Mechanism of Synergistic Cell Killing when Methotrexate Precedes Cytosine Arabinoside

STUDY OF L1210 AND HUMAN LEUKEMIC CELLS

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ABSTRACT Synergistic killing of L1210 cells occurs when methotrexate (MTX) is administered just before 1-β-D-arabinofuranosylcytosine (Ara-C). This phenomenon is dependent upon both the dose and time of exposure to MTX. Such increased killing of cells can be explained by the enhanced intracellular accumulation of Ara-C in cells exposed to MTX. This enhancement of Ara-C entry into cells was only observed when the dose of MTX was high enough (1, 10, and 100 μM) to result in free intracellular non-dihydrofolate reductase-bound MTX. At the highest doses of MTX (10 and 100 μM) Ara-C triphosphate was increased eightfold and deoxycytidine triphosphate was decreased by 50%. Therefore, the maximum synergistic cell kill when MTX precedes Ara-C may be the consequence of greater inhibition of DNA polymerase by the increased Ara-C triphosphate in the presence of the decreasing natural substrate of this enzyme, deoxycytidine triphosphate. Enhanced Ara-C accumulation after administration of MTX was also observed in human acute myelogenous leukemia cells.

INTRODUCTION

Methotrexate (MTX)[1] and 1-β-D-arabinofuranosylcytosine (Ara-C) are chemotherapeutic agents often used together for the treatment of human neoplasms. Considerable controversy exists over whether these drugs are synergistic (1–3) or antagonistic (4–6) when given together or sequentially.

One focus of attention has been the change in deoxycytidine triphosphate (dCTP) pools after MTX. This occurs because the phosphorylation of Ara-C by deoxycytidine kinase can be inhibited by dCTP (7, 8). Therefore, drugs which would lower dCTP pools would be expected to produce greater quantities of Ara-C monophosphate (Ara-CMP) and ultimately Ara-C triphosphate (Ara-CTP), the lethal form of Ara-C, and to be synergistic in their antitumor activity. Conversely, drugs which produced greater quantities of dCTP would be expected to generate less Ara-CTP and to be antagonistic when given with Ara-C. Also, because both nucleotides dCTP and Ara-CTP compete for DNA polymerase, alterations of these nucleotides can affect the inhibition of this enzyme by Ara-CTP (9).

This study was designed to examine the biological and biochemical consequences of MTX and Ara-C combinations in L1210 and human leukemic cells.

METHODS

Cells. L1210 murine leukemia cells were maintained as suspension culture in Fischer's medium plus 10% horse serum, transferred twice weekly, and kept at 37°C in a 5% CO_2 atmosphere. After obtaining informed consent, blood from untreated human leukemic patients was collected in heparinized tubes by venipuncture, suspended in equal volumes of 3% dextran, and sedimented at a 45° angle for 30 min to remove erythrocytes. Microscopic examination of the separated leukemic cells revealed a 1% persistence of erythrocytes. Separated erythrocytes did not appreciably concentrate Ara-C with or without MTX pretreatment; therefore, this 1% contamination does not significantly alter the validity of the experiments subsequently performed. The leukemic cells were transferred into incubation medium composed of 80% Fischer's culture medium, 10% 1.25 M Hepes buffer (pH 7.4), and 10% horse serum to achieve a cell concentration of 5×10^7 cells/ml.

Drugs. MTX was provided by the Drug Development Branch of the National Cancer Institute, Bethesda, Md. Ara-C was obtained from The Upjohn Co., Kalamazoo, Mich.

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[1] *Abbreviations used in this paper*: Ara-C, 1-β-D-arabinofuranosylcytosine; Ara-CMP, Ara-C monophosphate; Ara-CTP, Ara-C triphosphate; dCTP, deoxycytidine triphosphate; DHFR, dihydrofolate reductase; dTMP, deoxythymidine monophosphate; dTTP, deoxythymidine triphosphate; dUMP, deoxyuridine monophosphate; MTX, methotrexate; MTX_f, free intracellular MTX; THF, tetrahydrofolate; UdR, deoxyuridine.

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[^{3}H-(G)]Ara-C (13.2 mCi/mmol) and [6-^{3}H]deoxyuridine (UdR) (21.9 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. [3′,5′,9(*n*)-^{3}H]MTX (17 Ci/mmol) was procured from Amersham Corp., Arlington Heights, Ill.

Cloning. The biological antitumor effect of MTX and Ara-C was determined by cloning L1210 cells in soft agar by the technique we previously reported (10). After appropriate single drug exposure, the suspension culture was centrifuged at 100 *g* for 5 min at 37°C. The drug-containing supernate was discarded, the cell pellet was resuspended in drug-free Fischer's medium, and then recentrifuged as before. This washing procedure was repeated twice to remove any extracellular drug before cloning. In sequential drug studies, the second drug was added to the cell suspension after a variable exposure to the first drug. After the designated time, the washing sequence was repeated and the cells placed in cloning medium.

The cloning procedure involved placing 100 cells into 10-ml culture tubes which contained 2 ml of liquified agar (37°C) and 3 ml of drug-free Fischer's medium that contained 10% horse serum. The tubes were capped and placed upright and incubated at 37°C in a 5% CO_2 atmosphere. The agar-medium solution was of such viscosity as to prevent cell settling. Viability was defined by a cell's ability to divide and produce progeny, which after 7 d were visible as individual cell colonies or clones. Percent viability, then, is the ratio of clones formed from drug-treated suspension cultures to clones formed from untreated cultures times 100. The cloning efficiency of L1210 in this system was 85%.

