1977

Reversal of methotrexate-induced cytotoxicity by 5-methyl tetrahydrofolate : implications for chemotherapy

Attilio V. Granata
Yale University

REVERSAL OF METHOTREXATE-INDUCED CYTOTOXICITY BY 5-METHYL TETRAHYDROFOLATE: IMPLICATIONS FOR CHEMOTHERAPY

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Attilio V. Grandi

Signature of Author

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Date

Reversal of Methotrexate-Induced Cytotoxicity By 5-Methyl Tetrahydrofolate: Implications for Chemotherapy

Attilio V. Granata
B.S., Yale College, 1974

A thesis submitted to the Faculty of Medicine
in partial fulfillment of the requirements
for the degree of Doctor of Medicine

Department of Medicine
Yale University
1977

Dedication

This thesis is dedicated to the memory of

Ann Magoun

and

John Arnold Crotta

Acknowledgements

To Barbara Moroson, Barbara Stanley, Judy Uhoch, Sanha Panichajakul, and all in the Tissue Culture Lab for their help, cooperation, and cheerfulness,

To Wendy Sawicki for tremendous assistance in teaching me to perform the UdR studies,

To Craig Lindquist, Kevin Scanlon, Ed Cadman, and all in JRB's lab for continuing advice and support,

To Arlene Cashmore for expert charts and graphs,

To Anne Esposito for utmost patience while without her typewriter,

And especially to Joseph R. Bertino, M.D., for infinite encouragement, consolation, patience, and criticism,

I wish to offer my sincerest thanks for a beautiful year.

Attilio V. Granata
2/10/1977

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Introduction

In a study recently to detect biochemical differences between malignant and nonmalignant cells to render the former more susceptible to chemotherapy[7], Sugimura et al.[114] showed the inability of Walker 256 rat breast carcinosarcoma cells to grow on a methionine-deficient diet. Halpern et al.[49] extended this work by demonstrating that L1210 mouse leukemia cells and J111 human leukemia cells also required preformed methionine to grow. Normal cells, alternatively, grew well in methionine-free medium as long as the important substrates and co-factors for methionine biosynthesis were present, namely, homocysteine, 5-methyl tetrahydrofolate (5MTHF), and vitamin B_{12}. The enzyme methionine synthetase, with vitamin B_{12} as co-factor, catalyzes the methylation of homocysteine by 5MTHF to yield methionine and tetrahydrofolate (THF):

$$\begin{array}{c}\text{Homocysteine} \\ + \\ \text{5MTHF}\end{array} \longrightarrow \begin{array}{c}\text{Methionine} \\ + \\ \text{THF}\end{array}$$

This difference between normal and malignant cells was exploited to the point where it was observed that Walker 256 tumor cells and nonmalignant mouse liver fibroblasts placed in culture together grew differently depending upon the methionine content of the medium. If grown in methionine-deficient medium, all malignant cells died within a few weeks, while normal cells thrived. If the medium contained

methionine, however, the malignant cells rapidly outgrew the normal ones, and the latter shortly all died[51].

Assay of methionine synthetase activity in several cell lines showed decreased absolute activity as well as decreased percent enzyme present as holoenzyme, as compared with assay of nonmalignant cells[2]. Halpern postulated in this work that deficient ability to synthesize methionine might account at least in part for the dependence of malignant cells on preformed methionine in the growth medium.

Since the methionine biosynthesis reaction is also closely involved with folate metabolism, Halpern looked at the reversibility of methotrexate (MTX) toxicity in normal and malignant cells in vitro[50]. MTX is a potent folic acid analog that causes inhibition of THF production and thus disruption of folate metabolism. It was found in these studies that leucovorin (LV), a reduced folate often given as an antidote or "rescue" agent following administration of MTX to tumor patients, readily reversed the effects of MTX on growth inhibition in both normal and malignant cells. 5MTHF, however, appeared to be capable of reversing MTX toxicity only in normal cells, which presumably possessed enough methionine synthetase activity to form methionine from 5MTHF and release enough THF from this reaction to counteract the folate block produced by MTX.

The results of these studies, while preliminary, seemed exciting. We began work to further elaborate the nature of 5MTHF's effect on cells treated with MTX.

In this study, we sought to verify the potentially useful differen-

tial reversal of normal vs. malignant cells by 5MTHF through an investigation of the effects of LV and of 5MTHF on the incorporation of radio-labelled deoxyuridine (^{3}H UdR) into DNA using MTX-treated cells. Mouse leukemia cells (L1210, L5178Y), human acute leukemia cells (acute lymphoblastic, acute myeloblastic), and normal human bone marrow cells were studied. We used this MTX-sensitive assay to discover whether malignant cells could be rescued by 5MTHF as compared to normal cells, using LV as well for comparison.

In addition, we studied the effects of MTX with either LV or 5MTHF on the growth of L1210 and L5178Y leukemia cells and of Walker 256 rat breast carcinosarcoma cells during a period of several days in tissue culture. Evidence was sought, therefore, from both short-term and long-term experiments using human and rodent cells in vitro.

Review of the Literature

Methotrexate (MTX) is a potent inhibitor of folate and one-carbon metabolism which is extremely useful in the chemotherapeutic management of malignant disease. In order to better understand the relationship between this inhibition and its resulting derangements in DNA, RNA, and protein synthesis which lead to cell death, it will be useful to briefly review the properties and pathways of folic acid and folate intermediates.

Folate Metabolism

Folic acid (pteroylmonoglutamic acid) consists of a pteridine ring attached to para-amino benzoic acid and glutamic acid[45] (Figure 1). The 2- and 4-positions of the pteridine ring are substituted, as indicated in the figure, by an amino group and a hydroxy group, respectively. The 6-position of the ring is the point of attachment of the amino end of p-amino benzoic acid, and the carbonyl end of this acid is attached via an amide linkage to glutamic acid.

The 5- through 8-positions of the pteridine ring are of special importance, for each of these atoms may be reduced by addition of one hydrogen atom, yielding 5,6,7,8-tetrahydrofolic acid, or tetrahydrofolate (THF). This is the principal oxidation-reduction level at which most folate reactions occur. One-carbon substitutions at the 5- and/or 10-positions result in several important reduced folates,

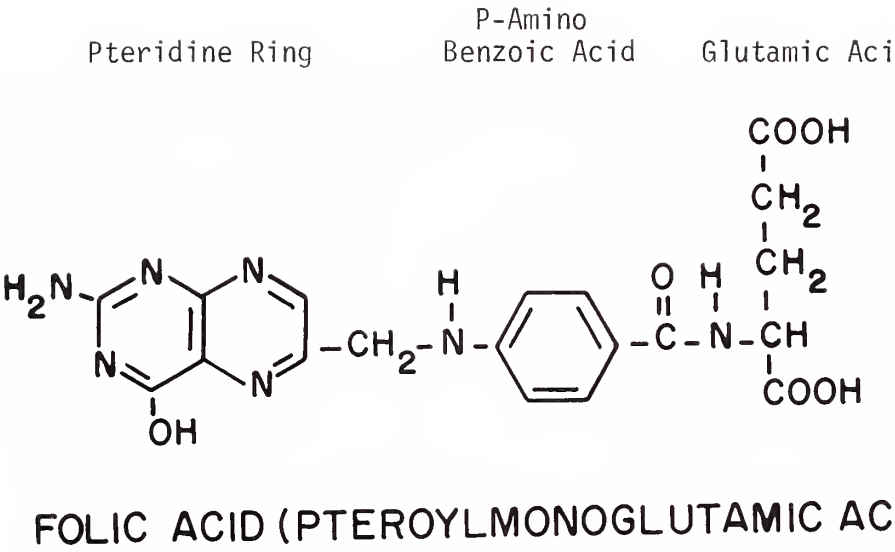


Figure 1. The Structure of Folic Acid.

Reduction of the pteridine ring yields 7,8-Dihydrofolate (DHF) or 5,6,7,8-Tetrahydrofolate (THF).

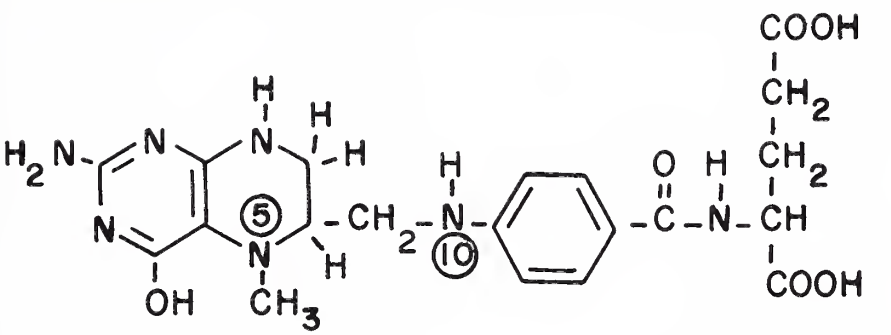


Figure 2. The Structure of N^5-Methyl Tetrahydrofolic Acid (5MTHF).

A one-carbon group is substituted at the 5-position. This group is donated to homocysteine to form methionine, with THF remaining.

Substitution of a formyl (-CHO) group at the 5-position yields N^5-Formyl THF, or LV.

Other possible intermediates include N^{10}-Formyl THF, N^5,N^{10}-Methenyl THF, and N^5,N^{10}-Methylene THF. The latter two molecules contain a one-carbon group bridging the 5- and 10-positions.

such as N^5-Methyl THF (5MTHF, Figure 2), N^5-Formyl THF (citrovorum factor, folinic acid, leucovorin, LV), N^{10}-Formyl THF, N^5,N^{10}-Methenyl THF and N^5,N^{10}-Methylene THF.

Folic acid is first reduced to 7,8-Dihydrofolic acid, or dihydrofolate (DHF), then to THF. The enzyme catalyzing both reductions is dihydrofolate reductase (E.C. 1.5.1.5, DHFR, labelled "A" in Figure 3). The same enzyme performs both reductions, using NADPH in each case as a co-factor[34,121].

Pure folic acid makes up less than 10% of dietary folates[95]. Most forms present in a typical American diet consist of reduced folates, often containing polymers of several glutamic acid moieties rather than the single monoglutamate[20,45,92].

A principal metabolic function of THF is to donate a one-carbon unit from serine to form glycine and N^5,N^{10}-Methylene THF, as in Figure 3. This enzyme is a vitamin B_6-requiring transhydroxymethylase[45], and is an important source for the regeneration of glycine from serine.

N^5,N^{10}-Methylene THF engages in two important reactions: an essentially irreversible reduction via NADPH to produce 5MTHF[70], or the donation of its one-carbon unit to deoxyuridylic acid (dUMP). The latter reaction forms thymidylic acid (dTMP), which after becoming further phosphorylated to dTTP is incorporated into DNA as a pyrimidine nucleotide[64,117]. The enzyme catalyzing the conversion of dUMP to dTMP is thymidylate synthetase (Figure 3, "B"). A product of this reaction is DHF, which must be again reduced to the THF reduction level

