Cancer Chemother Pharmacol (1990) 27: 60–66

Cancer Chemotherapy and Pharmacology

Biochemical interactions between methotrexate and 1-β-D-arabinofuranosylcytosine in hematopoietic cells of children: a Pediatric Oncology Group study*

Edward M. Newman[1], Doris G. Villacorte[1], Anna Maria Testi[1], Robert A. Krance[1], Michael B. Harris[2], Y. Ravindranath[3], and Donald Pinkel[1]**

[1] Division of Pediatrics, City of Hope National Medical Center, Duarte, CA 91010, USA
[2] Pediatric Division, Mount Sinai School of Medicine, New York, NY 10029, USA
[3] Children's Hospital of Michigan, Wayne State University School of Medicine, Detroit, MI 48201, USA

Received 6 April 1990/Accepted 14 June 1990

Summary. Children with acute lymphocytic leukemia (ALL) in remission were treated with overlapping sequential infusions of methotrexate (MTX) and 1-β-D-arabinofuranosylcytosine (araC) as part of continuation therapy. The doses and the sequence were chosen to mimic conditions that produced greater than additive antineoplastic activity with these two drugs in preclinical studies. To assess the potential for the drug combination to exhibit greater than additive effect in vivo, we investigated several biochemical parameters that had been associated with synergism in vitro. Because the patients were in remission, the intracellular parameters could only be measured in cytologically normal hematopoietic cells. We observed that (1) the mean plasma concentrations of MTX and araC were above those required to obtain a greater than additive cytotoxicity with the two drugs in tissue culture; (2) MTX did not have a significant antipurine effect in bone marrow mononuclear cells; (3) the mean intracellular concentration of deoxycytidine triphosphate (dCTP) was significantly lower after treatment with the drug combination than after therapy with araC alone; and (4) the ratio of araC triphosphate (araCTP) to dCTP was 2.6 times higher after treatment with the combination than after araC alone. These results indicate that it is possible to achieve in patients the biochemical conditions associated with the greater than additive antineoplastic activity of MTX and araC in vitro.

Introduction

Several preclinical studies have shown synergism, usually defined in those studies as representing a greater than additive effect, between the antineoplastic activities of methotrexate (MTX) and 1-β-D-arabinofuranosylcytosine (araC) [1, 3, 4, 11, 13, 16]; others have shown antagonism [2, 10, 13, 18]. Jackson and Harkrader [13] could discern a pattern in the reports of synergism and antagonism, which enabled them to define biochemical parameters associated with synergism. Synergism was more often observed at higher, cytocidal concentrations of the two drugs than at lower, cytostatic concentrations [13]; moreover, the degree of synergism increased with longer MTX preincubation times [3]. In several tissue-culture lines, the interaction between MTX and araC has correlated better with the intracellular level of deoxycytidine triphosphate (dCTP) and the ratio of araC triphosphate (araCTP) to dCTP than with the absolute level of araCTP [13, 18]. When MTX lowered the intracellular concentration of dATP through inhibition of purine synthesis, dCTP accumulated and antagonism ensued. When the intracellular concentration of dATP was maintained, MTX treatment decreased the content of dCTP, which resulted in synergism. Thus, changes in the endogenous levels of the deoxyribonucleoside triphosphates (dNTPs) are an important consideration in the interaction between MTX and araC (Fig. 1).

Based on this preclinical background, the Pediatric Oncology Group initiated a pilot protocol to test the feasibility of treating childhood non-T, non-B acute lymphocytic leukemia (ALL) with a regimen that included sequential intermediate-dose MTX and araC in the continuation phase. A

* Supported in part by grants from the National Cancer Institute, National Institutes of Health (CA-38053; CA-33572, CA-32278, CA-38859, CA-29691, and CA-30969). Preliminary reports on the biochemical data were published by E. M. N., A. M. T., and D. P. in *Proc Am Assoc Cancer Res 24:* 133 (1983) and those on the clinical data, by R. A. K., E. M. N., D. P., R. B. R., M. B. H., Y. R., and A. I. F. in *Proc Am Soc Clin Oncol 3:* 201 (1984)

Abbreviations: ALL, acute lymphocytic leukemia; *araC*, 1-β-D-arabinofuranosylcytosine; *araCTP*, araC triphosphate; *araU*, 1-β-D-arabinofuranosyluracil; *dNTPs*, deoxyribonucleoside triphosphates; *MTX*, methotrexate; *TCA*, trichloroacetic acid

** *Current addresses:* Cattedra di Ematologia, University La Sapienza, 00161 Rome, Italy; R. A. K., Children's Hospital of Orange County, Orange, CA, 92668; M. B. H., Hackensack Medical Center, Hackensack, NJ, 07601; D. P., Department of Pediatrics, University of Texas, M. D. Anderson Cancer Center, Houston, TX, 77030.

