Fludarabine Infusion Potentiates Arabinosylcytosine Metabolism in Lymphocytes of Patients with Chronic Lymphocytic Leukemia

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ABSTRACT

Our previous work has shown that incubation of K562 cells or lymphocytes from patients with advanced chronic lymphocytic leukemia (CLL) with arabinosyl-2-fluorodeoxyadenosine (ara-A) potentiates the rate of arabinosylcytosine 5'-triphosphate (ara-CTP) synthesis during subsequent treatment with arabinosylcytosine (ara-C). To test the biochemical modulation of ara-CTP in a clinical setting, we designed a protocol to administer fludarabine (Fludara, F-ara-AMP) and ara-C in a pharmacologically directed sequence for patients with CLL refractory to conventional fludarabine therapy. ara-C was infused in seven patients with progressive CLL at a dose rate that maximizes ara-CTP accumulation (9.5 g/m² during 2 h). Fludarabine (30 mg/m² during 30 min) was infused 20 h later, followed by a second, identical dose of ara-C at 24 h, when the concentration of F-ara-A 5'-triphosphate (F-ara-ATP) was maximal in CLL cells. Comparison of ara-CTP pharmacokinetics in circulating CLL cells demonstrated that the ara-CTP area under the curve increased by a median of 1.5-fold (range, 1.1- to 1.7-fold) after fludarabine infusion. Plasma pharmacokinetics indicated that neither the median steady-state ara-C concentrations nor the levels of its deamination product arabinosyluracil were significantly affected by fludarabine infusion. The median rate of ara-CTP elimination was slightly faster after fludarabine treatment (76 h versus 59 h), suggesting that catalysis of ara-CTP was not responsible for the increased ara-CTP area under the curve. The rate of ara-CTP accumulation by CLL cells after fludarabine infusion, however, was increased by a median of 1.3-fold in seven of the eight patients (range, 1.2- to 1.8-fold); the peak occurred within 1 h of the end of the infusion. In vitro incubation of leukemic lymphocytes with F-ara-A before ara-C also showed a median 1.3-fold increase in the rate of ara-CTP accumulation. Thus, infusion of fludarabine before ara-C augments ara-CTP metabolism in leukemic lymphocytes. This knowledge should be considered in the design of combination chemotherapy.

INTRODUCTION

Fludarabine and ara-C are two important arabinosyl analogues used in the treatment of CLL and acute myelogenous leukemia, respectively (1-4). To act as antileukemia agents, these antimetabolites have to be phosphorylated to their active cytotoxic 5'-triphosphates (F-ara-AMP and ara-CTP) (5-7). The rate-limiting step in the synthesis of these triphosphates is the initial phosphorylation to the respective monophosphates, which is catalyzed by dCyd kinase (8-11).

Previous studies demonstrated that when K562 human leukemia cells were incubated with the concentration of nucleoside analogue that saturates the rate of triphosphate formation, ara-CTP accumulated to 100 μM in cells, whereas the maximal cellular F-ara-ATP concentration exceeded 500 μM (12). Sequential incubation with ara-C followed by F-ara-A slowed the F-ara-ATP accumulation rates. This indicated that intracellular ara-CTP inhibited dCyd kinase or possibly residual ara-C competed with F-ara-A for this enzyme, consequently lowering the rate of phosphorylation of F-ara-A. Reversal of this sequence, however, resulted in an enhanced rate of ara-CTP accumulation. This potentiation of ara-CTP accumulation by intracellular F-ara-ATP was apparently due to an indirect effect of F-ara-ATP on dCyd kinase, achieved by reducing the deoxynucleotide pools that regulate the enzyme and possibly to a direct effect of F-ara-ATP on the activity of dCyd kinase (12).

These observations were extended to patients with CLL receiving fludarabine therapy. Accumulation of ara-CTP increased 1.7-fold in lymphocytes obtained after fludarabine therapy when leukemic lymphocytes isolated before and after therapy were incubated in vitro with 100 μM ara-C (13). Because the indirect effect of F-ara-ATP on deoxynucleotide pools would be minimal in these quiescent lymphocytes, a direct effect of F-ara-ATP is a likely explanation for the augmentation of ara-C phosphorylation.

