40 μM dThd was administered to N_1S_1 cells 1 h after 1 μM MTX, there was no significant reversal of cytotoxicity. However, when the dThd was added to the growth medium 2 h before MTX the cells continued dividing, though at a reduced rate, for 48 h. Results of similar experiments with three cell lines are summarized in Table 1. In the nonmalignant BF5 and W1L2 cell lines, addition of 40 μM dThd 1 h after 1 μM MTX gave a partial rescue. In the malignant N_1S_1 hepatoma cells it did not. In all three cell lines a 2-h pretreatment with dThd before the addition of MTX gave better protection. Thus the com-

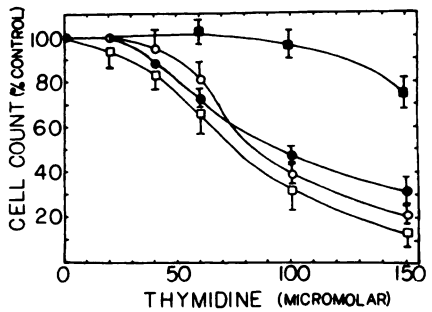

FIG. 1. *Growth of cultured cell lines in the presence of thymidine* Cultures were established with 5×10^5 cells in 10 ml and counted after 48 h. Open circles, N_1S_1 hepatoma; closed circles, W1-L2 lymphoblasts; open squares, BF5 fibroblasts; closed squares, 8999S hepatoma.

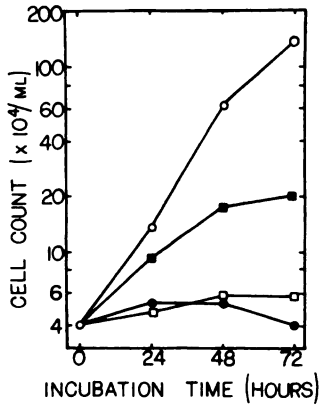

FIG. 2. *Growth of N_1S_1 hepatoma cells in the presence of methotrexate and thymidine*

Open circles, control; closed circles, MTX, 1 μM; open squares, MTX, 1 μM, followed by dThd, 40 μM, after 1 h; closed squares, dThd, 40 μM, followed by MTX, 1 μM, after 2 h.

bination of dThd rescue following MTX had partial selectivity against the malignant N_1S_1 cells, but this selectivity was lost with dThd pretreatment. Table 2 shows results obtained with Morris 8999S cells, which (in contrast to the thoroughly dedifferentiated N_1S_1 cells) are moderately well-differentiated hepatoma cells. In this line 40 μM dThd gave no protection from MTX, regardless of whether it was added before or after the MTX. This lack of effect of dThd was probably a consequence of rapid dThd breakdown in these cells (8). Like other well-differentiated hepatomas this tumor has partially retained the hepatic function of thymidine catabolism, even under conditions of thymidine starvation. Even pretreatment with 100 μM dThd gave only a comparatively minor degree of protection from MTX toxicity.

MTX selectivity depends partly upon the fact that this drug exerts both an antithymidylate effect and an antipurine effect, and the relative magnitudes of these two modes of toxicity differ between cell types (3, 4, 6). One way of estimating the degree of antithymidylate and antipurine effects is to measure the deoxyribonucleoside triphosphates (dNTPs) in treated cells. Since the differences between results of dThd administration before and after MTX were most marked in N_1S_1 cells, we used this

TABLE 1

Effects of thymidine rescue and thymidine pretreatment on methotrexate cytotoxicity

Values are means ± SEM for quadruplicate cultures. Culture conditions were as described under Materials and Methods, and cells were counted on a Coulter counter, Model ZBI.

Treatment	Cell doublings in 72 h		
	BF5 fibroblasts	W1-L2 lymphoblasts	N_1S_1 hepatoma
None	2.7 ± 0.5	3.3 ± 0.7	5.1 ± 0.7
MTX (1 μM)	0	0	0
MTX (1 μM) + dThd (40 μM) 1 h after MTX	1.6 ± 0.4	1.1 ± 0.3	0
MTX (1 μM) + dThd (40 μM) 2 h before MTX	2.4 ± 0.2	1.9 ± 0.4	2.3 ± 0.1

TABLE 2

Effects of thymidine rescue and thymidine protection from methotrexate in well-differentiated hepatoma 8999S

Triplicate cultures were grown, except for controls which were quadruplicates. All experimental groups gave results significantly different from control ($P < 0.05$).