Offprint requests to: Edward M. Newman (8205), Pediatric Oncology Group, 4949 West Pine Blvd., Suite 2A, St. Louis, MO 63108, USA

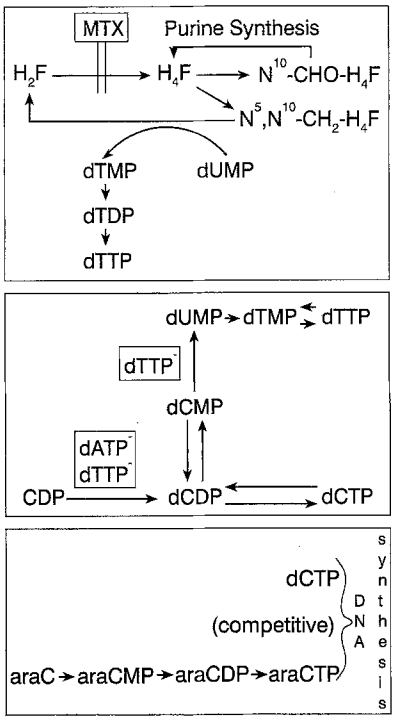

Fig. 1. Rationale for sequential treatment with MTX and araC. *Top:* inhibition of dihydrofolate reductase by MTX leads to a decrease in dTTP and to decreased de novo synthesis of purines. During prolonged exposure to MTX, cells may accumulate MTX polyglutamates, which could also directly inhibit enzymes required for dTMP and purine synthesis. *Center:* the concentration of dCTP is influenced by the concentrations of the other dNTPs through allosteric control of ribonucleotide reductase and dCMP deaminase. *Bottom:* araCTP is competitive with dCTP as both an inhibitor of and a substrate for DNA synthesis. Decreased levels of dCTP compete less effectively with the antimetabolite

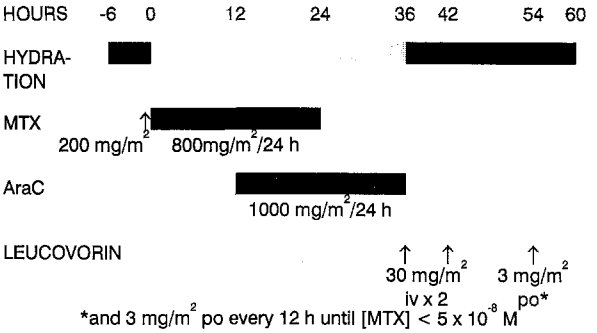

Fig. 2. Scheme for each course of intermediate-dose MTX and araC. The hydration solution contained 30 mEq/l $NaHCO_3$. The total volume of the intravenous fluids and the dose of $NaHCO_3$ and of leucovorin were adjusted when necessary for best clinical management. MTX and araC doses were not altered

dose of 1 g/m^2 MTX, divided into a 200-mg/m^2 bolus and an 800-mg/m^2 intravenous infusion over 24 h, with leucovorin rescue given at 36 h, was reported to be safe and to produce a mean steady-state level of 15.8 μM MTX [7], which was above the minimal concentration (1 μM) that is synergistic with araC in vitro [3]. This MTX infusion was combined with a 24-h intravenous infusion of 1 g/m^2 araC, which started after 12 h pretreatment with MTX and ended just prior to leucovorin rescue. Our objective in the pharmacology studies was to determine whether the hallmarks associated with synergism in vitro could be attained in the plasma and the hematopoietic cells of patients treated with this regimen.

Patients and methods

Reagents. The following drugs and other chemicals were obtained from the specified sources: tetrahydrouridine and MTX, Drug Synthesis and Chemistry Branch, DTC, NCI (Bethesda, Md.); araC, The Upjohn Company (Kalamazoo, Mich.); leucovorin, Lederle Laboratories (Pearl River, N.Y.); 1-β-D-arabinofuranosyluracil (araU), Sigma Chemical Company (St. Louis, Mo.); HPLC-grade ammonium dihydrogen phosphate and acetonitrile, Fisher Scientific Company (Fair Lawn, N.J.); 1,1,2-trichlorotrifluoroethane and tri-*n*-octylamine, Aldrich Chemical Company (Milwaukee, Wis.); redistilled trifluoroacetic acid, Brian Clark, Division of Immunology, City of Hope. Other chemicals were of reagent grade.