Based on these studies, we hypothesized that sequential combination of fludarabine and ara-C would potentiate the accumulation of ara-CTP in lymphocytes of patients with CLL. To test this hypothesis, the accumulation of ara-CTP by leukemic lymphocytes after infusion of ara-C alone was compared with ara-CTP pharmacokinetics following fludarabine infusion in the same patient. Modulation of ara-CTP accumulation in vitro was compared and correlated with the results from the same patients during therapy to analyze the mechanism behind the potentiation of ara-CTP metabolism in quiescent leukemic lymphocytes. A preliminary report of these results has been presented (14).

PATIENTS AND METHODS

Patients. Eleven patients with CLL refractory to conventional fludarabine therapy were treated with this protocol between April and November 1990. Eight patients, 3 females (36-74 years of age; median, 55 years) were selected for the present study on the basis of adequate number 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 during the course of their disease) and the percentage of lymphocytes in the bone marrow (>40%). All patients were assigned a Rai stage (3 patients were stage 1, and 5 patients were stage 4). Prior to therapy, the median number of WBCs was 70,000/μl (range, 13,000-214,000/μl). All patients had been refractory to previous treatment with alkylating agents and to fludarabine alone.

Protocol. The first course of the treatment plan (Fig. 1) included one dose of 0.5 g/m² ara-C and one dose of 30 mg/m² fludarabine followed by a second identical dose of ara-C. The subsequent courses consisted of one dose of fludarabine and one dose of ara-C given at least 2 weeks after the first course. In the first course, ara-C was given twice to evaluate the effect of fludarabine on the pharmacokinetics of ara-CTP.
in circulating leukemic lymphocytes. The dose rate of ara-C, 0.5 g/m²2
for 2 h, produced ara-CTP accumulation rates similar to those
observed after high-dose ara-C therapy (3 g/m² during 2 h) in the same
patients (15, 16). These observations suggested that dCyd kinase activity
was saturated at plasma ara-C levels (>7 μM) achieved with inter-
mediate-dose ara-C, thus, the lower dose had the potential to produce
equivalent responses but with less toxicity. The median elimination t½
of ara-CTP in CLL cells is 6.6 h (15), indicating that the concentration of
ara-CTP 20 h after ara-C infusions would be low in leukemia cells
and thus unlikely to affect the metabolism of fludarabine infused at
that time. This provided the rationale to schedule the fludarabine
infusion 20 h after the start of the ara-C. Previous clinical studies using
fludarabine at a dose rate of 25–30 mg/m²/d for patients with progres-
sive CLL resulted in a >50% response rate (1). Because this dose is
well tolerated, we selected 30 mg/m² as the dose of fludarabine for the
present protocol. Earlier studies demonstrated that, at this dose (25–
30 mg/m²/d), the F-ara-ATP concentration peaked within 4 h in CLL
lymphocytes (17, 18). Because our previous investigations using human
leukemia cells indicated that the augmentation of ara-CTP accumula-
tion is directly related to the cellular concentration of F-ara-ATP (12),
we reasoned that ara-C infusions given 4 h after fludarabine infusions
would optimize ara-CTP modulation. Based on these studies, we ad-
ministered fludarabine 20 h after the first ara-C infusion; the second
ara-C dose was given 4 h after fludarabine. Thus, each patient served
as his or her own control for the effect of cellular F-ara-ATP on ara-
CTP pharmacokinetics.

Drugs. Fludarabine was supplied by Berlex Biosciences, Inc. (Ala-
meda, CA) as a sterile, lyophilized powder free of antibacterial preserv-
avatives, ara-C was commercially available as Cytosar-U from the Upjohn
Co. F-ara-A and chlorodeoxyadenosine were provided by Dr. V. L.
Narayanan, Drug Synthesis and Chemistry Branch, National Cancer
Institute (Bethesda, MD). Hydroxyurea, ara-C, and ara-CTP were
obtained from Sigma Chemical Co. (St. Louis, MO). Lilly Research
Laboratories (Indianapolis, IN) provided difluorodeoxycytidine. All
other chemicals were reagent grade.

