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Intracellular pharmacodynamic studies of the synergistic combination of 6-mercaptopurine and cytosine arabinoside in human leukemia cell lines

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Abstract Selective combinations of purine and pyrimidine analogs increase remission rates in pediatric patients with relapsed leukemias. The combination of 6-mercaptopurine (6-MP) and cytosine arabinoside (ara-C) may exhibit synergism similar to that observed for fludarabine and ara-C and may diminish the potential for development of resistance since the two drugs are activated by separate enzymatic pathways. To determine the efficacy of the combination against human leukemia cells, we investigated the time-concentration relationships of the drugs given alone or in combination to the resultant cytotoxicity. To determine whether the combination leads to enhanced activity of deoxycytidine kinase (dCk), the rate-limiting enzyme in ara-C activation, we characterized the cellular dCk in CCRF/CEM/0, CCRF/CEM/ara-C/7A, and CCRF/CEM/ara-C/3A monoclonal cells before and after treatment with 6-MP. CCRF/CEM/0 (wild type), CCRF/CEM/ara-C/7A (≈50% ara-C-resistant as determined by ara-C sensitivity assay and dCk characterization), and CCRF/CEM/ara-C/3A (≈90% resistant to ara-C) human leukemia cells were incubated with various concentrations of 6-MP and ara-C given alone or in combination. Cell survival, inhibition of DNA synthetic capacity (DSC), ara-CTP anabolism, and dCk enzymatic characteristics were studied. Incubation of CEM/0 cells with 6-MP for 24 h, followed by ara-C for 48 h, increased cell-growth inhibition by approximately 0.5–1 $\log_{10}$, corresponding to 5- to 10-fold synergism, as compared with ara-C alone after identical drug incubation in all cell lines. Simultaneous administration showed no synergism, whereas reversal of the sequence produced an antagonistic effect. The ara-CTP levels were 2- to 3.5-fold and 3- to 5-fold higher in CEM/0 and CEM/ara-C/7A cells, respectively, in cells exposed to 6-MP followed by ara-C than in those exposed to ara-C alone at the same concentrations. Furthermore, a progressive increase in ara-CTP levels was noted in CEM/0 cells exposed to increasing concentrations of 6-MP followed by 10 or 20 μ*M* ara-C. A significant decrease in DSC was observed upon treatment of wild-type and ara-C-resistant cells with 6-MP and ara-C. The combination of 6-MP and ara-C exhibits significant sequence-specific synergism in both wild-type and partially ara-C-resistant leukemia cell lines. The combination also exerts collateral sensitivity in the ara-C-resistant cell lines. 6-MP pretreatment may play a role in enhancing ara-C activation, thus producing drug synergism in sensitive and resistant leukemia cell lines.

Key words Synergism · 6-Mercaptopurine
Cytosine arabinoside · Drug resistance · Human leukemia

Abbreviations *6-MP* 6-mercaptopurine · *ara-C* cytosine arabinoside · *ara-CMP* cytosine arabinoside monophosphate · *ara-CTP* cytosine arabinoside triphosphate · *TIMP* thioinosine monophosphate · *HGPRT* hypoxanthine-guanine phosphoribosyl transferase · *PRPP* 5-phosphoribosyl-1-pyrophosphate · *AMP* adenylic acid · *SAMP* adenylosuccinate · *XMP* xanthylate · *TGMP* thioguanylic acid *DSC* DNA synthetic capacity · *dCk* deoxycytidine kinase *RR* ribonucleotide reductase · *AUC* area under the curve *PCA* perchloric acid

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Introduction

Ara-C, a pyrimidine analog, is the 2′-alpha-hydroxy ribose (arabinoside) derivative of deoxycytidine [1–3]. The anti-

leukemic activity of ara-C has long been established, making it an important agent in the treatment of both pediatric and adult patients with acute and chronic leukemias as well as non-Hodgkin's lymphoma [2–9]. The activity of this drug is dependent on the phosphorylation of ara-C to ara-CMP by cytoplasmic deoxycytidine kinase (dCk), which is the rate-limiting step in the final formation of ara-CTP [10–12]. Ara-CTP exerts its effect by inhibiting DNA polymerases and by being incorporated into the newly synthesized DNA strand, mostly in the intrastrand positions [13–16]. Studies have shown that the levels of cellular ara-CTP and the duration of its retention are strongly correlated with clinical response [17–20].

6-MP is a purine analog of hypoxanthine that competes with the latter for inosinic acid phosphorylase [21]. The biological activity of 6-MP depends on its intracellular conversion to thioinosine monophosphate (TIMP) by hypoxanthine-guanine phosphoribosyl transferase (HGPRT) in the presence of 5-phosphoribosyl-1-pyrophosphate (PRPP) [21, 22]. TIMP is also further converted into thioguanylic acid (TGMP), which is responsible for the inhibition of three key enzymes in purine biosynthesis, namely, amidotransferase, HGPRT, and inosinate dehydrogenase [22, 23].

It has been demonstrated that 6-MP can be incorporated into cellular DNA in the form of thioguanine (TG), which is responsible for the delayed cytotoxic effect of the drug [24, 25]. Like other thiopurines, 6-MP acts on multiple loci and inhibits RNA and DNA synthesis, both early in the de novo synthesis and at the purine nucleotide interconversion steps [21].

The combination of 6-MP and ara-C decreases leukemic cell survival in acute myeloblastic leukemia (AML) patients in a manner similar to that observed for fludarabine phosphate and ara-C [26–28]. The combination could be synergistic at the DNA level by inhibiting the DNA polymerases in both the purine and pyrimidine sites and/or may be synergistic at the ribonucleotide reductase (RR) level via a mechanism similar to that of fludarabine/ara-C [18, 29]. The coadministration of 6-MP and ara-C could diminish the development of resistance to either drug subclone, since they are activated by separate enzymatic systems to their respective triphosphates [30–32]. This presents a significant advantage over other treatment combinations where both drugs are activated by the same kinase.

