Modulation of Arabinosylcytosine Metabolism by Arabinosyl-2-Fluoroadenine in Lymphocytes From Patients With Chronic Lymphocytic Leukemia: Implications for Combination Therapy

By Varsha Gandhi, Billie Nowak, Michael J. Keating, and William Plunkett

Our previous studies indicated that K562 cells loaded with arabinosyl-2-fluoroadenosine 5'-triphosphate (F-ara-ATP) accumulated arabinosylcytosine 5'-triphosphate (ara-CTP) at a threefold higher rate compared to the control cells. In the present study lymphocytes were obtained from patients with chronic lymphocytic leukemia before and after F-ara-A monophosphate therapy. The rate of ara-CTP accumulation after in vitro ara-C incubation was compared in lymphocytes obtained prior to therapy without any other manipulation, after ex vivo F-ara-ATP (100 μmol/L) treatment, and after in vivo F-ara-A monophosphate therapy. Lymphocytes showed a 2.2-fold (n = 23) and 1.7-fold (n = 23) median increase in the cellular concentration of ara-CTP after an ex vivo incubation with 100 μmol/L F-ara-A and 20 to 24 hours after the first dose (25 or 30 mg/m²) of F-ara-A monophosphate in vivo treatment, respectively. Although the rates of F-ara-ATP and ara-CTP accumulation varied among patients, a relationship was observed in individuals between the cellular concentration of F-ara-ATP at the beginning of the ara-C incubation and ara-CTP accumulation. These studies strongly suggest that a protocol designed to administer F-ara-A monophosphate prior to ara-C infusion will augment ara-CTP accumulation by leukemia cells.

MULTIPLE DRUG TREATMENT of malignant diseases has resulted in an increased frequency of response to chemotherapy and prolongation of disease-free survival. Combination drug therapy would ideally use the optimal schedule and doses of drugs for reinforcement of the therapeutic efficacy of one or more of the agents. Since combinations of chemotherapeutic drugs are necessary to potentially cure established cancers, it is rational to use drugs that interact via biochemical modulation to enhance the cell kill and thus to increase the cure rate.

We studied the combination of 9-β-D-arabinofuranosycytosine (ara-C) and 1-β-D-arabinofuranosyl-2-fluoroadenine (F-ara-A) in K562 human leukemia cells to establish a biochemical rationale for the use of these drugs in combination. Ara-C is a deoxyctydine analogue effective in the treatment of leukemia.24 After conversion to its monophosphate by the enzyme deoxycytidine kinase (dCK), ara-C is converted to the 5'-triphosphate (ara-CTP), a cytotoxic metabolite.25 Studies have indicated that the level and retention of ara-CTP in human leukemia cells in vitro or during therapy,10 are directly related to clinical response. Since intracellular deoxynucleoside triphosphates (dNTP) inhibit dCK,11,12 drugs capable of depleting or reducing these metabolites might be expected to potentiate the metabolism of nucleoside analogues, such as ara-C. Therapeutic synergism between ara-C and agents that block ribonucleotide reductase, ultimately reducing the dNTP pools, has been demonstrated in animal tumors and human neoplastic cells.3,14 F-ara-A, a deoxyadenosine analogue that is also phosphorylated by dCK,15,16 accumulates as its cytotoxic 5'-triphosphate (F-ara-ATP),17,18 which is an effective inhibitor of ribonucleotide reductase.9,20 Our previous studies using F-ara-A and ara-C in a sequential combination resulted in a higher rate of ara-CTP accumulation.2,14 The potentiation of ara-CTP accumulation by intracellular F-ara-ATP was apparently due to the indirect effect of F-ara-ATP on dCK by reducing the dNTP pools that regulate the enzyme and possibly to a direct effect of F-ara-ATP on the activity of dCK.2

Patients with chronic lymphocytic leukemia (CLL) have been entered in a Phase II trial with the monophosphate of F-ara-A (F-ara-AMP). The F-ara-AMP is rapidly dephosphorylated in vivo to F-ara-A,21 which is taken up by leukemic cells and accumulated intracellularly as F-ara-ATP. This protocol provided the opportunity to study the accumulation of ara-CTP in lymphocytes obtained from patients before and after treatment with F-ara-AMP. Comparisons were made in the ability of leukemic lymphocytes from each patient to accumulate ara-CTP in vitro after no other manipulation following ex vivo incubation with F-ara-A or 20 to 24 hours after therapy with F-ara-AMP.