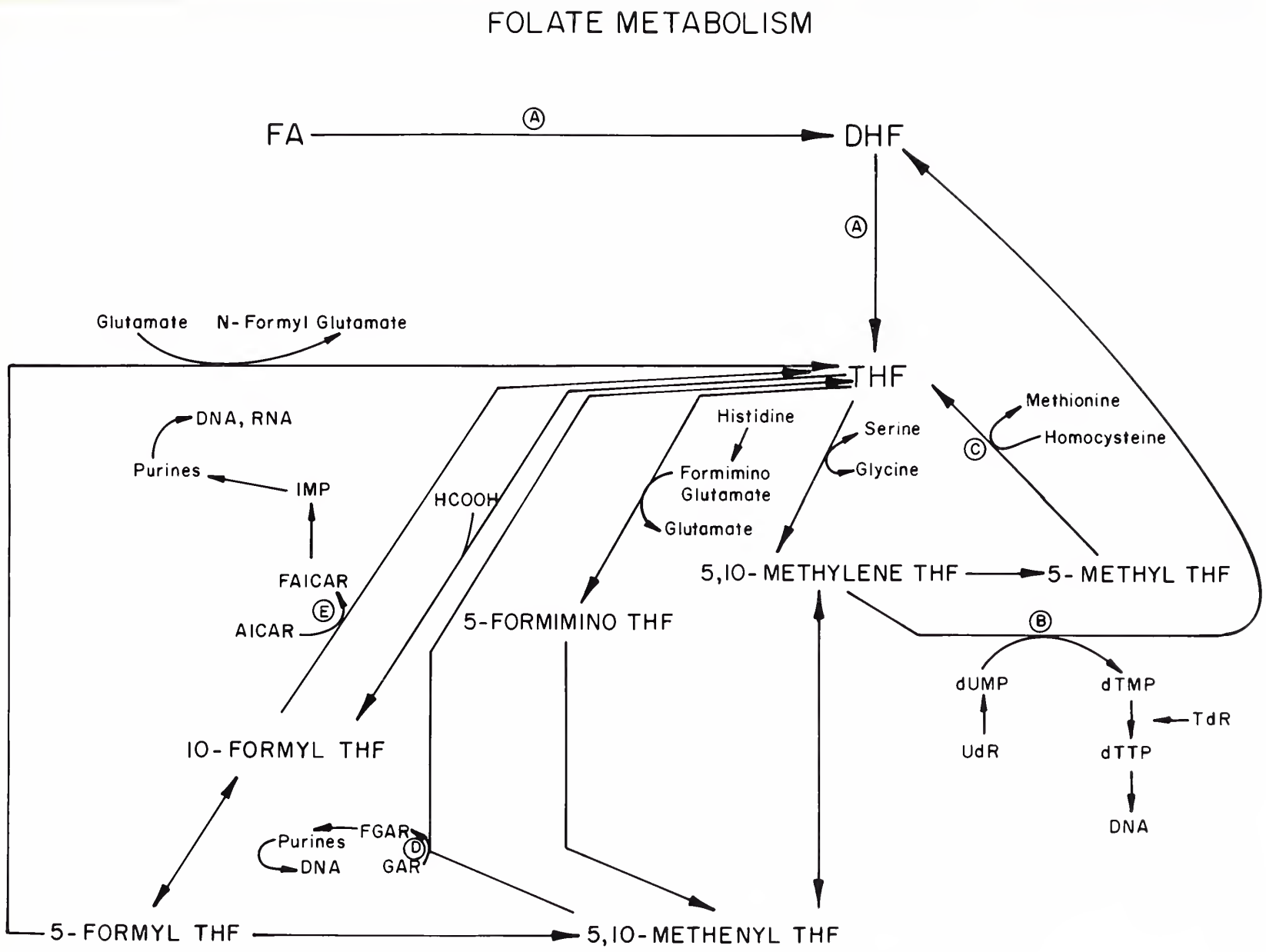


Figure 3. The Principal Pathways of Folic Acid Metabolism.

to proceed further in folate pathways.

Thymidine deoxyribonucleoside (TdR) is able to bypass the thymidylate synthetase enzyme, as indicated in Figure 3. TdR is directly phosphorylated to dTMP by a kinase, and subsequently enters DNA synthesis as dTTP.

The principal role of 5MTHF is the methylation of homocysteine to form methionine and THF[6,76,100]. The enzyme catalyzing this reaction, methionine synthetase (E.C. 2.1.1.13, N^5-Methyl THF:Homocysteine Methyltransferase, Figure 3, "C"), requires vitamin B_{12} in the methylcobalamin form as a co-factor[27,45].

Other Folate Pathways

In addition to participation in serine, glycine, homocysteine and methionine metabolism, reduced folates also participate in the catabolism of histidine (Figure 3). The reduction of histidine to formiminoglutamic acid enables the latter molecule to donate its formimino moiety to THF, yielding glutamic acid and N^5-Formimino THF. The latter product is cyclo-deaminated, as indicated in the figure, to produce N^5,N^{10}-Methenyl THF.

Reduced folate derivatives participate in the de novo synthesis of purines at two different stages. N^5,N^{10}-Methenyl THF, the product of histidine catabolism just described, donates its one-carbon group to glycineamide ribonucleotide (GAR), producing the formylated ribonucleotide FGAR, shown in Figure 3, "D". This step causes incorporation of the carbon at the 2-position of the purine ring.

A few steps later in the build-up of the ring, N^{10}-Formyl THF

loses its formyl group to 4-amino-5-imidazole carboximide ribotide (AICAR), again yielding a formylated product, FAICAR (Figure 3, "E"). FAICAR is dehydrated to yield the common precursor of both purines, inosinic acid[56]. In this conversion from AICAR to FAICAR, the carbon at the 8-position of the purine ring is incorporated.

A final important folate intermediate is LV. Silverman et al.[102] described the enzymatic transfer of its formyl group to glutamic acid, resulting in N-formyl glutamate and the regeneration of THF. In addition, Nahas et al.[88] found that L1210 mouse leukemia cells were able to rapidly metabolize ^{14}C-labelled LV formyl groups through the other two principal LV conversion routes, forming N^5,N^{10}-Methenyl THF and N^{10}-Formyl THF (Figure 3).

Methionine Synthetase

The methionine synthetase reaction, discussed previously, is a crucial source of the amino acid for protein synthesis when preformed methionine is lacking in the medium. The ability to produce methionine via this reaction, in turn, is critically dependent upon the presence of vitamin B_{12} to sufficiently activate the enzyme.

Rats grown on a methionine-free diet containing homocysteine but lacking vitamin B_{12} did not grow nearly as well as those on the same diet supplemented with the vitamin[5]. Mangum[80] showed similar results in several normal and malignant cell lines grown *in vitro*, and Kutzbach et al.[74] found that methionine synthetase activity was totally dependent upon the presence of B_{12}.

It is currently believed that activation of the enzyme in the presence of vitamin B_{12} is probably due to conversion of inactive apoenzyme to functional holoenzyme[2,55,71]. In baby hamster kidney cells grown *in vitro*, addition of the vitamin to medium containing homocysteine in place of methionine resulted in a sudden four-fold increase in enzyme activity[69]. Similarly, Gawthorne and Smith[25] showed that in vitamin B_{12}-deficient ewes methionine synthetase activity in liver was almost totally absent.

Although methylcobalamin is the form of B_{12} required by the enzyme, most mammalian cells are able to utilize the cyano and hydroxy forms of the vitamin, presumably by converting them to the methyl form[14]. There have been reported in the literature several cases of congenital deficiencies in ability of human cells to properly transport or utilize vitamin B_{12}, resulting in homocystinemia, homocysteinuria, and hypomethioninemia[86,87].

5MTHF, a substrate in the methionine synthetase reaction, appears to be the predominant reduced folate form in mammalian serum and cytoplasm[52]. Administration of ^{14}C-labelled 5MTHF to L1210 and L5178Y mouse leukemia cells results in rapid transfer of the methyl group to non-folate compounds (i.e., methionine), although 87% of ^{3}H-labelled THF attached to the methyl group was still in the form of 5MTHF after 60 minutes, illustrating that there is an appreciable intracellular pool of 5MTHF[91]. In this study the vast majority of reduced folates were metabolized through the methionine synthetase pathway during the

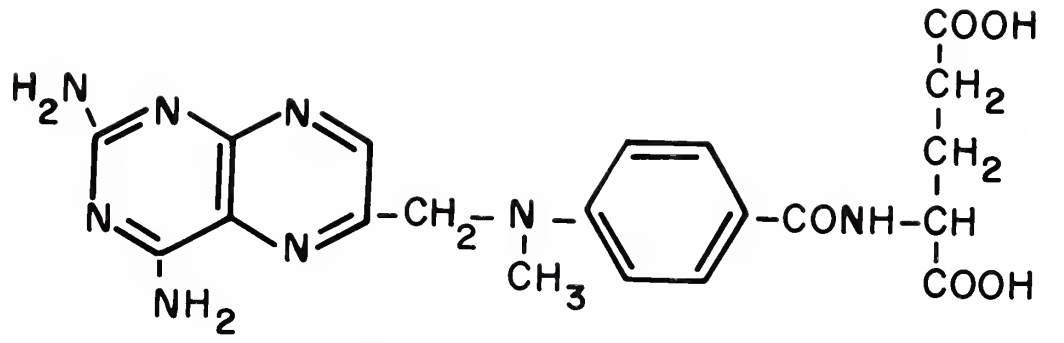
assay, and about a third engaged in the thymidylate biosynthetic pathway. These results indicate that the 5MTHF species appears to be the principal folate accumulating to any substantial degree, and that there is an extremely rapid intracellular turnover of reduced folates.

The form in which folates are metabolized has been intensively studied. Moran et al.[85] found that derivatives in logarithmically growing L1210 cell cultures were entirely in the form of polyglutamates. The authors noted that all enzymes catalyzing intermediary folate metabolic pathways are capable of utilizing polyglutamyl as well as monoglutamyl folates, and an exception to this has never been found. Thus it is felt that polyglutamyl folates may not only be a storage form but perhaps a principal form in which folates undergo metabolism.

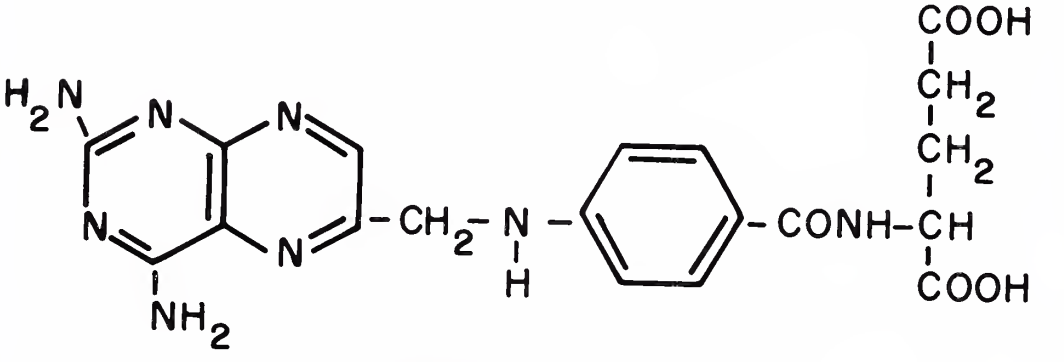
Structure and Mechanism of Folate Antagonists

The intimate role of reduced folates in DNA, RNA, and protein synthesis is helpful in the design of chemotherapy using antimetabolites. Several structural analogs of folic acid have been developed in the past 25 years, of which the most potent are probably aminopterin, amethopterin (MTX), and 3',5'-Dichloro MTX[9,12] (Figure 4). Each of these analogs is a 4-amino derivative of folic acid.

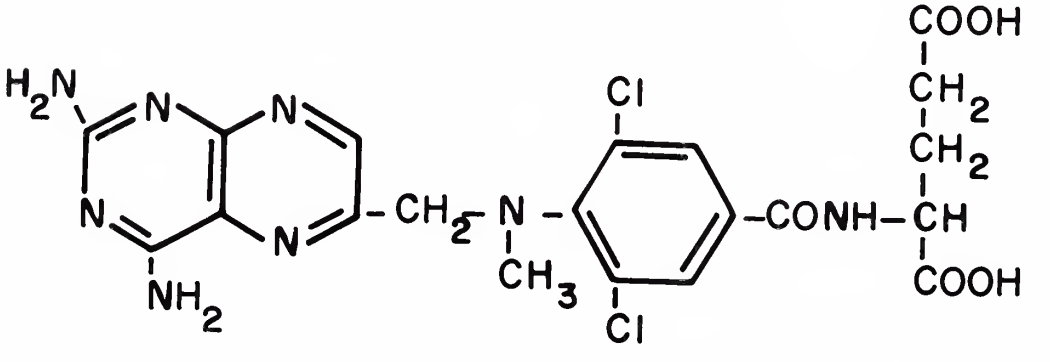
The function of an antimetabolite is to compete with a true metabolic intermediate for an enzyme binding site in an effort to either decrease the rate of catalysis of the true substrate, or to



METHOTREXATE



AMINOPTERIN



DICHLOROMETHOTREXATE

Figure 4. The Structure of 3 Principal Folate Antagonists.

All of these structural analogs of folic acid have 4-amino substitutions, while MTX and Dichloro-MTX have N^{10}-Methyl groups as well.

MTX binds almost irreversibly to DHFR, preventing adequate reduction of folic acid and DHF to THF by the enzyme.

bind to the site in such a way as to render the enzyme useless. The mechanism of the antifolate drug MTX is probably related to its extremely tight binding and consequent inhibition of DHFR[94,118]. The K_i of MTX for DHFR is on the order of 10^{-11} M, and it binds tightly enough to persist in mouse liver and kidney tissue for up to 8 months[118].