The present report describes biochemistry and pharmacology studies demonstrating the synergistic potential of 6-MP and ara-C in inhibiting the growth of wild-type (CEM/0) human leukemia cells and a cell line that is 50% resistant to ara-C (CEM/ara-C/7A). These studies are significant for they may serve as a model for pediatric leukemias refractory to ara-C. We also report on survival studies done in a cell line that is approximately 90% resistant to ara-C (CEM/ara-C/3A). We investigated (1) the degree of growth inhibition of these cells, (2) the inhibition of DNA synthetic capacity (DSC) by each of these drugs given alone and in combination, (3) the effect of 6-MP on the cellular anabolism of ara-C, and (4) the effect on dCk-specific activity. These studies produced promising results, which will be also examined in a phase I clinical study (CCG-0933).

Materials and methods

Cells. The in vitro culture of the established human leukemia cell lines CEM/0 (wild type), CEM/ara-C/7A, and CEM/ara-C/3A was performed in accordance with procedures reported earlier [33, 34]. The ara-C-resistant cell lines were recently developed in our laboratory and were rendered partially resistant by repeated treatments with ara-C as discussed elsewhere [35]. CEM/0 wild-type cells were treated with 1 μM ara-C every 24 h for 72 h. The monoclonal colonies were isolated and recultured in liquid media. CEM/ara-C/7A and CEM/ara-C/3A were two of these clonal outgrowths (clone 7 and clone 3, respectively). Pharmacological and enzymatic dCk determinations were performed in all the monoclonally derived cultures. These were conducted to determine sensitivity to ara-C and the relative percentage of dCk activity as compared with wild-type CEM/0 cells as described elsewhere [34, 36]. CEM/ara-C/7A was shown to be approximately 50% resistant to ara-C and demonstrated a 50% decrease in dCk activity as compared with the control. CEM/ara-C/3A was approximately 90% resistant to ara-C and had 10% dCk activity relative to the control values.

Determination of 50% growth-inhibitory concentrations. To determine the concentrations of drug required to inhibit the growth of cells by 50% (IC_{50} values), ara-C, 6-MP, or both were added to 24-well plates containing 1 ml growth media with a known number of cells (2×10^5) in various molar ratios, namely, 1:1, 10:1, 100:1, 1000:1, 0.1:1, 0.01:1, and 0.001:1. The number of viable cells detected per well over a 2- to 3 day period versus time were plotted. The isobologram method and median-effect principle were used to obtain the IC_{50} values [36, 37].

Drug-synergy studies. For the drug-synergism studies, cells and drug solutions were placed in two 24-well plates (6×8 matrix) as reported earlier [37–39]. Experiments were performed using various concentrations above and below the IC_{50} of 6-MP (100-fold dilutions, 10^{-4}–10^{-8} M) as well as intermediate concentrations (2- to 4-fold dilutions). The ara-C concentrations used ranged from 10^{-5} to 10^{-9} M.

The ara-C anabolism studies performed in subsequent experiments utilizing concentrations of 10–20 μM produced significant results in terms of ara-CTP augmentation by 6-MP. These experiments were repeated in triplicate. After a total of 72 h incubation (6-MP×24 h, ara-C×48 h) the cells were counted in triplicate and the appropriate statistical calculations were conducted, the survival data being expressed as percentages of control data.

The isobologram method and the median-effect principle were then used to determine quantitatively the level of drug synergism between 6-MP and ara-C in vitro. The trypan-blue exclusion test and clonogenic assay were also performed to correct for cell viability. The number of trypan-blue-stained intact cells (defective membrane permeability) were subtracted from the cell-counter values as being nonviable cells.

Various sequences of treatment combinations (6-MP followed by ara-C, ara-C followed by 6-MP, and simultaneous administration of 6-MP and ara-C) and various incubation periods of 6-MP alone and ara-C alone (12–72 h) were studied. These in vitro studies were designed to simulate clinical settings where the washing step is omitted.

Clonogenic assay of cell viability. CEM/0 and CEM/ara-C/7A cells that had been exposed to varying concentrations of 6-MP and ara-C were allowed to recover in drug-free media in suspension for 24 h, before the actual clonogenic assay was done. This assay was performed to measure the ability of the surviving cells to form colonies and grow in soft agar as described earlier [34]. A comparison of the IC_{50} values derived from the converted percentage of survival data with the results of this clonogenic assay was done.

DNA synthetic capacity. To determine DNA synthetic capacity, the amount of tritiated thymidine ([^{3}H]-Tdr) incorporation into DNA was measured by a scintillation counter in cells exposed to one or both drugs. The results were expressed as percentages of control values (untreated cells) [33].

Cellular anabolism. To determine cellular anabolism, aliquots of CEM cells (wild type and partially resistant) were incubated in the presence of various concentrations of 6-MP, ara-C, or both. After perchloric acid (PCA) extraction, the nucleotide and nucleoside extracts were then analyzed by high-performance liquid chromatography (HPLC) using a strong anion-exchange (SAX-10) column to separate the different species of phosphorylated anabolites [33].

The equation used to calculate the micromolar ara-CTP levels was as follows:

$$\frac{\text{AUC}}{\text{RF}} \times \text{F} = \text{cellular ara-CTP concentration } (\mu M),$$

where *RF* is the conversion factor to convert the AUC value into nanomoles and *F* is the multiplication factor to convert the mean channel number (mean cell size) into the cellular concentration.