Treatment	Cell doublings in 96 h	
None	2.3 ± 0.6	
MTX (1 μM)	0	(All dead)
MTX (1 μM) + dThd (40 μM) 1 h after MTX	0	(All dead)
MTX (1 μM) + dThd (40 μM) 2 h before MTX	0	(All dead)
MTX (1 μM) + dThd (100 μM) 1 h after MTX	0.4 ± 0.3	
MTX (1 μM) + dThd (100 μM) 2 h before MTX	0.7 ± 0.2	

cell line to examine the effects of these two regimens on dNTP pools (Table 3). BF5 fibroblasts were also examined for purposes of comparison, since in this line dThd gives good protection from MTX when administered either before or after. In N_1S_1 cells, MTX had a potent antipurine effect, since dGTP was reduced to 38% of control, while dTTP was decreased to 46% of control. Thymidine after MTX restored the dTTP pool, but not the dGTP pool. However, pretreatment of the cells with dThd before addition of MTX not only blocked the depletion of dTTP but also largely prevented the decrease in dGTP. In the fibroblasts the antipurine affect of MTX was less marked, which may explain why a partial thymidine rescue was possible in these cells. In these cells dThd pretreatment completely prevented the antipurine effect of MTX (Table 3).

Table 4 shows results of measurement of dUMP pools in three of the cell lines. These results suggest that the different effects of dThd rescue and dThd pretreatment are mediated through the dUMP pool. MTX alone caused a greatly increased dUMP pool, and dThd alone caused decreased dUMP. When dThd was administered 1 h after MTX the dUMP after a further 3 h was still high. When dThd administration preceded MTX by 2 h, dUMP after a further 4 h was still low. In cells of hepatoma 8999S (in which, unlike results with the other cell lines, dThd pretreatment gave no protection from MTX) dThd pretreatment, although reducing the rate of dUMP accumulation consequent upon MTX treatment, still did not prevent the cellular dUMP pool from increasing above the control level by 4 h after MTX treatment. The lesser effect of dThd on dUMP pools in 8999S cells was consistent with the previously observed rapid catabolism of dThd in this line (8). In the other cell lines it appeared that dThd pretreatment before MTX might antagonize the MTX by depleting the dUMP pool and thus decreasing the rate of the thymidylate synthetase reaction. In the absence of thymidylate synthetase the tetrahydrofolate pool cannot be depleted, so MTX is without effect. Additional evidence for this explanation of the sequence dependence for the MTX + dThd combination is shown in Table 5, which gives results of measurements of dihydrofolate in treated cells; the enzymatic assay method used gave a measure of total dihydrofolates, including polyglutamate forms. In un-

TABLE 3

The effect of methotrexate with thymidine rescue or thymidine pretreatment on deoxynucleoside triphosphates

Data are means + SEM of determinations on triplicate cultures. dNTPs were extracted and assayed as described under Materials and Methods. Extracts were prepared 4 h after addition of MTX or, for cultures treated with dThd alone, 4 h after dThd addition.

Cell line	Treatment	dNTP (nmol/10^9 cells)			
		dTTP	dATP	dCTP	dGTP
N_1S_1 Hepatoma	Control	52 ± 3	19 ± 2	14 ± 2	9.2 ± 1.2
	MTX (1 μM)	24 ± 2	17 ± 4	27 ± 5	3.5 ± 0.8
	dThd (40 μM)	119 ± 23	14 ± 2	10 ± 1	8.3 ± 2.1
	MTX (1 μM) + dThd (40 μM) 1 h after MTX	161 ± 16	4 ± 1	9 ± 3	4.1 ± 0.4
	MTX (1 μM) + dThd (40 μM) 2 h before MTX	138 ± 14	17 ± 3	9 ± 2	7.4 ± 0.5
BF5 Fibroblasts	Control	28 ± 6	12 ± 2	22 ± 3	8.5 ± 1.6
	MTX (1 μM)	9 ± 1	19 ± 1	14 ± 1	6.1 ± 2.1
	dThd (40 μM)	92 ± 15	11 ± 1	18 ± 2	9.2 ± 0.9
	MTX (1 μM) + dThd (40 μM) 1 h after MTX	65 ± 13	9 ± 1	13 ± 5	7.0 ± 1.6
	MTX (1 μM) + dThd (40 μM) 2 h before MTX	105 ± 12	17 ± 3	12 ± 2	8.8 ± 1.3

treated cells dihydrofolate was below the limit of detection, but following treatment large amounts of dihydrofolate could be detected in N_1S_1 cells. If the MTX was added after a 2-h pretreatment with dThd, no dihydrofolate could be detected, indicating that in the presence of dThd the rate of the thymidylate synthetase reaction was decreased, perhaps because of the limitation of its substrate, dUMP, documented in Table 4. However, when MTX preceded dThd by 1 h, dihydrofolate accumulated, and it may be inferred that tetrahydrofolates were depleted, with the resulting inhibition of *de novo* purine biosynthesis.