Inhibition of DHFR by MTX is at least partially responsible for causing lethal depletion of intracellular reduced folates, disrupting nucleic acid and protein metabolism and killing the cell. The mechanism of cell death appears to be due at least in part to decreased levels of intracellular thymidine following treatment with MTX, preventing efficient DNA synthesis and resulting in the so-called "thymidineless death". As stated previously, the folate product in the thymidylate synthetase reaction is DHF, not THF. This product must be reduced to THF in order to further proceed with folate metabolism: inhibition of DHFR by MTX, therefore, prevents regeneration of THF and thus ultimately halts DNA synthesis[15].

Numerous theories have been proposed to explain in detail the cause of death in the absence of thymidine[33,98], all of which deal generally with decreased synthesis and increased degradation of DNA. TdR, which can be phosphorylated by a kinase directly to dTTP (Figure 3), is able to reverse MTX toxicity in cells[16,24,115].

Inhibition of folate reduction would also be expected to affect other pathways, such as those of purine biosynthesis. Hryniuk et al.[61] emphasized the importance of MTX-induced inhibition of de novo purine biosynthesis as contributing to cell death, although the dominant

mechanism is probably thymidineless death[59,60,67]. The relative extents to which thymidine depletion and purine depletion each contribute to overall cell kill are not yet fully known.

In any case, MTX kills cells in the S phase of their growth cycle, during the period of DNA synthesis[31,43,44,75,112]. It is a general principal of antimetabolite chemotherapy that rapidly and uncontrollably proliferating cells are more susceptible to drugs that interfere with DNA synthesis than are slowly dividing or resting cells[17,58].

Chemotherapy with Antifolates and Differential Effects of MTX on Normal and Malignant Cells

It was Farber[32] who first used antifolates in the treatment of neoplastic disease, observing dramatic responses to administration of aminopterin in children with acute leukemia. In an effort to further characterize the mechanism of aminopterin and related agents, Jukes et al.[68] noted symptoms of folic acid deficiency in animals fed these drugs. Philips et al.[96] found that 4-amino folate derivatives were especially useful as antagonists, and Skipper[111] showed that administration of 4-amino antifolates impaired nucleic acid synthesis in mice.

Goldin[36] did extensive work on the management of L1210 leukemia with antifolates, and found that MTX was the most effective of the 4-amino derivatives. He pointed out the need for early treatment and developed optimum dose schedules for chemotherapy of mouse leukemia: frequent low-dose treatment was better for late, advanced malignancies,

while intermittent high-dose was the treatment of choice early in disease[37].

Administration of MTX is toxic to all rapidly proliferating cells, including those of the gastrointestinal mucosa, skin, and bone marrow[8] as well as those that are malignant. Consequently, workers have sought techniques designed to protect normal tissues as much as possible from fatal effects of MTX, while preserving anti-tumor effects.

Burchenal[18] attempted to administer various folate intermediates to counteract MTX effects, and found that LV was much more effective than folic acid in this regard[19]. Since his work, administration of LV "rescue" following MTX treatment has permitted the administration of much higher doses of the antifolate with reduced toxicity and higher therapeutic index in a variety of malignancies, including acute leukemia, cervical carcinoma, choriocarcinoma, osteogenic sarcoma, breast and lung malignancies, and tumors of the head and neck[1,8,10,11,25,28,30,57,66,79,97,116].

LV probably rescues by several means. Both LV and MTX compete for the same cell membrane carrier for transport into the cell[88], and thus the mechanism of LV's reversal of toxicity probably has much to do with competing for cellular uptake of MTX or with the enhancement of MTX efflux from the cell, as well as with serving as a reduced folate antidote to a folate-starved cell[40].

MTX Transport

MTX enters cells through a temperature-dependent process that

resembles active transport[39-41,103-107]. Inside the cell, there are at least two different pools of MTX[38]: a small amount of the drug is tightly bound and unexchangeable, while a more substantial quantity, if allowed to accumulate, is present in an osmotically active state in intracellular water. This latter pool of MTX is freely exchangeable with external medium through the membrane transport system. In addition, a very small amount of the drug is adsorbed on or near the membrane, and is probably of minor significance in terms of MTX action.

MTX is transport rapidly and linearly into L1210 and other cells, following Michaelis-Menten kinetics[38]. Eventually a saturated intracellular level is reached, which is the steady state level - influx and efflux of drug are equal. In the absence of competitive inhibitor for uptake, this state is usually achieved within 20 to 30 minutes. L1210 cells are able to achieve a ratio of internal to external MTX levels of up to 1.29 to 1, consistent with an energy-requiring process[38,44]. As the external drug concentration increases, this ratio diminishes even though the actual amount of MTX transported into the cell increases.

During the linear portion of the uptake phase, the tightly-binding sites are presumably filled first, followed by the accumulation of free intracellular drug. Cells that are loaded with MTX and then resuspended in MTX-free medium rapidly lost this free or exchangeable portion (within 15 to 20 minutes), and a constant amount of MTX remained tightly bound in the cell.

LV and folic acid are competitive inhibitors of MTX transport, the former substance much more potent than the latter[110]. Addition of large amounts of LV to cells in steady state with external MTX results in counter-transport (efflux) of the freely exchangeable portion of intracellular MTX. For example, addition of 125 μM LV to cells at equilibrium with an external MTX level of 2 μM results in a rapid exit of exchangeable MTX within 15 to 20 minutes[38]. An even more potent competitive inhibitor of transport is 5MTHF[40], with a K_i less than half that of LV (see Table I).

Preloading cells with less than enough MTX to fully saturate the tightly-binding sites does not result in appreciable efflux of MTX either when the cells are resuspended in MTX-free media or if large amounts of a competitive inhibitor of transport are added to the suspension[38]. It is postulated that the high-affinity binding sites first occupied by MTX at low levels represent DHFR, especially in view of the observation that the maximum amount of intracellular MTX for which no efflux can be induced, approximately 9 nanomoles per gram of cells (dry weight), is comparable to the measured level of DHFR in these cells, 10.5 nanomoles per gram (dry weight).

In addition to tightly binding and inactivating DHFR, MTX may also inhibit folate uptake by malignant cells and stimulate efflux of intracellular folates. Similarly, LV and 5MTHF may reverse cell toxicity not only by providing reduced folates, but also by competing for uptake of MTX as well as by enhancing efflux of drug.

Table Ia. MTX Transport Parameters (L1210)

K_m MTX (μM)	Inhibitor	K_i Inhibitor (μM)
3.13		
	Folic acid	228
	LV	4.41
	5MTHF	1.74

Table Ib. MTX Distribution Ratios (L1210, Steady State)

$[MTX]_{ext}$ (μM)	$[MTX]_{int}$ (μM)	$[MTX]_{int}/[MTX]_{ext}$
0.44	0.54	1.223
2.2	1.55	0.710

(Ref: 110)

The Role of Free Intracellular MTX

Although the principal target of MTX is DHFR, saturation by the drug of all enzyme binding sites does not result in complete cessation of DNA synthesis, nor does intracellular accumulation of MTX halt when DHFR is fully inhibited. To achieve inhibition of 50% of UdR incorporation into DNA in mouse L cell fibroblasts, free intracellular MTX levels of 0.2 to 0.4 μM were required[42]. Sirotnak and Donsbach[108] found that an equimolar ratio of MTX to DHFR in cells caused inhibition of only 20% of UdR incorporation into DNA: an intracellular level of at least 1 μM was required for 50% inhibition in these L1210 cells. Interestingly enough, in these cells an external MTX level of 0.4 μM was required simply to enable complete saturation of all DHFR binding sites, and levels below 0.1 μM externally had no effect whatsoever on inhibition of UdR into DNA.

White et al.[120] have demonstrated that uptake of radio-labelled formate into DNA, RNA, and protein of L cell fibroblasts is only slightly inhibited by MTX levels just sufficient to saturate the high-affinity binding sites. To achieve 50% inhibition of incorporation of formate into DNA, RNA, and protein required free intracellular drug levels of 0.3, 1.0, and 3.0 μM, respectively. Stokstad et al.[113] previously suggested that conversion of DHF to THF may continue in the absence of exchangeable MTX, since MTX toxicity can be reversed by administration of DHF[83].

These studies were interpreted to indicate that either a "low affinity" form of the enzyme DHFR was present, which was not as

markedly inhibited by MTX as the high affinity form, or that even in circumstances when the enzyme was completely in the E-I complex, addition of sufficient substrate (DHF or DHF polyglutamate) enabled generation of enough THF to allow thymidylate synthesis to continue, since DHFR is not the rate-limiting enzyme for this synthesis. In support of the former hypothesis is the fact that several forms of DHFR have been shown to exist in both bacterial and mammalian cell lines[29,46,84]. Another explanation may lie in direct inhibition of thymidylate synthesis by MTX, which occurs at much higher MTX levels[108].

There is no firm agreement at this time on the nature or the existence of a specific low-affinity binding site. Accumulation of free MTX above DHFR levels is, however, regarded as crucial for attainment of full toxicity.

MTX Transport and Responsiveness of Cells to MTX

There have been noted in several studies correlations between the ability of human leukemia and other tumor cells to transport MTX into the cell and responsiveness of the tumor to MTX, both *in vivo*[72] and *in vitro*[73]. Sirotnak and Donsbach[109], in studies of mice bearing L1210 leukemia, showed that these cells accumulated 12 to 40 times as much MTX as there was intracellular DHFR, with persistence of intracellular free drug for at least 24 hours. Small intestine epithelial cells, on the other hand, were only able to accumulate drug to 5 to 8 times the cells' DHFR content and in these cells free drug persisted for no longer than 4 hours. Similarly, MTX levels in normal

liver were lower and persisted for a shorter time than in liver infiltrated with leukemic cells.

Since normal tissues and tissues from responsive and unresponsive tumors contain roughly the same amount of DHFR[3,13,48,62,81,82,99,119], and since DHFR from normal and tumor tissues is inhibited to roughly the same extent by MTX[3,13,53,62,65,82,99,119], the ability of malignant cells to maintain higher free MTX levels for a longer period of time than non-malignant cells is probably of crucial significance to toxicity observed with MTX.

A further correlation between MTX transport and responsiveness was demonstrated in five variably responsive tumor cell lines, S180, P288, P388, Ehrlich's ascites tumor, and L1210. Sirotnak and Donsbach[110] showed that in these lines the K_m value for MTX influx was directly in accord with each cell line's response to the drug: the more easily MTX was transported, the more sensitive the cell was to the drug. In all of these lines, steady state levels were reached within 40 to 50 minutes, and the authors were able to calculate the values for the K_m of MTX and for the K_i of the competitive inhibitors folic acid, LV, and 5MTHF (Table Ia). They also report the drug distribution ratios for two different external MTX levels, given in Table Ib.

All of the parameters studied reveal that MTX transport exhibits concentrative uptake, temperature dependence, and concentration dependence of the drug distribution ratio, which are all in accord with an active transport mechanism. Several workers have attempted to

isolate the membrane carrier protein for MTX and folates. Huennekens[63] has found a protein with a molecular weight of 29,000 daltons from the membrane of Lactobacillus casei which may be the carrier. He postulates that approximately 1000 molecules of glucose must be metabolized for each molecule of folate or folate derivative transported by the carrier protein.

^{3}H UdR Uptake Studies

In this group of experiments, cells from mice, in vitro tissue cultures, and patients were incubated with MTX and various concentrations of LV or of 5MTHF. After a period of equilibration, ^{3}H UdR was added to the cells and the uptake of label into DNA, via metabolism of UdR to dUMP, dTMP, and dTTP (Figure 3), was monitored as a function of time for the different conditions employed. Presumably, healthier cells are better able to synthesize DNA than those affected by MTX, and this assay was therefore felt to be a reliable indicator of the reversal of toxicity by either LV or by 5MTHF.

Materials and Methods

Calcium leucovorin and methotrexate were obtained from Lederle Laboratories Division, American Cyanamid Co. (Pearl River, N.Y.). Chromatographically pure N^{5}-Methyl THF was obtained through the courtesy of Dr. John Mangum, and from the Sigma Chemical Co. (St. Louis) as the barium salt, purity > 90%. The barium ion was removed by precipitation in solution by $NaHCO_3$ and Na_2HPO_4 at 0^{o} C., and purity was verified by checking the absorption maximum for 5MTHF at 290 mμ[47,89].

Eagle's Basal Medium (with Earle's salts and glutamine, EBM), Fischer's medium for leukemic cells of mice, and horse serum were obtained from the Grand Island Biological Co. (Grand Island, N.Y.).