HPLC assay of nucleotide anabolites. The neutralized PCA extracts were assayed using a Waters Associates HPLC system. The elution buffers were solvent A (H_2PO_4, 0.005 *M*, pH 2.8) and solvent B (H_2PO_4, 0.75 *M*, pH 3.50) run at a combined flow rate of 2 ml/min. Under these conditions, efficient separation of nucleosides/bases and of mono-, di-, and triphosphate anabolites has been reported [33, 34]. Using this technique, ara-CTP eluted in the triphosphate region 2 min after CTP. HPLC reverse-phase chromatography was used to determine the stability of both 6-MP and ara-C under the tissue-culture conditions.

Extraction, partial purification, and characterization of dCk. The standard procedures for extraction, purification, and characterization of dCk were performed as previously described [34, 36]. In brief, 2×10^8 cells were lysed and precipitated with streptomycin sulfate. HPLC protein separation using Waters Protein Pak SP 5PW was followed by determination of enzyme activity [36].

Probit analysis/isobologram and median-effect principle. Probit transformation was done on all data and the results were expressed as percentages of control values as previously described [37–39]. The probit values were used in constructing the cell-survival graphs so as to linearize the sigmoidicity of the dose-response curve, thereby reducing the error [40]. The probit values were also included in the actual computations used for constructing the isobolograms. This allowed us to perform a single-order regression analysis of the dose-response data, which was necessary for extrapolation of the IC_{50} values.

The isobologram and median-effect equations were used to determine synergism, summation, or antagonism between 6-MP and ara-C [38]. The combination index (CI), determined from the isobologram and median-effect equations, was used to evaluate synergism, summation, or antagonism between 6-MP and ara-C.

Isobologram method. The isobologram method involves the use of the following equation:

$$\text{CI} = (A_c/A_e) + (B_c/B_e),$$

where A_e and B_e are the doses of compound *A* and *B* alone that are required to inhibit a system by *x*% and A_c and B_c are the concentrations of compounds *A* and *B* in combination that inhibit *x*% of the system.

Median-effect equation. The median-effect principle involves the use of the following equation:

$$f_a/f_u = (D/D_m)^m,$$

where *D* is the dose, f_a is the fraction of the system affected by dose *D*, f_u is the fraction of the system unaffected by dose *D*, D_m is the dose required to produce the median effect (analogous to the IC_{50}), *m* is the Hill-type coefficient signifying sigmoidicity of the dose-effect curve, $f_a + f_u = 1$, $D = D_m\,[f_a/(1-f_a)]^{1/m}$, and $log(f_a/f_u) = m\log(D) + m\log(D_m)$.

For calculation of the CI for mutually exclusive drugs,

$$\text{CI} = (D)_1/(D_x)_1 + (D)_2/(D_x)_2.$$

For calculation of the CI for mutually nonexclusive drugs,

$$\text{CI} = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + (D)_1 \times (D)_2/(D_X)_1 \times (D_X)_2.$$

For mutually exclusive or nonexclusive drugs, when $CI<1$, synergism is indicated; when $CI = 1$, additivity is indicated; and when $CI>1$, antagonism is indicated.

Results

Biology studies and IC_{50} determinations

The effects of increasing concentrations of 6-MP and ara-C on the growth inhibition of CEM/0 cells as compared with those of either drug alone is demonstrated in Fig. 1. This figure shows the survival curves generated for CEM/0 cells treated with 6-MP alone, ara-C alone and the combination of 6-MP and ara-C. The growth of CEM/0 cells was inhibited by 50% (IC_{50}; probit = 5) at a concentration range of between 10^{-6} and 2×10^{-6} *M* 6-MP given for 24 h ($X_9 = 1.76\pm0.71\mu M$) as shown by probit analysis of several data corrected for viability with the trypan-blue exclusion test. The mean IC_{50} values calculated after exposure to 6-MP alone for 48 and 72 h were 4.4×10^{-7} and 3.75×10^{-7} *M*, respectively. We also verified these results by the clonogenic assay, which produced IC_{50} values identical to those obtained with the trypan-blue exclusion test.

However, the clonogenic assay experiments showed that cells that appeared to survive at high drug concentrations ($>10^{-7}$ *M*) as shown by the trypan-blue exclusion test did not yield colonies. Experiments in which the incubation

Fig. 1 Concentration-response curves generated for 6-MP alone, ara-C alone and the combination of 6-MP and ara-C in CEM/0 cells. Exponentially growing cells were treated with 6-MP alone for 24 h, with ara-C alone for 48 h, and with the combination of 6-MP and ara-C given sequentially (6-MP×24 h, ara-C×48 h). Results were adjusted for cell viability by the trypan-blue exclusion test, expressed as a percentage of control values and transformed statistically into probit values for determination of IC_{50} values. Triplicate plates were performed as described in Materials and methods

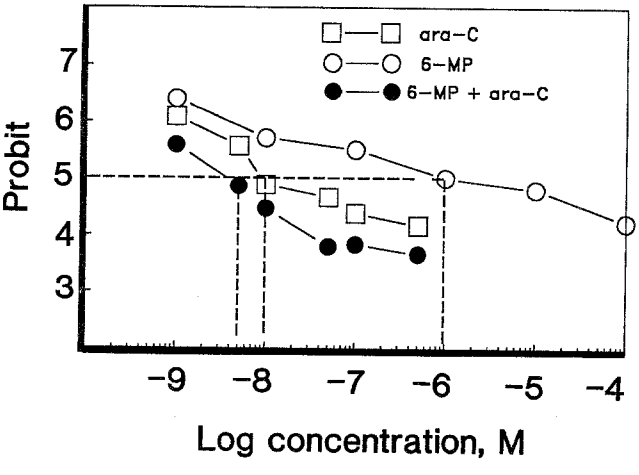

Table 1 IC_{50} values determined following treatment with 6-MP alone, ara-C alone, and their combination in CEM/0 cells[a]