Patients. The patients who received MTX and araC while in remission were participants in a Pediatric Oncology Group pilot study. The study was approved by the institutional review board responsible for human investigations at each participating center. Informed consent was obtained in writing from the parents or guardians of all patients. The characteristics of the patient population, details of the full treatment regimen, and the clinical toxicology have been presented elsewhere (Kranee et al., submitted for publication). In all, 7 patients received one course of araC without MTX followed by five courses of MTX and araC; 26 children were scheduled to receive six courses of intermediate-dose MTX and araC. All patients were in complete remission at the time of treatment. The intracellular nucleotide levels were measured in cytologically normal hematopoietic cells isolated from bone-marrow aspirates. Four additional patients in second or subsequent relapse of ALL were given intermediate-dose MTX and araC with the intent of inducing remission or alleviating symptoms. Mononuclear cells isolated from the bone marrow of relapsing patients consisted mostly of leukemic blasts.

Treatment plan. Only the schedule for MTX and araC courses is described. These courses were given in addition to, not in lieu of, standard treatment for this disease (Krance et al., submitted for publication). Figure 2 shows the scheme for each course of MTX and araC. A course of araC alone involved a continuous intravenous infusion of 1,000 mg/m^2 over 24 h.

Plasma concentrations of araC, araU, and MTX. Blood was collected in ice-cold heparinized tubes, to which 10 μl 10^{-2}M tetrahydrouridine had been added to inhibit deamination of the araC. Plasma was separated, protein was precipitated with trichloroacetic acid (TCA), and the acid-soluble fraction was neutralized (pH >>>>5.0) with tri-*n*-octylamine in 1,1,2-trichlorotrifluoroethane [14]. Then, 50–100 μl deproteinized plasma was chromatographed on a 4.6- × 250-mm Vydac 201HS 5-μm C_{18} column (The Separations Group, Hesperia, Calif.) at a flow rate of 1.2 ml/min. After a 6-min isocratic elution with 7.5 mM ammonium phosphate buffer (adjusted to pH 2.5 with trifluoroacetic acid), a linear gradient was run over 20 min in 7.5 mM ammonium phosphate with 25% acetonitrile buffer (adjusted to pH 2.9 with trifluoroacetic acid) and elution was continued with the latter buffer for 5 min. The system volume between the gradient-proportioning valve and the detector contributed an additional 7-min delay between the programmed start of the gradient at the valve and the observed start at the detector. Typical retention times were 4.7 min for araC, 8.4 min for araU, and 27 min for MTX. AraC and araU were quantitated by peak areas at 280 nm and MTX, by peak area at 303 nm. The areas obtained were converted to

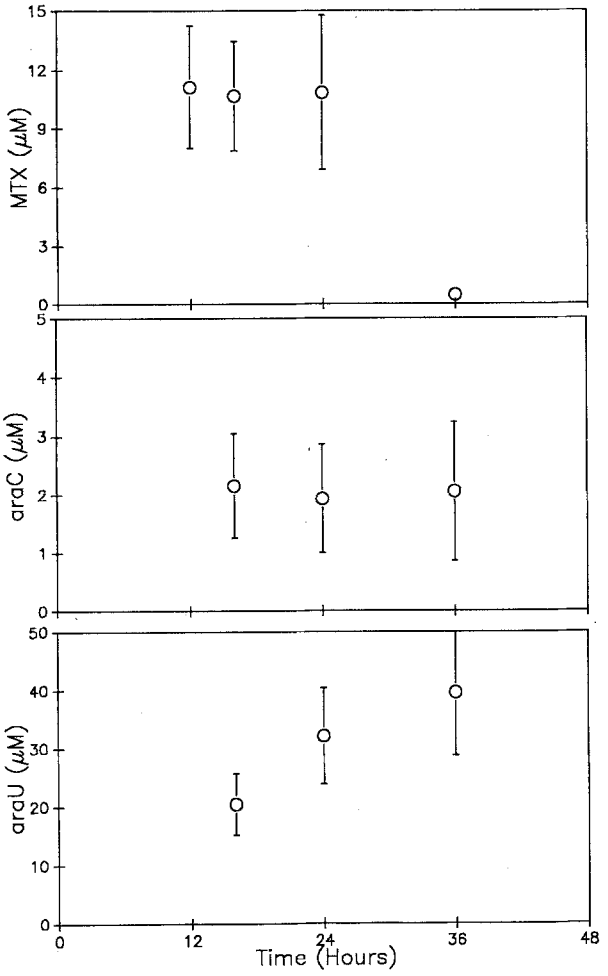

Fig. 3. Plasma concentrations of MTX, araC, and araU. Each data point represents the mean ± SD for 149–169 observations obtained from a total of 170 courses given to 33 patients

molarity by their comparison with the areas of external standards. Absorbance at 254 nm was also monitored.