Blood Samples for Clinical Pharmacology. To determine the phar-
macokinetics of ara-C in plasma and ara-CTP in intact leukemic
lymphocytes, 40-ml blood samples were obtained before therapy, and
10-ml samples were obtained at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, and
12 h after the first ara-C infusion. Ten-ml samples were also drawn just
before the fludarabine infusion at 20 h and at 20.5, 21.5, 22.5, and 23.5
h to determine the cellular pharmacology of F-ara-ATP and ara-CTP.
The second ara-C dose was infused over 2 h starting at 24 h, and blood
samples were obtained at 24, 24.5, 25, 25.5, 26, 26.5, 27, 28, 30, 32,
34, 36, and 44 h. All blood samples were collected in Vacutainer tubes
containing heparin and 5 μmol tetrahydrouridine to inhibit cytidine/
deoxyctydine deaminase. Although F-ara-A is relatively resistant to
adenosine deaminase, the inhibitor erythro-9-(2-hydroxy-3-
nonyl)adenine was added to a final concentration of 1 μM as a precau-
tion. The tubes were placed in an ice water bath before being transported
to the laboratory for processing. Control studies have demonstrated
that leukemia cells are stable under these conditions with respect to
size, membrane integrity, and cellular nucleotide content for at least
15 h (13).

Plasma Pharmacology. To determine the ara-Cₘ and ara-U levels,
the blood samples were centrifuged to separate plasma, ara-C and ara-
U concentrations were determined by reverse-phase HPLC as described
before (19, 20). The plasma ara-Cₘ is the mean of the values of the
samples taken 1 and 2 h after the start of the ara-C infusion. Because
ara-U levels at these times were very different, they are presented as 1-
and 2-h values.

Cellular Pharmacology. After removal of plasma, the blood samples
were diluted with phosphate-buffered saline, and mononuclear cells
were isolated by Ficoll-Hypaque density-gradient centrifugation pro-
cedures described before (20). Normal nucleotides and arabinosyl nu-
cleotides were extracted from lymphocytes by HClO₄ (20). ara-CTP
and F-ara-ATP were separated from ribonucleoside triphosphates by
anion-exchange chromatography on a Partisil-10 SAX column by gra-
dient elution with NH₄H₂PO₄ and were quantitated at λ₂₅₄nm by electronic
integration with reference to response factors derived from external
standards (21). The intracellular concentrations of nucleotides were
calculated and expressed as the quantity of nucleotides contained in the
extract from a given number of cells of a determined mean volume.
This calculation assumes that these nucleotides are uniformly distrib-
uted between the total cell volume. The lower limit of sensitivity of this assay
is 25 pmol in an extract of 2 × 10⁷ cells, corresponding to a cellular
concentration of 5 μM.

In Vitro Studies. Leukemic lymphocytes were washed with phos-
phate-buffered saline and suspended in RPMI-1640 medium containing
10% fetal calf serum. The cultures were kept at 37°C in a humidified
incubator containing 5% CO₂. Previous studies indicated that lympho-
cytes obtained, processed, and maintained this way are viable for >24
h as indicated by trypan blue dye exclusion and that their cellular
nucleotide levels are not affected (13). The lymphocytes were incubated
with or without 5 μM F-ara-A for 4 h, washed, and incubated with 10
μM ara-C and 10 μM tetrahydrofuryldine to inhibit deamination of ara-
C. Samples were taken at 0.5, 1, 1.5, 2, 3, and 4 h and processed to
alyze the accumulation of ara-CTP.

To analyze the effect of other ribonucleotide reductase inhibitors on
the phosphorylation of ara-C, leukemic lymphocytes isolated from
patients before therapy were incubated with no drug or with 5 mM
hydroxyurea, 10 μM [8-³H]chlorodeoxyadenosine (Moravek Biochemi-
ticals Inc., Brea, CA), 10 μM [5-³H]difluorodeoxyuridine (Lilly Research
Laboratories, Indianapolis, IN), or 10 μM [8-³H]ara-A (Amersharm
Corp., Arlington Heights, IL). After 3 h, the lymphocytes were washed
and incubated with 10 μM [2-³H]ara-C (Moravek) and 10 μM tetrahy-
drofuryldine for 4 h. Samples were taken every hour for 4 h and assayed
for ara-CTP accumulation.

