Cancer Chemother Pharmacol (1985) 15: 214–219

Cancer Chemotherapy and Pharmacology

Lack of enhanced cytotoxicity of cultured L1210 cells using folinic acid in combination with sequential methotrexate and fluorouracil*

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Summary. Previous studies from this laboratory have demonstrated that treatment of cultured cells with sequential methotrexate (MTX) and fluorouracil (FUra) leads to synergistic cell killing in several murine and human neoplasms in vitro. In this study leucovorin (folinic acid, LCV) was added to the MTX/FUra combination with the intention of generating elevated levels of methylenetetrahydrofolate to promote the formation of a stable fluorodeoxyuridylate-thymidylate synthetase ternary complex, thereby augmenting the cytotoxicity of the MTX-FUra sequence. The addition of 10 or 100 μ*M* LCV concurrently with or after 10 μ*M* FUra following MTX (1 μ*M*) pretreatment did not augment the inhibition of L1210 cell growth or the clonigenicity compared with MTX prior to FUra without LCV. The effects of LCV schedulling on the sequential MTX and FUra-induced inhibition of thymidylate synthesis were measured by examining the rate of [6-^{3}H] dUrd incorporation into the acid-precipitable cell fraction and by direct quantitation of the thymidylate synthetase ternary complex. Combination of 100 μ*M* LCV with 10 μ*M* FUra after 1 μ*M* MTX resulted in significantly more ternary complex formation than did 1 μ*M* MTX before 10 μ*M* FUra alone. The inhibitory effects of FUra on thymidylate synthetase in the presence of MTX, however, could not be augmented by LCV as determined by [6−^{3}H] incorporation into acid-precipitable material, nor did the addition of LCV result in increased cytotoxicity. Factors other than the inhibition of DNA synthesis may be critical to the cytotoxicity of sequential MTX and FUra in L1210 cells.

Introduction

Methorexate (MTX) and fluorouracil (FUra) are often used for the treatment of human colorectal and breast cancer. FUra is a base analogue which must be converted to nucleotides for cytotoxicity. FUra may interfere with DNA synthesis by inhibiting thymidylate synthetase (TS) as fluorodeoxyuridylate (FdUMP), or fluorouridine triphosphate (FUTP) may become incorporated into RNA, thereby disrupting RNA processing [16]. Covalent binding of FdUMP to TS is necessary for inhibition of dTMP formation and requires the reduced folate $N^{5,10}$-methylenetetrahydrofolate (CH_2FAH_4) [11, 29]. MTX, by inhibiting dihydrofolate reductase, prevents the regeneration of CH_2FAH_4). Administration of MTX before FUra could therefore prevent formation of this stable ternary, FdUMP-CH_2FAH_4-TS, complex because of the diminished levels of CH_2FAH_4 subsequent to MTX [32].

Previous reports from this laboratory indicate that sequential MTX and FUra exposure leads to synergistic cell killing in several murine and human neoplasms in vitro [1–3, 8–10]. Other workers have observed therapeutic synergism with in vivo systems as well [18, 22, 26]. The mechanism of this synergistic cell killing is not understood; however, we have shown that cells exposed to MTX accumulate more FUra. This was a consequence of the antipurine effect of MTX induced by the depleted CH_2FAH_4 levels and the subsequently elevated intracellular levels of 5-phosphoribosyl-1-pyrophosphate (PRPP), a co-substrate in the conversion of FUra to FUMP.

Both the increase in PRPP and FUra accumulation could be prevented by administering leucovorin (5-formyltetrahydrofolate, LCV) after MTX but before FUra at concentrations that rescued cells from the inhibitory effects of MTX [10]. LCV replenishes CH_2FAH_4 and circumvents the effect of MTX [24, 31]. Therefore, if it were given after the FUra was trapped within the MTX-treated cells it could promote the formation of a stable FdUMP-TS ternary complex. Ullman et al. [32] reported that 'physiologic' levels of CH_2FAH_4 are insufficient for optimum binding of FdUMP to TS. They demonstrated that when the LCV concentration in the culture medium was raised from 0.01

* This work was supported in part by grant CH-145 from the American Cancer Society and by grants CA-27130 and CA--08341 from the National Cancer Institute. LLD was supported by Postdoctoral Training Grant S-T32-CA09085-08 from the National Institutes of Health and EC was the recipient of a Cancer Research Award from the American Cancer Society