Nucleotide pools. The ribonucleotide pools were evaluated of logarithmically growing L1210 cells (100 ml at 2–4 × 10^5 cells/ml) exposed to 3 h of MTX at concentrations from 0.01 to 100 μM. After exposure to MTX, Ara-C (1.1 μCi/nmol) was added, achieving a concentration of 0.5 μM. 1 h later, the cells were centrifuged at 1,000 *g* for 10 min at 4°C, the supernate discarded, and the cell pellet washed twice with 0.9% NaCl at 4°C before being precipitated in 2 ml of 10% TCA. The supernate that contained the ribonucleotides was extracted three times with 6 ml of ether to remove the TCA and stored at −20°C until all samples could be evaluated on the same day. Separation of the triphosphate ribonucleotides was by high-pressure liquid chromatography on a Du Pont model 640 system (Du Pont Co., Instruments Div., Wilmington, Del.) with a linear gradient (0.01–1.0 M) of sodium phosphate buffer (pH 3.31) with a flow rate of 0.9 ml/min on a 25-cm × 4.6-mm Partisil SAX (10 μm particle size) column. Absorbance was recorded at 254 and 280 nm, and 30-s collections were obtained for radiochromatogram correlations. The data is expressed as picomoles of Ara-CTP per 10^6 cells.

dCTP and deoxythymidine triphosphate (dTTP) isolation and quantitation were performed by the method of Solter and Handschumacher (11). Suspension cultures of L1210 cells dividing at a logarithmic rate were studied after a 3-h exposure to MTX (0.01–100 μM).

UdR incorporation. Logarithmically growing L1210 cells were centrifuged as before and resuspended to a cell concentration of 1–5 × 10^7 cells/ml. At designated times after adding [^{3}H]UdR (21.9 Ci/mmol), 0.04 ml of the cell suspension was placed in harvesting plates (Microbiological Associates, Walkersville, Md.) and automatically suctioned onto glass filter strips by use of a MASH cell harvester (Microbiological Associates). The acid-insoluble fraction was then precipitated on the filter strips with a 10-ml solution of 10% TCA and washed twiced with distilled H_2O to remove any nonprecipitated label. After room drying, the cell-precipitated areas were placed in glass minicounting vials, 3 ml of OCS added (Amersham Corp.), and radioactivity quantitated with a liquid scintillation spectrometer.

Drug uptake studies. 0.01–100 μM of MTX was added to suspension cultures of L1210 in logarithmic growth (inoculated at 1 × 10^4 cells/ml and used at 2–5 × 10^5 cells/ml). After 1, 3, and 6 h, 250 ml of the cell suspension was centrifuged at 100 *g* for 5 min. The drug-containing supernate was then used to resuspend the cells to a 6-ml vol at a concentration of ≅3 × 10^7 cells/ml. This cell suspension was placed in a 25-ml stoppered flask and incubated in a 37°C shaker water bath. A 0.3-ml solution of [^{3}H]Ara-C (1.1 μCi/nmol) was then added, giving a final Ara-C concentration of 0.5 μM. 1 ml of the cell suspension was removed at designated times and centrifuged at 1,000 *g* for 5 min in a Wintrobe sedimentation tube. The supernate was removed, and radioactivity was determined in a 0.2-ml sample (drug in medium). The inside of the tube that contained the cell pellet was washed gently twice without disturbing the cell pellet with 0.9% NaCl at 4°C to remove any radioactivity remaining on the side walls of the tube. The undisturbed cell pellet was then precipitated with 0.5 ml of 3.5% $HClO_4$, releasing the intracellular radiolabeled drug into this supernate which was removed after repeat centrifugation of cellular debris at 1,000 *g* for 5 min, and radioactivity was determined in a 0.1-ml sample (drug in cells). The ratio of intracellular drug was calculated as previously described by Handschumacher and Fischer (12) after appropriate corrections for drug loss from the cell pack during the washing procedure and for the interstitial space of the cell pack which had drug-containing medium. The fraction of the cell pellet which contained medium was 30% of the cell pack volume as determined by [^{14}C]inulin (1 μCi/100 μg). The identical procedure was performed with [^{3}H]MTX to determine the intracellular accumulation of MTX. The intracellular accumulation of [^{3}H]Ara-C in human leukemia cells was performed in the same fashion. Identical cloning procedures as outlined above were performed on a sample of cells which had been concentrated from 2–5 × 10^5 to 2–5 × 10^7 cells/ml before adding Ara-C for 1 h. This was to establish that the concentration of cells for the duration of the drug uptake studies did not adversely affect cell viability.

The intracellular accumulation of [^{3}H]Ara-C is expressed both as the ratio of the concentration of Ara-C inside the cells to that in the medium and as picomoles per 10^6 cells. Approximately 90% of the intracellular Ara-C is in the form of nucleotide derivatives; only 10% being the unmetabolized nucleoside, Ara-C.

Free intracellular MTX. L1210 suspension cultures were exposed to MTX (0.01–100 μM) for variable times during logarithmic growth (250 ml at 2–5 × 10^5 cells/ml). The cells were separated from the drug-containing medium by centrifuging at 1,000 *g* for 10 min at 4°C. 1.0 ml of a Tris/KCl solution (0.05 M/0.1 M, pH 7.0), which contained 10% glycerol, was added to the cell pellet. The cells in this suspension were lysed, releasing intracellular MTX into the solution by freeze-thawing three times. The cellular debris was sedimented by centrifuging at 10,000 *g* for 10 min. The supernate was removed, and the quantity of unbound or free intracellular MTX (MTX_f) was determined by the enzymatic inhibition of dihydrofolate reductase (DHFR) method previously described (13). The same enzymatic method was used to determine the specific activity of DHFR. Protein content was established by the method of Lowry et al. (14).

RESULTS

L1210 cells

Cloning. The biological antitumor effect of MTX and Ara-C was evaluated in the soft agar cloning system.

When MTX was present in the suspension culture at 0.1, 1, and 10 μM for 9 h before cloning in drug-free medium, the resulting percent viability was 100, 50, and 20%, respectively. Administration of Ara-C (1 μM) for 1 h before cloning resulted in 100% cell viability; when MTX was present in the suspension culture from 1 to 8 h before the 1-h exposure to Ara-C, the result was a greater killing of cells (Fig. 1). When Ara-C preceded the MTX for 1 h, viability was identical to that observed with MTX alone.