MATERIALS, PATIENTS, AND METHODS

Materials. F-ara-A was provided by Dr V.L. Narayanan, Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). F-ara-AMP for clinical use was supplied by the National Cancer Institute as a sterile, lyophilized powder (200 mg/vial) free of antibiotic preservatives; it was reconstituted with sterile water. Ara-C and ara-CTP were obtained from Sigma Chemical Co (St Louis, MO). All other chemicals were reagent grade.

Patients. Patients with CLL were treated with F-ara-AMP therapy during 1985 to 1988. Twenty-four patients, 8 females, ages 43 to 82 years (median, 59 years) were selected for the present study on the basis of adequate numbers of circulating lymphocytes, laboratory preparedness, and informed consent to participate in the investigation. CLL was diagnosed based on the number of lymphocytes in blood (>10,000/μL at some time of course of their disease).
and the percentage of lymphocytes in the bone marrow (>-4%). All patients were assigned a Rai stage (2 patients each stage 1; 2; 10 patients each stage 3, 4). Prior to therapy, the median hemoglobin was 10.4 g/dL (range 6.3 to 13.5 g/dL), number of platelets was 151,000/µL (range 9,000 to 352,000/µL), and number of white blood cells (WBCs) was 150,000/µL (range 28,000 to 594,000/µL).

Most patients had previous treatment with antileukemic agents and were refractory to all therapies received before they entered this trial. F-ara-AMP was given intravenously (IV) at a dose of 25 or 30 mg/m² as a 30-minute infusion daily for 5 days. The dose was repeated every 3 to 4 weeks. Peripheral blood samples were obtained before and 20 to 24 hours after the first dose of F-ara-AMP.

**Lymphocytes.** Blood samples (10 mL) were drawn into sterile heparinized tubes and immediately placed in an ice bath. Mononuclear cells, which were mainly leukemic lymphocytes, were isolated from whole blood by Ficol-Hypaque density-gradient centrifugation procedures. Lymphocytes were counted, and the mean cell volume was determined with a Coulter counter (Coulter Electronics, Hialeah, FL) equipped with a cell-size analyzer. The cells were washed twice with phosphate-buffered saline (PBS) and were suspended in RPMI 1640 medium containing 10% fetal calf serum (FCS, GIBCO, Grand Island, NY). The cultures were kept at 37°C in a humidified incubator containing 5% CO². Lymphocytes obtained from healthy donors processed and maintained as above were viable for more than 24 hours as indicated by trypan blue dye-exclusion test.

**Pharmacologic studies.** Lymphocytes obtained from patients before F-ara-AMP treatment were divided into two portions. The first was incubated with 100 µmol/L F-ara-A ex vivo and after 2 or 3 hours was washed into drug-free medium. Aliquots taken from this and the other portion were HClO4 extracted to analyze F-ara-ATP and ribonucleoside triphosphates. The remaining cells in each portion and a third portion comprised of lymphocytes obtained 20 to 24 hours after in vivo F-ara-AMP therapy were then incubated in vitro with 100 µmol/L ara-C and the cytidine deaminase inhibitor tetrahydrouridine (500 µmol/L). After 2 or 4 hours, lymphocytes were washed and HClO4 extracted to analyze cellular ara-CTP concentrations. Detailed kinetic studies were conducted on lymphocytes from four patients. For these analyses, cells obtained before treatment were divided into three portions. The first was incubated with 100 µmol/L ara-C and tetrahydrouridine. Samples were taken at 0, 0.5, 1, 1.5, 2, 3, and 4 hours. The second portion was treated with 100 µmol/L F-ara-A for 2 hours, washed, and incubated with ara-C for 4 hours, and samples were taken as mentioned above. The third portion was divided into three, and each was treated with F-ara-A (100 µmol/L) for 0.5, 1, or 1.5 hours to accumulate different concentrations of F-ara-ATP. Subsequently cells were washed and incubated with 100 µmol/L ara-C for 2 hours, after which ara-CTP concentrations were determined. Twenty to 24 hours after the first F-ara-AMP dose, lymphocytes were isolated from the same four patients and incubated with ara-C (100 µmol/L) for 4 hours. Samples were taken at the same time intervals described for the pretreatment samples to determine the rate of ara-CTP accumulation.