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Abbreviations: MTX, methotrexate; FUra, 5-fluorouracil; LCV, d,l-N^5-formyltetrahydrofolic acid, calcium salt (folinic acid, leucovorin); FdUMP, 5-fluoro-2'-deoxyuridylate; FUTP, 5-fluorouridine-5-triphosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; CH_2FAH_4, $N^{5,10}$-methylenetetrahydrofolate; dUMP, 2'-deoxyuridylate; dTMP, thymidylate; TS, thymidylate synthetase; PBS, phosphate-buffered saline: NaCl, 8.0 g; KCl, 0.2 g; Na_2HPO_4, 1.15 g; KH_2PO_4, 0.2 g in 1 l H_2O, pH 7.4; FdUrd, 5-fluoro-2'-deoxyuridine

to 1 μ*M* this produced a three-fold increase in the sensitivity of L1210 cells to FdUrd. Other workers have used this knowledge to potentiate the cytocidal effects of FUra in vitro [12, 33, 34].

Several studies in tumor-bearing mice have shown that the recovery of TS activity is a critical factor associated with recovery from the effects of FUra in vivo [19, 20, 27]. However, the addition of LCV to FUra in animal studies has not uniformly enhanced the efficacy of FUra [18, 21]. Phase I clinical trials of FUra with high-dose LCV have recently been undertaken [7, 23].

On the basis of these investigations, we felt it would be of considerable interest to give LCV after sequential administration of MTX and FUra and to evaluate the regenerated TS-CH_2FAH_4-FdUMP complex and TS activity. Expansion of the MTX-depleted CH_2FAH_4 pools in such a manner might result in stabilization of the ternary complex and augment the cytotoxicity of the MTX-FUra sequence further than previously described.

Materials and methods

Drugs. [6−^{3}H]FUra (2.1 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, Ill). [6−^{3}H]FdUrd (20 Ci/mmol was obtained from Moravek Biochemicals (City of Industry, Calif). The [6−^{3}H]dUrd (15 Ci/mmol) came from New England Nuclear (Waltham, Mass). The MTX was obtained from Lederle Parenterals, Inc. (Pearl River, NY), and FUra was purchased from Hoffman-La-Roche (Nutley, NJ). The LCV and all other nonradiolabeled compounds were purchased from Sigma (St. Louis, Mo).

Cells. L1210 murine leukemia cells were maintained as stationary suspension cultures in Fischer's medium plus 10% horse serum, transferred twice weekly, and kept at 37° C in a 5% CO_2 atmosphere. Tests performed every 3 months for mycoplasma contamination were negative. All experiments were performed with cells which had been inoculated at $1-3\times10^4$ cells/ml and had been in the logarithmic phase of growth for 48 h, which corresponds to $1-2\times10^5$ cells/ml. Enumeration of cells was performed with a model ZBI Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla).

Cell viability. To assess the biological effects of the MTX/FUra/LCV combinations, L1210 cells that had been growing in the logarithmic phase for 48 h to a density of $1-1.5\times10^5$ cells/ml were treated with MTX (1.0 μ*M*) 3 h before and then concurrently with a 1-h exposure to FUra (1, 10, 20 μ*M*). Concentrations of 10 μ*M* and 100 μ*M* LCV were added as indicated. Wherever LCV was added after FUra exposure the cells were centrifuged at 1000 *g* for 5 min at 37° C, the supernatant was discarded, and the cell pellet was resuspended in drug-free medium and then recentrifuged as before to wash any extracellular drug from the cells. The cell pellet was then finally resuspended in fresh medium and LCV added. Following all drug treatments and in controls, cells were washed again twice before cloning in soft agar, techniques previously described by workers in this laboratory [10] being used. The cloning efficiency in this system was 80%–90%.

Total intracellular FUra accumulation. Logarithmically growing cells were exposed for 3 h to 1 μ*M* MTX ±10 or 100 μ*M* LCV. Following drug exposure, 50 ml of the cell suspension was centrifuged at 1000 *g* for 5 min at 37° C, and the cell pellet was gently resuspended in 2 ml of the initial, drug-containing supernatant, to which was added 0.1 ml 1 *M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4, HEPES buffer). This concentration procedure was done to reduce the amount of radiolabeled FUra required to perform the accumulation studies. The cell suspension was transferred to a 25-ml stoppered flask and placed in a 37 °C shaking water bath. [6−^{3}H]FUra was then added to a concentration of 1 or 10 μ*M* and the total intracellular FUra accumulation of ribo- and deoxyribonucleotides was according to a previously described microfuge method [10].

dUrd Incorporation. Cells were prepared as before for the FUra accumulation studies and at designated times after adding [6−^{3}H]dUrd to a concentration of 1 μ*M*, 0.025 ml of the cell suspension was processed according to the method we have previously described [10]. Under normal conditions, the role of radiolabel incorporated into the acid-precipitable material (DNA) is an indication of the intracellular activity of TS.