Activities of dihydrofolate reductase and thymidylate synthetase in the four cell lines are tabulated in Table 6. Dihydrofolate reductase did not correlate with growth rate, and did not vary by more than a factor of 2 between the various cell lines. Thymidylate synthetase activity was highest in the N_1S_1 cells. In the W1-L2 cells it was one-third of the N_1S_2 value, and in the still more slowly growing BF5 cells thymidylate synthetase activity was less than 20% of that in N_1S_1 cells. If the thymidylate synthetase activities of BF5, W1-L2, and N_1S_1 cells (Table 6) are compared with the degree of rescue from MTX given by dThd 1 h later (Table 1) it is seen that the degree of rescue is greatest in BF5 cells (which have the lowest thymidylate synthetase activity) and least in N_1S_1 cells (which have high thymidylate synthetase activity). Accumulation of dihydrofolate in treated cells also correlated with thymidylate synthetase activity. Dihydrofolate was not detectable in untreated cells of any of the lines. After 12 h in the presence of 0.05 μM MTX, the measured dihydrofolate contents of cultures were: N_1S_1, 5.5 nmol/10^9 cells; W1-L2, 2.3 nmol/10^9 cells; BF5, 1.7 nmol/10^9 cells; 8999S, 1.5 nmol/10^9 cells. Since the thymidylate synthetase reaction is the only known source of dihydrofolate in these treated cells (if folic acid reduction is blocked by MTX) and further reduction of dihydrofolate is blocked by MTX, the rate of dihydrofolate accumulation may be a measure of the cellular rate of thymidylate synthetase.

DISCUSSION

The use of 5-formyltetrahydrofolate (leucovorin) following high-dose MTX treatment is an established

TABLE 4

Effects of methotrexate with thymidine rescue or thymidine pretreatment on deoxyuridylate pools

The values are means + SEM for triplicate cultures. Extraction and assay procedures are described under Materials and Methods. Extracts were prepared 4 h after addition of MTX or, for cultures treated with dThd alone, 4 h after addition of dThd.

Treatment	dUMP (nmol/10^9 cells)		
	BF5	N_1S_1	8999S
None	15 ± 2	17 ± 2	9 ± 1
MTX (1 μM)	204 ± 34	168 ± 33	87 ± 15
dThd (40 μM)	1 ± 1	1 ± 0.3	8 ± 2
MTX (1 μM) + dThd (40 μM) 1 h after MTX	58 ± 9	45 ± 6	36 ± 3
MTX (1 μM) + dThd (40 μM) 2 h before MTX	3 ± 0.5	2 ± 0.6	20 ± 5

TABLE 5

Dihydrofolate in N_1S_1 hepatoma cells treated with MTX and thymidine

Values are means for triplicate cultures. Dihydrofolate was extracted and measured enzymatically as described under Materials and Methods.

Treatment[a]	Dihydrofolate (nmol/10^9 cells)
None	<0.2[b]
MTX (0.05 μM) 12 h	5.5 ± 1.1
dThd (40 μM) 2 h, then MTX (0.05 μM) 12 h	<0.2[b]
MTX (0.05 μM) 1 h, then dThd (40 μM) 11 h	3.5 ± 0.7
dThd (40 μM) 12 h	<0.2[b]

[a] In these experiments MTX was used at a nonlethal concentration to minimize cell lysis. Assays were performed 12 h after addition of MTX to the medium.

[b] Below the limit of detection for the assay.

method of optimizing MTX selectivity (27). Hill *et al.* (28) have pointed out that leucovorin protection (simultaneous administration of MTX and leucovorin) had biochemical consequences different from those of leucovorin rescue, where leucovorin was administered after MTX. The continuing interest in the use of thymidine to increase MTX selectivity (29) prompted the present study of the sequence dependence for these two agents. The results clearly show that dThd pretreatment strongly antagonized the antitumor effect of MTX in a rapidly growing rat hepatoma line, but that administration of dThd following MTX did not antagonize the MTX (Table 1, Fig. 2). However, in two nonmalignant cell lines, though dThd pretreatment gave maximum protection of cells from MTX, dThd after MTX gave partial protection, for up to 72 h. After this period, although cell growth had been partially inhibited, most cells were alive, as shown by the fact that transfer of cells to drug-free medium caused a return of the growth rate of the BF5 and W1-L2 cells to near normal. This was not the case with BF5 or W1-L2 cells treated with MTX alone, which at 1 μM was >99.9% lethal after 72 h. Thus administration of dThd 1 h after MTX gave a rescue in these cells. That did not happen in cultures of N_1S_1 hepatoma cells; both 1 μM MTX alone and MTX followed by dThd resulted in complete cell death after 72 h. However, dThd pretreatment before MTX gave protection of N_1S_1 cells. These results suggest that although dThd pretreatment gave maximum protection of normal cells, dThd rescue (dThd after MTX) maximized therapeutic selectivity, since it gave good protection of normal cells but no protection of the malignant hepatoma cells.