Drug	Drug-combination ratio	IC_{50} (*M*)	Combination index (CI)[b]	CI[c]	Clonogenic assay IC_{50} (*M*)
6-MP×24 h		1.76×10^{-6}			1.00×10^{-6}
6-MP×48 h		4.40×10^{-7}			
6-MP×72 h		3.75×10^{-7}			
ara-C×24 h		4.25×10^{-8}			
ara-C×48 h		1.50×10^{-8}			2.00×10^{-8}
ara-C×72 h		7.50×10^{-9}			
6-MP×24 h/ ara-C×24 h	1:1	5.90×10^{-9}	0.28	0.30	
6-MP×24 h/ ara-C×48 h	1:1	5.30×10^{-9}	0.12	0.19	5.00×10^{-9}
ara-C×24 h/ 6-MP×24 h	1:1	7.50×10^{-8}	42.86	45.01	
ara-C×24 h/ 6-MP×48 h	1:1	8.25×10^{-8}	57.14	50.25	
6-MP + ara-C×48 h	1:1	3.00×10^{-8}	1.86	1.08	

[a] Data represent mean values for triplicate determinations per concentration. Five concentration-survival points were used for linearization and estimation of IC_{50} values

[b] CI values obtained in cells incubated with 6-MP followed by ara-C without the washing step as stated in Materials and methods

[c] IC values obtained in cells incubated with 6-MP followed by ara-C with the washing step as stated in Results. These data were not statistically significantly different from those obtained in experiments carried out without the washing step

Table 2 IC_{50} values determined following treatment with 6-MP alone, ara-C alone, and their combination in CEM/ara-C/7A cells[a]

Drug	Drug-combination ratio	IC_{50} (*M*)	Combination index (CI)[b]	CI[c]	Clonogenic assay IC_{50} (*M*)
6-MP×24 h		2.13×10^{-6}			2.00×10^{-6}
6-MP×48 h		2.00×10^{-6}			
6-MP×72 h		1.98×10^{-6}			
ara-C×24 h		3.60×10^{-7}			3.00×10^{-8}
ara-C×48 h		7.14×10^{-8}			
ara-C×72 h		7.00×10^{-8}			
6-MP×24 h/ ara-C×24 h	1:1	1.00×10^{-7}	0.19	0.21	
6-MP×24 h/ ara-C×48 h	1:1	6.42×10^{-9}	0.04	0.06	2.00×10^{-8}
ara-C×24 h/ 6-MP×24 h	1:1	4.00×10^{-7}	15.15	14.79	
ara-C×24 h/ 6-MP×48 h	1:1	2.80×10^{-7}	12.33	11.58	
6-MP + ara-C×48 h	1:1	5.62×10^{-7}	0.93	1.03	

[a] Data represent mean values for triplicate determinations per concentration. Five concentration-survival points were used for linearization and estimation of IC_{50} values

[b] CI values obtained in cells incubated with 6-MP followed by ara-C without the washing step as stated in Materials and methods

[c] CI values obtained in cells incubated with 6-MP followed by ara-C with the washing step as stated in Results. These data were not statistically significantly different from those obtained in experiments carried out without the washing step

periods with 6-MP ranged from 12 to 24 to 36 to 48 to 72 h showed that a period of 24–72 h was optimal in inhibiting leukemic cell growth with minimal variability in the results. A 24-h incubation period was chosen for future experimentation using 6-MP in light of lesser toxicities demonstrated in vivo. Cells exposed to 6-MP for 12–36 h showed near-identical drug synergism with ara-C. Experiments were performed using various concentrations above and below the IC_{50} of 6-MP given for 24 h (10^{-4}–10^{-8} *M*) as well as concentrations intermediate between those previously mentioned. Drug concentrations intermediate between 10^{-4} and 10^{-8} *M* showed no significant difference in percentage of survival values or in the estimation of IC_{50} values.

CEM/0 cells treated with ara-C alone for 48 and 72 h produced a mean IC_{50} of 1.50×10^{-8} and 7.50×10^{-9} *M*, respectively, whereas that determined for ara-C alone given for 24 h was 4.25×10^{-8} *M*. Identical IC_{50} values were obtained in experiments where a washing step was included between the administration of each of the drugs.

Simultaneous administration of both drugs yielded a minimal cytotoxic effect, possibly an additive effect, as determined by the median-effect principle. Ara-C followed by 6-MP did not show drug synergism; instead, an antagonistic effect was observed, especially at high concentrations of both drugs as judged by the median-effect method (Table 1). In contrast, sequential exposure of cells to 6-MP for 24 h followed by ara-C for 48 h, inhibited cell growth

