ABSTRACT

Arabinosylcytosine (ara-C) is a cytotoxic agent with major activity against acute leukemias. To exert this effect, it must first be phosphorylated to its active 5'-triphosphate, ara-CTP, which is incorporated into DNA. Our previous studies demonstrated that preincubation with arabinosyl-2-fluoroadenine (F-ara-A) increased the rate of ara-CTP accumulation in leukemia cells when incubated with 10 μM ara-C. Such concentrations of ara-C are readily obtained during intermittent bolus infusions of ara-C, and clinical trials were conducted using fludarabine in combination with 2-h infusions of intermediate-dose ara-C. During continuous infusion of ara-C, however, serum ara-C levels are <10 μM. Because the effectiveness of ara-C depends on the levels of intracellular ara-CTP and its incorporation into DNA, we sought to investigate the influence of fludarabine on pharmacodynamics of ara-C at concentrations of ara-C achieved during continuous infusion. Using the K562 human leukemia cell line, we established that incubation with 30 μM F-ara-A was able to modulate intracellular dNTP pools and achieve maximum enhancement of ara-CTP levels at all concentrations of ara-C tested (0.3–10.0 μM). The relative enhancement of ara-CTP concentrations ranged from 2.2- to 2.8-fold. Combination of F-ara-A with 1.0 and 3.0 μM ara-C also increased the incorporation of ara-CTP into DNA. To model the influence of F-ara-A on continuous infusion ara-C, cells were incubated with 1 μM ara-C alone or in combination with F-ara-A. The F-ara-A-incubated cells accumulated effective intracellular concentrations of F-ara-ATP, which resulted in greatly increased intracellular ara-CTP levels. These studies demonstrate the capacity of clinically attainable concentrations of F-ara-ATP to enhance the formation of ara-CTP at concentrations of ara-C that are achieved during a continuous infusion schedule. Given the important role intracellular ara-CTP concentrations and ara-CMP incorporation into DNA have on the ultimate cytotoxic capacity of ara-C against acute myelogenous leukemia blasts, these studies suggest a promising pharmacological model for improving the efficacy of the continuous infusion ara-C regimen.

INTRODUCTION

The clinical efficacy of ara-C4 in the therapy of patients with AML was first demonstrated more than 25 years ago (1). Although many drugs and biologicals have been evaluated since that time, it remains one of the most effective chemotherapeutic agents in use against hematological malignancies (2–5). For this reason, novel strategies are still sought to optimize the use of cytarabine, particularly in combinations with other effective agents. For ara-C to exert its cytotoxicity, it must first be phosphorylated intracellularly by the rate-limiting enzyme dCyd kinase and subsequently to its 5'-triphosphate, ara-CTP (6, 7). Ara-CTP then competes with dCTP for incorporation into DNA (8). Numerous in vitro studies have demonstrated that the amount of ara-CMP incorporated into cellular DNA is a predictor of loss of clonogenicity in human leukemia cells (9–11). Although technically possible (12), it is extremely difficult to reliably and reproducibly measure the incorporation of ara-CMP into the DNA of circulating human leukemic myeloblasts during therapy. Thus, alternative predictors of the clinical cytotoxic effect of ara-C have been sought.

In the in vitro setting, the major determinant of ara-CMP incorporation into DNA was found to be the product of the intracellular ara-CTP concentration and time (11). This observation has been confirmed in patients in vivo where statistically significant correlations have been demonstrated between the intracellular pharmacokinetics of ara-CTP and clinical response to single-agent high-dose ara-C therapy given either on an intermittent schedule (13, 14) or by continuous infusion (15). Similarly, very low incorporation of ara-C into DNA in vitro was predictive of an adverse outcome with subsequent ara-C-based therapy in vivo (16).