Enhanced Ara-C entry. One mechanism of this synergism in cell kill might be enhanced accumulation of Ara-C in MTX-treated cells. The accumulation rate of Ara-C into the acid-soluble fraction of cells was examined. A 1-h exposure to MTX (1 μM) did not alter the Ara-C accumulation from the control rate. However, after a 3-h exposure to MTX (1 μM) the rate of Ara-C accumulation was twice that of the control rate. Ara-C accumulation was increased further to three times the control rate after a 6-h exposure to MTX (1 μM) (Fig. 2).

Similar experiments were done after a 3-h exposure to MTX at concentrations from 0.1 to 100 μM. With increasing MTX concentrations there was an increasing rate of accumulation of Ara-C. Of note, however, is that the rate of Ara-C accumulation was actually less than

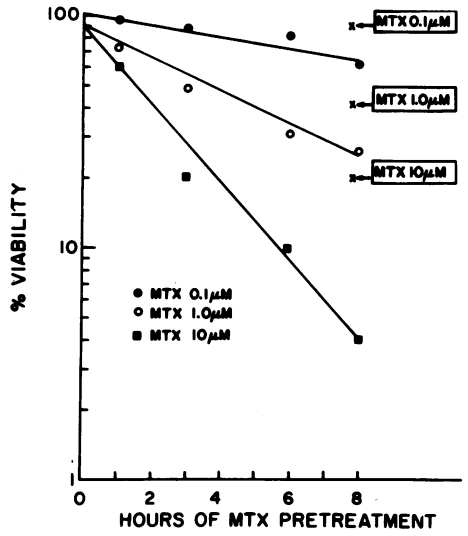


FIGURE 1 Viability of L1210 cells. To logarithmically growing L1210 cells was added MTX at the designated concentrations. The abscissa is the time of MTX exposure before adding Ara-C (1 μM). 1 h after the addition of Ara-C, the cells were cloned as described in Methods. The experiment was done in triplicate three times, and the variability was <5%. Viability after 9 h of only MTX at 0.1, 1.0, and 10 μM is indicated in the boxes. The Ara-C (1 μM) control for 1 h resulted in 100% viability and is not shown. At all doses of MTX pretreatment, synergistic cell killing was observed which was maximum at the highest dose of MTX. At each dose of MTX, this synergistic cell kill was enhanced by increasing the exposure time to MTX.

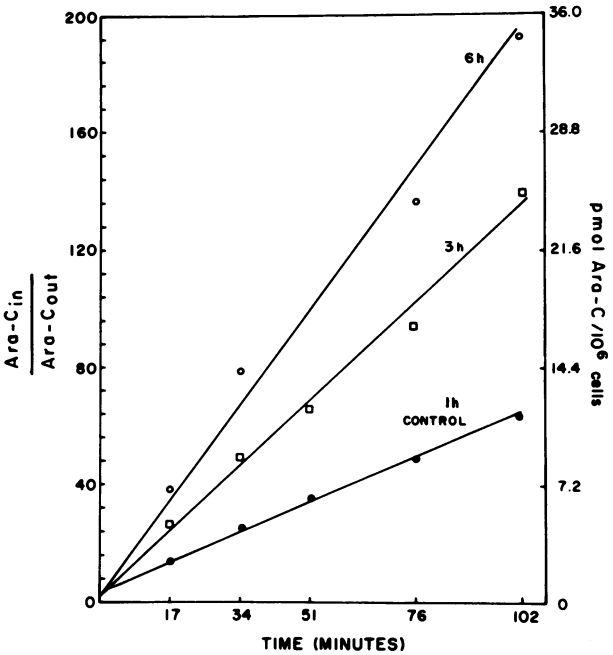


FIGURE 2 Rate of intracellular accumulation of [^{3}H]Ara-C in L1210 cells. Accumulation of [^{3}H]Ara-C was examined in logarithmically growing cells after the addition of 1 μM of MTX for 1, 3, and 6 h to the suspension cultures. The accumulation of intracellular Ara-C is expressed as a ratio of the amount of Ara-C in the cell to the amount of Ara-C in the medium on the left ordinate and as picomoles of Ara-C per 10^6 cells on the right ordinate. The rate of [^{3}H]Ara-C accumulation in the acid-soluble fraction after a 1 h exposure to MTX was identical to control (●). Augmentation of this rate was seen after 3 h of MTX (□) and was maximum after a 6-h exposure of MTX (○). Therefore, the augmented entry of Ara-C into L1210 cells after MTX treatment is time dependent.

control rates when MTX was 0.01 and 0.1 μM (Fig. 3). It is of interest that in previous reports demonstrating antagonism between MTX and Ara-C the MTX concentration used was 0.1 μM in L5178Y cells by Tattersall and Harrap (5) and 0.02 μM in L1210 cells by Grindey and Nichol (6). Our observation that the rate of entry of Ara-C is less in L1210 cells at these concentrations of MTX could account for their findings. The data in Figs. 2 and 3, therefore, indicate that the time of exposure to and concentration of MTX are important factors which lead to enhanced Ara-C accumulation in L1210 cells. This augmentation of Ara-C accumulation by MTX can be prevented by administration of leucovorin in concentrations which reverse [^{3}H]UdR inhibition (data not shown). This information indicates that the effect of MTX in producing greater accumulation of Ara-C occurs at similar concentrations of MTX as those required for depletion of tetrahydrofolate and the inhibition of the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). Because it has been shown that

the inhibition of the methylation of dUMP requires free intracellular MTX in spite of the high affinity of DHFR for MTX (15, 16), it would be expected that the augmentation of Ara-C uptake by MTX might also require MTX_f.

During the experiment outlined in Fig. 3, MTX_f was examined. MTX_f was only present when extracellular concentrations of MTX were 1, 10, and 100 μM. The relationship between these free intracellular levels of MTX and the rate of Ara-C accumulation in cells is presented in Fig. 4. Augmentation of Ara-C accumulation did not occur until free intracellular levels of MTX were present (MTX_f).