After nucleotide extraction by HClO4, the acid-soluble fractions were neutralized with potassium hydroxide. Supernatants were stored at -20°C until chromatographic analysis was performed. F-ara-ATP and ara-CTP were separated from natural nucleotides by HPLC using an anion-exchange Partisil-10 SAX column by gradient elution with NH₄H₂PO₄ and were quantitated at 262 nm. Quantitation was determined by electronic integration and reference to preprogrammed response factors. The intracellular concentration of these nucleotides was expressed as the quantity of ara-CTP or F-ara-ATP contained in the HClO₄-soluble fraction extracted from a given number of cells of a determined mean volume. Such a calculation presumes that the nucleotides are distributed uniformly in total cell water.

**RESULTS**

The accumulation of ara-CTP was studied in lymphocytes containing F-ara-ATP that were obtained from patients with CLL. The concentration of ara-CTP accumulated in vitro by leukemic lymphocytes was analyzed 0.5 hours after incubation with ara-C (100 µmol/L) and tetrahydrouridine (500 µmol/L) in cells obtained from untreated patients, following preincubation with F-ara-A (100 µmol/L), and after the first dose of F-ara-AMP (25 or 30 mg/m²; Table I). Lymphocytes obtained from patients before therapy exhibited substantial heterogeneity in the concentration of accumulated ara-CTP (median, 241 µmol/L; range 55 to 712 µmol/L). After lymphocytes were pretreated with F-ara-A ex vivo, the ara-CTP concentration increased in the cells of all patients, with a median of 538 µmol/L (range 79 to 1042 µmol/L). The median intracellular concentration of F-ara-ATP in these lymphocytes at the start of ara-C incubation was 237 µmol/L (range 44 to 714 µmol/L). The level of F-ara-ATP in lymphocytes obtained 20 to 24 hours after the first F-ara-AMP dose was significantly lower (median 9 µmol/L; range 2 to 37 µmol/L) compared to lymphocytes incubated ex vivo. However, these relatively small concentrations were associated with potentiation of ara-CTP accumulation in 19 of 23 patients by 1.7-fold (range 0.9 to 6.1-fold).

To determine if the increased ara-CTP accumulation was observed in lymphocytes obtained from patients who were receiving a second or third dose of F-ara-AMP, three patients (Nos. 3, 4, and 9) were studied again when they came for subsequent doses. Although the absolute values were different, each patient’s cells showed an augmentation of ara-CTP accumulation after F-ara-A incubation or F-ara-AMP treatment (Table I; patients 3b, 4b, 9b). In addition, cell samples from 10 patients exposed to F-ara-A ex vivo or F-ara-AMP during therapy were analyzed for ara-CTP accumulation after incubation with ara-C for 4 hours. Compared to the respective 2-hour values, 12 of 15 samples (8 ex vivo and 7 in vivo) increased ara-CTP levels during the additional 2-hour incubation (data not shown).

To study in detail the relationship between F-ara-ATP and ara-CTP accumulation, kinetic studies of arabinosyl nucleoside metabolism were conducted separately in four patients. The rate of F-ara-ATP accumulation over 2 hours was linear in the lymphocytes of all four patients when incubated with 100 µmol/L F-ara-A (Fig 1). The rate of F-ara-ATP accumulation, however, varied between 27 µmol/L/hour and 116 µmol/L/hour. The maximum cellular concentration of F-ara-ATP varied between 50 µmol/L and >200 µmol/L, indicating substantial heterogeneity among patients.

The accumulation of ara-CTP was studied up to 4 hours in three different conditions. First, control lymphocytes obtained from patients before the F-ara-AMP treatment were incubated with 100 µmol/L ara-C and 500 µmol/L tetrahydrouridine (Fig 2A). Two patients (nos. 23 and 24) accumulated ara-CTP at a linear rate up to 4 hours. Accumulation of ara-CTP in patients 21 and 22 was linear for

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Table 1. Accumulation of Ara-CTP by CLL Lymphocytes

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<th>Patient</th>
<th>Ara-CTP, p.mol/L</th>
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Median 241 538 460 2.2 1.7 237 9
Range 55-712 79-1042 50-1241 1.3-4.6 0.9-6.1 44-714 2-37

*Fold increase = ara-CTP in F-ara-A or F-ara-AMP--treated cells/ara-CTP in untreated cells.
†=analyzed after second or third dose.