Based on these studies demonstrating the importance of ara-CTP formation and retention on treatment outcome, our laboratory (17, 18), and others (19) have investigated potential means to favorably modulate ara-CTP metabolism. One of the most promising methods of biochemical modulation identified was the capacity of pretreatment with F-ara-A to enhance the

4 The abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine or cytarabine; AML, acute myelogenous leukemia; dCyd, deoxycytidine; dNTP, deoxynucleoside triphosphate; F-ara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine.
rate of ara-CTP accumulation in a human leukemia cell line (20). Mechanistic studies suggest that the major mechanism by which F-ara-A augments the intracellular anabolism of ara-C to ara-CTP is through an enhanced rate of ara-C-CTP accumulation (20, 21) due to both a direct stimulatory effect of F-ara-ATP on dCyd kinase and indirectly through reduced concentrations of dCTP, which acts as a feedback inhibitor of dCyd kinase activity. Subsequent clinical studies have demonstrated the effectiveness of this strategy in circulating AML blasts (21), chronic lymphocytic leukemia lymphocytes (22), or leukemic lymphoblasts (23) where F-ara-A is administered as a more soluble form, fludarabine (the monophosphate of F-ara-A).

These studies of ara-C modulation by F-ara-A or fludarabine were carried out at concentrations of ara-C (10 μM or above) that saturate the rate of ara-CTP formation in human leukemia cells in vitro and during therapy (24). This concentration of ara-C can easily be attained or exceeded in vivo with intermittent high-dose or intermediate dose ara-C therapy (24, 25). However, serum concentrations of ara-C are generally less than 10 μM when maximally tolerated continuous infusion schedules are administered (26). Continuous infusion schedules of single-agent ara-C have been extensively used for treatment of acute leukemias (27–29) and have demonstrated clinical efficacy in previously untreated AML (30). Thus, the present study aimed to explore the capacity and to quantify the influence of F-ara-A on the incorporation of ara-CMP into DNA. The results provide a rational basis for the clinical application of intermittent bolus fludarabine along with continuous infusion ara-C in the treatment of patients with relapsed or refractory AML.

MATERIALS AND METHODS

Cells. The K562 cell line (31) was obtained from the American Type Culture Collection (Rockville, MD). Maintenance procedures for the cultures, Mycoplasma analysis, and growth characteristics of this cell line have been published previously (20). Cells were maintained in exponential growth in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum in 5% CO₂ in fully humidified room air. Cell density and size were determined with a Coulter counter (Coulter Electronics, Hialeah, FL).

Chemicals. ara-C and ara-CTP were purchased from Sigma Chemical Co. (St. Louis, MO). [5-³H]ara-C (11.7 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). F-ara-A was produced by alkaline phosphatase treatment of ara-C, which was obtained from Berlex Laboratories (Richmond, CA). F-ara-ATP was synthesized by Sierra Bioresearch (Tucson, AZ). All other chemicals were reagent grade.

Cellular Pharmacology. Cells were incubated with aliquots of standard solutions of ara-C or F-ara-A to yield desired drug concentrations. Incubations were maintained for 3 to 4 h, and aliquots were removed at hourly intervals. In studies of sequential drug exposure, the cells were washed three times in three volumes of ice-cold PBS (8.1 g NaCl, 0.22 g KCl, 1.14 g Na₂HPO₄, and 0.27 g KH₂PO₄/liter H₂O, pH 7.4), and then resuspended in fresh medium, to which the appropriate concentration of the second drug was added. At the completion of the final incubation, the reaction was stopped by the addition of three volumes of ice-cold PBS. After centrifugation, the cell pellet was extracted with HClO₄ as previously described (32). Ara-CTP and F-ara-ATP were separated from ribonucleoside triphosphates by high-pressure liquid chromatography on an anion exchange Partisil-10 SAX column (Waters Associates, Milford, MA) as previously described (33). Nonradioactive ara-CTP and F-ara-ATP were quantified at 262 nm by electronic integration with reference to external standards. The [³H]ara-CTP peak was collected, and radioactivity was quantitated with a liquid scintillation counter. 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 nucleotides are uniformly distributed in total cell water.