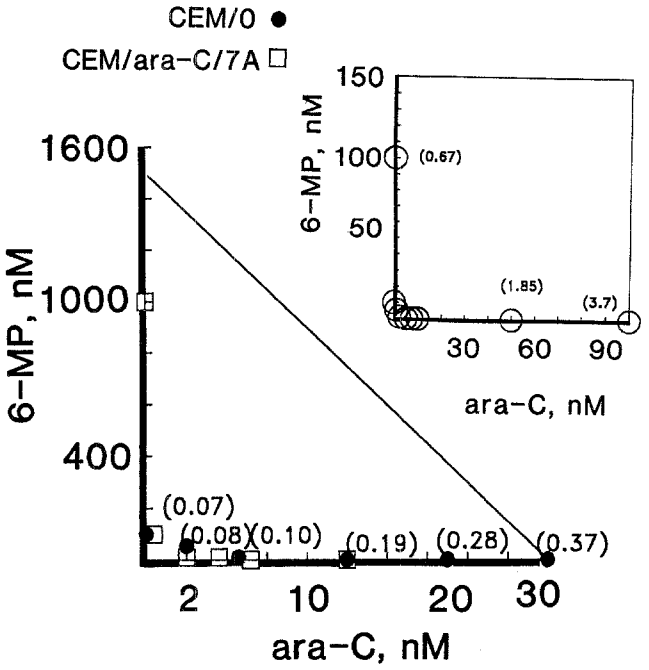

Fig. 2 Isobologram of 6-MP and ara-C in CEM/0 and CEM/ara-C/7A cells. In CEM/0 cells the IC_{50} value for 6-MP×24 h was 1.5 μ*M*, whereas that for ara-C×48 h was 0.03 μ*M*. The IC_{50} values for 6-MP and ara-C were used to construct a line to show the synergism between the two drugs in both cell lines (CI < 1) in relation to their individual IC_{50} values. In CEM/ara-C/7A cells the IC_{50} values for 6-MP and ara-C were 5 and 0.1 μ*M*, respectively. *Inset*: The complete isobologram for CEM/0 cells, showing synergism as well as some antagonism at higher concentrations of ara-C

by approximately 0.5 $\log_{10}$ as compared with ara-C alone (IC_{50}, 5.3×10^{-9} *M*).

CEM/ara-C/7A, a cell line that is approximately 50% resistant to ara-C, was developed in our laboratory to simulate resistant cells in patients with relapsed leukemias who have been treated with ara-C. The same pattern of synergism between 6-MP and ara-C was demonstrated in this cell line (Table 2, Fig. 2). The cell lines that were 50% or 90% resistant to ara-C were 1.22-, 4.55-, and 5.28-fold more resistant to 6-MP (Tables 1, 2). The mean IC_{50} values calculated for 6-MP given alone for 24 and 48 h were 2.13×10^{-6} and 2.00×10^{-6} *M*, respectively. The mean IC_{50} value determined for ara-C given alone for 24 h was 3.60×10^{-7} *M*, whereas that calculated for ara-C given alone for 48 h was 7.14×10^{-8} *M*. After the sequential administration of 6-MP followed by ara-C, the IC_{50} ranged from 1×10^{-8} to 2.83×10^{-9} M, with the mean value being 6.42×10^{-9} *M* (Table 2). Cell survival was greater in CEM/ara-C/7A cells after administration of this regimen than in CEM/0 cells as determined by the clonogenic assay, indicating their partial resistance to ara-C (Tables 1, 2). However, the sequential exposure of these leukemic cells to 6-MP and ara-C produced about 1 $\log_{10}$ difference, or approximately 10-fold synergism relative to ara-C alone or greater drug synergism as compared with CEM/0 cells.

In CEM/ara-C/3A cells, the mean IC_{50} values determined for 6-MP given for 24 and 48 h were 4.39×10^{-5} and 2.80×10^{-5} *M*, respectively (Table 3). The mean IC_{50} value calculated for 6-MP given for 72 h was 1.56×10^{-5} *M*. The IC_{50} values determined for ara-C given for 24 and 48 h were 1.87×10^{-6} and 1.6×10^{-6} *M*, respectively, whereas that found for ara-C given for 72 h was 1.75×10^{-6} *M*. After the sequential administration of 6-MP for 24 h followed by ara-C for 48 h, the IC_{50} was reduced to 2.28×10^{-7} *M*

Fig. 3 Effect of ara-C concentration on ara-CTP accumulation after pre-treatment with 6-MP in CEM/0 cells. Cells were treated with 6-MP for 24 h followed by ara-C for 48 h. PCA extraction of nucleosides/nucleotides was done as described in Materials and methods. The results represent mean values (±SD) for triplicate determinations

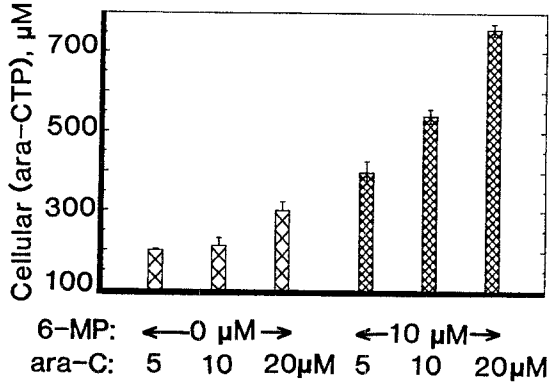

Table 3 IC_{50} values determined following treatment with 6-MP alone, ara-C alone, and their combination in CEM/ara-C/3A monoclonal cells[a]

Drug	Drug-Combination ratio	IC_{50} (*M*)	Combination index (CI)[b]	CI[c]
6-MP×24 h		4.39×10^{-5}		
6-MP×48 h		2.80×10^{-5}		
6-MP×72 h		1.56×10^{-5}		
ara-C×24 h		1.87×10^{-6}		
ara-C×48 h		1.60×10^{-6}		
ara-C×72 h		1.75×10^{-6}		
6-MP×24 h/ ara-C×24 h	1:1	1.85×10^{-7}	0.54	0.49
6-MP×24 h/ ara-C×48 h	1:1	2.28×10^{-7}	0.32	0.37
ara-C×24 h/ 6-MP×24 h	1:1	5.00×10^{-5}	7.49	7.78
ara-C×24 h/ 6-MP×48 h	1:1	5.62×10^{-5}	5.56	5.02
6-MP + ara-C×48 h	1:1	2.57×10^{-6}	1.28	1.33