To help establish that MTX_f is necessary for enhanced Ara-C uptake, the MTX_f was allowed to efflux from cells by transferring them to drug-free medium. MTX (10 μM) was added to logarithmically growing suspension cultures of L1210 cells. After a 3-h exposure to this concentration of MTX, the cells were removed from the MTX-containing medium, washed twice with 0.9% NaCl, and then resuspended in drug-free medium. [^{3}H]Ara-C accumulation into the acid-soluble fraction was determined without washing the cells, immediately after this washing procedure, and 1, 2, and 3 h later. Increasing amounts of MTX_f effluxed out of the cells with time. As seen in Fig. 5, the enhancement of [^{3}H]Ara-C accumulation in the acid-soluble cell fraction decreased with time after the cells were placed in MTX-free medium. At the time of these [^{3}H]Ara-C uptake studies, a proportion of the cells were removed and

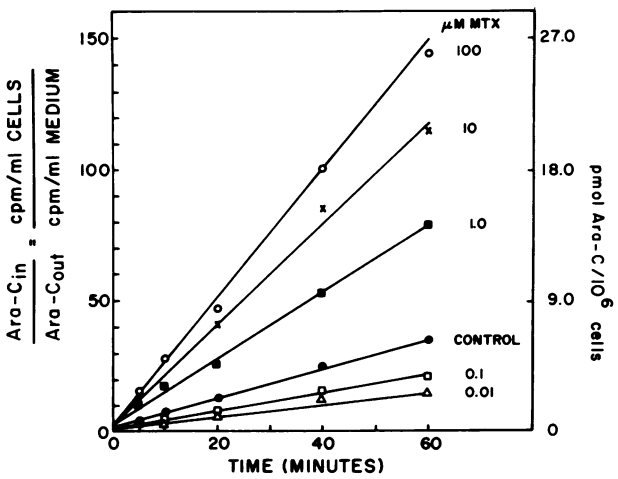


FIGURE 3 Accumulation of [^{3}H]Ara-C into logarithmically growing L1210 cells after a 3-h pretreatment of MTX at varying concentrations. The intracellular accumulation of [^{3}H]Ara-C into the acid-soluble fraction was determined as in Fig. 2. The rate of this accumulation was less than the control rate when MTX in the medium was 0.01 and 0.1 μM. However, the rate of [^{3}H]Ara-C accumulation was enhanced after 1, 10 and 100 μM MTX. Therefore, the augmentation of Ara-C accumulation into L1210 cells is not only time dependent (Fig. 2) but also dose dependent.

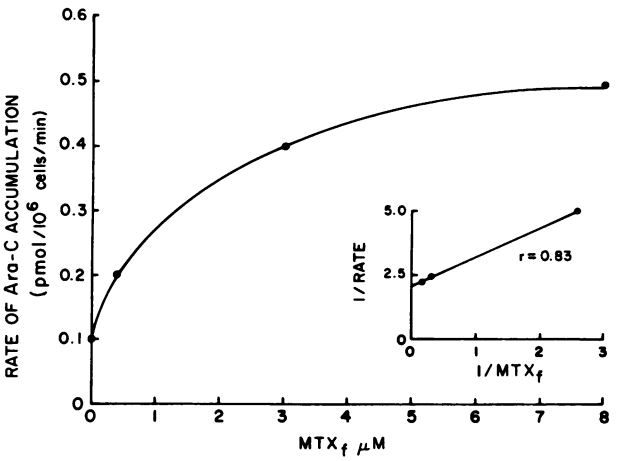


FIGURE 4 The rate of [^{3}H]Ara-C accumulation into cells (pmol/10^6 cells per min) vs. the MTX_f. The concentration of MTX_f (non-DHFR bound) present in the cells from Fig. 3 was evaluated as described in Methods. The maximum rate of Ara-C accumulation occurred after the highest molar concentration of MTX in the medium (100 μM), which corresponds to 8 μM of MTX_f. A plot of 1/rate of Ara-C accumulation vs. $1/MTX_f$ was linear, which indicates that the enhanced accumulation of Ara-C into these MTX-treated cells resembles first-order kinetics.

the MTX_f was determined. The same relationship between [^{3}H]Ara-C entry and MTX_f was observed as just described. The presence of MTX_f was necessary for augmented Ara-C accumulation in cells.

If the biochemical results of the washout experiments are a true reflection of the biological synergism observed in Fig. 1, then such synergism should not occur upon repeat of the cloning experiments after washing of the MTX-treated cells with drug-free medium and allowing a 3-h time period for efflux of MTX_f before adding Ara-C. Indeed, Fig. 6 demonstrates that this washing of MTX-treated cells prevents synergistic killing.

To confirm that MTX_f is absolutely necessary for enhanced Ara-C accumulation, [^{3}H]Ara-C uptake studies were repeated in two MTX-resistant lines of L1210. These mutant lines of L1210 were developed by continuous exposure to MTX. The R line had normal MTX transport, but the specific activity of DHFR was 0.076 μmol tetrahydrofolate (THF)/min per mg protein, 13.5 times the specific activity of the wild or MTX-sensitive (S) line, which was 0.0056 μmol THF/min per mg protein. The RR line did not actively transport MTX. In addition, the specific activity of DHFR was 0.315 μmol THF/min per mg protein. As seen in Fig. 7, augmented [^{3}H]Ara-C accumulation in the R cells occurred only at MTX concentrations which resulted in MTX_f. MTX_f was never present in the RR cell line even at the highest extracellular MTX concentration (100 μM); nor did MTX enhance the rate of Ara-C accumulation in these resistant cells. Therefore, MTX_f