Determination of Deoxynucleotides. K562 cells (5 × 10⁵) were obtained before and 3 h after incubation with the different concentrations of F-ara-A. These cells were extracted by 60% methanol for determination of dNTPs. The DNA polymerase assay as modified by Sherman and Fyfe (34) was used to quantitate dNTPs in the cell extracts. DNA polymerase I (U. S. Biochemical Corporation, Cleveland, OH) was used to start a reaction in a mixture that contained 100 mM HEPES buffer (pH 7.3), 10 mM MgCl₂, 7.5 μg BSA, and synthetic oligonucleotides of defined sequences as templates annealed to a primer, [2,8-³H]dATP (16.7 Ci/mmol; Moravek Biochemicals) or [methyl-³H]dTTP (47 Ci/mmol; ICN Radiochemicals, Irvine, CA), and either standard dNTP or the extract from 1 to 3 × 10⁶ cells. Reactants were incubated for 1 h and applied to filter discs; after washing, the radioactivity on the discs was determined by liquid scintillation counting.

ara-C Incorporation into DNA. ara-C incorporation into cellular DNA was determined following incubations with varying concentrations of [³H]ara-C with and without prior incubation with F-ara-A. The ara-C stock solution used for these experiments was at a concentration of 1.0 mM, with a specific activity of 500 μCi/ml. At the completion of incubations, cells were washed twice in three volumes of ice-cold PBS and lysed with digestion buffer [100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.1 mg/ml proteinase K, and 0.5% SDS]. Following a 12-h incubation at 50°C, DNA was extracted using a standard phenol/chloroform/isooamyl alcohol method (35). DNA was quantified by determining absorption at 260 nm and [³H]ara-C content measured using a liquid scintillation counter. The amount of ara-C incorporated into DNA was quantitated and expressed as pmol ara-C per mg of cellular DNA.

RESULTS

Influence of F-ara-A Concentration on ara-CTP Accumulation. Previous work has established that F-ara-ATP accumulation is saturated following incubation of cells with F-ara-A at concentrations of ≥300 μM and that this pretreatment enhances subsequent ara-CTP accumulation in cells incubated with 10 μM ara-C (17). To establish the minimum concentration of F-ara-A needed to achieve this potentiation, we incubated cells with 10–100 μM F-ara-A for 3 h, followed by incubation with 10 μM ara-C. Fig. 1 demonstrates that K562 cells preincubated with F-ara-A accumulated ara-CTP at an increased rate compared to that achieved with ara-C alone. At 10 μM F-ara-A,
there was a marginal increase in ara-CTP accumulation. Pretreatment with 30–100 μM F-ara-A potentiated ara-CTP at similar levels. This suggests that a 3-h incubation with 30 μM F-ara-A results in optimal potentiation of ara-CTP formation. Incubation with greater concentrations of F-ara-A do not further enhance ara-CTP formation. All subsequent combination experiments used a 3-h incubation with 30 μM F-ara-A. Preliminary experiments demonstrated that washing and resuspension of cells alone did not significantly alter the accumulation of ara-CTP (data not shown).

Dose-dependent Accumulation of F-ara-ATP and Effect on dNTP Pools. As reported previously, the potentiation of the rate of ara-CTP accumulation by F-ara-ATP was due to a direct effect of F-ara-ATP on dCyd kinase and an indirect mechanism (through alterations in the dNTP pool) on this enzyme. Hence, F-ara-ATP levels and its effect on endogenous deoxyribonucleotides were determined in cells treated with indicated concentrations of F-ara-A for 3 h (Table 1). Incubation with F-ara-A resulted in a dose-dependent increase in F-ara-ATP formation. Levels of F-ara-ATP were 20 μM or less at exogenous F-ara-A levels of 10 μM or lower. At 30 μM or higher concentrations of F-ara-A, ≥50 μM F-ara-ATP was achieved after 3 h of incubation. Purine deoxyribonucleotides were affected at 10 μM level of intracellular F-ara-ATP; dATP was 51% of control and dGTP was 70% of the pretreatment value. At higher concentrations of F-ara-ATP, all dNTPs except dTTP were lowered. Concentrations of dATP and dCTP were lowered most significantly: a 60–70% reduction after a 3-h incubation with 100 μM F-ara-A which accumulated 175 μM F-ara-ATP.