[a] Data represent mean values for triplicate determinations per concentration. Five concentration-survival points were used for linearization and estimation of IC_{50} values

[b] CI values obtained in cells incubated with 6-MP followed by ara-C without the washing step as stated in Materials and methods

[c] CI values obtained in cells incubated with 6-MP followed by ara-C with the washing step as stated in Results. These data were not statistically significantly different from those obtained in experiments without the washing step

Table 4 dCk determination in CEM cells[a]

Cells	Treatment	K_m (μM)	V_{max} (cpm h^{-1} mg protein)	Relative increase in V_{max}
CEM/0	Control	24.88 ± 1.10	2.50E5	
CEM/ara-C/7A	Control	27.11 ± 3.30	2.30E5	
CEM/ara-C/3A	Control	36.18 ± 1.18	1.70E4	
CEM/0	10 μM 6-MP	18.58 ± 1.1	2.80E5	1.20-fold
CEM/ara-C/7A	10 μM 6-MP	15.87 ± 2.2	3.75E5	1.50-fold
CEM/ara-C/3A	10 μM 6-MP	23.06 ± 0.96	9.40E4	5.50-fold

[a] dCk activity was determined before and after 6-MP treatment in CEM/0 and CEM/ara-C/7A cells with ara-C serving as the variable substrate. Cells (2×10^8) were treated with 10 μM 6-MP×48 h, dCk extraction and purification were performed, and the results were compared with control values. The initial velocities were determined by varying the ara-C concentration and by using HPLC-purified leukemic cell dCk at fixed concentrations of ATP and Mg^{2+}. The reaction volume was 25λ and the incubation time was 1 h

(Table 3). The data show that in the leukemic cell lines partially resistant to ara-C, a significantly increased level of drug synergism was observed with the combination of 6-MP and ara-C (collateral sensitivity).

Ara-CTP anabolism after 6-MP and ara-C treatment

Pretreatment of CEM/0 cells with increasing concentrations of 6-MP (1–10 μM) followed by exposure to 5–20 μM ara-C, the latter concentration achieving saturation of this drug, resulted in a linear increase in ara-CTP concentration from 550 to 770 μM (Fig. 3). These data indicate that a 6-MP concentration as low as 1 μM can induce some augmentation ($P \leq 0.02$) of cellular ara-CTP, which can be linearly enhanced by increasing the 6-MP concentration. An increase of 5–10 μM 6-MP did not appreciably augment the cellular ara-CTP production. The same pattern was demonstrated in CEM/ara-C/7A cells exposed to the combination of these drugs (data not shown).

Upon exposure to a constant 10-μM concentration of 6-MP followed by a variable concentration of ara-C within the therapeutic range, 5–20 μM, the intracellular ara-CTP concentration in CEM/0 cells linearly ranges from approximately 400 to 770 μM as compared with the values obtained after treatment of CEM/0 cells with the same concentrations of ara-C alone for 48 h (200 ± 3.64 and 212 ± 20 μM; Fig. 4). These ara-CTP levels are approximately 2- to 3.5-fold higher than those achieved in cells exposed to ara-C alone. The data suggest that further augmentation of ara-CTP production can be achieved by increasing the ara-C concentration. The ara-CTP levels were significantly lower (≈50%) in the CEM/ara-C/7A cells than in the CEM/0 cells. However, upon pretreatment of the cells with 5 or 10 μM 6-MP followed by 1–20 μM ara-C, we observed a linear increase of 3- to 5-fold higher ara-CTP cellular concentrations over control values in a manner similar to that seen in CEM/0 cells (data not shown).

These data suggest that a pretreatment of CEM cells with 2–5 μM 6-MP is needed to augment cellular ara-C activation to ara-CTP. The cells need to be exposed to ara-C at 15–20 μM to obtain the maximal biochemical activation effect. An approximately 2:1 to 3:1 ratio of cellular ara-CTP concentration was generated in CEM/0 and CEM/ara-C/7A cell lines upon 6-MP treatment, confirming that CEM/ara-C/7A cells are partially resistant to ara-C (Figs 3, 4).

Kinetic characterization of dCk

To investigate further the augmentation of ara-C anabolism to ara-CTP, dCk assays and protein characterizations were performed in both leukemic cell lines before and after 6-MP treatment to determine whether there is a difference in the Michaelis constant (K_m), the Michaelis-Menten rate constant for high substrate concentration, and the maximal

Fig. 4 Correlation between cellular ara-CTP levels in CEM/0 cells exposed to increasing concentrations of 6-MP (1–10 μM) followed by 20 μM ara-C. The results represent the mean values (±SD) for triplicate determinations

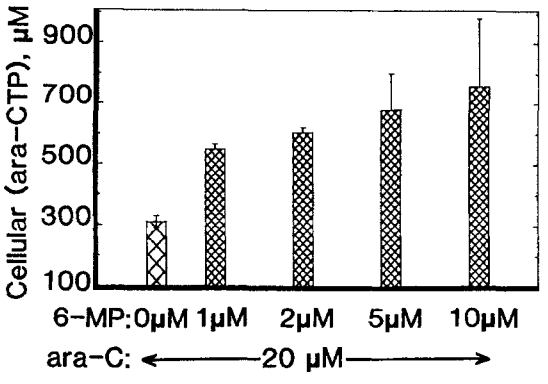

Table 5 DNA synthetic capacity (*DSC*) expressed as a percentage of control values in CEM/0 cells