Calculations and Statistical Analysis. The levels of ara-C and F-
ara-ATP obtained by HPLC analysis were normalized based on endog-
aneous NTPs. The means of each NTP in all samples were determined
each individual, and the deviation from the mean for each NTP was
cluded. The observed cellular concentrations of F-ara-ATP and ara-
CTP were normalized at each time point by multiplying by the average
development of all four NTPs.

The rate of ara-CTP accumulation in leukemia cells was calculated
by linear regression analysis forcing a 0-h value. For the first ara-C
dose, 0 h was computed as 0 μM, and for the second ara-C dose, the
cellular ara-CTP concentration at 24 h was computed as the pretreat-
ment value. Generally, the ara-CTP accumulation rate was linear up to
3 h during therapy and up to 4 h in vitro. The AUC for the accumulation
of ara-CTP in leukemia cells was estimated by triangulation, whereas
the AUC for the elimination of ara-CTP was calculated by integration
as previously described (22). The total AUC in the leukemia cells was
the sum of these calculations. The ara-CTP AUC observed after the
second ara-C dose was corrected by subtraction of the calculated resid-
ual ara-CTP AUC of the first dose from the values of the second dose.
Similar corrections were made to calculate the peak ara-CTP and the
plasma ara-U concentrations associated with the second ara-C dose.

Pharmacological data obtained during first and second doses of ara-
C were compared using the two-tailed, paired t test. Rectangular
hyperbola, nonlinear regression analysis was used to fit the curves for the
relationship between the concentration of F-ara-ATP and the potentia-
the ara-CTP rate or AUC by using GraphPAD lupini, version
3.0 (Intuitive Software for Science, San Diego, CA).

RESULTS

Effect of Fludarabine Infusion on Plasma ara-C and ara-U. The ara-Cₘ showed little variation among individuals (range,
7–22 μM), with median values for the ara-C infusions before and after fludarabine of 12 and 13 μM, respectively (Table 1). To evaluate the effect of fludarabine infusion within individuals, we compared the ara-Cₜₐₜ value observed during the two ara-C doses. The median ratio between the second and first dose of ara-C was 0.9 (range, 0.7–1.4), indicating that fludarabine infusion did not influence the plasma pharmacology of ara-C (P = 0.36). Plasma concentrations of ara-U generated during the two ara-C doses were greater than those of ara-C and increased during the infusion (Table 2). However, fludarabine infusion did not change ara-U concentrations significantly (P = 0.40 for 1 h and 0.29 for 2 h). The ratio of ara-U plasma concentrations was also similar at 3 and 4 h after the start of the ara-C infusions (data not shown). Because the concentration of ara-C in plasma during the first ara-C infusion was >7 μM, the rate of ara-CTP accumulation was expected to be maximal during this dose (15, 16). This allowed us to question whether prior fludarabine infusion would potentiate the pharmacokinetics of ara-CTP.

Effect of Fludarabine Infusion on the Cellular Pharmacokinetics of ara-CTP. To illustrate our approach, the pharmacokinetics of ara-CTP in circulating leukemic lymphocytes during the first therapy cycle are presented in Fig. 2. Inspection of these data indicates that greater concentrations of ara-CTP were accumulated in cells when ara-C was administered after fludarabine than when ara-C was infused before fludarabine. Following this approach, we characterized the action of fludarabine on ara-CTP concentrations by comparing the rates of ara-CTP accumulation, the peak cellular ara-CTP concentrations, the rates of ara-CTP elimination, and the intracellular AUC of ara-CTP during and after each ara-C infusion in eight patients.

The peak, elimination rate, and AUC of ara-C were compared in lymphocytes from patients before and after fludarabine infusion (Table 3). The peak ara-C concentration was achieved within 1 h of the end of ara-C infusion, except for patients 1 and 5, in whose lymphocytes ara-CTP levels reached maximum at 2 and 4 h after the second dose, respectively. The peak ara-CTP during the first dose ranged from 144 to 401 μM, whereas during the second dose of ara-C peaks were higher, ranging from 232 to 632 μM. As indicated by the ratio of the peak values after and before fludarabine, the peak ara-CTP concentration was elevated significantly by fludarabine infusion (P = 0.007).

