Chlorodeoxyadenosine and Arabinosylcytosine in Patients With Acute Myelogenous Leukemia: Pharmacokinetic, Pharmacodynamic, and Molecular Interactions

By Varsha Gandhi, Elihu Estey, Michael J. Keating, Antoine Chrucallah, and William Plunkett

The effectiveness of arabinosylcytosine (ara-C) for the treatment of acute myelogenous leukemia (AML) depends on the formation of its active metabolite, the triphosphate of ara-C (ara-CTP). Using biochemical modulation strategies to increase the accumulation of ara-CTP in leukemia blasts, a clinical protocol was designed combining 2-chlorodeoxyadenosine (CdA), an inhibitor of ribonucleotide reductase, and ara-C for adults with AML. The protocol stipulated an infusion of 1 g/m² of ara-C over 2 hours on day 1. A continuous infusion of CdA (12 mg/m²/d) begun 24 hours later and continued for 5 days. Identical doses of ara-C were administered on days 3, 4, 5, and 6. Pharmacokinetic and pharmacodynamic interactions between CdA and ara-C during therapy were investigated. To complement these studies, molecular actions of the triphosphate of ara-C and CdA on DNA extension by human DNA polymerase α in an in vitro model system was conducted. In the circulating leukemia blasts of 7 of the 9 patients studied, ara-CTP pharmacokinetics showed a median 40% increase in the rate of ara-CTP accumulation after 24 hours of CdA infusion. The ex vivo effect of CdA on accumulation of ara-CTP in AML blasts was similar to that during therapy except that the enhancement was less. The ara-CTP accumulation. The intracellular accumulation of ara-CTP is a multistep process, the first step of which, phosphorylation of ara-C to its monophosphate by deoxycytidine kinase, is rate-limiting. Our previous investigations showed that arabinosyl-2-fluoroadenine (F-ara-A, the nucleoside of fludarabine) increases the activity of this crucial enzyme, leading to a higher rate of ara-CTP accumulation and consequently a greater AUC in K562 cells, in lymphocytes from patients with chronic lymphocytic leukemia, and in AML blasts during this combination therapy. Mechanistic studies suggested that inhibition of ribonucleotide reductase by fludarabine triphosphate plays a major role in augmenting the activity of deoxycytidine kinase.

This successful modulation provided us a rationale to use other inhibitors of ribonucleotide reductase to be combined with ara-C. 2-Chlorodeoxyadenosine ( cladribine, CdA) was selected based on the following facts: (1) The triphosphate of CdA (CdATP) is a more potent inhibitor of ribonucleotide reductase than is fludarabine triphosphate. (2) CdA, used as a single agent, has been effective in the treatment of leukemias, including acute leukemias, in vitro incubation of AML blasts with CdA followed by ara-C produced a higher rate of ara-CTP accumulation than did ara-C alone.

The primary objective of the present work was to test the hypothesis that CdA can increase ara-CTP accumulation by biochemical modulation in circulating leukemia blasts from patients with AML. A second objective was to determine the pharmacodynamic and molecular actions of this combination. We met these objectives by testing this combination as a phase II trial for patients with AML who had relapsed or failed to respond to initial therapy. A report detailing patient characteristics and clinical responses to these treatments has appeared elsewhere.

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PATIENTS AND METHODS

Drugs

For clinical use ara-C (cytosar-U) and CdA (cladribine) were obtained commercially from the Upjohn Co and from Ortho Biotech (Raritan, NJ), respectively. Tetrahydrouridine was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, MD). For ex vivo and in vitro investigations, ara-C and ara-CTP were purchased from Sigma Chemical Co (St Louis, MO). All other chemicals were reagent grade.

Patients

Seventeen patients with AML refractory to initial therapy or in relapse were treated with this protocol. For pharmacokinetic investigations, nine patients were studied on this protocol. These nine patients had received ara-C-containing regimens as their primary therapy, but each patient’s disease had relapsed (4 in first salvage, 1 in second salvage, and 4 in third or higher salvage). The patients were selected on the basis of adequate numbers of circulating blasts (>5,000/μL) to conduct the investigation, laboratory preparedness, and informed consent to participate on the pharmacology studies. They were informed about the investigational nature of this program in accord with institutional policies and they signed an informed consent document.

Protocol

On day 1 patients received a 2-hour infusion of 1 g/m² ara-C. Starting on day 2, CdA was given as a continuous infusion for 5 days at a dose of 12 mg/m² daily. On day 3, ie, 1 day after starting CdA infusion, a second identical dose of ara-C was administered. Three more daily infusions of ara-C were given to complete one course of therapy. Pharmacokinetic studies were conducted during and after the first two ara-C infusions.