^{3}H UdR (25 Ci/mmol, 1.00 mCi/ml) was obtained from the New England Nuclear Co. (Boston). Mice used for harvesting tumor cells were BDF_1 males from Jackson Laboratories (Bar Harbor, Me.).

Approximately 10^7 L1210s (the designation "s" refers to the cells being MTX-sensitive) murine leukemia cells were injected intraperitoneally into groups of 3 mice and harvested at log phase on day 4 by intraperitoneal injection and aspiration of normal saline. L1210s cells and L5178Y murine leukemia cells from tissue culture were grown in Fischer's medium with 10% horse serum. Human malignant cells were blasts obtained from the peripheral blood of patients with acute leukemia who were untreated, or, in one case, 9 days post-chemotherapy. "Normal" bone marrow was aspirated from patients with non-hematologic tumors undergoing diagnostic work-ups: all marrow slides had normal morphology.

Murine cells were collected by centrifugation and resuspended in EBM with 10% horse serum and 0.2% sodium heparin. Human cells were obtained in heparinized Vacutainer tubes and allowed to sediment for 60 minutes in two volumes of 3% dextran in normal saline, after which the serum and buffy coat layers were removed. These were centrifuged, and the leukemic cells were resuspended in EBM, horse serum, and heparin.

MTX was used at a concentration of 2 μM except one case in which 10 μM was used. LV and 5MTHF were at concentrations of 4 μM, 40 μM, and 400 μM. Since both LV and 5MTHF were present as racemic mixtures with only the L-diastereomers active, the actual molar ratios used with respect to MTX were 1, 10, and 100.

In "simultaneous rescue" experiments, MTX and rescue drug (LV or 5MTHF) were present in solution together from the start of the experiment. 1 ml of cell solution containing approximately 3 x 10^7 cells was added to a 10 ml Erlenmeyer flask containing the appropriate amount(s) of concentrated drug(s) for that particular condition. These cells were allowed to preincubate for 30 minutes under gentle agitation in a water bath at 37^o C.

In "delayed rescue" experiments, cells were first incubated with MTX for up to 60 minutes, after which concentrated LV or 5MTHF was added, followed by equilibration for an additional 30 minutes. Controls containing no drug, MTX alone, and occasionally LV or 5MTHF alone were always included with both simultaneous and delayed rescue experiments.

After preliminary incubation as above, the cells in each flask were labelled with 100 µl of ^{3}H UdR solution made by adding 90 µl of labelled UdR to 1 ml of medium. While the cells continued to gently agitate at 37^o C., 0.2 ml aliquots were withdrawn from the flasks at four or five time points up to 60 minutes after labelling. These samples were added to individual centrifuge tubes containing 5 ml of 10% trichloroacetic acid at 0^o C. After overnight precipitation the tubes were centrifuged at 800 x g for 5 minutes in an International Equipment Co. PR2 refrigerated centrifuge.

The pellets were washed by aspiration of supernatant, resuspension in fresh cold trichloroacetic acid, and vortex agitation. After re-centrifugation and repeated washing for a total of 3 cycles, the final

pellet was dissolved in 0.4 ml of NCS solubilizer. This was added to a counting vial containing 15 ml of liquid scintillation cocktail made by adding 20 g PPO, 75 mg POPOP, and 10 ml 100% ethanol to 3800 ml (8 pints) of toluene. The samples were allowed to cool and were then counted for tritium in a Beckman LS230 liquid scintillation spectrometer.

In addition to the above procedures, certain experiments were run with and without methionine present in the medium. In such cases, cells were initially washed with saline and resuspended in methionine-free, folate-free Fischer's medium and 10% horse serum, to which d,l-homocysteine (20 mg/l) and cyanocobalamin (2 mg/l) had been added. Those conditions with methionine had 100 mg/l of l-methionine in solution as well. Incubations and uptakes were carried out as above.

A final group of experiments was carried out in which delayed rescue cells were washed free of MTX after the initial 60 minute incubation. They were suspended and centrifuged in normal saline at 0^{o} C. for a total of two washings. Resuspension was in MTX-free medium containing rescue drug.

Results

For each condition, four or five time points were obtained. The ideal slope, as computed by the method of least squares, of the resulting line showing the incorporation of ^{3}H UdR into the trichloroacetic acid-insoluble fraction over time was calculated. The efficacies of LV and of 5MTHF at the various concentrations in reversing MTX-produced inhibition

of UdR uptake were computed by determining the ratio of the rescue uptake slope to the slope of the control condition, in which no drug was used. This ratio is reported as "% control", and was compared to inhibition caused by MTX alone. Degree of fit was determined by computation of $\underline{r}$ values: the majority of lines had $\underline{r} > 0.98$, and virtually all had $\underline{r} > 0.95$.

Two figures displaying uptake rates for a typical experiment using cells from L1210s and human ALL lines are shown in Figures 5 and 6. Tables II and III give the complete results obtained with murine and human cells, respectively. In all cases the rescue values (% control) are to be compared to the % control values in the presence of MTX alone in order to evaluate reversal. The control slope is, by definition, 100%.

In all cell lines with the exception of those washed free of MTX before rescue (Table II, E and F), incorporation of UdR into DNA was inhibited to less than 20% of control values by 2 μM MTX, and all human leukemia cells were inhibited to less than 5% of control values. Simultaneous addition of LV or of 5MTHF resulted in relief of this inhibition in all cell lines, usually as a function of concentration of the reduced folate. In general, a minimal amount of reversal was produced by equimolar concentrations of LV or 5MTHF. When the concentration was 10 times that of MTX, there was partial relief of inhibition, and at 100 times the MTX concentration almost complete or better than complete reversal occurred. The reversal noted with 5MTHF was equal

Figures 5 and 6. Reversal of MTX Inhibition of UdR Incorporation into DNA by LV and by 5MTHF, in L1210s (Figure 5) and in Human ALL Cells (Figure 6).

These figures illustrate the results of two typical UdR uptake experiments. In each figure, chart (A) shows the results of simultaneous rescue experiments, in which MTX and LV or MTX and 5MTHF were present in solution together from the start of incubation. Chart (B) in each figure shows the results of delayed rescue experiments, in which LV or 5MTHF was added to cells after a 60 minute incubation in MTX with no other drug added to medium.

In all charts, line (1) represents the control rate of uptake, in which no drug was added, and line (2) represents uptake in the presence of 2 μM MTX alone. Lines (3), (4), and (5) show uptake rates in the presence of MTX and LV, with LV present in molar ratios of 1, 10, and 100, respectively, to MTX. Lines (6), (7), and (8) represent uptake rates with 5MTHF present, instead of LV, in the same molar ratios to MTX.

Notice that increasing the concentration of LV or of 5MTHF results in progressively better incorporation of UdR into DNA over time.

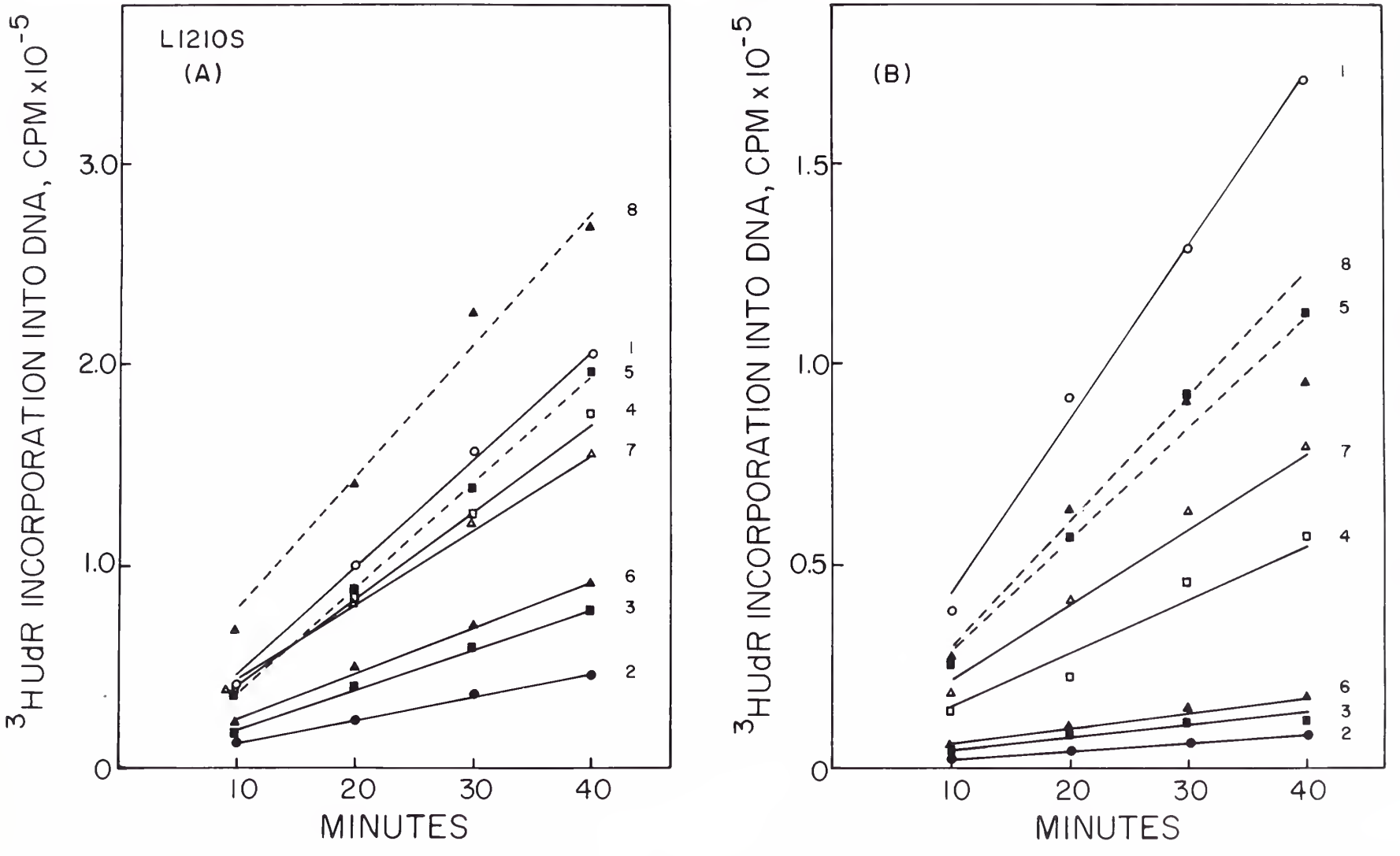


Figure 5. Reversal of MTX Inhibition of UdR Incorporation into DNA by LV and 5MTHF (L1210s). See Legend.