Comparison of the ara-CTP AUCs indicated a significant (P = 0.009) 1.5-fold increase during the second dose of ara-C (range, 1.1- to 1.7-fold, Table 3). To determine whether the increased intracellular exposure to ara-CTP was due to a decrease in the rate of ara-CTP elimination, the t₀ of ara-CTP retention was compared before and after fludarabine infusion (Table 3). These values ranged from 3.4 to 12.7 h among the eight patients during the first course of ara-C and from 3.7 to 10.1 h during the second course, indicating that fludarabine infusion did not decrease the rate of ara-CTP elimination; in fact, the median elimination rate was slightly greater after fludarabine infusion (Table 3), although the paired elimination rates were not significantly different (P = 0.25). Taken together, these results demonstrate that the increase in the ara-CTP AUC was due to a higher rate of anabolism of ara-C rather than to a slower rate of catabolism of the active metabolite ara-CTP.

The rates of ara-CTP accumulation before and after fludarabine infusion are summarized in Table 4. All patients showed a significant increase in the rate of ara-CTP accumulation during the second ara-C infusion (P = 0.0013) except for patient 5. The median increase in the rate of ara-CTP accumulation following fludarabine infusion was 1.3-fold. The fold increase in the rate of ara-CTP accumulation during therapy ranged from 1.2 to 1.8. To compare it with the fold increase in the rates of ara-CTP accumulation in vitro, leukemic lymphocytes were obtained from each patient before therapy and divided into two cultures. One served as control and was incubated in drug-free medium for 4 h, whereas the other culture was incubated with 5 μM F-ara-A to accumulate F-ara-ATP. Both were washed and incubated with 10 μM ara-C, and kinetics of ara-CTP accumulation were analyzed. Although there was heterogeneity in the absolute values among patients, each patient's cells showed increased ara-CTP accumulation after F-ara-A incubation. Table 4 indicates a significant (P = 0.016) median 1.3-fold increase (range, 1.1- to 2.5-fold) after F-ara-A incubation in vitro. The median increases in the ara-CTP accumulation rates are similar during therapy and in vitro; however, there was not a strong correlation for individuals (r = 0.35). Also, the accumulation of ara-CTP in vitro was much less than that during therapy. This is unexpected, since the plasma concentration of ara-C was similar to that in the medium. Second, the ara-C concentration remained >7 μM for 2 or 3 h in the plasma but for 5 h in the medium. Conversely, the

### Table 1 Effect of fludarabine on ara-Cₜₐₜ

<table>
<thead>
<tr>
<th>Patient</th>
<th>Before</th>
<th>After</th>
<th>Ratio after to before</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22 ± 0.7</td>
<td>20 ± 19.8</td>
<td>0.9</td>
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<tr>
<td>2</td>
<td>12 ± 5.1</td>
<td>14 ± 1.4</td>
<td>1.2</td>
</tr>
<tr>
<td>3</td>
<td>9 ± 3.5</td>
<td>13 ± 1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>4</td>
<td>11 ± 0.7</td>
<td>13 ± 0.7</td>
<td>1.2</td>
</tr>
<tr>
<td>5</td>
<td>10 ± 0.7</td>
<td>7 ± 6.4</td>
<td>0.7</td>
</tr>
<tr>
<td>6</td>
<td>17 ± 0.0</td>
<td>12 ± 1.4</td>
<td>0.7</td>
</tr>
<tr>
<td>7</td>
<td>18 ± 0.7</td>
<td>12 ± 4.5</td>
<td>0.7</td>
</tr>
<tr>
<td>8</td>
<td>9 ± 0.3</td>
<td>7 ± 0.2</td>
<td>0.8</td>
</tr>
</tbody>
</table>