Lymphocytes were obtained from patients before F-ara-AMP infusion and subjected to no further manipulation (None), or incubated with 100 μmol/L F-ara-A (ex vivo), or 20 to 24 hours after infusion of F-ara-AMP (in vivo). Cells were washed, incubated with 100 μmol/L ara-C and 500 μmol/L tetrahydrouridine for 2 hours. Intracellular levels of ara-CTP and F-ara-ATP were determined as described in Materials, Patients, and Methods.

Abbreviation: ND, not determined.

only 2 hours. The maximum level of intracellular ara-CTP at 4 hours varied between 100 and 300 μmol/L (Fig 2A).

In the second condition, lymphocytes obtained from patients before F-ara-AMP therapy were washed and incubated ex vivo with 100 μmol/L F-ara-A for 2 hours. Cells were washed and incubated with ara-C as mentioned for the control lymphocytes. After the F-ara-A treatment, lymphocytes accumulated ara-CTP at higher rates to greater concentrations than before F-ara-A incubation (Fig 2B). The highest level of ara-CTP was two to three times more than that of the control cells (200 to 800 μmol/L). Except for patient 23, all accumulated ara-CTP at a linear rate for up to 2 hours. Patient 23 also accumulated the lowest concentration of F-ara-ATP (Fig 1) and ara-CTP in control conditions (Fig 2A).

In the third condition, lymphocytes from the same four patients were obtained 20 to 24 hours after the first dose of F-ara-AMP therapy. These were incubated with ara-C, as were the control cells. The rate of ara-CTP accumulation was linear up to 2 hours in all four patients (Fig 2C) and was higher than in control cells (Fig 2A), although less than the F-ara-A–pretreated cells (Fig 2B). The maximum concentration of ara-CTP was between 350 to 450 μmol/L after 4 hours. The intracellular level of F-ara-ATP in these four patients at the start of the ara-C incubation was substantially less in in vivo (5 to 15 μmol/L) than in ex vivo F-ara-A–treated cells (52 to 233 μmol/L).

To evaluate the relationship between the cellular concentration of F-ara-ATP and the accumulation of ara-CTP, lymphocytes from these four patients were incubated with F-ara-A for increasing times (0, 0.5, 1, 1.5, and 2 hours) to accumulate different concentrations of F-ara-ATP, were washed to drug-free medium, and were incubated for 2 hours with ara-C. Figure 3 presents the relationship of ara-CTP and F-ara-ATP derived from such incubations for each patient. The cells of patients 21 and 22 showed a plateau of ara-CTP accumulation after 100 μmol/L intracellular F-ara-ATP. Patient 23 did not accumulate more than 60 μmol/L F-ara-ATP and did not reach the saturation for ara-CTP accumulation. Little stimulation of ara-CTP accu-
DISCUSSION

Previously we have demonstrated that K562 cells loaded with F-ara-ATP accumulated ara-CTP at a greater rate to higher cellular concentrations than did cells incubated with ara-C alone. Additional experiments indicated a direct relationship between the cellular concentration of F-ara-ATP and the enhancement of ara-CTP synthesis. Stimulated accumulation of ara-CTP in cells loaded with F-ara-ATP was also observed in the present study when fresh clinical specimens were analyzed. Lymphocytes isolated from patients with chronic lymphocytic leukemia had a 2.2-fold increase in ara-CTP accumulation when they were pretreated with F-ara-A ex vivo. Also, leukemic lymphocytes recovered from patients after infusion of 25 or 30 mg/m² of F-ara-AMP accumulated higher (1.7-fold) ara-CTP when incubated in vitro with 100 µmol/L ara-C for 2 hours, compared to a similar incubation before F-ara-AMP treatment (Table 1). This metabolic enhancement is of interest because previous studies demonstrated strong correlations between cytotoxicity and cellular levels of arabinosyl nucleotide in experimental systems. In addition, the accumulation and retention of ara-CTP in human acute leukemia cells in vitro or during therapy have been correlated with clinical response.