The three compounds of interest were separated from 5-formyltetrahydrofolate, 5-methyltetrahydrofolate, 7-hydroxymethotrexate, 4-deoxy-4-amino-10-methylpteroic acid, and normal plasma constituents, with the following exception. In a few samples, an endogenous compound did not separate well from araC. The 280/254 ratio of this uncharacterized substance was quite different from that of araC, and the peak areas at 280 and 254 nm were used to solve two equations with two unknowns for the araC concentration. Recoveries of known amounts of araC and araU added to plasma were quantitative. MTX recovery was 61.6%±1.6% (mean ± SD, $n = 6$); plasma MTX concentrations were corrected for this loss.

Intracellular nucleotide levels. Bone-marrow aspirates containing sodium citrate as an anticoagulant were mixed with an equal volume of RPMI 1640 and separated over ficoll with sodium diatrizoate (Organon Teknika, Durham, N.C.) for 15 min at 550 *g*. The mononuclear cells at the interface were washed with ice-cold RPMI 1640, resuspended in 1 ml of medium, and counted. The cells were extracted with TCA and the ribonucleotides were quantitated by anion-exchange HPLC as previously described [15]. However, a minimum of 100 μl 0.6 M TCA was used in the extraction. AraCTP and dNTPs were quantitated after periodate oxidation of the ribonucleotides [8]. Because of the small volume of acid-soluble extract, the concentrations of some stock reagents were decreased such that the minimal volume to be measured was 4 μl.

Table 1. Ribonucleoside triphosphate content of human hematopoietic cells following treatment with araC alone or sequential MTX and araC in the same patients

	araC alone[a]	MTX/araC[a]	3 h after MTX/araC[b]
UTP[c]	368 ± 28	421 ± 26	437 ± 32
CTP[c]	119 ± 11	122 ± 9	131 ± 12
ATP[c]	2,417 ±276	2,565 ±220	2,903 ±252
GTP[c]	393 ± 41	429 ± 33	473 ± 32
Purines[d]	84.9% ± 0.6%	84.3% ± 0.5%	85.4% ± 0.5%
Number of samples	7	14	14

[a] The bone marrow aspirate was obtained at the end of the araC infusion.
[b] The bone marrow aspirate was obtained 3 h after the end of the araC infusion, which was also 3 h after the start of leucovorin rescue
[c] pmol/10^6 cells, mean ± SE
[d] 100 × (ATP+GTP) / (UTP+CTP+ATP+GTP), mean ± SE

Results

Plasma concentrations of araC, araU, and MTX

Plasma concentrations of drugs were determined for a total of 170 courses of MTX and araC given to 33 patients (Fig. 3). The mean steady-state concentration of MTX, taken as the median of values obtained at 12, 16, and 24 h for each course, was 10.9±2.6 μM. The minimal steady-state concentration during any course was 5.8 μM; the maximal value was 23.1 μM. By 36 h (12 h after the end of the MTX infusion), the mean plasma concentration had fallen to 0.5 μM. The mean steady-state concentration of araC, taken as the median of values obtained at 16, 24, and 36 h for each course, was 2.1±0.7 μM. The minimum was 0.4 μM; the maximum was 3.9 μM. The concentration of araU, the deamination product of araC, did not reach steady-state during the 24-h infusion of araC. The mean concentration of araU was 40 μM at the end of the infusion.

Intracellular pools of ribonucleoside triphosphates

Bone-marrow aspirates were obtained from seven patients within 30 min of the end of the araC infusion without MTX. Following the MTX/araC courses in the same seven children, aspirates were obtained within 30 min of the end of the araC infusion (before leucovorin rescue) or at 3 h after the end of the araC infusion and the beginning of leucovorin rescue. The ribonucleoside triphosphate content of hematopoietic cells obtained under these three conditions were compared (Table 1). Neither pretreatment with MTX nor leucovorin rescue significantly altered any of the four ribonucleoside triphosphate pools. Of particular importance in this study, there was no change in the relative amounts of purine and pyrimidine nucleotides. The data obtained after the MTX/araC infusions in this small group of patients (Table 1) was representative of the data from all patients (Table 2).