### Table 2 Effect of fludarabine on the plasma concentration of ara-U

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<td>0.9</td>
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<td>89</td>
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<tr>
<td>After</td>
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<tr>
<td>Ratio after to before</td>
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<td>0.9</td>
</tr>
<tr>
<td>Before</td>
<td>22</td>
<td>70</td>
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<tr>
<td>After</td>
<td>27</td>
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</tr>
<tr>
<td>Ratio after to before</td>
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<td>0.9</td>
</tr>
<tr>
<td>Before</td>
<td>37</td>
<td>63</td>
</tr>
<tr>
<td>After</td>
<td>32</td>
<td>61</td>
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<tr>
<td>Ratio after to before</td>
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<td>0.9</td>
</tr>
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<td>Before</td>
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</tr>
<tr>
<td>After</td>
<td>19</td>
<td>47</td>
</tr>
<tr>
<td>Ratio after to before</td>
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<td>0.9</td>
</tr>
<tr>
<td>Before</td>
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<td>52</td>
</tr>
<tr>
<td>After</td>
<td>19</td>
<td>25</td>
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<td>Ratio after to before</td>
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<td>0.9</td>
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<tr>
<td>Before</td>
<td>31</td>
<td>60</td>
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<tr>
<td>After</td>
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<tr>
<td>Before</td>
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<td>42</td>
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<tr>
<td>After</td>
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<td>28</td>
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<tr>
<td>Ratio after to before</td>
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<tr>
<td>Before</td>
<td>34</td>
<td>39</td>
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<tr>
<td>After</td>
<td>30</td>
<td>52</td>
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Table 3 Effect of fludarabine on the ara-CTP peak, elimination, and intracellular AUC

<table>
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<th>Patient</th>
<th>Before</th>
<th>After</th>
<th>Ratio after to before</th>
<th>$t_{1/2}$ of ara-CTP elimination (h) Before</th>
<th>After</th>
<th>Ratio after to before</th>
<th>ara-CTP AUC (µM/h) Before</th>
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<td>1.1</td>
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<td>5.1</td>
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<td>10.0</td>
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<td>4.8</td>
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<td>2659</td>
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<td>1.0</td>
<td>1144</td>
<td>1873</td>
<td>1.6</td>
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</table>

Table 4 Comparison of the rate of ara-CTP accumulation before and after fludarabine treatment in vivo and in vitro

<p>| F-ara-ATP and ara-CTP were quantitated by HPLC assay and the rates of ara-CTP accumulation were calculated. |
|--------------------------------------------------|-------------------------------------------------|------------------------------|</p>
<table>
<thead>
<tr>
<th>Patient</th>
<th>Before (µM/h)</th>
<th>After (µM/h)</th>
<th>Ratio after to before</th>
<th>F-ara-ATP (µM)</th>
<th>Before (µM/h)</th>
<th>After (µM/h)</th>
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intracellular level of F-ara-ATP in these seven patients at the start of the ara-C exposure was substantially less during therapy (7-37 µM) than in cells treated with F-ara-A in vitro (43-198 µM).

Relationship between F-ara-ATP and Potentiation of ara-CTP. Our previous in vitro studies using K562 cells and leukemic lymphocytes suggested a relationship between the concentration of F-ara-ATP and ara-CTP described by a rectangular hyperbola, reaching a maximum at 75-100 µM intracellular F-ara-ATP (12, 13). To determine whether a similar relationship existed in lymphocytes during therapy, the levels of F-ara-ATP at the time of ara-C infusion and the potentiation of ara-CTP accumulation were compared. The curve generated by fitting the data to a rectangular hyperbola indicated that the increase in the ara-CTP AUC was maximal at 20 µM intracellular F-ara-ATP (Fig. 3A). Although the relationship was similar in vitro, the saturation concentration was higher than that observed during therapy (Fig. 3B). Although an exact value is difficult to determine because of the data distribution, Fig. 3A indicates that the concentration required for maximal potentiation was between 50 and 100 µM. Nevertheless, these results from eight patients are consistent with values observed in K562 cells (12) and leukemic lymphocytes from individuals receiving multiple F-ara-ATP concentrations (13). Thus, the F-ara-ATP concentration needed for the augmentation of ara-CTP accumulation during therapy was less than that predicted by in vitro studies.