Ability of F-ara-A to Modulate ara-CTP Formation at Low Concentrations of ara-C. Cells were incubated with 30 μM F-ara-A for 3 h, washed, and then exposed to 0.3–10.0 μM ara-C for 3 h. The resulting intracellular levels of ara-CTP were between 2.2- and 2.8-fold higher than those attained with the corresponding concentration of ara-C alone, i.e., without prior F-ara-A exposure (Fig. 2). In these experiments, the mean (±SE) intracellular F-ara-ATP concentration attained at the completion of the 3-h incubation with 30 μM F-ara-A was 75 (±11 μM), confirming that an F-ara-ATP concentration adequate to achieve optimal potentiation of ara-CTP metabolism was present. These data suggest that, in addition to the established capacity of F-ara-A to modulate ara-CTP accumulation at ara-C concentrations that saturate the rate of ara-CTP accumulation, F-ara-A can also modulate the accumulation of ara-CTP when ara-C is present at lower concentrations (0.3–3 μM).

Influence of F-ara-A on ara-CMP Incorporation into DNA. Cells were incubated with 30 μM F-ara-A for 3 h, washed, and then incubated with varying concentrations of [3H]ara-C for 3 h. At the completion of the incubation, the cell suspension was divided into separate aliquots for DNA isolation and nucleoside triphosphate quantitation as described. In spite of the ability of F-ara-A pretreatment to enhance intracellular ara-CTP accumulation at all concentrations of ara-C studied, there was a dose-dependent effect on ara-CMP incorporation into DNA (Fig. 3). At intermediate concentrations of ara-C (1.0 and 3.0 μM), F-ara-A pretreatment resulted in a mean 1.8- and 1.9-fold increase in the quantity of ara-CMP incorporated into cellular DNA, respectively. At the extreme low (0.3 μM) and high (10 μM) concentrations of ara-C, F-ara-A pretreatment did not discernibly influence ara-CMP incorporation into DNA. The mean ratios of ara-CMP incorporation for F-ara-A pretreated to control cells were 1.09 and 0.94 at 0.3 and 10 μM ara-C, respectively.

Modeling of the Influence of F-ara-A on Continuous Infusion ara-C. The preceding studies demonstrated the capacity of F-ara-A to enhance both intracellular ara-CTP accumulation and ara-CMP incorporation into cellular DNA at concentrations (1 and 3 μM) of ara-C, which are attained in plasma during therapy with well tolerated and efficacious dose of 1500 mg/m²/day continuous infusion of ara-C (26, 30). Additionally, the F-ara-ATP concentration (50 μM) required for this biochemical modulation is achieved in circulating leukemia blasts during therapy with a 30-mg/m² infusion of fludarabine, the dose used in the clinic (21). To model the influence of intermittent bolus F-ara-A administration on continuous infusion ara-C, aliquots of K562 cells were incubated for 10 h with 1 μM ara-C along with 50 μM tetrahydrodoridine to minimize loss of ara-C through deamination. Following attainment of steady-state intracellular ara-CTP concentrations at 3 h, half of the cells were coincubated with 30 μM F-ara-A. Portions of the cell mixtures from each flask were collected at hourly intervals for measurement of intracellular ara-CTP concentrations. Accumulation of ara-CMP reached a plateau after incubation with 1 μM ara-C (Fig. 4), and continued incubation with ara-C in the absence of F-ara-A demonstrated a true steady-state level of ara-CTP. However, addition of F-ara-A to the parallel culture resulted in a continuous increase in ara-CTP accumulation up to 10 h. Compared to the concentration of ara-CTP in cells incubated with ara-C alone, the level of ara-CTP at 10 h was 2.5-fold higher in cells coincubated with F-ara-A. Similar experiments conducted with 3 μM F-ara-A in combination with 1 μM ara-C resulted in a 1.5-fold increase in the accumulation of ara-CTP at 10 h (data not shown).