Leukemic lymphocytes incubated with 100 µmol/L ara-C accumulated a median ara-CTP concentration of 241 µmol/L (range 55 to 712 µmol/L). The heterogeneity in ara-CTP accumulation among patients may represent variation in the activity of dCK. In general, peripheral lymphocytes and bone marrow cells from patients with CLL possess a high activity of ara-C kinase, now known to be dCK, compared with the normal marrow and WBCs. This is consistent with the greater ability of CLL lymphocytes to accumulate ara-CTP after infusion of high-dose ara-C (3 g/m² over 2 hours; peak = 625 µmol/L, n = 22) relative to acute leukemia in relapse (peak = 375 µmol/L, n = 41) and chronic myelogenous leukemia in blast crisis (peak = 243 µmol/L, n = 15). Although the activity of cytidine/deoxycytidine deaminase is low in leukemia cells from...
patients with CLL compared with other leukemia cells and normal cells, tetrahydrouridine was included in the in vitro incubation with ara-C to ensure uniform ara-C concentrations. Furthermore, potentiation of ara-CTP accumulation appears to be due to increased anabolism rather than an effect on dephosphorylation. Therefore the high ara-CTP accumulation by CLL lymphocytes reflects high dCK activity that can be potentiated even further in cells containing F-ara-ATP.

The mechanism of potentiation of ara-CTP accumulation by F-ara-ATP is attributed to two main factors. First, a direct effect of F-ara-ATP on the activity of dCK has been observed. Second, the indirect effect mediated by F-ara-ATP inhibition of ribonucleotide reductase, resulting in a decrease of dNTP pools, has been associated with an increase in ara-C phosphorylation.

Peripheral blood lymphocytes have relatively low levels of dNTPs. Furthermore, the activity of ribonucleotide reductase in these cells was below the limit of detection. If this is the case with lymphocytes from patients with CLL, the indirect effect of F-ara-ATP on dNTP pools would be minimal. This suggests that F-ara-ATP may directly affect the activity of dCK. Additional evidence for an increase of the activity of dCK in cells containing F-ara-ATP was obtained by studying the metabolism of 2',2'-difluorodeoxycytidine, a nucleoside analogue that also requires deoxycytidine kinase for phosphorylation. CLL lymphocytes loaded with F-ara-ATP accumulated 2',2'-difluorodeoxycytidine triphosphate to twofold greater concentrations compared to similar incubation in untreated lymphocytes from the same patient (unpublished results). This indicates that the effect of F-ara-ATP is not restricted to ara-C; rather it potentiates anabolism of nucleoside analogues that use dCK for phosphorylation.

It is difficult to extrapolate from the in vitro metabolism of a substrate to the in vivo metabolism of the same compound. One important difference we noticed with the lymphocytes was that the in vitro accumulation of F-ara-ATP was much higher than in vivo accumulation of F-ara-ATP. The concentration of F-ara-A in vitro was 100 μmol/L, whereas after the first dose of 20 to 25 mg/m² of F-ara-AMP, the peak level of F-ara-A in plasma was between 1.4 and 2.5 μmol/L. Therefore the higher accumulation of F-ara-ATP in vitro probably reflects the higher concentration of F-ara-A. In fact, patients who received higher doses (50 to 125 mg/m²) achieved proportionately greater peak levels of F-ara-ATP.

Second, lymphocytes treated with F-ara-AMP in vivo accumulated similar ara-CTP levels (median 460 μmol/L), as did cells incubated ex vivo with F-ara-A (median 538 μmol/L; Table 1). However, the median concentration of F-ara-ATP at the time of ara-C incubation was only 9 μmol/L in in vivo treated cells compared with 237 μmol/L in ex vivo-treated cells (Table 1). This indicates that the relationship of the F-ara-ATP level to the potentiation of ara-CTP accumulation was quantitatively different in vivo compared with ex vivo (Fig 3).

Patients with CLL treated with F-ara-AMP at a dose of 25 or 30 mg/m², some of whom participated in the present investigations, achieved complete (13%) and partial remissions (44%) without untoward toxicity seen at higher doses. Potentiation of ara-CTP accumulation in lymphocytes obtained from these patients was proportional to the cellular concentration of F-ara-ATP (Fig 3). Consistent with the previous pharmacology studies, in the present investigation, lymphocytes during therapy demonstrated that the peak cellular concentration of F-ara-ATP (median 19 μmol/L, range 6 to 52 μmol/L, n = 23) was achieved 4 hours after the beginning of the 30-minute infusion (unpublished observations). These data predict that infusion of ara-C 4 hours after F-ara-AMP administration will achieve the maximum potentiation of ara-CTP accumulation. This strategy is being employed in the design of a new protocol using F-ara-A and ara-C in combination for treatment of CLL.

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