Indications of a Direct Effect of F-ara-ATP on dCyd Kinase in CLL Cells. As mentioned previously, our mechanistic studies (12, 13) indicated that in K562 cells the potentiation of ara-CTP by F-ara-ATP was due to both a direct and an indirect effect on dCyd kinase. Peripheral blood lymphocytes have relatively low levels of dNTPs, and the activity of ribonucleotide reductase in these quiescent cells was below the limit of detection (23, 24). If one assumes that this holds for lymphocytes from patients with CLL, the indirect effect of F-ara-ATP on dNTP pools would be minimal. This suggested that F-ara-ATP may directly affect the activity of dCyd kinase.

To evaluate the participation of an indirect effect on the
potentiation of ara-CTP accumulation, leukemic lymphocytes obtained from patients with CLL were incubated for 3 h with the known inhibitors of ribonucleotide reductase: hydroxyurea, chlorodeoxyadenosine, difluorodeoxycytidine, and F-ara-A. Control lymphocytes were left in drug-free medium for 3 h. All cultures were washed and incubated with ara-C, and ara-CTP accumulation was measured hourly for 4 h. As predicted, preincubation with either hydroxyurea (Fig. 4), chlorodeoxyadenosine, or difluorodeoxycytidine (for clarity not shown) did not increase the rate of accumulation of ara-CTP. Conversely, the cells pretreated with F-ara-A showed a 20% increase \((P<0.005)\) in the rate of ara-CTP accumulation. These results are consistent with the hypothesis that the potentiation of ara-CTP in CLL cells was not due to lowering of deoxynucleotide pools. Rather, it is likely that F-ara-ATP has a more direct action on the processes that control the rate of ara-C anabolism.

DISCUSSION

This study showed that accumulation of ara-CTP in leukemic lymphocytes during therapy was potentiated by infusion of fludarabine. The results obtained through this investigation suggest the following conclusions. (a) This in vivo study confirms our previous in vitro investigations of leukemic lymphocytes and extends them to lymphocytes obtained from patients during therapy. (b) Mechanistic studies allowed us to conclude that the modulation of ara-CTP accumulation by leukemic lymphocytes was not due to inhibition of ribonucleotide reductase and subsequent lowering of deoxynucleotides by F-ara-ATP; instead F-ara-ATP may have stimulated ara-C phosphorylation directly. (c) Biochemical modulation of the cytotoxic metabolite ara-CTP with this combination sequence may be applied to the treatment of leukemia in which ara-C is used as a major therapy. These conclusions will be discussed with a view to developing strategies to optimize ara-C therapies.

Potentiation of ara-CTP Pharmacology by Fludarabine during Therapy. Previously, we demonstrated that K562 cells loaded with F-ara-ATP accumulated ara-CTP at a greater rate and achieved higher cellular concentrations than did cells incubated with ara-C alone (12). Additional experiments indicated a direct relationship between the cellular concentration of F-ara-ATP and the enhancement of ara-CTP synthesis. Increased accumulation of ara-CTP in cells loaded with F-ara-ATP has also been observed in fresh clinical specimens (13). Lymphocytes isolated from patients with CLL had a 2.2-fold increase in ara-CTP accumulation when they were pretreated with F-ara-A in vitro. Also, leukemic lymphocytes recovered from patients after infusion of 25 or 30 mg/m² of F-ara-AMP accumulated higher (1.7-fold) concentrations of ara-CTP when incubated in vitro with 100 \(\mu\)M ara-C for 2 h, compared to a similar incubation before F-ara-AMP treatment (13). The design of the present protocol permitted the demonstration of similar potentiation of ara-CTP metabolism in CLL cells by fludarabine during therapy.

Extrapolation from the in vitro metabolism of a substrate to the in vivo metabolism and modulation of the same compound is certain to be confounded by pharmacological factors. Quantitative differences in the level of enhancement of ara-CTP potentiation were observed between this clinical study and previous in vitro studies. Nevertheless, the general features of potentiation of ara-CTP accumulation in leukemic lymphocytes by F-ara-ATP were observed in seven of eight patients. In contrast, previous pharmacological studies in patients with CLL receiving two serial doses of ara-C demonstrated that neither the accumulation rate nor the AUC of ara-CTP changed.