The data shown in Table 4 indicate that these effects may be explained by the modulation of the cellular dUMP pool in the presence of dThd. As previously reported (21) dThd caused marked decreases in cellular dUMP pools, which would limit the rate of the thymidylate synthetase reaction. It is the depletion of tetrahydrofolate cofactors by the thymidylate synthetase reaction that is responsible for the antipurine effect of MTX. The effect was sequence dependent because when MTX was administered first it caused a large accumulation of dUMP (20, 21) (Table 4) which even after subsequent addition of dThd was sufficient to sustain the thymidylate synthetase reaction long enough to result in a considerable accumulation of dihydrofolate (Table 5).

The combination of MTX with dThd rescue has been shown to give significant prolongation of survival in rats bearing the hepatoma 8999 under conditions where MTX alone was ineffective (30). Future studies will examine the time-sequence dependence of the combination in this *in vivo* system.

TABLE 6

Activities of dihydrofolate reductase and thymidylate synthetase in cultured cell lines

Activities are means ± SEM for triplicate cultures. Assay procedures are listed under Materials and Methods.

Cell line	Activity (nmol/min/mg protein)	
	Dihydrofolate reductase	Thymidylate synthetase
N_1S_1 Hepatoma	1.9 ± 0.4	0.44 ± 0.10
8999S Hepatoma	3.2 ± 0.5	0.09 ± 0.02
W1-L2 Lymphoblasts	1.6 ± 0.2	0.15 ± 0.02
BF5 Fibroblasts	2.5 ± 0.2	0.08 ± 0.03