DISCUSSION

Ara-C remains the basis of standard cytotoxic treatments for most patients with AML. Despite more than 25 years of
Influence of Fludarabine on Pharmacodynamics of Continuous Infusion ara-C

Table 1 Accumulation of intracellular F-ara-ATP after a 3-h incubation with indicated concentrations of exogenous F-ara-A and its effect on the deoxynucleotide pool

<table>
<thead>
<tr>
<th>F-ara-A (μM)</th>
<th>F-ara-ATP</th>
<th>dATP</th>
<th>dCTP</th>
<th>dGTP</th>
<th>dTTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.4 ± 0.5</td>
<td>24.8 ± 0.6</td>
<td>13.6 ± 0.3</td>
<td>7.6 ± 0.3</td>
<td>73.6 ± 3.3</td>
</tr>
<tr>
<td>3</td>
<td>20.8 ± 0.8</td>
<td>12.7 ± 0.4</td>
<td>11.7 ± 0.6</td>
<td>5.3 ± 0.1</td>
<td>53.9 ± 2.3</td>
</tr>
<tr>
<td>10</td>
<td>53.8 ± 0.9</td>
<td>9.1 ± 0.2</td>
<td>12.2 ± 0.3</td>
<td>4.1 ± 0.2</td>
<td>68.7 ± 3.4</td>
</tr>
<tr>
<td>30</td>
<td>59.6 ± 0.4</td>
<td>9.8 ± 0.3</td>
<td>11.1 ± 0.4</td>
<td>4.9 ± 0.3</td>
<td>73.1 ± 3.2</td>
</tr>
<tr>
<td>100</td>
<td>174.9 ± 8.1</td>
<td>7.3 ± 0.4</td>
<td>9.6 ± 0.3</td>
<td>5.3 ± 0.4</td>
<td>74.3 ± 3.3</td>
</tr>
</tbody>
</table>

Fig. 2 Influence of F-ara-A preincubations on ara-CTP accumulation at different concentrations of ara-C. Parallel cultures of cells were incubated with no drug or 30 μM F-ara-A for 3 h, washed, and then incubated with the indicated concentrations of [3H]ara-C for an additional 3 h. Nucleotides were extracted and separated by high-pressure liquid chromatography. Columns, means of duplicate determinations from three to six experiments. Bars, SE.

At present, it is not possible to quantify the relative contributions of these components to the observed clinical efficacy of the combination (36, 37). Preliminary studies using an in vitro model system demonstrated that incorporation of both ara-CTP and F-ara-ATP in a DNA primer by human DNA polymerase α results in more than 99% inhibition of further extension (38).

The results we obtained in vitro using human leukemic blasts in this study suggest that the influence of F-ara-A on ara-CMP incorporation into DNA are related to the concentration of ara-C. At the extremes of ara-C concentrations studied, 0.3 and 10.0 μM, pretreatment with 30 μM F-ara-A did not discernibly influence ara-CMP incorporation into DNA. This was despite the fact that ara-CTP concentrations were increased more than 2-fold. It is likely that at 0.3 μM ara-C, the difference in the concentrations of ara-CTP (10 μM without and 25 μM with F-ara-A pretreatment) was not great enough to materially alter subsequent incorporation of ara-CMP into DNA. It is also possible that the relatively small changes in ara-CMP incorporation at low ara-C concentrations may not be detectable.

Prior studies suggested that ara-C incorporation into DNA does not continue to increase above levels attained following incubation with 5–10 μM ara-C in mouse L cells, despite continuing increments in ara-CTP concentrations (8). This system differs from human leukemic cells, however, in which ara-CTP concentrations do not continue to increase with exposure to concentrations of ara-C above 10 μM but may indeed diminish due to