Table 2. Ribonucleoside triphosphate content of human hematopoietic cells following treatment with sequential MTX and araC

	MTX/araC[a]	3 h after MTX/araC[b]
UTP[c]	434 ± 23	429 ± 40
CTP[c]	118 ± 7	113 ± 8
ATP[c]	2,605 ±147	2,798 ±240
GTP[c]	408 ± 23	471 ± 69
Purines[d]	84.2% ± 0.3%	85.4% ± 0.3%
Number of samples	73	54

[a] The bone marrow aspirate was obtained at the end of the araC infusion
[b] The bone marrow aspirate was obtained 3 h after the end of the araC infusion, which was also 3 h after the start of leucovorin rescue
[c] pmol/10^6 cells, mean ± SE
[d] 100× (ATP+GTP) / (UTP+CTP+ATP+GTP), mean ± SE

Table 3. AraCTP and deoxyribonucleoside triphosphate content of human hematopoietic cells following tretment with araC alone or sequential MTX and araC in the same patients

	araC alone[a]	*P*[b]	MTX/araC[a]	3 h after MTX/araC[c]
araCTP	39 ±5[d] (18 –60) *n* = 7	0.253	53 ±8 (11 –120) *n* = 14	56 ±11 (6 –161) *n* = 14
dCTP	13 ±2 (6 –26) *n* = 7	0.056	7 ±2 (2 –16) *n* = 8	11 ± 3 (2 –35) *n* = 10
dTTP	32 ±6 (20 –50) *n* = 4	<0.001	7 ±1 (3 –10) *n* = 7	16 ±3 (2 –36) *n* = 10
dATP	15 ±3 (8 –22) *n* = 4	0.991	15 ±2 (6 –24) *n* = 8	24 ±6 (8 –70) *n* = 10
araCTP/dCTP	3.6 ±0.7 (1.4 –6.6) *n* = 7	0.007	9.1 ±1.5 (2.5 –15) *n* = 8	6.1 ±1.3 (3 –15) *n* = 10

[a] The bone marrow aspirate was obtained at the end of the araC infusion
[b] Two-tailed *t*-test of araC alone vs sequential MTX and araC
[c] The bone marrow aspirate was obtained 3 h after the end of the araC infusion, which was also 3 h after the start of leucovorin rescue
[d] All data represent the mean ± SE, expressed in units of pmol/10^6 cells, except for the ratio araCTP/dCTP, which is unitless. The values in parentheses are the minima and maxima for the detectable samples; *n* is the number of samples in which the component was detected

Intracellular pools of dNTPs and araCTP

The dNTPs were measured under three conditions: at the end of an infusion of araC alone, at the end of a combined MTX/areC infusion, and 3 h after an MTX/araC infusion (Table 3). Only one sample was obtained after each course. The probabilities presented in the table were derived from the combined data from all relevant courses. Unlike araCTP, which was detectable in all aspirates, each dNTP could not always be detected. Because the number of cells obtained differed among the bone-marrow aspirates, the detection limit on a per-cell basis varied among the samples; therefore, only the detectable levels were considered. The most important deoxynucleotide, dCTP, was detected in all samples obtained after the administration of araC alone.

Table 4. AraCTP and deoxyribonucleoside triphosphate content of human hematopoietic cells following treatment with sequential MTX and araC

	MTX/araC[a]	*P*[b]	3 h after MTX/araC[c]
araCTP	51 ±3[d] (11 –156) *n* = 73	0.034	40 ±4 (5 –161) *n* = 55
dCTP	5.8 ±0.5 (0.9 –20) *n* = 57	0.047	8 ±1 (1.3 –40) *n* = 45
dTTP	7.4 ±0.7 (1 –28) *n* = 44	<0.001	13 ±2 (2 –46) *n* = 37
dATP	19 ±2 (5 –56) *n* = 50	0.768	20 ±2 (5 –70) *n* = 35
araCTP/dCTP	9.3 ±0.6 (2.5 –28) *n* = 57	<0.001	6.1 ±0.4 (2.3 –15) *n* = 45

[a] The bone marrow aspirate was obtained at the end of the araC infusion
[b] Two-tailed *t*-test
[c] The bone marrow aspirate was obtained 3 h after the end of the araC infusion, which was also 3 h after the start of leucovorin rescue
[d] All data represent the mean ± SE, expressed in units of pmol/10^6 cells, except for the ratio araCTP/dCTP, which is unitless. The values in parentheses are the minima and maxima for the detectable samples; *n* is the number of samples in which the component was detected