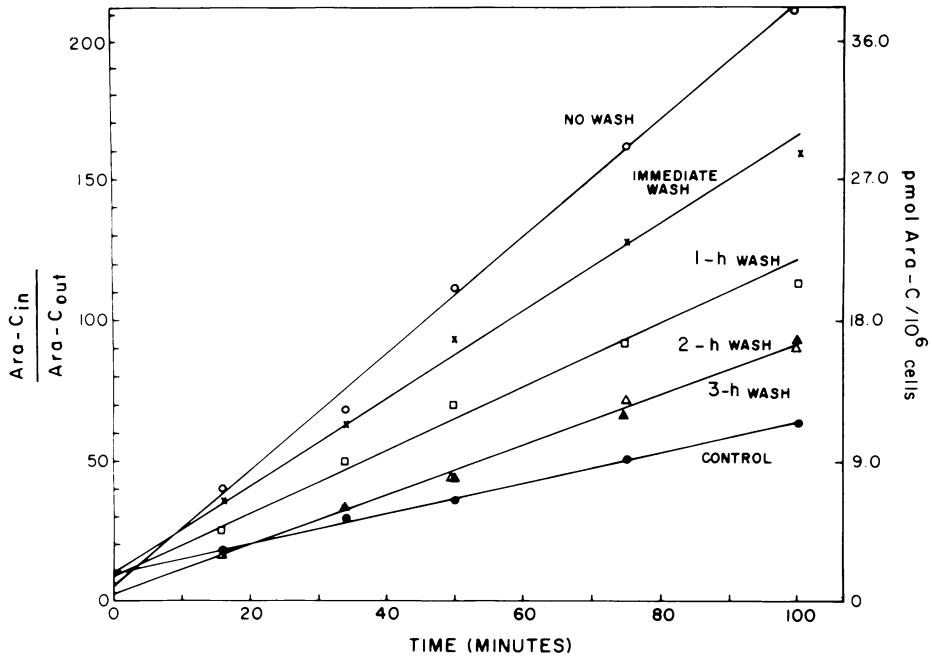
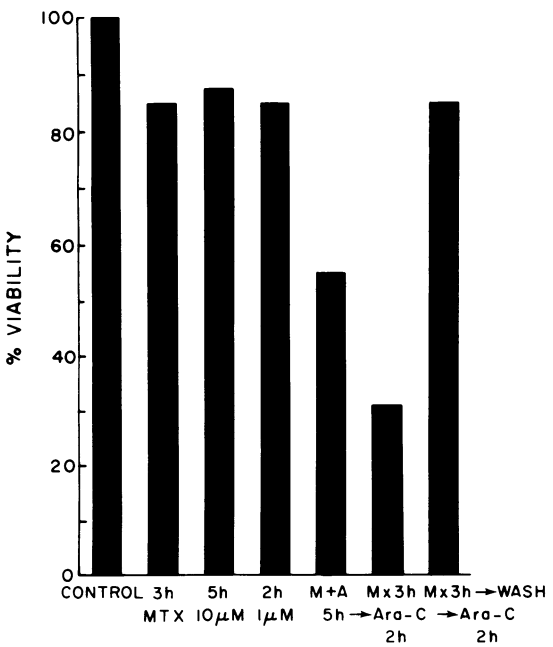


FIGURE 5 Accumulation of [^{3}H]Ara-C in logarithmically growing L1210 cells at various times after removal from MTX (10 μM)-containing medium. Those cells which remained in the MTX medium had the greatest rate of accumulation of [^{3}H]Ara-C (○). The remaining cells were removed from the MTX medium by centrifugation and washed twice in fresh drug-free medium, and the rate of [^{3}H]Ara-C accumulation was subsequently determined after various time intervals in drug-free medium. The rate of [^{3}H]Ara-C accumulation was decreased in all of these conditions; immediately after the washing procedure (X), 1 (□), 2 (△), and 3 h (▲) later. Although the enhanced rate of accumulation decreased after the washing and placing of cells in drug-free medium, it never returned to the control rate (●). MTX_f was also determined just before the addition of [^{3}H]Ara-C; the decreasing rate of [^{3}H]Ara-C accumulation correlated with the decreasing MTX_f as shown with the previous experiment in Fig. 4.



must be present for enhanced intracellular accumulation of Ara-C to occur.

To reconfirm that this observation of enhanced [^{3}H]Ara-C accumulation in cells and the degree of inhibition of the conversion of dUMP to dTMP occur at the same concentration of MTX, the relationship

FIGURE 6 Viability of L1210 following MTX (M) (10 μM) and Ara-C (A) (1 μM): The effect of removing cells from MTX-containing medium before adding Ara-C. Repeat cloning experiments were done in triplicate twice as described in Fig. 1. The viability after a 3- and 5-h exposure to MTX was 85 and 87%, respectively. The viability after 2 h of Ara-C was 85%. When MTX and Ara-C were added simultaneously for a duration of 5 h before cloning, the viability decreased to 55%. If the Ara-C were added for 2 h after a 3-h pretreatment of MTX, the viability was decreased even further to 30%. However, if the cells which had been exposed to MTX for 3 h were washed free of their MTX-containing medium and placed in drug-free medium for 2 h before the addition to a 2-h exposure to Ara-C, the viability rose to 85%. This reversal of the synergistic killing of cells after the removal of cells from the MTX-containing medium confirms the biochemical information presented in Figs. 4 and 5: MTX_f is necessary for maximum killing of cells when Ara-C is given after MTX.