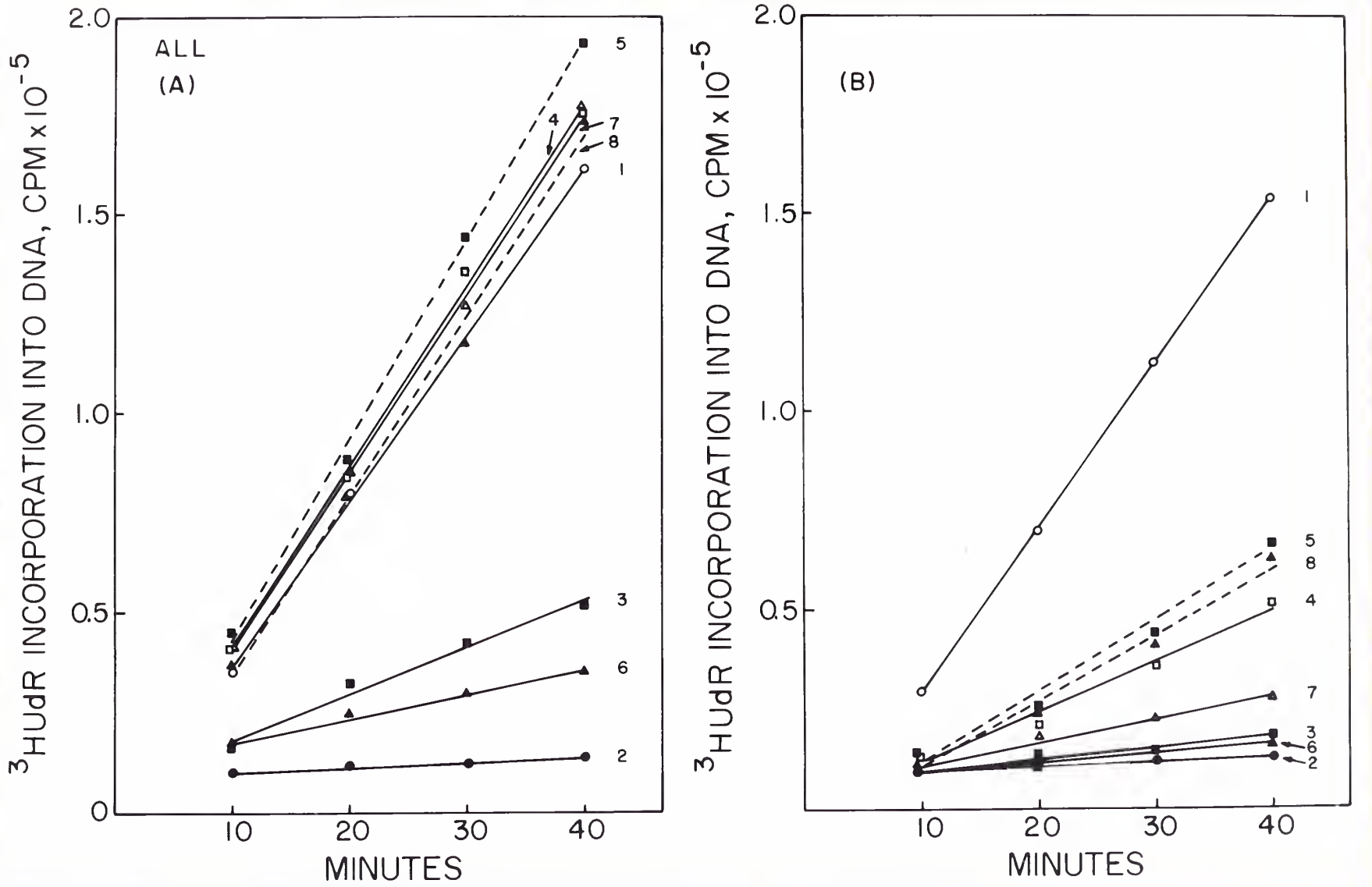


Figure 6. Reversal of MTX Inhibition of UdR Incorporation into DNA By LV and 5MTHF (Human ALL)
See Legend

Table II. Reversal of MTX Inhibition of UdR Incorporation Into DNA of Murine Tumor Cells by LV and by 5MTHF

CELL TYPE	^{3}H UDR INHIBITION BY 2 μM MTX (% Control)	REVERSAL (% Control) LV ADDED 4 μM	LV ADDED 40 μM	LV ADDED 400 μM	5MTHF ADDED 4 μM	5MTHF ADDED 40 μM	5MTHF ADDED 400 μM
A) L1210s from in vitro							
Simultaneous addition	9.5	12.5	31.3	45.1	11.2	28.0	110.9
60 minute delay	15.1	32.7	65.7	138.1	7.7	92.2	124.5
B) L1210s from in vivo							
Simultaneous addition	21.0	36.1	78.2	90.5	42.2	65.9	120.8
60 minute delay	4.7	8.2	29.5	62.3	8.0	42.4	74.2
C) L1210s from in vivo at 10 μM MTX							
Simultaneous addition	16.4 (10 μM)	29.8	50.5	108.4	30.3	68.6	116.0
60 minute delay	7.5 (10 μM)	2.9	2.1	51.2	0.5	2.9	57.2
D) L5178Y from in vitro							
Minus Methionine							
Simultaneous addition	3.1	3.6	16.6	74.1	4.4	17.6	91.0
60 minute delay	0.3	3.8	6.0	2.8	1.3	4.9	8.9
Plus Methionine (100 mg/l)							
Simultaneous addition	3.6	7.8	24.7	92.9	5.1	32.8	88.2
60 minute delay	0.3	0.3	1.4	5.1	0.7	1.9	13.8
E) L1210s from in vitro							
Simultaneous addition	35.1	28.3	78.7	103.2	45.8	108.6	98.3
30 minute delay, MTX washed from cells*	39.4	123.5	119.0	105.3	109.5	120.5	123.1
F) L5178Y from in vitro							
Simultaneous addition	24.2	35.6	103.7	105.9	45.0	114.5	72.0
30 minute delay, MTX washed from cells*	43.0	97.5	96.9	80.5	83.8	94.8	105.5
G) L1210s from in vitro, no MTX							
Uptake in LV or 5MTHF only	--	135.4	145.9	121.4	114.3	120.1	126.0

*(Uptake values for MTX-treated cells resuspended in EBM without LV or 5MTHF:

L1210s 129.1%
L5178Y 118.7%)

Table III. Reversal of MTX Inhibition of UdR Incorporation Into DNA of Human Cells by LV and by 5MTHF

CELL TYPE	^{3}H UDR INHIBITION BY 2 μM MTX (% Control)	REVERSAL (% Control) LV ADDED 4 μM	LV ADDED 40 μM	LV ADDED 400 μM	5MTHF ADDED 4 μM	5MTHF ADDED 40 μM	5MTHF ADDED 400 μM
A) Normal bone marrow							
Simultaneous addition	7.5	43.0	119.3	87.3	46.8	137.6	126.2
60 minute delay	19.0	33.3	87.7	24.3	30.0	35.2	147.5
B) Normal bone marrow							
Simultaneous addition	3.0	17.3	74.0	40.8	10.5	66.0	118.1
60 minute delay	1.5	6.5	22.5	19.1	3.1	9.4	5.6
C) Acute Lymphoblastic Leukemia							
Simultaneous addition	1.1	4.0	65.4	107.7	5.1	80.1	99.1
60 minute delay	0.1	0.2	0.6	1.9	0.3	1.0	1.4
D) Acute Lymphoblastic Leukemia							
Simultaneous addition	2.6	27.6	108.7	119.8	14.3	107.6	107.8
60 minute delay	2.4	7.3	31.3	43.8	6.1	14.1	40.6
E) Acute Lymphoblastic Leukemia, same as previous patient; 9 days post-Vincristine + Ara-C							
Simultaneous addition only	4.7	25.6	68.1	74.0	0.2	0.3	152.6
F) Acute Myeloblastic Leukemia							
Simultaneous addition	2.4	7.1	40.1	204.8	4.4	75.9	203.1
60 minute delay	1.2	1.6	4.8	3.5	1.4	1.9	1.3
G) Acute Myeloblastic Leukemia							
Simultaneous addition only	1.0	3.7	12.5	74.5	2.0	11.0	51.0
H) Acute Myeloblastic Leukemia							
Simultaneous addition only	0.5	1.9	7.0	64.6	1.4	9.8	98.9

to or greater than that provided by LV in most cases, although in one study using ALL cells (Table III, E), 5MTHF did not reverse significantly until 100 times the MTX concentration was used.

When rescue with LV or 5MTHF was delayed for up to 60 minutes, reversal of MTX inhibition was less, although there were again for the most part no significant differences in rescue rates. Importantly, there appeared to be no difference in reversal by 5MTHF of MTX inhibition of DNA synthesis when human leukemia cells and normal human marrow cells were compared (Table III, A and B vs. C-H).

In cells washed free of MTX (Table II, E and F), absolute rates of inhibition by MTX in both groups were much less than in other experiments. While there was a proportional increase in reversal of inhibition by 5MTHF and LV in the simultaneous rescue portions of these two studies, the delayed rescue experiments demonstrated uptake of UdR at or near control rates at all concentrations of LV or 5MTHF: resuspension in ordinary medium, without any rescue drug, caused the same effect (Table II, E and F, footnote).

There was no significant difference in recovery of UdR uptake rates in L5178Y cells incubated with and without methionine (Table II, D). Slight enhancement of simultaneous rescue rates in the plus-methionine experiment was seen over similar values in the minus-methionine experiment, and in both cases delayed rescue with 5MTHF produced much better uptake rates than delayed rescue with LV at 100 times the MTX concentration.

Table II, G shows results of a control experiment for LV and 5MTHF. Uptake of UdR into DNA is slightly improved over control values when these folates are added to cell suspensions in the absence of MTX, although there is no significant relationship between individual rates and type or amount of reduced folate added.

Further discussion and analysis of these studies will be found following the next section describing the tissue culture experiments.

Tissue Culture Studies

This group of experiments was performed to evaluate the growth of murine leukemia cells and Walker 256 rat breast carcinosarcoma cells *in vitro* in the presence of MTX and after simultaneous or delayed addition of LV or of 5MTHF. While cells in the previous set of experiments which studied UdR uptake were examined for a period of up to one hour, cells in tissue culture were permitted to grow for up to 5 days. By measuring increase in cell concentration over time, it was possible to judge the effects of various MTX-folate combinations upon cell growth.

Materials and Methods

All drugs used were obtained from the same sources as in the ^{3}H UdR uptake experiments, with the exception that all were sterilized by filtration through a 0.22 μ Millipore filter prior to use.

Cells from 5 or 6 day cultures were added to sterile Fischer's medium with 10% horse serum, then divided into various 100 ml culture bottles, one for each condition. Approximately 60 to 75 ml of cell suspension containing 2×10^{4} cells/ml were added to the bottles. Appropriate amounts of drug were then added for each of the conditions, and cell density was checked by counting in a Model B Coulter Counter.

The contents of simultaneous rescue bottles were immediately apportioned into 15 ml tissue culture tubes, 5 ml per tube, and placed

horizontally in a 5% CO_2 incubator at 37° C. Cells for delayed rescue experiments were kept in the 100 ml culture bottles containing MTX for from 4 to 24 hours. After this period, cells were gently centrifuged in a warm room at 37° C., washed with Fischer's medium plus 10% horse serum, and aseptically resuspended in MTX-free medium containing the appropriate amount of LV or of 5MTHF. After counting, these suspensions were apportioned into 15 ml culture tubes as described previously.

Each 24 hours, duplicate culture tubes for every condition were randomly removed from the incubator. Cell clumps were disrupted by pipetting, and cells were suspended in normal saline at appropriate dilutions and counted in the Coulter counter. Duplicate counts were taken and averaged for each tube. Average counts between the two tubes agreed to within 10%.

Three experiments were performed. In the first, L1210s, L5178Y, and Walker 256 cell lines were grown at a MTX concentration of 0.04 µM, a relatively low concentration. LV and 5MTHF were used at concentrations of 0.08 µM (equimolar) and 0.8 µM (10 times the MTX concentration). L-methionine was present at a concentration of 15 mg/l, and 10 mg/l of folic acid plus 0.5 µg/l of cyanocobalamin were also added. In the simultaneous rescue part of the experiment, MTX and rescue drug were present for the entire 5 day growth cycle, while cells in the delayed rescue part were suspended in MTX alone for 24 hours, then washed and resuspended in LV or 5MTHF for the remainder of the experiment.

The second experiment was performed using a higher MTX dose,

identical with that of the UdR experiments, 2 μM. LV and 5MTHF were again used at equimolar and at 10-fold ratios to MTX, and the cell lines employed were L1210s and L5178Y. A simultaneous rescue and a delayed rescue part were again performed, but the latter group was suspended in MTX alone for 4 hours before wash and resuspension in LV or 5MTHF. This high-dose experiment was performed in Fischer's medium with 10% horse serum, but at a slightly higher methionine concentration of 100 mg/l. In both this high-dose and the previous low-dose MTX experiments, controls were always used in which cells were grown with MTX alone, with no drug, or with LV or 5MTHF alone.

A third experiment was performed simply to determine the effects of external methionine concentration on growth of L1210s and L5178Y. Various concentrations of methionine were added to methionine-free, folate-free Fischer's medium with 10% dialyzed horse serum.

Results

All simultaneous rescue experiments began at identical initial cell concentrations, and thus cell number vs. time is plotted to reflect growth rate. In the delayed experiments, varying amounts of the initial cell population were recovered from the wash and resuspension following incubation in MTX. While the concentrations varied slightly from condition to condition, the initial values upon resuspension in LV or 5MTHF were all within the same decade on a logarithmic scale. To avoid confusion in comparing growth curves not beginning from exactly the same point, however, the delayed rescue values were converted

to total divisions since resuspension, calculated from the formula

$$\text{Total divisions} = \frac{\log_{10}(N_t/N_0)}{\log_{10}2}$$

where N_0 is the initial cell number (following resuspension in reduced folate) and N_t is the cell number at time t. Growth curves plotted in this way retain the original shape of a curve of cell number vs. time, but all curves began at 0 cell divisions, facilitating the comparison of the growth rates of each condition.