The dCTP content was lower immediately after the araC infusion when MTX pretreatment was included. Using $P \leq 0.05$ as the criterion for significance, the difference in dCTP was not quite significant in this small group of patients. dTTP levels were significantly lower after treatment with the combination than after the infusion of araC alone. Importantly, dATP values were the same in the presence or absence of MTX pretreatment. dGTP, which eluted last and represented the broadest peak in the chromatogram, could be detected so seldom that data on this compound was not included. Although the difference in the accumulation of araCTP at the end of the araC infusion given with or without MTX was not significant, the ratio of araCTP to dCTP was significantly higher when MTX was included, due to the lower level of dCTP. The differences in dTTP content and the ratio of araCTP to dCTP were also significant when MTX/araC courses were compared with araC courses in each individual using a paired two-tailed *t*-test (dTTP, $P = 0.008$; araCTP/dCTP, $P = 0.024$). The mean values for all parameters obtained at the end of the MTX/araC infusions in this subset of seven patients (Table 3) were close to those obtained for all patients (Table 4). The larger number of observations for MTX/araC courses in the complete set of patients decreased the likelihood that these differences were due to chance ($P < 0.001$ for the changes in dCTP, dTTP, and araCTP/dCTP).

At 3 h after the first dose of leucovorin, the dATP level appeared to have increased in the subset of seven patients (Table 3). However, the mean was strongly influenced by one observation that was >2 SD above the mean. The difference in dATP content at these two time points was

not statistically significant and was not indicative of what was observed in the complete group, in which there was no change (Table 4). The cells obtained 3 h after the first dose of leucovorin differed significantly in several respects from those obtained at the end of the araC infusion but before leucovorin rescue (Table 4): both dCTP and dTTP values were higher, araCTP content was lower, and the ratio of araCTP to dCTP was lower.

Intracellular pools of dCTP and araCTP in ALL cells at relapse

In the four patients treated while in relapse, intracellular araCTP accumulated to a level of 93 ± 16 pmol/10^6 cells (mean ± SE), nearly twice that obtained in patients in remission. dCTP could be detected in only two of the four samples, but sufficient cells were obtained in the other two samples to place a reasonable upper limit on the intracellular concentration of dCTP. The mean dCTP level was <5.1 pmol/10^6 cells and the mean ratio of araCTP to dCTP was >22.

Discussion

Our rationale for treating ALL with sequential intermediate-dose MTX and araC was based on the synergy observed between these two drugs in preclinical studies. The objective of the pharmacology and pharmacodynamics studies described in this report was to evaluate whether the biochemical hallmarks associated with synergism between MTX and araC in vitro were attainable in vivo. To place these studies in context, the preclinical background is briefly described below.

An early report of synergism between MTX and araC in L1210 murine leukemia noted that the synergism was dose- and sequence-dependent [1]. MTX (12 mg/kg) and araC (1200 mg/kg) given together were synergistic, and araCTP was retained to a greater extent by cells treated with the combination than by those exposed to araC alone [16]. The synergism in antineoplastic activity was attributed to the increased retention of araCTP because the intracellular formation of the latter was known to be required for araC cytotoxicity [5]. The same doses of these two drugs, given 1 day apart in either order, were no more effective in prolonging the life of tumor-bearing animals than was the first drug given alone [1, 16]. However, sequential treatment was successful if a much higher dose of MTX (200 mg/kg) was followed by araC (500 mg/kg) at 6 h and by folinic acid (20 mg/kg) at 24 h [4].

Not all preclinical models have consistently exhibited synergism. Even in L1210 leukemia, low doses of MTX and araC given in combination had less than additive effects [2]. The same was true in vitro at low, cytostatic concentrations [10]. In L5178Y murine leukemia cells, MTX and araC were also antagonistic in regard to growth inhibition [18]. However, when hypoxanthine was added to the culture medium as an exogenous purine source and a concentration of MTX high enough to kill cells (as determined by clonogenic potential in soft agar) was used, MTX enhanced the phosphorylation and the cytotoxicity of araC [11]. Synergism against L1210 cells also occurred between MTX and araC at cytocidal concentrations; the synergism was greater at higher concentrations of MTX and at longer MTX preincubation times [3]. The cytotoxicity correlated with an increase in the intracellular accumulation of araCTP. At MTX concentrations of ≥ 10 μM, the dCTP content of cells decreased. In fresh human acute myelogenous leukemia cells as well, the in vitro uptake of araC was increased by prior exposure to MTX [3]. Subsequently, hepatoma cell lines and a fibroblast line that differed in their responsiveness to the combination of MTX and araC were studied in detail [13]. Antagonism between MTX and araC occurred in cell lines in which MTX caused a significant inhibition of purine synthesis; in contrast, synergism occurred in lines in which the purine nucleotide pools wre not decreased by exposure to MTX. Moreover, the addition of a purine to the culture medium elicited synergism in cell lines that otherwise exhibited antagonism. In each case, antagonism was associated with an increase in intracellular dCTP, whereas synergism was associated with a decrease in dCTP.