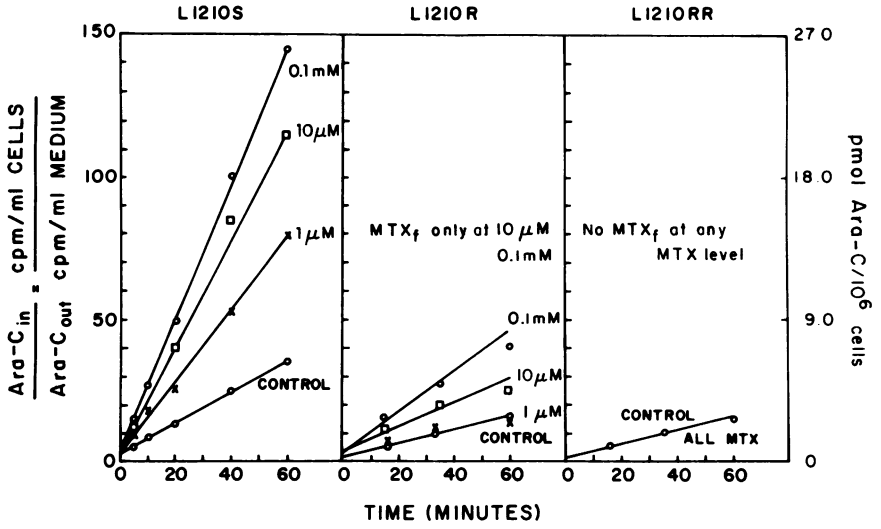


FIGURE 7 Intracellular accumulation of [^{3}H]Ara-C into MTX-resistant lines of L1210. (See text for details of these resistant lines). All cells were exposed for 3 h to varying concentrations of MTX. In the normal or MTX-sensitive line (L1210S) augmentation was greater after increasing MTX doses (×, 1 μM; □, 10 μM; ○, 0.1 mM). Enhanced entry of [^{3}H]Ara-C into L1210R cells occurred at MTX concentrations of 10 (□) and 100 μM (○). MTX_f in these L1210R cells was only present at these concentrations of MTX. Because of the higher specific activity of DHFR in this cell line, 1 μM of MTX was totally bound to DHFR. The L1210RR cells which did not transport MTX had no enhancement of Ara-C accumulation after MTX, and MTX_f was not present even at 0.1 mM of MTX.

of MTX_f and inhibition of [^{3}H]UdR incorporation into acid-insoluble material was determined (Fig. 8). The maximum inhibition of UdR incorporation into acid-insoluble material also occurred with 100 μM MTX in the medium and 8 μM of MTX_f.

Another method of examining the necessity of MTX_f for maximum inhibition of the coversion of dUMP to dTMP is to quantitate dTTP. Inhibition of DHFR by MTX prevents THF production; because of this reduced methyl donor pool (THF) the conversion of dUMP to dTMP is reduced, and, therefore, a reduction in dTTP follows. L1210 cells exposed to MTX (0.01–100 μM) for 3 h were studied. The intracellular pools of dTTP did not decrease until 10 and 100 μM of MTX had been given (Table I). There is little doubt that the greatest inhibition of dTMP synthesis occurs when the levels of MTX_f are greatest. Therefore, a general correlation exists between the concentration of MTX required to reduce dTTP pools and that required to enhance Ara-C accumulation. As previously mentioned, earlier studies reporting antagonism of MTX and Ara-C were performed with concentrations of MTX in the medium that would be insufficient to result in MTX_f levels and maximally inhibit dTMP synthesis.

Nucleotide pools

The lethal form of Ara-C is the triphosphorylated derivative, Ara-CTP. This nucleotide inhibits DNA polymerase and is considered to be the major site of action of Ara-C (17–19). The initial phosphorylation of Ara-C upon entry into the cell is to Ara-CMP by deoxycytidine kinase (7, 8). dCTP can influence the

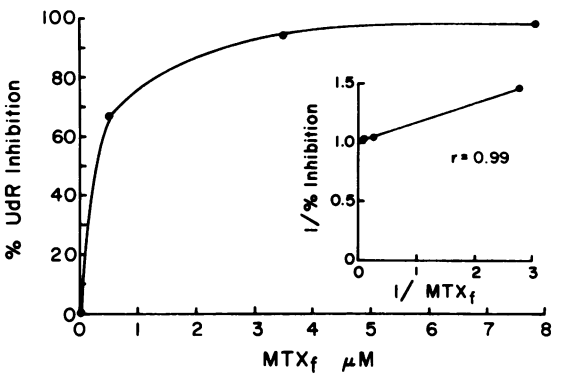


FIGURE 8 Inhibition of [^{3}H]UdR incorporation into the acid-insoluble fraction of L1210S cells vs. MTX_f. L1210 cells were exposed for 3 h at doses of MTX from 0.01 to 100 μM. The rate of appearance of [^{3}H]UdR in acid-insoluble material was determined, and the decrease from the control rate was calculated as percent inhibition (ordinate). Just before the addition of [^{3}H]UdR, a portion of the cells were examined for the amount of MTX_f (abscissa). With increasing MTX_f, the inhibition of [^{3}H]UdR incorporation into acid-insoluble material increased. A plot of 1/inhibition vs. $1/MTX_f$ is represented on the inset. Therefore, MTX_f is necessary for inhibition of [^{3}H]UdR incorporation into acid-insoluble material.

TABLE I
Changes in dTTP, dCTP, and Ara-CTP after Increasing Concentrations of MTX for 3 h

MTX × 3 h	dTTP	dCTP	Ara-CTP	Percentage of Ara-CTP of the total Ara-C in cells
μM	*pmol/10⁶ cells*			%
0	21	20	1.4	20
0.01	32	26	2.0	19
0.1	25	37	2.5	18
1.0	23	30	5.0	30
10.0	14	13	7.7	33
100.0	14	15	8.5	40

MTX at the doses indicated was added to logarithmically growing suspension cultures of L1210 (3×10^5 cells/ml), and after 3 h dTTP, dCTP, and Ara-CTP were quantitated as outlined in Methods. The maximum decrease of dTTP and dCTP occurred after 10 and 100 μM of MTX, which were also the conditions yielding the highest Ara-CTP levels. The maximum range from the mean values shown was ±5%.

initial phosphorylation step of Ara-C by inhibiting deoxycytidine kinase (7, 8) and also compete with Ara-CTP for DNA polymerase. Therefore it is important to evaluate alterations in Ara-CTP and dCTP pools in the context of MTX pretreatment.