Steps Involved in the Potentiation of ara-CTP Accumulation. The intracellular accumulation of ara-CTP is not representative of the rate of ara-C phosphorylation; rather, it is a combination of several processes. The repertoire of steps which are involved in the accumulation of ara-CTP includes the achievement of plasma concentrations of substrate (ara-C), transport into the cell, deamination of both the nucleoside and its monophosphate, the initial phosphorylation of ara-C to monophosphate, phosphorylation of ara-C monophosphate to di- and triphosphates, and eventually the dephosphorylation and elimination of ara-C nucleotides. Each of these parameters has been considered to determine the role of fludarabine.

The level of ara-C in plasma during the first dose of ara-C was similar to that during the second dose which was infused after fludarabine treatment (Table 1). At this exogenous level (>7 \(\mu\)M), the transport of ara-C is by a high-capacity, rapid transmembrane equilibration resulting in a similar intracellular concentration of ara-C (26). Deamination of ara-C to ara-U, which is a result of the activity of large organ deoxycytidine deaminase (27, 28), was substantial but was not different during the first and second doses of ara-C (Table 2). The rate-limiting step in the accumulation of triphosphate is the initial phosphorylation catalyzed by dCyd kinase (10, 11). The augmentation of the rate of ara-CTP accumulation during the second dose of ara-C indicated that phosphorylation of ara-C is the step influenced by F-ara-ATP (Table 4). The fact that the rate of ara-CTP elimination (Table 3) did not decrease after fludarabine infusion suggests that fludarabine affected only the anabolism of ara-C and not its catabolism.

Mechanisms for the Potentiation of ara-CTP Accumulation by Fludarabine. Potentiation of ara-C phosphorylation by F-ara-ATP can be attributed to two main factors: (a) a direct effect of F-ara-ATP on the activity of dCyd kinase (12) and (b) the indirect effect mediated by F-ara-ATP inhibition of ribonucleotide reductase (29, 30), which results in a decrease in the
dNTP pools, has been associated with an increase in ara-C phosphorylation (12, 31). Peripheral blood lymphocytes have relatively low levels of dNTP (23, 24). Furthermore, the activity of ribonucleotide reductase in these cells was below the limit of detection (23). If this is true of 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 dCyd kinase.

Our in vitro experiment using different inhibitors of ribonucleotide reductase was designed to study the role of these analogues in potentiation of ara-CTP metabolism. Since the results were negative with hydroxyurea, chlorodeoxyadenosine, and difluorodeoxycytidine, the indirect effect of F-ara-ATP on dCyd kinase is unlikely, emphasizing the importance of the direct effect in these quiescent cells. This finding leads to two predictions that can be evaluated in different settings: (a) in myelogenous leukemias, in which the percentage of cycling cells is much greater (32–34) than in CLL, the combination of fludarabine and ara-C would result in a greater increase in ara-CTP accumulation because both the direct and indirect effects of F-ara-ATP would affect ara-CTP pharmacology. The 3-fold increase in the ara-CTP rate observed in K562 cells (a cell line derived from a patient with chronic myelogenous leukemia) and a 2-fold increase in circulating leukemic blasts of patients with acute myelogenous leukemia are consistent with this postulate (12, 35); (b) if fludarabine treatment of quiescent lymphocytes can augment ara-CTP accumulation, we can predict that the cells in different phases of the cell cycle would show the same response. Indeed, our data demonstrated the capability of higher ara-CTP accumulation after F-ara-A incubation of K562 cultures enriched for cells in G1, S, and G2 + M phases (36).

Design of Future Protocols. The metabolic enhancement of ara-CTP is of interest because previous studies demonstrated strong correlations between cytotoxicity and cellular levels of arabinosyl nucleotides in experimental systems (22, 37). In addition, the accumulation and retention of ara-CTP in human acute leukemia cells in vitro (38) or during therapy (39–41) have been correlated with clinical response. As discussed above, the effect of fludarabine on ara-CTP accumulation would be greater in acute myelogenous leukemia. These strategies are being applied to the design of protocols using fludarabine and ara-C in combination for treatment of patients with acute leukemias and chronic myelogenous leukemia in blast crisis (35, 42, 43).

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REFERENCES

POTENTIATION OF ara-C METABOLISM BY FLUDARABINE

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Varsha Gandhi, Annette Kemena, Michael J. Keating, et al.


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