The results of the first experiment, at the low-dose MTX level, are shown on the following 2 pages (Figure 7). For all 3 cell lines, MTX alone inhibited growth in the simultaneous rescue group (curves (2), charts (A), (C), and (F)), while LV proportionally reversed this inhibition (curves (3) and curves (4)). 5MTHF did not appear to affect inhibition by MTX (curves (5) and curves (6)). In the delayed group, cells washed of MTX after 24 hours suspension in the drug all grew at approximately equivalent rates, roughly similar to control values (curves (1) vs. curves (3)-(6), charts (B), (D), and (F)). In addition, cells transferred from MTX to ordinary Fischer's medium grew at identical rates (curves (2)).

Table IV illustrates that cells actually increased in number, although slowly, in the low-dose concentration of MTX during the 24 hours of incubation in the delayed rescue experiment. The total number of cell divisions is reported and compared with growth of control cells suspended in MTX-free medium for 24 hours.

Figure 7. Growth of L5178Y, Walker 256, and L1210s Cells in 0.04 μM MTX Simultaneously with LV or with 5MTHF, or in MTX-Free Medium with LV or with 5MTHF Following a 24 Hour Exposure to 0.04 μM MTX.

Charts (A), (C), and (E) represent simultaneous rescue results using L5178Y, Walker 256, and L1210s cell lines, respectively. Charts (B), (D), and (F) represent delayed rescue results.

In the simultaneous rescue experiments, curves (1) show control growth (no drug added), and curves (2) show growth in MTX alone. Curves (3) and Curves (4) represent growth in the presence of MTX and LV, with LV present in molar ratios of 1 and 10, respectively, to MTX. Curves (5) and Curves (6) represent growth with 5MTHF, instead of LV, present at the same molar ratios, respectively.

In the delayed rescue experiments, curves (1) represent control growth, and curves (2) represent growth following resuspension in Fischer's medium and 10% horse serum after a 24 hour exposure to MTX. Curves (3) through Curves (6) represent growth in equimolar or 10-fold LV or 5MTHF, as in the simultaneous rescue experiments, following a 24 hour exposure to MTX. The control cells (Curves (1)) were never exposed to MTX, but were washed and resuspended as were the other delayed rescue conditions after 24 hours.

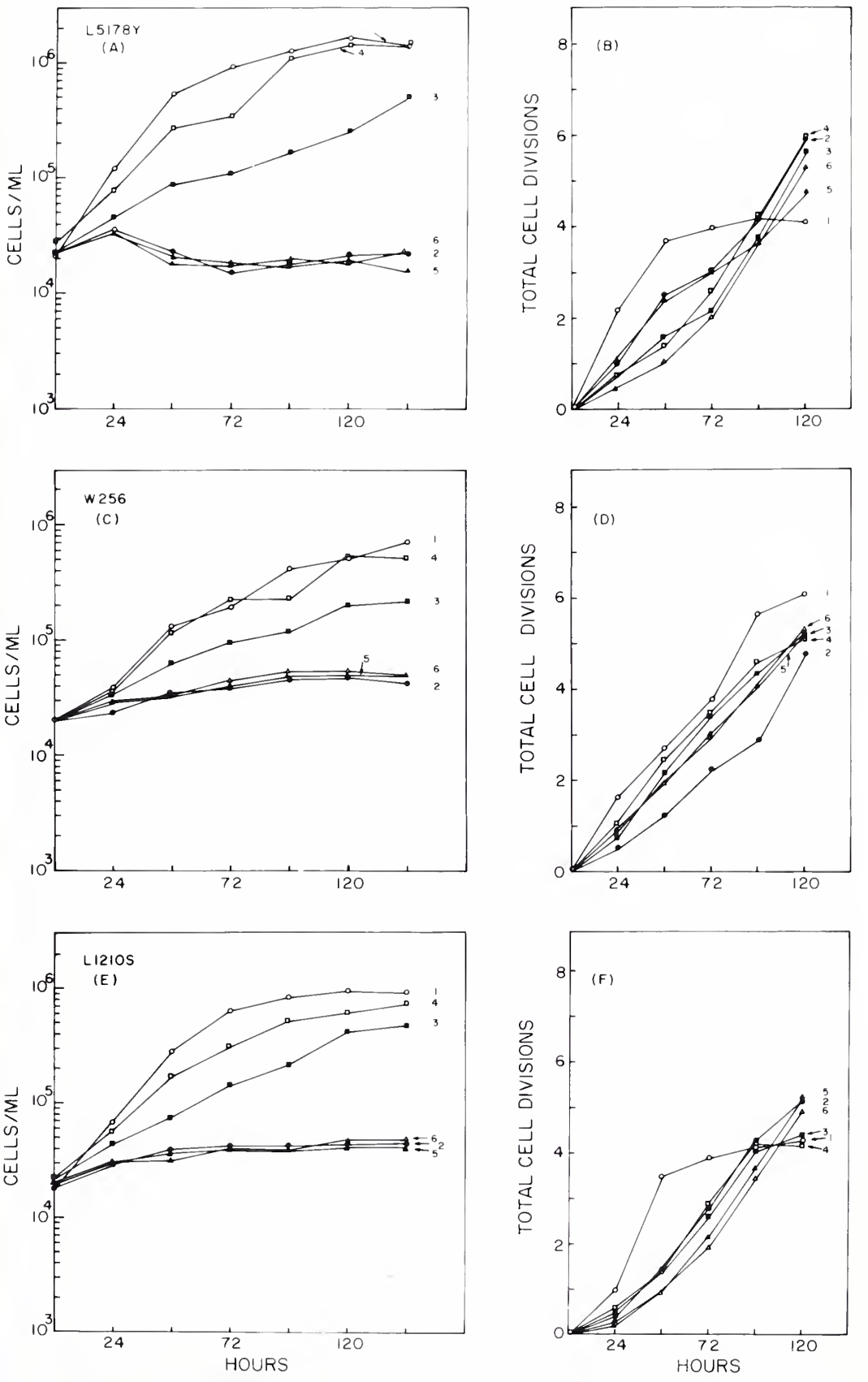


Figure 7. Low-Dose MTX Tissue Culture Experiment. See Legend.

Table IV. Growth of Cells During 24 Hours in 0.04 µM MTX

Cell Type	Divisions per 24 Hours Fischer's (MTX)	Fischer's (No MTX)
L5178Y	0.59	2.64
Walker 256	0.12	1.03
L1210s	0.30	1.63

The results of the high-dose experiment are shown in Figure 8. MTX at this concentration (2 μM) totally inhibited growth in all of the simultaneous rescue conditions, including at equimolar and 10-fold concentrations of LV and of 5MTHF (charts (A) and (D)). Removal of cells from MTX after 4 hours and resuspension in normal Fischer's medium plus horse serum resulted in only minimal growth (curves (2), charts (B) and (D)). Resuspension of the delayed rescue cells in LV produced excellent recovery at either equimolar or 10-fold ratios to MTX (curves (3) and curves (4)), but 5MTHF only minimally reversed inhibition of growth - equimolar 5MTHF resulted in very little recovery (curves (5)) while 10-fold 5MTHF (curves (6)) gave slightly better recovery, but not as good as with LV. Growth in LV or 5MTHF without MTX (charts (C) and (F)) revealed no appreciable effect of these reduced folates alone on growth rates.

The results of the methionine dependence experiment are shown in Figure 9. Normal Fischer's medium contains 100 mg/l of methionine, and growth of both cell lines in normal medium is not appreciably different from growth in medium with 10 mg/l or 50 mg/l of the amino acid. At levels below 10 mg/l, however, growth begins to fall off until there is no growth whatsoever at a methionine level of 0 (curves (8)).

Further discussion of these results and correlation with the UdR uptake results will be presented in the Discussion section following.

Figure 8. Growth of L1210s and L5178Y Cells in 2 μM MTX Simultaneously with LV or with 5MTHF, in MTX-Free Medium with LV or with 5MTHF Following a 4 Hour Exposure to 2 μM MTX, or in Medium Containing LV or 5MTHF Without MTX.

Charts (A) and (D) represent simultaneous rescue results using L1210s and L5178Y cell lines, respectively. Curves (1) show control growth with no drug added, and Curves (2) show growth with MTX alone added. Curves (3) and Curves (4) show growth in the presence of MTX and LV, with LV present at molar ratios of 1 and 10, respectively, to MTX. Curves (5) and Curves (6) show growth with 5MTHF, instead of LV, present at the same molar ratios to MTX.

Charts (B) and (E) represent delayed rescue results. Curves (1) show control growth with no exposure to MTX, and Curves (2) show growth of cells transferred to Fischer's medium and horse serum following a 4 hour exposure to MTX. Curves (3) through Curves (6) represent growth in equimolar or 10-fold LV or 5MTHF, as in the simultaneous rescue experiments, following a 4 hour exposure to MTX.

Charts (C) and (F) represent growth of cells with no MTX added. Curves (1) show growth in normal medium and horse serum, while Curves (3) through Curves (6) show growth at 4 μM and at 40 μM LV or 5MTHF, as above, with no MTX.

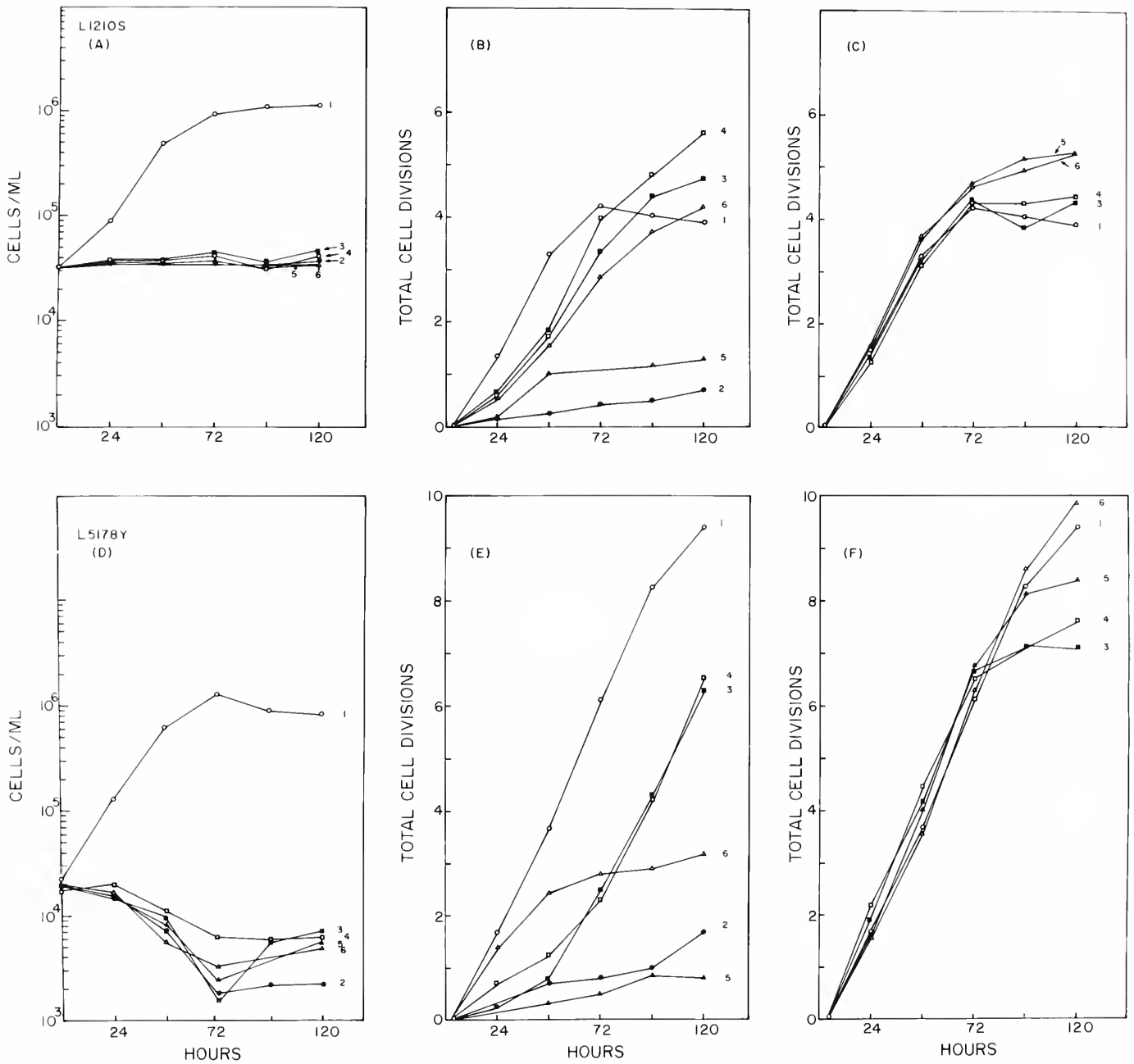


Figure 8. High-Dose MTX Tissue Culture Experiment. See Legend.