Ara-CTP levels were determined in L1210 cells exposed for 3 h to varying concentrations of MTX. Separation and isolation of Ara-CTP was achieved by high-pressure liquid chromatography and quantified by determining the radioactivity present in the fractions collected in the region of the Ara-CTP. Ara-CTP was markedly increased (eightfold) in those cells exposed to 100 μM of MTX and to a lesser extent at lower concentrations of MTX (Table I). Recovery of radiolabel was 99%, with 90% of this present in the nucleotide derivatives Ara-CMP, Ara-C diphosphate, and Ara-CTP.

The changes in the dCTP pools were biphasic. When concentrations of extracellular MTX were 0.01, 0.1, and 1 μM, dCTP values actually increased, but when MTX was 10 and 100 μM the dCTP pools decreased (Table I). A logical interaction of these two triphosphorylated nucleotides, Ara-CTP and dCTP, could help explain the synergistic killing observed in the initial cloning experiments (Fig. 1) and the accumulation of Ara-C into these cells. Specifically, after 0.01 and 0.1 μM MTX, the elevated dCTP pools could have inhibited deoxycytidine kinase and, therefore, the initial phosphorylation of Ara-C to Ara-CMP. This could explain the observed decrease of Ara-C accumulation in cells exposed to MTX at these lower concentrations. The markedly enhanced accumulation of Ara-C in cells exposed to 10 and 100 μM of MTX could partially be the consequence of the decreasing dCTP pools and, subsequently, of less inhibition of deoxycytidine kinase. Similarly, because both Ara-CTP and dCTP would be competing for DNA polymerase, the greatest inhibition of this enzyme would occur when the Ara-CTP was maximally increased and the dCTP was maximally decreased, precisely the situation when cells are exposed to 100 μM of MTX. Therefore, MTX is both antagonistic and synergistic with respect to Ara-C, dependent upon the pretreatment concentration of MTX.

Human leukemia cells

The effect of these interactions between MTX and Ara-C are useful clinically if the same alterations described in L1210 cells also occur in human cancer cells. Two patients with rapidly increasing acute myelogenous leukemic cell numbers were evaluated. The first patient received 1 g/M² MTX in a phase II study examining the efficacy of high doses of MTX. Immediately before the MTX injection, and 2 h later, peripheral blood was obtained by venipuncture, and the leukemic cells were removed as described in Methods. The inhibition of [³H]UdR into acid-insoluble material was determined to be certain that this dose of MTX did, in fact, inhibit methyl transfer to dUMP. The intracellular accumulation of [³H]Ara-C in these leukemic cells was performed as with L1210 cells above. The result was an enhanced entry and accumulation of [³H]Ara-C (Fig. 9). The second patient did not receive MTX, but rather the leukemic cells were removed and separated as before. One-half the cells were exposed for 4 h to MTX (1 μM), and the remaining one-half served as controls. The cells incubated in MTX-containing medium had an augmented [³H]Ara-C entry (Fig. 10), therefore the augmentation

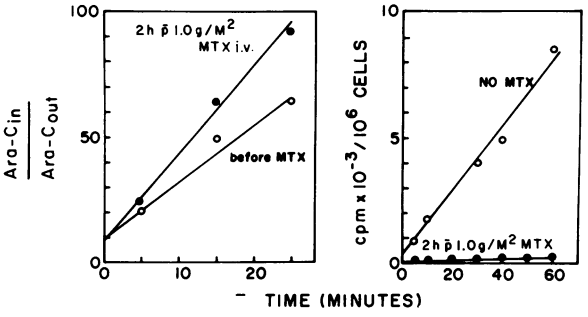


FIGURE 9 Augmentation of [³H]Ara-C uptake into human leukemia cells. A patient with acute myelogenous leukemia whose peripheral blast count was doubling daily had blood removed before and 2 h after receiving an intravenous dose of 1 g/M² of MTX. Leukemic cells were separated by centrifugation, and the uptake of [³H]Ara-C was determined (left). Simultaneously, the inhibition of [³H]UdR incorporation into acid-insoluble material was measured to verify that this dose of MTX had inhibited DNA synthesis (right). The rate of intracellular accumulation of [³H]Ara-C after MTX (●) was greater than the rate before MTX (○). This dose of MTX resulted in 99% inhibition of [³H]UdR incorporation into acid-insoluble material (−●). p̄, after.

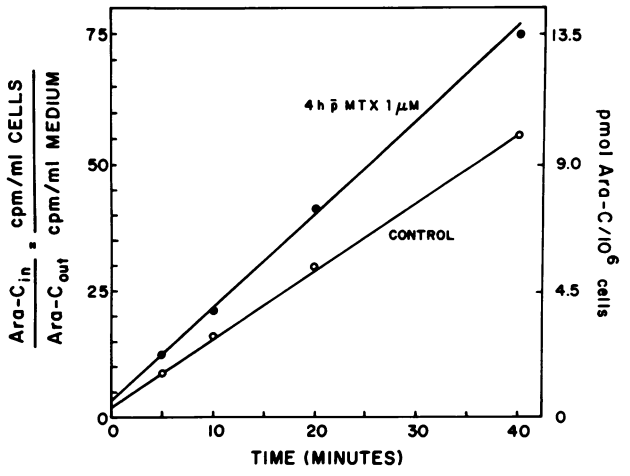


FIGURE 10 Augmentation of [^{3}H]Ara-C accumulation into human leukemia cells. Peripheral blood from a second patient with acute myelogenous leukemia who had a daily doubling leukemic cell count was obtained, and the leukemic cells were separated by centrifugation. The cells were divided into equal quantities (5×10^7 cells). 1 μM of MTX was added to one volume for 4 h. The other volume was the control (○). The accumulation of [^{3}H]Ara-C was greater in cells exposed to MTX (●). p̄, after.

of accumulation of Ara-C in human leukemic cells does occur after high doses of MTX. This presumably would lead to enhanced or synergistic killing of cells, however, this could not be evaluated with any certainty in these patients and must remain a presumption.