The probable link between the purines and the level of dCTP was dATP. The accumulation of dCTP was probably due to a loss of dATP's feedback inhibition of ribonucleotide reductase. When only the synthesis of thymidine nucleotides was significantly reduced by MTX, the level of dCTP was decreased. One mechanism by which this decrease in dCTP might have occurred involves a release of the inhibition by dTTP of dCMP deaminase. Increased activity of this deaminase would deplete levels of dCMP and its higher phosphates. This hypothesis also explains the earlier data obtained in murine leukemia lines. MTX has been shown to produce greater inhibition of de novo purine synthesis in L5178Y cells than in L1210 cells [19]; thus, only L5178Y would be expected to require the addition of a purine to obtain synergism between MTX and araC. The pivotal role of dCTP in the activity of the combination of MTX and araC is reasonable. As both an inhibitor and a substrate of DNA polymerase, araCTP competes with dCTP. Also, dCTP can inhibit deoxycytidine kinase, the first enzyme in the activation of araC.

The foregoing preclinical studies by other investigators not only provided the rationale for combining MTX and araC in the treatment of leukemia, but also suggested biochemical hallmarks of synergism that could potentially be observed in cells from patients undergoing treatment. These hallmarks include plasma concentrations of each drug greater than or equal to those that are synergistic in vitro, little or no difference in the cellular content of purine nucleotides, and an increase in the ratio of araCTP to dCTP. The steady-state plasma concentrations of MTX and araC were 10.9 μM and 2.1 μM, respectively; these concentrations were sufficient to obtain synergism in L1210 in vitro [3].

The potential antipurine effect of MTX was troublesome in the design of the current treatment regimen because of the association described above between lower intracellular dATP levels and antagonism with araC. Pretreatment with MTX did not reduce the levels of ATP, GTP, or dATP in this study as compared with araC therapy alone. Either MTX did not inhibit de novo purine synthesis

Appendix. Principal investigators of the Pediatric Oncology Group participating in this study

Institution	Investigator	Grant
City of Hope National Medical Center Duarte, California	Robert Krance	CA-32278
Children's Hospital of Michigan Detroit, Michigan	Y. Ravindranath	CA-29691
Mount Sinai School of Medicine New York, New York	Michael Harris	CA-38859
Roswell Park Memorial Institute Buffalo, New York	Martin Brecher	CA-28383
St. Christopher's Hospital for Chilren, Philadelphia, Pennsylvania	Robert Wimmer	CA-41573

in the bone marrow or the high concentration of hypoxanthine in the marrow [12] maintained the purine nucleotide levels through salvage. The net result of these two possibilities is the same. The decrease in purine nucleotides that is associated with antagonism in vitro was not observed in vivo at the MTX dose given.

The most direct evidence that conditions associated with a greater than additive effect were established in our patients came from measurement of the dCTP and araCTP pools. In vitro, a decrease in dCTP following treatment with MTX consistently leads to synergism [13]. In our study, dCTP content was significantly lower when MTX was included in the treatment regimen. A causal relationship between treatment with the antifolate MTX and the change in the dCTP pool was supported by the fact that dTTP was also significantly lower, as required by the proposed mechanism, and by the fact that leucovorin rescue partially restored the levels of both dTTP and dCTP.

We did not observe a significant increase in the absolute intracellular accumulation of araCTP. The distinction of antagonism vs synergism between MTX and araC in cell lines has correlated better with changes in the ratio of araCTP to dCTP than with araCTP accumulation [13]. This correlation with the relative rather than the absolute amount of araCTP has also been observed in the interaction of araC with other modulatory agents [9]. The in vitro studies used uniform cell lines. In the clinical setting, any modulatory effect of MTX would be superimposed on the heterogeneity in inherent sensitivity to araC among patients. In patients with leukemia, there is a wide variation in the accumulation and retention of araCTP, which has correlated with the clinical efficacy of araC-containing induction regimens [6, 17]. In our study, the ratio of araCTP to dCTP was significantly higher in treatment courses that included MTX.

Although it is reassuring that intracellular araCTP accumulated to a greater extent in relapsing patients than in those who were in remission, the limited data obtained from leukemic bone marrow must be interpreted with caution. No patient in relapse was treated with araC alone, which means that the role played by MTX in both the greater accumulation of araCTP and the higher ratio of araCTP to dCTP could not be determined. Furthermore, the behavior of undetected leukemic cells in a patient in remission may be very different from that of their progeny at the time of clinical relapse. There is no way to isolate and study the metabolism of araC in undetected leukemic cells, which we presume are present during clinical remission in patients who eventually relapse. Therefore, our data does not imply that the modulation of dNTPs by MTX that was observed in normal hematopoietic cells also occurred in any leukemic cells present in the marrow of patients in remission.