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REFERENCES

1. Hakala, M. T., S. F. Zakrezewski and C. A. Nichol. Relation of folic acid reductase to amethopterin resistance in cultured mammalian cells. *J. Biol. Chem.* **236:** 952–958 (1961).
2. Szybalska, E. H. and W. Szybalski. Genetics of human cell lines. IV. DNA-mediated heritable transformation of a biochemical trait. *Proc. Natl. Acad. Sci. USA* **48:** 2026–2034 (1962).
3. Tattersall, M. H. N., R. C. Jackson, S. T. M. Jackson and K. R. Harrap. Factors determining cell sensitivity to methotrexate: studies of folate and deoxyribonucleoside triphosphate pools in five mammalian cell lines. *Eur. J. Cancer* **10:** 819–826 (1974).
4. Borsa, J. and G. F. Whitmore. Studies relating to the mode of action of methotrexate. II. Studies on sites of action on L-cells *in vitro*. *Mol. Pharmacol.* **5:** 303–317 (1969).
5. Cohen, S. S. On the nature of thymineless death. *Ann. N. Y. Acad. Sci.* **186:** 292–301 (1971).
6. Hryniuk, W. M., G. A. Fischer and J. R. Bertino. S-Phase cells of rapidly growing and resting populations. Differences in response to methotrexate. *Mol. Pharmacol.* **5:** 557–564 (1969).
7. Hryniuk, W. M. Purineless death as a link between growth rate and cytotoxicity by methotrexate. *Cancer Res.* **32:** 1506–1511 (1972).
8. Jackson, R. C. and G. Weber. Enzyme pattern directed chemotherapy. The effects of combinations of methotrexate, 5-fluorodeoxyuridine and thymidine on rat hepatoma cells *in vitro*. *Biochem. Pharmacol.* **25:** 2613–2618 (1976).
9. Tattersall, M. H. N., B. Brown and E. Frei. The reversibility of methotrexate toxicity by thymidine with maintenance of antitumour effects. *Nature (Lond.)* **253:** 198–200 (1975).
10. Grindey, G. B., J. H. Semon and Z. P. Pavelic. Modulation versus rescue of antimetabolite toxicity by salvage metabolites administered by continuous infusion. *Antibiot. Chemother.* **23:** 295–304 (1978).
11. Straw, J. A., D. C. Talbot, G. A. Taylor and K. R. Harrap. Some observations on the reversibility of methotrexate toxicity in normal proliferating tissues. *J. Natl. Cancer Inst.* **58:** 91–97 (1977).
12. Harrap, K. R., G. A. Taylor and G. P. Browman. Enhancement of the therapeutic effectiveness of methotrexate and protection of normal proliferating tissues with purines and pyrimidines. *Chem.-Biol. Interact.* **18:** 119–128 (1977).
13. Plagemann, P. G. W. and H. E. Swim. Replication of mengovirus. I. Effect on synthesis of macromolecules by host cells. *J. Bacteriol.* **91:** 2317–2326 (1966).
14. Jackson, R. C., J. C. Williams and G. Weber. Enzyme pattern directed chemotherapy. Synergistic interaction of 3-deazauridine with D-galactosamine. *Cancer Treat.* **60:** 835–843 (1976).
15. Levy, J. A., M. Virolainen and V. Defendi. Human lymphoblastoid cells from lymph node and spleen. *Cancer* **22:** 517–524 (1968).
16. Tyrsted, G. The pool size of deoxyguanosine 5′-triphosphate and deoxycytidine 5′-triphosphate in phytohaemagglutinin-stimulated and non-stimulated human lymphocytes. *Exp. Cell Res.* **91:** 429–440 (1975).
17. Harrap, K. R. and R. M. Paine. Adenosine metabolism in cultured lymphoid cells. *Adv. Enzyme Regul.* **15:** 169–193 (1977).
18. Solter, A. W., and R. E. Handschumacher. A rapid quantitative determination of deoxyribonucleoside triphosphates based on the enzymatic synthesis of DNA. *Biochim. Biophys. Acta* **174:** 585–590 (1969).
19. Lindberg, V. and L. Skoog. A method for the determination of dATP and dTTP in picomole amounts. *Anal. Biochem.* **34:** 202–208 (1970).
20. Tattersall, M. H. N., R. C. Jackson, T. A. Connors and K. R. Harrap. Combination chemotherapy: the interaction of methotrexate and 5-fluorouracil. *Eur. J. Cancer* **9:** 733–739 (1973).
21. Jackson, R. C. The regulation of thymidylate biosynthesis in Novikoff hepatoma cells and the effects of amethopterin, 5-fluorodeoxyuridine, and 3-deazauridine. *J. Biol. Chem.* **253:** 7440–7446 (1978).
22. Jackson, R. C., L. I. Hart and K. R. Harrap. Intrinsic resistance to methotrexate of cultured mammalian cells in relation to the inhibition kinetics of their dihydrofolate reductases. *Cancer Res.* **36:** 1991–1997 (1976).
23. Jackson, R. C., D. Niethammer and F. M. Huennekens. Enzymic and transport mechanisms of amethopterin resistance in L1210 mouse leukemia cells. *Cancer Biochem. Biophys.* **1:** 151–155 (1975).
24. Whiteley, J. M., R. C. Jackson, G. P. Mell, J. H. Drais and F. M. Huennekens. Folate antagonists covalently linked to carbohydrates: synthesis, properties, and use in the purification of dihydrofolate reductase. *Arch. Biochem. Biophys.* **150:** 15–22 (1972).

25. Mathews, C. K. and F. M. Huennekens. Further studies on dihydrofolic reductase. *J. Biol. Chem.* **238:** 3436–3442 (1963).
26. Lomax, M. I. S. and G. R. Greenberg. A new assay of thymidylate synthetase based on the release of tritium from deoxyuridylate-5-^{3}H. *J. Biol. Chem.* **242:** 109–113 (1967).
27. Chabner, B. A., and D. G. Johns. Folate antagonists, in *Cancer: A Comprehensive Treatise* (F. F. Becker, ed.). Plenum, New York, 363–377 (1977).
28. Hill, B. T., L. A. Price and J. H. Goldie. Selective folinic acid (CF) protection as opposed to CF rescue in L5178Y cells. *Proc. Am. Assoc. Cancer Res.* **18:** 24 (1977).
29. Ensminger, W. D. and E. Frei. The prevention of methotrexate toxicity by thymidine infusion in humans. *Cancer Res.* **37:** 1857–1863 (1978).
30. Jackson, R. C. and D. Niethammer. Folate enzymes in rat hepatomas: implications for antifolate therapy, in *Chemistry and Biology of Pteridines* (R. L. Kisliuk and G. M. Brown, eds.). Elsevier, Amsterdam, 665–670 (1979).

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