DISCUSSION

MTX and Ara-C are synergistic in killing L1210 cells when MTX precedes Ara-C. This phenomenon is dependent upon the dose of extracellular MTX and time of exposure before adding Ara-C. This sequential drug synergism is most likely the consequence of the enhanced intracellular accumulation of Ara-C which occurs after MTX treatment. Enhanced Ara-C accumulation occurred with those concentrations of MTX which induced inhibition of methyl transfer to dUMP and reduced dTTP production, which was dependent upon achieving increased MTX_f. The maximum enhancement of Ara-C accumulation in cells was only observed at doses of MTX which resulted in the highest MTX_f. This fact probably accounts for the confusing reports previously recorded by others evaluating the interaction of MTX and Ara-C, because the doses used in these communications were insufficient to produce MTX_f (4–6).

The consequence of using high doses of MTX (10 and 100 μM) which might account for the observed antitumor synergism between MTX and Ara-C is twofold. First, at these doses of MTX, the levels of intracellular Ara-CTP, the lethal form of Ara-C, is increased considerably (eightfold, after a 3-h exposure to 100 μM of MTX). Second, after following these same high doses of MTX, the dCTP pools are decreased. The importance of this latter finding is that dCTP is a feedback inhibitor of deoxycytidine kinase, the enzyme responsible for the initial phosphorylation of Ara-C to Ara-CMP (7, 8), and the dCTP also competes with Ara-CTP for DNA polymerase (9). It is therefore not surprising that the maximum synergistic activity of these two drugs occurred when 100 μM of MTX preceded Ara-C, the condition in which there were both increased quantities of Ara-CTP and decreased quantities of dCTP.

Given the maximum doses of MTX used in these studies (10 and 100 μM), which inhibited nearly completely [^{3}H]UdR incorporation into the acid-insoluble fraction, it would be expected that the concentrations of dTTP should approach zero. This was not observed, nor have others documented these theoretically low levels after inhibitory doses of MTX (20, 21). This could be a result of the fact that because the doubling time of L1210 cells is 12 h, many of the cells exposed to 4 h of MTX would not be synthesizing DNA and, therefore, dTTP in this fraction of the cell population would not be depleted.

The increase in dCTP pools after lower doses of MTX had been observed by others as well (21, 22). A logical explanation is lacking. However, the lowered dCTP pools that resulted after the higher doses of MTX (10 and 100 μM) are probably the result of the deamination of deoxycytidine nucleotides. Maley and Maley (23) have demonstrated that dTTP is an effective inhibitor of deoxycytidylate deaminase and that [^{14}C]deoxycytidine could readily be incorporated into DNA thymidine of rat embryos. Fridland (20) has recently documented that the major contribution to the increasing dUMP pools in response to MTX inhibition are the deoxycytidine nucleotides. A probable explanation for the lowered dTTP and dCTP pools at these high doses of MTX is that the decrease in dTTP after MTX results in greater deamination of the deoxycytidine nucleotides to deoxyuridylate. Therefore, the major effect of MTX on Ara-C accumulation may be mediated by these alterations of dTTP and dCTP.

An unusual aspect of the Ara-C accumulation is that the rate of accumulation was linear up to 102 min. Most drugs reach saturable levels within 60 min, and our MTX uptake experiments were saturable by 45 min. However, these unusual kinetics with Ara-C have been observed and reported previously. Chu and Fischer (24) found a linear accumulation of radiolabeled Ara-C in L5178Y cells up to 4 h. Their cells, like ours, were in logarithmic growth (3×10^5 cells/ml) and the concentration of Ara-C (3.3 μM) was also similar to ours. We have also performed experiments with L5178Y cells and found that the biochemical alterations induced

by MTX and subsequent Ara-C accumulations are nearly identical to those in L1210 cells. The explanation of this prolonged linear Ara-C uptake may be related to the fact that Ara-C accumulation, which is a consequence of the phosphorylation of this drug, is only occurring significantly in those cells which are in DNA synthesis. Only in these cells would the MTX have the effects described on dTTP and dCTP. In individual cells, Ara-C accumulation may indeed be saturable, but as the fraction of the total cell population which enters DNA synthesis increases, Ara-C accumulation of the entire population of cells increases. Because the doubling time of L1210 cells is 12 h, one could expect that the maximum quantity of Ara-C accumulated in the total cell population might not be achieved until all cells have reached the DNA synthesis portion of their cell cycle, or 12 h. For example, MTX, whose intracellular accumulation is limited by membrane transport and which is independent of cell cycle, is saturable by 45 min.

It is important to recognize from these studies that this augmented Ara-C accumulation, which is dependent upon MTX_f, can be reversed by allowing the MTX_f to efflux from the cells and also by administering leucovorin in doses which reverse inhibition by MTX of [^{3}H]UdR incorporation into the acid-insoluble cell fraction. The clinical correlation of this observation is obvious. If synergistic killing of human cancer cells is to occur, high doses of MTX must be given which result in MTX_f in tumor cells; then Ara-C would be administered before the MTX_f effluxes from the cells and also before leucovorin rescue is given.

Augmentation of Ara-C accumulation in human acute myelogenous cells occurred after high doses of MTX paralleling our observations of MTX-enhanced Ara-C accumulation in L1210 cells. Because MTX is an S-phase specific drug, one would expect that the synergistic interactions between MTX and Ara-C would primarily occur in cells undergoing active DNA synthesis. The augmentation of Ara-C accumulation in plateau-phase L1210 growth cells was only a 20% enhancement as compared with the eightfold enhancement in logarithmically growing cells—a finding consistent with this hypothesis. Therefore, this drug combination would not be expected to be nearly as useful for the treatment of tumors with a small growth fraction as it would be for those tumors with a high growth fraction, such as rapidly proliferating leukemia or lymphoma. We have, in fact, employed this information in designing drug therapy which includes high doses of MTX followed by high doses of Ara-C for the treatment of diffuse histiocytic lymphoma. Toxicity has been minimal.

Administration of high doses of MTX, when preceding ARA-C, results in enhanced killing of rapidly growing L1210 cells. The biochemical consequences of this drug sequence are lower dCTP pools and higher Ara-CTP levels, which can account for the synergistic antitumor effects.

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