Thymidylate synthetase ternary complex. The TS ternary complex formation in cell cultures were determined by means of a method we developed and described in detail in a separate publication on methods [16]. Briefly, the ternary complex was isolated by gel exclusion chromatography. Appropriate controls have been done to assure the accuracy of this method [16].

Incorporation of FUra into RNA. At the indicated times, ice-cold 100 μ*M* dipyridamole in PBS was added to similarly drug-treated cultures that also included [6−^{3}H]FUra. The samples were then centrifuged and washed, and the RNA was extracted from the acid-precipitable fraction as described previously [10]. Radioactivity in the RNA fractions was then related micrograms to of D-ribose by the orcinol reaction [6].

Statistics. Student's two-tailed *t*-test for the statistical evaluation of matched-pair data was used to assess the differences between treatment groups [30].

Results

Cytotoxicity of LCV combined with MTX and FUra

Assessment of cell viability by cloning in soft agar after exposure to MTX before FUra with and without LCV confirmed that, under these conditions, addition of LCV to the MTX/FUra sequence did not augment cytotoxicity. LCV administered for 1 of 3 h immediately after the MTX and FUra combination (MTX → FUra → LCV) also failed to reduce L1210 cell clonigenicity below that obtained with sequenced MTX and FUra. MTX at 1 μ*M* resulted in 80% viability, 10 μ*M* FUra resulted in 72% viability, and the sequence of MTX before FUra caused a reduced cell viability of 2.6%, but the addition of LCV did not enhance the cytotoxicity.

FUra accumulation

The intracellular accumulation of [6−^{3}H]FUra metabolites by L1210 cells exposed to MTX ±LCV is shown in Fig. 1.

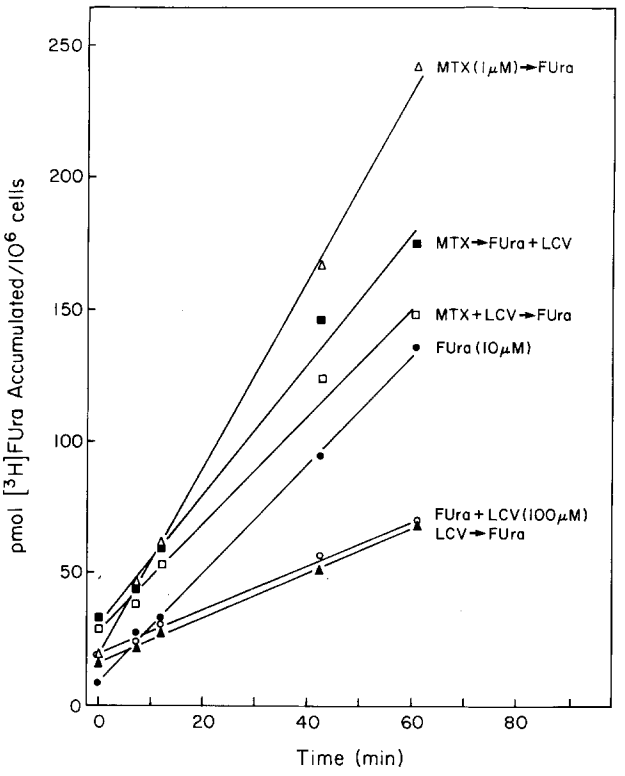

Fig. 1. Total intracellular accumulation of [6−^{3}H]FUra by L1210 cells exposed to MTX ± LCV. Uptake of 10 μ*M* FUra by logarithmically growing L1210 cells was linear for at least 60 min. All MTX exposures were for 3 h, after which time the cell suspension was centrifuged and the cell pellet was resuspended in 2 ml of the initial drug-containing supernatant with HEPES buffer added. This supension was placed in a 37°C shaking water bath and [6−^{3}H]FUra ± 100 μ*M* LCV was then added. Over the course of 1 h, aliquots of the cell suspension were placed in microfuge tubes containing perchloric acid overlayed with a silicone/mineral oil mixture. The tube was then centrifuged, quickly frozen, and cut at the liquid interfaces, and the radioactivity in the cell pellet and medium fractions was quantitated. The arrow indicates the 3-h pretreatment period. Each *point* represents triplicate experiments