Drug concentration (μ*M*)	DSC (% of control)	
	6-MP	ara-C
Untreated	100.00 ± 5.67	100.00 ± 20.79
10.00	12.20 ± 4.79	2.66 ± 0.62
5.00	16.64 ± 3.54	4.46 ± 0.27
1.00	55.70 ± 16.17	4.86 ± 0.03
0.10	88.38 ± 20.10	21.74 ± 8.93
6-MP + ara-C[a]:		
Untreated	100.00 ± 14.74	
0.1 + 0.01	27.63 ± 5.82	
0.1 + 0.10	3.10 ± 0.05	
0.1 + 0.50	6.00 ± 0.09	
0.1 + 1.00	1.06 ± 0.03	
0.1 + 5.00	1.11 ± 0.07	
0.1 + 10.00	8.05 ± 0.07	
0.1 + 20.00	5.19 ± 0.16	
1.0 + 0.01	11.49 ± 0.06	
1.0 + 1.00	7.80 ± 0.03	
1.0 + 20.00	1.60 ± 0.12	
2.0 + 0.01	6.60 ± 0.05	
2.0 + 1.00	2.30 ± 0.09	
2.0 + 20.00	1.30 ± 0.05	
5.0 + 0.01	5.01 ± 0.07	
5.0 + 1.00	1.00 ± 0.06	
5.0 + 20.00	1.60 ± 0.10	
10.0 + 0.01	4.14 ± 0.04	
10.0 + 1.00	1.20 ± 0.11	
10.0 + 20.00	1.30 ± 0.01	

[a] Four additional data points at between 0.01 and 20 μ*M* ara-C produced DSC values within the range reported in this table

volume (V_{max}) for ara-C substrate between the respective kinases. The kinetic data were fitted by an Adapt II computer program to the Michaelis-Menten equation to obtain the values of the parameters.

Table 4 shows that there was a significant difference between the K_m and V_{max} of the three cell lines before treatment with 6-MP ($P < 0.05$). Upon treatment with 6-MP for 48 h, the K_m of dCk in the CEM/ara-C/7A cell line decreased from approximately 27 to 15 μ*M* with a concurrent 1.5-fold increase in V_{max}, whereas the K_m of dCk in the CEM/ara-C/3A line decreased from approximately 36 to 23 μ*M* with a 5.5-fold increase in V_{max}. In CEM/0 cells there was also a demonstrable decrease in K_m from 24.9 to 18.6 μ*M*, but the increase in V_{max} was less significant ($P < 0.05$; Table 4). Thus, these studies show that 6-MP treatment does exert a qualitative effect on dCk characteristics in a cell-free system, which could explain the enhanced biological activity and pharmacology determinations of ara-CTP after 6-MP and ara-C treatment. Further studies are under way to investigate this molecular mechanism of dCk activation.

Inhibition of DNA synthetic capacity

DNA synthetic capacities in CEM/0 cells treated with varying concentrations of both drugs were determined as shown in Table 5. CEM/0 cells treated with 6-MP alone for 24 h required 100 μ*M* of the drug to decrease the DNA synthetic capacity (DSC) to < 10% of the control value. On the other hand, cells treated with ara-C alone for 48 h required 0.5 μ*M* ara-C to decrease the DSC to < 10% of the control value. When 6-MP was given for 24 h followed by ara-C for 48 h, only 0.1 μ*M* of both 6-MP and ara-C was required to achieve the same degree of inhibition of DNA synthesis. At an ara-C concentration of 0.01 μ*M*, only 2 μ*M* 6-MP was needed to decrease the DSC to 6.6% of the control value, which confirms the marked synergism of the two drugs seen in the biology experiments.

In CEM/ara-C/7A cells, the DSC was inhibited to approximately 50% at a 6-MP concentration of 100 μ*M* given for 24 h. At ara-C concentrations in the range of 0.5–1.0 μ*M*, the DSC was inhibited to 10% of the control value. Higher ara-C concentrations decreased proportionally the DSC values. The sequential administration of 6-MP and ara-C also decreased the DSC to < 10% of the control value in a manner similar to that shown for the wild-type CEM/0 cells. Higher concentrations of 6-MP (2, 5, and 10 μ*M*) in combination with increasing concentrations of ara-C likewise diminished the DSC significantly in a synergistic manner.

Discussion

The combination of fludarabine and ara-C exhibits significant drug synergism in leukemic cell lines as well as in cells from patients with relapsed leukemias [27–29, 42]. These studies showed that results obtained in in vitro experiments using leukemic cell lines were reproduced in ex vivo experiments using leukemic cells from both pediatric and adult patients. These results encouraged us to design a phase I clinical trial using continuous infusions of fludarabine and ara-C in pediatric patients in relapse. The phase I study was followed by a phase II trial that achieved remission rates higher than those obtained in other trials utilizing these two drugs in different protocol designs [26, 42]. Since both ara-C and fludarabine are activated by dCk at their rate-limiting step, we considered that 6-MP and ara-C, which are activated by separate kinase mechanisms, may also be synergistic. We therefore tested the effects of the sequential administration of 6-MP and ara-C in an in vitro human leukemia model consisting of cell lines sensitive and partially resistant to ara-C.