preclinical and clinical evaluation in patients with AML, optimal application of ara-C remains elusive. Pioneering studies established the quantitative relationship between ara-C incorporation into cellular DNA and cytotoxicity (9–11), and the means of enhancing this end point are keenly sought. One of the limiting factors has been the difficulty of measuring ara-C incorporation into leukemic cellular DNA during therapy in vivo. The best predictive index readily measured in clinical samples is the intracellular ara-CTP concentration, which has been demonstrated to be strongly correlated with clinical response to ara-C therapy (13–15). Enhancement of ara-CTTP accumulation into cellular DNA and cytotoxicity (9–11), and the means of improving clinical results with ara-C. One such method of modulating used clinically is the combination of fludarabine with ara-C in a timed sequential manner (20, 21). This approach enhanced intracellular ara-CTP concentrations above that attainable using ara-C alone at intermediate or high-dose infusion levels. However, F-ara-ATP also has independent actions upon enzymes critical for DNA synthesis, which may inhibit the subsequent incorporation of the ara-CTP into DNA. Such an inhibition would presumably also diminish the cytotoxicity attributable to ara-C. Of course, F-ara-ATP may also have direct cytotoxic actions itself on AML blasts independent of its modulation of ara-C metabolism.
a direct inhibitory effect of ara-C on dCyd kinase activity (39). Ross et al. (40) have also demonstrated that the relative efficiency of ara-C incorporated into DNA diminishes with increasing ara-C concentrations. This suggests that further increments in intracellular ara-CTP concentrations will have an increasingly smaller influence on ara-C incorporation into DNA. It is also uncertain what, if any, negative influence the intracellular F-ara-ATP ATP might have had on incorporation of ara-C into DNA because F-ara-ATP incorporation results in chain termination (41). Regardless of a theoretical inhibitory effect, the net outcome of pretreatment with 30 µM F-ara-A followed by exposure to 1.0 or 3.0 µM ara-C was a marked enhancement of ara-C incorporation into DNA. Previous studies have demonstrated this to be the best predictor of loss of clonogenicity, and it is reasonable to hypothesize that treatment strategies that optimize ara-C incorporation into DNA will also improve clinical outcome.

Continuous infusion schedules of ara-C have been used in the clinic in an attempt to exploit the S-phase specificity of ara-C and to avoid the detrimental effect of rapid clearance of ara-CTP from leukemic blasts, which allows the resumption of DNA synthesis (42). A recent analysis of the long-term outcome of patients with AML treated with single-agent continuous infusion ara-C has shown this approach to be effective in obtaining durable remissions (30). Using intermittent bolus schedules of high-dose or immediate dose ara-C concentrations of ara-C (10 µM or above) that saturate the rate of cellular ara-CTP accumulation were readily achieved in plasma of most patients. In contrast, the dose escalation during continuous infusion schedules is limited by toxicity, particularly mucositis. Reported schedules with tolerable toxicity are between 1.5 and 3 g/m²/day for 36 to 96 h. Such schedules attain mean plasma ara-C steady-state concentrations of between 1 and 7 µM (26, 28, 29). It is clear that the leukemic blasts of many patients receiving these continuous infusion schedules will be exposed to suboptimal levels of plasma ara-C to achieve the maximum rate of ara-CTP accumulation.

The current studies provide a potential means of reducing the likelihood of exposure of leukemic blasts to suboptimal ara-CTP levels during continuous infusion schedules. We have shown that clinically attainable intracellular concentrations of F-ara-ATP are able to enhance both the formation of ara-CTP, and, more important, the quantity of ara-C incorporated into leukemic cellular DNA when exposed for prolonged periods to 1.0 µM ara-C. This concentration approximates that attained with the clinical application of continuous infusion of ara-C at 1.5 g/m²/day (26). The above data along with our previous experience with fludarabine provide a rationale for combining a continuous infusion high-dose ara-C schedule (30) with bolus dose of fludarabine. Such combination has provided responses in pediatric patients with relapsed acute leukemias (43).

Based on the present investigation and other studies with fludarabine and intermediate dose intermittent infusion of ara-C, a clinical protocol has been designed using a continuous infusion schedule for ara-C administration, with daily bolus doses of fludarabine for adults with relapsed AML. The validity of the in vitro studies reported here are being evaluated by measuring the intracellular F-ara-ATP and its influence on cellular steady-state ara-CTP concentrations. We expect that those patients whose leukemic blasts synthesize sufficient quantities of F-ara-ATP will demonstrate the predicted enhancement of intracellular ara-CTP levels. This protocol will provide the opportunity to seek correlations between clinical efficacy of this pharmacologically directed treatment approach with pharmacokinetic and pharmacodynamic parameters.

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