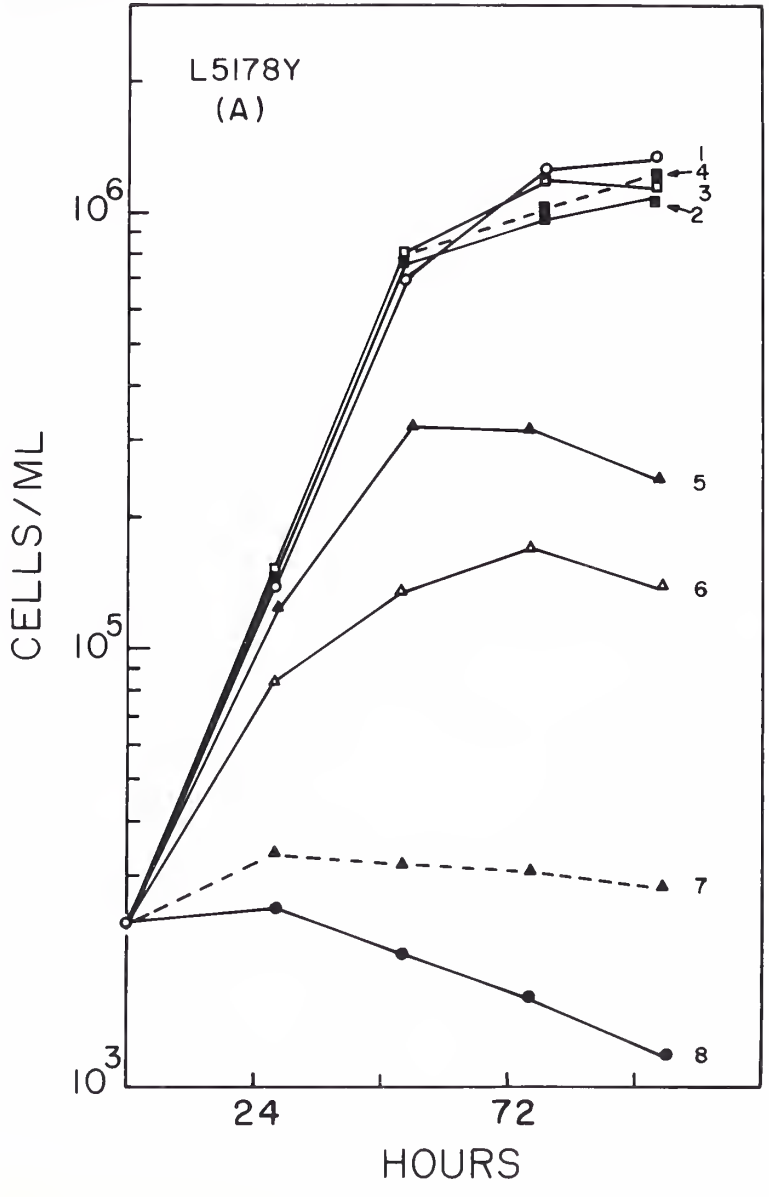
Figure 9. Growth of L5178Y and L1210s Cells in Methionine-Free Medium with Varying Amounts of Methionine Added.

Chart (A) represents growth of L5178Y cells, and Chart (B) of L1210s cells.

Curves (1) show control growth in normal Fischer's medium (100 mg/l methionine) while all other curves show growth in methionine-free medium to which the following amounts of methionine have been added:

Curves (2)	100 mg/l	Curves (5)	1 mg/l
Curves (3)	50 mg/l	Curves (6)	0.5 mg/l
Curves (4)	10 mg/l	Curves (7)	0.1 mg/l

Curves (8) 0 mg/l



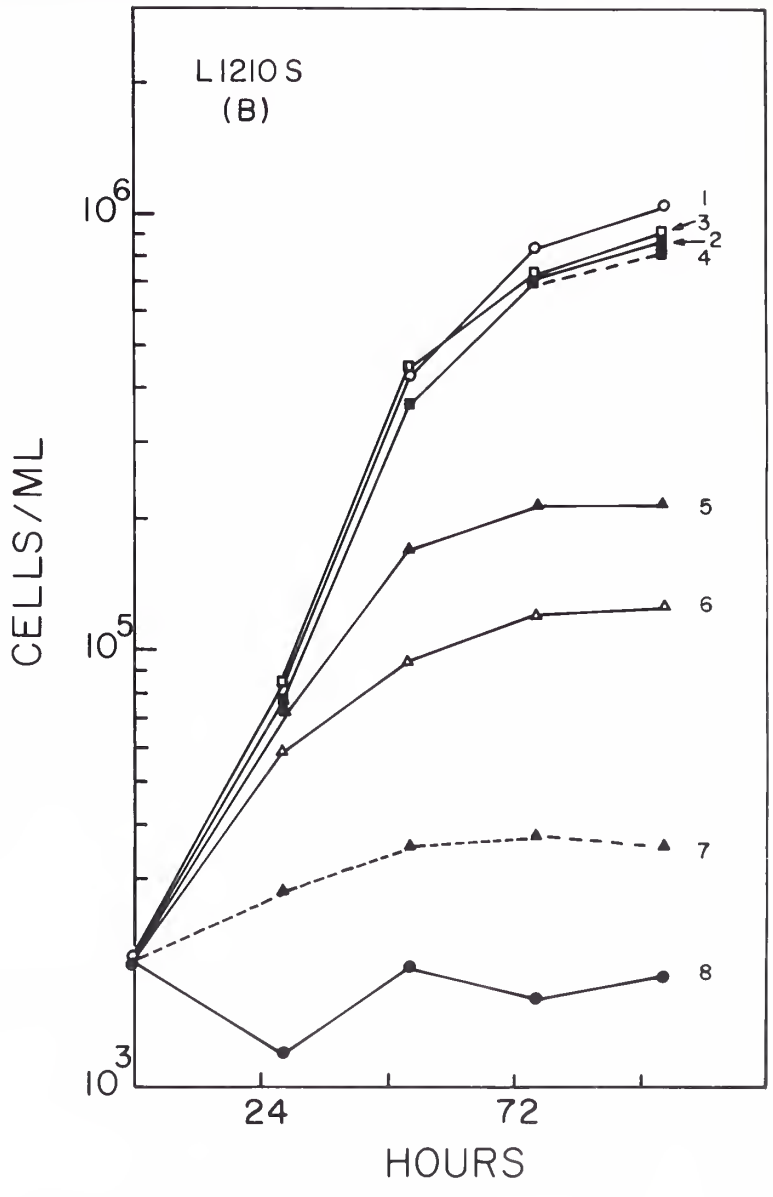


Figure 9. Methionine Dependence Tissue Culture Experiment.
See Legend.

Discussion

While 5MTHF did not appear to preferentially reverse MTX-induced inhibition of DNA synthesis in normal cells vs. malignant cells studied in UdR uptake experiments (Tables II and III), a marked degree of differential reversal was suggested by two of the tissue culture experiments, specifically the low-dose simultaneous rescue experiment (Figure 7, Charts (A), (C), and (E)) and the high-dose delayed rescue experiment (Figure 8, Charts (B) and (E)). Although normal cells could not be included in the long-term tissue culture growth studies primarily because of longer generation times, the differences in reversibility of MTX-induced growth inhibition between LV and 5MTHF seen in these two studies were significant. Both experiments in which differential reversal by 5MTHF was suggested were performed using MTX levels and drug exposure times which were intermediate between two extremes. At these two extremes, preferential reversal of malignant cells by LV as compared with 5MTHF was not seen (Figure 7, Charts (B), (D), and (F); Figure 8, Charts (A) and (D)).

DNA Synthesis Studies

In the studies described in Tables II and III, all of the uptake experiments were performed at a MTX level of at least 2 μM, which should have enabled achievement of a steady state intracellular drug level well within the 60 minute incubation time during which cells

were exposed to MTX (p. 17, paragraph 1).

That free intracellular MTX is required for maximal inhibition of DNA synthesis is exemplified by studies E and F (Table II). When cells were washed free of MTX and resuspended in MTX-free medium, resumption of DNA synthesis occurred at or near control rates regardless of the type or even presence of rescue drug (LV or 5MTHF) added after the cells had been washed. This effect, in view of the previous discussion of MTX transport and the need for intracellular free drug for maximal toxicity, is presumably due to efflux of intracellular drug into the medium during washing.

Both LV and 5MTHF are competitive inhibitors of MTX uptake. Although the ultimate steady state concentration of MTX intracellularly will be the same at any given external MTX level, the addition of increasing amounts of competitive inhibitor will slow the velocity of MTX influx, necessitating a longer period for achievement of steady state levels and of the highest attainable levels of free MTX inside the cell.

Using simple Michaelis-Menten equations for the velocity of an enzyme-substrate reaction with and without the presence of competitive inhibitor, it is relatively simple to calculate v_R/v, the ratio of velocity of reaction with inhibitor present (at R times the substrate concentration) to velocity with no inhibitor present.

The algebraically derived formula is as follows: if [I] is the concentration of competitive inhibitor and [S] the concentration of

substrate (MTX), with [I] = R[S], the ratio is

$$\frac{v_R}{v} = \frac{K_i(K_m + 1)}{K_i(K_m + 1) + R(K_m)}$$

In this equation, K_m is the Michaelis constant for the carrier and MTX, and K_i is the inhibitor constant for competitive inhibitor, LV or 5MTHF (Table I). Thus for concentrations of competitive inhibitor equal to 1, 10, and 100 times the MTX concentration, as used in the UdR experiments, it is possible to calculate the theoretical fraction of uninhibited velocity of uptake occurring in the presence of one particular concentration of competitive inhibitor with a specific K_i. These results for the rescue drugs LV and 5MTHF present at the indicated molar ratios to MTX are given in Table Va. The inverse of these values, given in Table Vb, shows the number of times longer required for the achievement of steady state MTX levels in the presence of inhibitor as compared to the 20 to 30 minutes required with no inhibitor. In these tables, 5MTHF appears to be an even better competitive inhibitor for transport across the membrane than LV: its K_i is less and thus a longer period of time is required to reach steady state than at an equal concentration of LV.

Using these calculations, we observe that in the UdR experiments and in the tissue culture experiments there will result a decreased velocity of MTX transport into the cell whenever LV or 5MTHF is present in the medium <u>simultaneously</u> with MTX, such as the simultaneous rescue

Table Va. Fractional MTX Uptake Velocity With Competitive Inhibitor (L1210)

Inhibitor	v_R/v R=0	R=1	R=10	R=100
5MTHF	1	0.357	0.053	0.006
LV	1	0.585	0.124	0.014

Table Vb. Number of Times Longer Required To Achieve MTX Steady State With Inhibitor (L1210)

Inhibitor	R=0	R=1	R=10	R=100
5MTHF	1	2.80	18.9	167
LV	1	1.71	8.1	71

experiments. This decrease in velocity of uptake, as suggested in Tables Va and Vb, will vary with the magnitude of the concentration difference between inhibitor and drug. If inhibitor is therefore present at equal molar ratios to MTX, achievement of steady state MTX levels will ideally require 1.7 to 2.8 times longer to achieve than without inhibitor; at 10 times the MTX concentration, 8 to 19 times longer, and at 100 times the MTX concentration, 71 to 167 times longer. These values are calculated neglecting other minor factors affecting transport, but are indeed illustrative of the probable magnitude of the effects of competitive inhibitor on MTX uptake.

At 2 μM MTX, which in L1210 cells would cause achievement of an intracellular steady state drug level of about 1.5 μM (Table I), it would ideally take several hours to a few days to achieve this level at the various molar ratios of LV and 5MTHF to MTX used in the uptake experiments. The reversal reported in the simultaneous rescue experiments in Table II and III is therefore due in large part to competition by LV or by 5MTHF with MTX for uptake into the cell: higher rates of UdR incorporation into DNA at LV or 5MTHF levels equal to 10 or 100 times the MTX level probably represent <u>prevention</u> of toxicity rather than actual <u>recovery</u> from toxicity, or "rescue". In some cases, however, incorporation still does not quite return to control rates at the highest levels of LV or 5MTHF used, indicating at least a partial effect of MTX on inhibiting UdR incorporation.