As previously reported from this laboratory, the greatest accumulation of FUra nucleotides occurred following MTX pretreatment, which increased total FUra nucleotides from 136 pmol/10^6 cells to 242 pmol/10^6 cells at 1 h after 1 μ*M* [^{3}H]-FUra addition. Inclusion of 100 μ*M* LCV concurrently with FUra following MTX pretreatment reversed the enhanced FUra effects of the MTX pretreatment by 50%. When LCV was given before or simultaneously with FUra there was less intracellular accumulation of [6−^{3}H]FUra, a consistent observation without a clear explanation.

Effect of LCV on sequential MTX and FUra at thymidylate synthetase

The maximum inhibition of [6−^{3}H]dUrd-incorporation into the acid-insoluble material occurred after MTX pretreatment. FUra after MTX exposure produced inhibition comparable to that obtained with MTX alone. LCV added 30 min after sequential MTX-FUra did not increase the inhibition caused by MTX alone, indicating that the inhibition of thymidylate synthesis was already at its maximum after 1 μ*M* MTX (Table 1).

Table 1. Effect of drug treatment[a] on the incorporation of [6–^{3}H] dUrd into L1210 cell DNA

Antimetabolite (μ*M*)		% of Control [6–^{3}H] dUrd incorporation[b]		
MTX	FUra	−LCV	+LCV	
			10 μ*M*	100 μ*M*
0	0	100	108.4 ± 3.2[c]	118.2 ± 26.2
1	0	2.7 ± 1.2	6.4 ± 3.8	12.0 ± 1.0
0	10	19.6 ± 2.6	12.0 ± 2.4	21.2 ± 2.7
0	20	11.6 ± 2.9	–	10.4 ± 2.4
1	10	2.8 ± 1.4	5.2 ± 1.6	10.8 ± 1.3

[a] Exposure times: MTX, 4 h; FUra, 1 h; LCV, 1 h. For drug combination studies MTX was given 3 h before addition fo FUra, LCV, or concurrent FUra and LCV

[b] Determined by calculating the slope of [6–^{3}H] dUrd incorporation over 45 min into acid-precipitable materials (control 1.0 pmol/10^6 cells)

[c] Each value represents the mean ± SD of two to five experiments

An increase in the amount of FUra added from 10 to 20 μ*M* did not increase TS complex formation; neither did the addition of LCV concomitantly with FUra at either dose (Table 2). This observation is consistent with the intracellular activity of TS recorded in Table 1. Pretreatment of L1210 cells with MTX resulted in less TS ternary complex formation. LCV restored TS ternary complex formation to that observed with FUra alone. Despite the increased ternary complex formation when LCV was given after sequential MTX-FUra, this addition of LCV sequence was not more cytotoxic than sequential MTX and FUra alone. This indicates that the enhanced TS complex formation was not adding substantially to the toxic effect of the drug combination.

Table 2. Inhibition of thymidylate synthetase following treatment[a] with combinations of MTX, FUra, and LCV

	% Ternary complex formed[b] in FUra-treated cells				
	−LCV		+LCV (100 μ*M*)		
[MTX] (μ*M*)	10 μ*M*	20 μ*M*	10 μ*M*	20 μ*M*	*P* value[c]
0	71.3 ± 5.4[d]	76.4 ± 5.4	69.2 ± 4.2	79.0 ± 6.4	N.D.
1	42.8 ± 15.1	–	67.7 ± 5.8	–	<0.05
P value	<0.05		N.D.		