We thus established, for the first time in patients, three findings indicative of a greater than additive effect between MTX and araC: cytocidal drug concentrations, the lack of an antipurine effect, and a decrease in dCTP content. These biochemical results, together with the toxicity and efficacy data from the clinical portion of the study, support further evaluation of this combination in childhood ALL. The Pediatric Oncology Group is conducting such an evaluation in a comparative phase III study.

References

1. Avery TL, Roberts D (1974) Dose-related synergism of cytosine arabinoside and methotrexate against murine leukemia L1210. Eur J Cancer 10: 425
2. Burchenal JH, Dollinger MR (1967) Cytosine arabinoside (NSC-63878) in combination with 6-mercaptopurine (NSC-755), methotrexate (NSC-740), or 5-fluorouracil (NSC-19893) in L1210 mouse leukemia. Cancer Chemother Rep 51: 435
3. Cadman E, Eiferman F (1979) Mechanism of synergistic cell killing when methotrexate precedes cytosine arabinoside, study of L1210 and human leukemic cells. J Clin Invest 64: 788
4. Cadman EC, Lindquist C, Eiferman F (1977) Enhanced intracellular cytosine arabinoside uptake following MTX pretreatment: a biochemical study of human leukemia and experimental tumor cells. Proc Am Assoc Cancer Res 18: 216
5. Creasey WA (1975) Arabinosylcytosine. In: Sartorelli AC, Johns DG (eds) Antineoplastic and immunosuppressive agents. (Handbook of experimental pharmacology, vol 38). Springer, Berlin Heidelberg New York, p 232
6. Estey E, Plunkett W, Dixon D, Keating M, McCredie K, Freireich EJ (1987) Variables predicting response to high dose cytosine arabinoside therapy in patients with refractory acute leukemia. Leukemia 1: 580
7. Evans WE, Hutson PR, Stewart CF, Cairnes DA, Bowman WP, Rivera G, Crom WR (1983) Methotrexate cerebrospinal fluid and serum concentration after intermediate-dose methotrexate infusion. Pharmacol Ther 33: 301
8. Garrett C, Santi DV (1979) A rapid and sensitive high pressure liquid chromatography assay for deoxyribonucleotide triphosphates in cell extracts. Anal Biochem 99: 268
9. Grant S, Bhalla K, Graham G (1987) Effect of thymidine and hydroxyurea on high-dose ara-C metabolism and cytotoxicity in highly ara-C resistant human leukemic cells. Proc Am Assoc Cancer Res 28: 325
10. Grindy GB, Nichol CA (1972) Interaction of drugs inhibiting different steps in the synthesis of DNA. Cancer Res 32: 527
11. Hoovis ML, Chu MY (1973) Enhancement of the antiproliferative action of 1-β-D-arabinofuranosylcytosine by methotrexate in murine leukemic cells (L5178Y). Cancer Res 33: 521
12. Howell SB, Mansfield SJ, Taetle R (1981) Thymidine and hypoxanthine requirements of normal and malignant human cells for protection against methotrexate cytotoxicity. Cancer Res 41: 945
13. Jackson RC, Harkrader RJ (1981) Synergistic and antagonistic interactions of methotrexate and 1-β-D-arabinofuranosylcytosine in hepatoma cells. Biochem Pharmacol 30: 223

14. Khym JX (1975) An analytical system for rapid separation of tissue nucleotides at low pressures on conventional anion exchangers. Clin Chem 21: 1245
15. Pogolotti AI, Nolan PA, Santi DV (1981) Methods for the complete analysis of 5-fluorouracil metabolites in cell extracts. Anal Biochem 117: 178
16. Roberts D, Peck C, Hilliard S, Wingo W (1979) Methotrexate-induced changes in the levels of 1-β-D-arabinofuranosylcytosine triphosphate in L1210 cells. Cancer Res 39: 4048
17. Rustum YM, Preisler HD (1979) Correlation between leukemic cell retention of 1-β-D-arabinofuranosylcytosine 5′-triphosphate and response to therapy. Cancer Res 39: 42
18. Tattersall MHN, Harrap KR (1973) Combination chemotherapy: the antagonism of methotrexate and cytosine arabinoside. Eur J Cancer 9: 229
19. Tattersall MHN, Jackson RC, Jackson STM, Harrap KR (1974) Factors determining cell sensitivity to methotrexate: studies of folate and deoxyribonucleotide triphosphate pools in five mammalian cell lines. Eur J Cancer 10: 819