The delayed rescue experiments represent conditions in which MTX has presumably reached steady state concentrations, since cells have

been permitted to equilibrate with MTX alone before the addition of reduced folate. Again, addition of large amounts of LV or of 5MTHF (100 times the MTX level) would probably cause substantial efflux of free intracellular MTX and thus result in significant reversal. Earlier (p. 18, paragraph 1) it was noted that 125 μM LV added to L1210 cells in equilibrium with 2 μM MTX caused complete efflux of the freely exchangeable portion of MTX within 20 minutes[38]. The lower levels of rescue drug used in the delayed rescue experiments (i.e., equimolar and 10-fold compared with MTX) probably reverse MTX inhibition also by this mechanism of stimulating efflux of intracellular drug, but probably to a lesser extent. Pharmacologic rescue, i.e., repletion of the reduced folate supply, may play a more significant role in reversal seen at these lower levels.

Human acute leukemia cells may be less permeable to MTX than animal cells. Recently, Bender et al.[4] have found that at least 160 minutes were required for 1 μM MTX to produce the steady state levels seen in human AML blasts, and that efflux from preloaded cells required 80 to 120 minutes. As a result, some of the uptake values given in Table III for delayed rescue of human cells may reflect preservation of DNA synthetic ability by insufficient time allowed for achievement of the fullest possible inhibition of UdR into DNA.

In summary, three principal modes of "reversal" of MTX toxicity are possible when reduced folates such as LV or 5MTHF are administered to cells in the UdR uptake experiments. Under conditions where folate

is added simultaneously with MTX to cells, especially at higher molar ratios of folate to MTX, there is probably significant competition for transport by the folates, and cells are protected from rapid achievement of high intracellular MTX levels. Under delayed rescue conditions, moderate or large quantities of LV and 5MTHF will again affect transport, but probably more by stimulation of efflux of free intracellular MTX to an appreciable extent. Finally, in the delayed rescue conditions in which 5MTHF or LV are added at lower concentrations, pharmacologic rescue probably contributes significantly to the reversal observed, primarily since the other two factors cannot predominate to as great an extent.

In the majority of the experiments performed and reported in Tables II and III, 5MTHF and LV are approximately equivalent in causing reversal through each of the above mechanisms, and work equally well in normal and in malignant cells in vitro. In short term culture, therefore, where DNA synthesis is used as a parameter reflecting cell viability, 5MTHF does not appear to preferentially rescue only normal cells from MTX.

Inhibition of Cell Growth in Tissue Culture

Two experiments were performed in which cell growth over 5 days was used as a parameter reflecting viability. The low-dose experiments used an MTX level of 0.04 μM, and the high-dose experiment used an MTX level of 2 μM. These results, as previously described, are shown in Figures 7 and 8, respectively.

Two extremes of growth patterns were seen in these experiments. Cells incubated for 24 hours with low doses of MTX were able to resume normal growth rates immediately upon removal of MTX from the medium. In contrast, cells grown at the higher dose of MTX for a full 5 day period in the presence of equimolar or 10-fold LV or 5MTHF did not grow appreciably better than cells in MTX alone. In the former case (Figure 7, Charts (B), (D), and (F)), the MTX level times exposure time appeared to be inadequate for substantial cell kill. In the latter case (Figure 8, Charts (A) and (D)), the MTX level times exposure time appeared to be severe enough to enable total cessation of growth even if LV or 5MTHF were present simultaneously with antifolate at 10 times its concentration.

Chello and Bertino[23] showed that exposure of L5178Y cells to only 1 μM MTX for 6 hours resulted in a 95-97% loss of viability. In view of this observation, it is probable that levels reached in the simultaneous high-dose experiment were high enough to kill most of the cells. Intracellular MTX levels reached during the delayed part of the low-dose experiment, on the other hand, are probably insufficient to completely inhibit DHFR: at MTX levels below 0.1 μM externally, there is no inhibition of UdR incorporation into DNA of L1210 cells, and DHFR is not fully saturated[108]. Approximately 10% to 20% of the DHFR binding sites in L1210 cells will in fact be occupied by MTX during steady state with an external drug concentration of 0.04 μM, as in the low-dose experiment[108]. In human lymphoblasts, Chello et al.[22] showed that a 0.01 μM external MTX concentration

caused no growth inhibition, and that approximately six times this concentration was necessary to effect even a 50% inhibition. As Table IV illustrates, cell number actually increased in all three lines between the start and end of the 24 hour incubation in low-dose MTX.

LV and 5MTHF do not work differently in reversing toxicity and growth inhibition in the simultaneous high-dose experiment or in the delayed low-dose experiment. In the former case, MTX levels are probably high enough to significantly inhibit growth even in the presence of 10 times as much reduced folate. In the latter case, the simple removal of MTX from the cells after 24 hours results in growth resumption, even if they are resuspended in ordinary medium containing no LV or 5MTHF (Curves (2), Charts (B), (D), and (F), Figure 7).

Differential rescue between LV and 5MTHF is seen in the other two major groups of experiments, high-dose delayed and low-dose simultaneous rescue. Presumably the former condition, resulting in exposure of cells to the high-dose level for only 4 hours followed by complete removal of MTX, is not toxic enough to kill most cells, and the latter condition, in which low-dose drug is permitted to remain with the cells for 5 days rather than for only 24 hours, results in levels high enough to at least substantially inhibit growth during the 5 day period (Curves (2), Charts (A), (C), and (E), Figure 7).

In both of these cases, simultaneous low-dose and delayed high-dose, LV at equimolar or 10-fold amounts compared with MTX enables substantial resumption of cell growth in the face of MTX. Neither equimolar nor 10-fold 5MTHF causes any resumption of growth whatsoever over the MTX control curve in the low-dose simultaneous experiment, while 10-fold 5MTHF in the high-dose delayed experiment results in some relief of growth inhibition but not nearly as well as LV. Equimolar 5MTHF in the high-dose experiment cannot relieve the inhibition to any extent (see Charts (A), (C), and (E) in Figure 7, and Charts (B) and (E) in Figure 8).

Therefore, where the presence of LV vs. 5MTHF does make a difference in counteracting MTX inhibition of growth in tissue culture (i.e., in the simultaneous rescue low-dose and delayed rescue high-dose experiments), 5MTHF does not rescue cells as well as MTX. We have not used normal cells for comparison in tissue culture, but the facts obtained thus far are in support of Halpern's findings regarding rescuability of malignant cells by LV vs. 5MTHF[50].

Halpern et al.[2,49] suggested that lower levels of the enzyme methionine synthetase found in malignant cells did not allow sufficient regeneration of THF from 5MTHF to counteract inhibition of DHFR by MTX. Normal cells, presumably possessing higher activity of the enzyme[5,69,86,87], could better regenerate reduced folate and resume normal growth. We initiated our studies to determine if this difference between rescue of normal and malignant cells by 5MTHF could be demonstrated in short

and long term cultures of various mammalian neoplastic cells.

In short term culture, studying the incorporation of UdR into DNA of malignant rodent and human cells and in non-malignant human marrow stem cells, 5MTHF and LV appeared comparable in their ability to reverse MTX toxicity through the combined efforts of competitive inhibition for uptake, enhancement of free intracellular MTX efflux, and pharmacologic rescue. In long term tissue culture, at levels of MTX dose and exposure time between two extremes, malignant cell growth was significantly better reversed by LV than 5MTHF.

This differential rescue effect may be due either to better competition for uptake by LV than 5MTHF, to better enhancement of efflux of MTX, to better pharmacologic rescue, or to a combination of these. The first two alternatives are unlikely as major causes of this differential effect, since the K_i of 5MTHF for the membrane carrier is at least half the value of LV's. Furthermore, UdR studies at high levels of rescue drug in simultaneous experiments and in delayed experiments reveal comparable effects of LV and 5MTHF on the handling of MTX by the membrane transport system.

In light of these facts, the remaining cause of differential rescue may lie in preferred utilization of LV over 5MTHF by the cell. Such a difference in utilization may like in a deficiency of methionine synthetase in malignant cells, or in other factors.

Recently, Hoffman and Erbe[55] have shown that several malignant cell lines including Walker 256 and two virally transformed human cell

lines have an absolute requirement for methionine for growth, while the nonmalignant cells studied grew well in methionine-deficient medium supplied with homocysteine. However, the malignant cell lines were found to be capable of synthesizing methionine from homocysteine at rates at least as high as those of normal cells, so long as a trace amount of exogenous methionine was present. Thus, while exogenous methionine is required for growth by malignant cells, the reason for this requirement appears not to be deficient ability to synthesize methionine from homocysteine. Also, it is known that L5178Y cells behave similarly: in the presence of high concentrations of vitamin B_{12}, especially with the B_{12} transport protein TC-II, folate requirements for these cells are met entirely by low concentrations of either 5MTHF or LV[21], despite the fact that this cell line has a requirement for exogenous methionine that cannot be spared by addition of vitamin B_{12}, TC-II, 5MTHF, and homocysteine.

Since deficient methionine synthetase activity and thus diminished potential to regenerate THF from 5MTHF is probably not a major factor in accounting for differential rescue by 5MTHF, the difference must lie in other aspects of utilization of the molecule. Recently, with the demonstration of the importance of polylgutamyl forms of reduced folates in mammalian cells[26,78,85], Hoffbrand et al.[54] have shown that far more radioactivity is found in the form of polyglutamate residues when labelled LV rather than labelled folic acid is administered to dividing human lymphocytes. Even though MTX decreased the rate of

polyglutamate formation from folic acid, there was no effect whatsoever by MTX on incorporation of LV monoglutamate into polyglutamate folate derivatives. Also, addition of labelled 5MTHF to dividing human lymphocytes resulted in no detectable polyglutamate radioactivity for up to 72 hours[77], suggesting that polyglutamyl forms of folate cannot be made directly from 5MTHF. The differential ability of LV to rescue malignant cells better than 5MTHF may lie to some extent in preferential incorporation of LV into the reduced folate polyglutamate pool, thus enabling it to more rapidly assume a metabolically active form.

In vivo studies by Mead[83] several years ago demonstrated that 5MTHF administered simultaneously with MTX to L1210-bearing mice caused reversal of antileukemic activity of MTX, comparable to that obtained with equivalent doses of LV. Delayed rescue of from 12 to 24 hours using both LV and 5MTHF in vivo showed reversal of toxic effects of MTX to approximately equal extents, and these authors concluded that "there is apparently no advantage to be obtained by the use of prefolic A (5MTHF) instead of citrovorum factor under these experimental conditions". The doses of MTX used in this work, however, produced serum levels of 0.1 mM or better, far outside the realm of concentrations used in the current studies in which we observed differential rescue effects.

Sauer and Jaenicke[101] recently demonstrated that 5MTHF at a concentration of 1 mM could reverse the effects of 10^4 times less MTX in

human lymphoblastic cells *in vitro* with respect to both inhibition of cell growth as well as with respect to changes in incorporation of TdR into DNA. These studies, however, were only performed in the simultaneous rescue fashion, and it is apparent that 10^4 times more competitive inhibitor (5MTHF) would seriously hinder uptake of MTX by the cells.

The current studies suggest several further investigative procedures to evaluate differential rescue of MTX by 5MTHF. Duplicating to some extent the tissue culture studies in which the results were obtained, it may be likely that simultaneous infusion of low doses of MTX (in the range of 0.04 μM) with equimolar 5MTHF over an extended period of time may be a viable protocol to test *in vivo* for improved response to L1210 and other tumors in mice. Similarly, infusion of higher doses of MTX, on the order of a serum concentration of 2 μM, followed within 4 to 6 hours by equimolar 5MTHF in a continuous infusion for several days may be also a worthwhile chemotherapeutic protocol to investigate *in vivo*.

It is known that LV or folic acid given orally or intravenously is rapidly converted to a large extent to 5MTHF, the major folate transport form in serum[90,93]. However, the molar ratios of 5MTHF and LV as compared with MTX used in current moderate-dose and high-dose protocols seldom exceed 0.25 to 0.50.

The two possible test protocols outlined above will be far more difficult to achieve and maintain *in vivo* due to time required for distribution of drug in total body water as well as effects of

metabolism and excretion, but a relatively great deal is known about the fate of MTX and reduced folates in man, so it should be possible to devise protocols in which an average, constant concentration could be maintained within reasonable limits for the periods outlined above.

Low-dose or moderate-dose MTX chemotherapy followed by equimolar 5MTHF rescue appears to be a viable new protocol to further evaluate chemotherapeutic management of animal and human neoplasms, especially the leukemias.

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