It has been shown that 1–10 μ*M* 6-MP given as a prolonged intravenous infusion for 24 h achieves greater bioavailability than that given by the oral route [30]. The intravenous route bypasses the problem associated with the valuable bioavailability of this drug. A clinical study of continuous-infusion high-dose 6-MP (1000 mg/m^2 given over 24 h) followed by low-dose continuous infusion of ara-C (500 mg/m^2 given over 24 h for 4 days) was recently done in children with relapsed leukemias using a 6-MP continuous-infusion design identical to that described earlier [26, 30]. The dose of ara-C used in this study

achieved an average plasma steady-state level of only 1.3 μ*M*, which is approximately 8-fold lower than the target concentration for this drug. All children with acute nonlymphocytic leukemia (ANLL) developed a complete remission (CR), but only 1 of 12 patients with acute lymphocytic leukemia (ALL) achieved a CR. These findings indicate that the ara-C doses given in this study may be sufficient for the treatment of ANLL but not for that of ALL, which requires higher (10 μ*M*) ara-C concentrations to induce significant cell killing [33].

In this study, we tested the effects of low (1–5 μ*M*) and high (10–20 μ*M*) ara-C concentrations against three human leukemia cell lines (CEM/0, CEM/ara-C/7A, and CEM/ara-C/3A) to determine the optimal concentration for both ANLL and ALL cells. The combination of 6-MP + ara-C showed sequence-specific synergism in both wild-type (CEM/0) and partially ara-C-resistant (CEM/ara-C/7A and CEM/ara-C/3A) human leukemic cell lines (Tables 1–3 and Fig. 2). In the latter two cell lines, a greater level of drug synergism was obtained, indicating a case of collateral sensitivity, where partial resistance to a drug leads to greater cytotoxicity by another drug or by a combination of drugs. This is very important in the clinical setting, since most relapsed pediatric patients with leukemia have been treated with ara-C and are considered to be clinically refractory to this agent [33, 36, 39].

Cells must be exposed to 6-MP for a significant amount of time to produce the synergistic augmentation in ara-C anabolism. Concurrent administration did not favor synergism, and reversing the sequence of the drugs produced antagonism. Synergism was achieved even at low (from 10^{-8} to 10^{-6} *M*) 6-MP and ara-C concentrations. Further verification by clonogenic assay showed a similar level of synergism with a 1:1 ratio of cell kill as compared with the trypan-blue test at the IC_{50} values. This finding correlated with the marked inhibition of DSC seen after treatment with various concentrations of the combination as compared with either drug alone. This also reflected the enhancement of ara-C cytotoxicity, especially at 20 μ*M*.

Ara-CTP levels in both CEM/0 and CEM/ara-C/7A cells increased linearly with increasing concentrations of both drugs. A concentration of 5–10 μ*M* 6-MP was required to achieve maximal augmentation of cellular ara-C anabolism to ara-CTP. These concentrations of 6-MP and ara-C were readily obtained in patients' plasma during treatment with these agents in commonly used protocols (continous infusion of 6-MP at 1 g/m^2; loading bolus + continuous infusion of ara-C) [30, 36]. When combined with low ara-C concentrations, 6-MP concentrations lower than 5 μ*M* produced minimal ara-CTP augmentation, thus failing to explain the drug synergism seen in another study against acute myeloblastic leukemia (AML) [26], which used intermediate ara-C doses. We demonstrated that the sequential exposure of leukemic cells to 6-MP and therapeutic concentrations of ara-C led to progressive enhancement of ara-CTP anabolism that was directly related to 6-MP concentrations and to greater ara-C concentrations [31, 32].

Furthermore, dCk analysis of both the wild-type and the partially ara-C-resistant cells treated with 6-MP showed enhancement of dCk activity as demonstrated by a decrease in the affinity to large concentrations of the substrate (K_m) and a subsequent increase in V_{max} in both cell lines. The exact mechanism by which 6-MP mediates the activation of dCk is not known and has to be investigated further. We postulate that 6-MP may play a role in decreasing dCk feedback inhibition by decreasing the levels of dCTP and dNTPs in general in a manner similar to that observed for fludarabine [29]. 6-MP and its cellular anabolites have been shown to inhibit purine triphosphate pools, ATP and GTP, in MOLT F4 human leukemic cells [41]. This may in turn be associated with the inhibition of cell growth and cell viability and may inhibit dNTP pools as well. Kowal and Grindey [43] have shown that an increase in the ratio of dTTP to dATP, or dUTP to dATP, or both would reduce dCk feedback inhibition, thereby increasing the rate of ara-C activation. Another possible mechanism could be due to perturbations of DNA-methylation patterns, which could enhance expression of the dCk gene. Further studies are needed to ascertain this mechanism. Obviously, the sequence-specific administration of these drugs is essential for drug synergism as seen in fludarabine + ara-C studies [27, 28, 42]. Therefore, the sequence of administration of purine and pyrimidine drugs becomes pivotal in the design of new clinical protocols.

The significant inhibition of growth of the leukemic cells, even in partially resistant cell lines, is a significant development in the treatment of patients with refractory leukemias who have been exposed to ara-C and invariably exhibit varying degrees of ara-C resistance. Since drug synergism was achieved, even at low drug concentrations, this regimen offers a promising alternative therapeutic regimen for relapsed ALL leukemia in pediatric patients. The role of 6-MP in enhancing dCk activity is definitely an important area to explore in patients' leukemic blasts. It would be very interesting to determine whether 6-MP treatment of ara-C-resistant cell lines, which have previously been shown to have reduced dCk expression, may lead to enhanced mRNA expression of this enzyme. This will be the subject of future investigations involving the probing of leukemic cell DNA and RNA in both CEM cells and leukemic blasts of pediatric patients with the human dCk probe recently cloned in our laboratory.

A new clinical protocol, which will soon be implemented through the Children's Cancer Group (CCG), will examine this synergism in pediatric patients with relapsed leukemias. This protocol has been designed to achieve average steady-state concentrations of 5 μ*M* 6-MP and 10–20 μ*M* ara-C, which have previously been shown to produce optimal cell killing in human leukemic cells with tolerable host toxicity.

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