[a] Exposure times: MTX, 4 h; FUra, 1 h; LCV, 1 h. For drug combination studies, MTX was given 3 h before and 1 h concurrently with FUra, LCV, or FUra and LCV

[b] Following drug treatment, cells were exposed for 30 min to [6–^{3}H] FdUrd, washed, resuspended in buffer, and sonicated to disrupt the cells, and released thymidylate synthetase complex was analyzed by steric exclusion chromatography. Control, LCV, MTX (1 μ*M*), and MTX prior to LCV-treated cells had 0 ± 3.1% (SD) thymidylate synthetase ternary complex formation during drug treatment (prior to [6–^{3}H] FdUrd exposure). Absolute amounts total TS were 869 ± 22 fmol/10^6 cells

[c] Student's *t*-test for the statistical evaluation of matched-pair data was used to assess the differences between treatment groups. ND indicates no difference

[d] Each value represents the mean ± SD of duplicate samples from three separate experiments

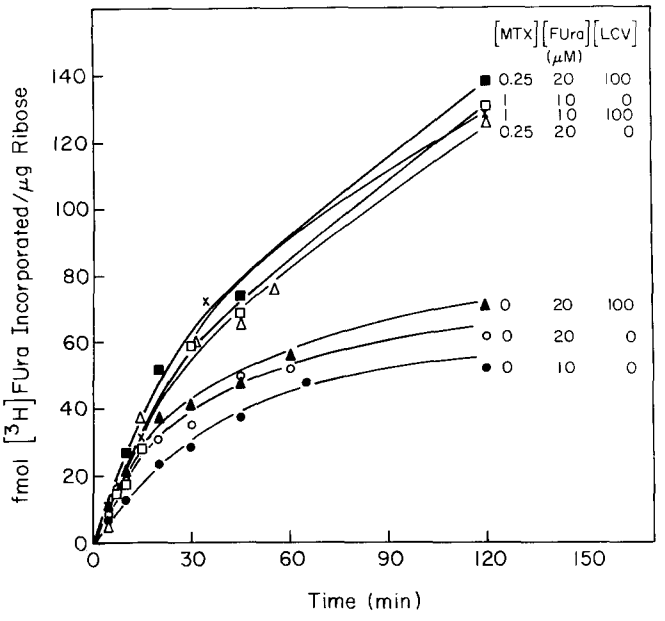

Fig. 2. Incorporation of [6−^{3}H]FUra into total intracellular RNA. Logarithmically growing L1210 cells were pretreated with MTX (0.25 or 1 μ*M* (3 h before exposure to 10 or 20 μ*M* FUra containing a tracer amount of [6−^{3}H]FUra ± LCV (100 μ*M*). At various times after addition of the radiolabel, dipyridimole was added and cells were assayed as described in *Materials and methods* to quantitate total intracellular [6−^{3}H]FUra incorporation into RNA. Each *point* represents duplicate samples

Intracellular FUra incorporation into RNA

Since MTX has been shown in this laboratory to increase PRPP levels and, consequently, the incorporation of FUra into RNA when given before FUra [10], the effects of MTX, when given before FUra ± LCV combinations, on the intracellular incorporation of FUra into RNA were examined, and the results are shown in Fig. 2. Incorporation of FUra into RNA following exposure to 20 μ*M* FUra was not enhanced by the simultaneous addition of LCV. Cells pretreated with MTX prior to 10 or 20 μ*M* FUra had equivalent amounts of FUra incorporated into RNA, as did concurrent LCV with FUra following MTX. Thus, addition of LCV had no effect on either the initial rate or the total incorporation of FUra residues into RNA following MTX pretreatment.

Discussion

Previous communications from this laboratory have reported synergistic enhancement of the cytotoxicity of FUra as a result of pretreatment with MTX in mouse [8, 10] and human [1, 3, 8] cells in culture. Such augmented cell kill was associated with the increased anabolism of FUra, which was attributed to the antipurine effects of MTX resulting in elevated levels of PRPP, the co-substrate required for the conversion of FUra to FUMP. FUra is thought to exert its antiproliferative action primarily via two mechanisms: the incorporation of FUTP into RNA and the inhibition of TS by FdUMP. Some reports have proposed that the effect of MTX prior to FUra on L1210 cell viability is due to intracellular dihydrofolate polyglutamate trapping (as a result of MTX inhibition of dihydrofolate reductase), which could augment the extent of binding of FdUMP to TS [13, 14]. Other studies, however, suggest that it is the incorporation of FUra into RNA which is largely responsible for the cytocidal activity of the MTX-FUra combination in L1210 cells.

Since adequate levels of CH_2FAH_4 are required for the formation of the most stable FdUMP-TS ternary complex, it was hoped that inclusion of LCV at some optimal time after the MTX-FUra sequence could promote the inhibition of dTMP synthesis by stabilizing the ternary complex without significantly reducing the antipurine effects of MTX, which elevate PRPP levels. Inhibition of [6−^{3}H]dUrd incorporation into the acid-precipitable fraction was greatest with MTX alone. Administration of FUra after MTX did not promote further inhibition of [6−^{3}H]dUrd incorporation. LCV addition to this combination did not promote a greater inhibition. When the FdUMP-TS ternary complex was quantitated, however (Table 2), it was found that less complex formation occurred when MTX preceded FUra than when FUra was given alone, suggesting an antagonistic interaction of these two drugs at TS. LCV, when given with FUra following the MTX pretreatment, however, did increase the TS complex formation to the amount observed following FUra alone. In spite of the regeneration of TS-FdUMP-CH_2FAH_4 complex, the addition of LCV after the FUra in the MTX-FUra sequence did not result in greater cytotoxicity than was observed with the MTX-FUra sequence alone.

The finding that the accumulation of [6−^{3}H] dUrd into acid-precipitable material, which is an indirect determination of the conversion of dUMP to dTMP by TS, was not further enhanced by the addition of LCV when given after the sequence of MTX and FUra, in spite of more complex formation, could be explained in the following manner. TS activity is inhibited in one of two ways. The depletion of CH_2FAH_4 secondary to MTX inhibition of DHFR will reduce the availability of the carbon source required for the conversion of dUMP to dTMP. FUra, as FdUMP, will directly inhibit TS in the presence of CH_2FAH_4 by MTX, then when the CH_2FAH_4 pools are repleted by leucovorin in the presence of FUra the activity of TS remains inhibited, now by the formation of the ternary complex FdUMP-TS-CH_2FAH_4. Therefore, if maximum inhibition of TS is achieved by the depletion of CH_2FAH_4 by MTX then the direct inhibition of TS by the subsequent addition of FUra (FdUMP) and LCV (CH_2FAH_4) should be no more effective in preventing dTMP formation. In addition, it has recently been shown that increased phosphorylase activity might result in response to the inhibition of TS activity by MTX, which could result in conversion of dURD to other nucleosides or the base uracil, in which case the [6−^{3}H] dUrd incorporation into acid-insoluble material following MTX could be spurious [28]. However, over the time course of our experiments, this possibility is unlikely.

Since less FdUMP was bound to TS after MTX, and presumably TS activity was maximally inhibited by MTX independent of the addition of FUra, then the MTX-FUra cytotoxic synergy could be the result of other FUra effects. It must be noted that the duration of the TS complex formation and the rate at which dUMP levels increased were not examined. Since no enhanced cytotoxicity was noted with the doses and timing of the drugs used in this study,

we felt that the measurement of these parameters would not have provided additional useful information to explain our results.

It must be stressed that whereas LCV did not augment the synergistic cytotoxicity of sequential MTX and FUra in L1210 cells, human cells may be more responsive to such combinations. Evans et al. [12] demonstrated that inhibition of TS could become growth limiting in human carcinoma Hep-2 cells if 10 μM LCV was administered with FUra, which stabilized the FdUMP-TS ternary complex. In these cells CH_2FAH_4 levels were apparently inadequate to bind to all the TS. Houghton et al. [19], using xenographs of human colorectal adenocarcinomas in mice, have made similar observations that CH_2FAH_4 intracellular levels appeared suboptimum, but they noted that LCV was not very useful as a promoter of greater in vivo inhibition of TS. It seems, therefore, that some cell lines have adequate CH_2FAH_4 for maximal complex formation (L1210); some have inadequate CH_2FAH_4 but can convert LCV to active forms (i.e., Hep-2); while still others have inadequate CH_2FAH_4 and cannot use LCV (i.e., human adenocarcinoma xenographs). The interactions of MTX, FUra, and LCV are obviously more complex than we currently understand [10, 13, 25, 32]. Drug interactions and mechanisms of action may vary from one cell line to another. Certainly, the information we have collected with the L1210 cell line, in which we have done extensive cytotoxic and biochemical investigations, suggests that the regenerated and stabilized TS-FdUMP-CH_2FAH_4 complex may be less important for the cytotoxicity observed when MTX precedes FUra than other unexplained effects of the drug combination.

Acknowledgements. We would like to thank Ms. Barbara Stanley for her excellent technical assistance and Ms. Hillary Raeffer for assisting in the preparation of this manuscript.

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Received December 3, 1984/Accepted May 7, 1985