Blood Samples for Clinical Pharmacology

To determine the pharmacokinetics of ara-C in plasma and ara-CTP in circulating blasts patients treated on this protocol, 40-mL blood samples were obtained before therapy and 10-mL samples were taken at 0.5, 1, 1.5, 2, 2.5, 3, and 4 hours after the start of therapy (first ara-C infusion). Ten-milliliter samples were also drawn just before the CdA infusion at 24 hours to determine the cellular pharmacology of ara-CTP. Starting at 48 hours, the second ara-C dose was infused over 2 hours, and blood samples were obtained at 48, 48.5, 49, 49.5, 50, 50.5, 51, 52, and 72 hours. All blood samples were collected in vacutainer tubes containing heparin and 500 μmol/L tetrahydrouridine to inhibit cytidine deaminase. The tubes were placed in an ice-water bath before being transported to the laboratory for processing. After centrifugation to remove plasma, the cell pellet was resuspended in phosphate-buffered saline (8.1 g NaCl, 0.22 g KCl, 1.14 g NaH₂PO₄, and 0.27 g KH₂PO₄ per liter of H₂O, pH 7.4), and mononuclear cells were isolated by Ficoll-Hypaque (Sigma) density-gradient centrifugation procedures. Control studies have shown that leukemia cells under these conditions are stable for at least 15 hours with respect to size, membrane integrity, and cellular nucleotide content.

Cellular Pharmacology

Normal and arabinosyl nucleotides were extracted from blasts by HClO₄. Ara-CTP was separated from ribonucleoside triphosphates by anion-exchange chromatography on a Partisil-10 SAX column (Whatman, Clifton, NJ) and quantified at 280 nm by electronic integration with reference to external standards. 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 distributed in total cell volume. The lower limit of sensitivity of this assay is 10 pmol in an extract of 2 × 10⁶ cells, which corresponds to a cellular concentration of about 5 μmol/L. The efficiency of this assay measured by extracting and quantitating known amounts of radioactive nucleotides was greater than 95%. The inter- and intra-assay variability was less than 10%.

Ex Vivo Studies

Blood samples were obtained from patients before and 24 hours after 2-CdA infusion. Mononuclear cells were isolated as above, and 5 to 5 × 10⁶ cells were incubated with 10 μmol/L [³H]ara-C (specific activity 3 × 10⁶ dpm/μmol) for 3 hours. All samples were processed as described above to quantitate the accumulation of [³H]ara-CTP. Radioactivity in the ara-CTP peak was quantitated by liquid scintillation counting. The intracellular concentrations of nucleotides were calculated and expressed as described above. With this specific activity of [³H]ara-C, the lower limit of detection was less than 0.2 μmol/L. For both in vivo and ex vivo comparison of ara-CTP pharmacokinetics before and after CdA, 10% was generally the standard deviation; hence a ratio of less than 0.9-fold was considered a decrease and 1.1-fold was taken as an increase in the accumulation of ara-CTP.

Determination of dNTPs

Samples obtained at 0, 24, and 48 hours were processed to obtain mononuclear cells. These cells were extracted by 60% methanol for determination of dNTPs. The DNA polymerase assay as modified by Sherman and Fye was used to quantify dNTPs in the cell extracts. DNA polymerase I (US Biochemical Corp, Cleveland, OH) was used to start a reaction in a mixture that contained 100 mmol/L HEPES buffer, pH 7.3, 10 mmol/L MgCl₂, 7.5 μg bovine serum albumin (BSA), and synthetic oligonucleotides of defined sequences as templates annealed to a primer, [³H]dATP or [³H]dGTP, and either standard dNTP or the extract from 1 or 2 × 10⁶ leukemia cells before and after therapy. Reactants were incubated for 1 hour and applied to filter discs; after washing, the radioactivity on the discs was determined by liquid scintillation counting.

Inhibition of DNA Synthesis

Leukemia cells (2 × 10⁶) were obtained before and at the indicated times after start of therapy. These were incubated with 1.0 μCi [³H]thymidine for 30 minutes to determine DNA synthesis. The cells were collected on 25-mm glass fiber discs (prewetted with 1% sodium pyrophosphate) by filtration and then washed two times with 4 mL of ice-cold 0.4 N HClO₄ and twice with 2 mL of ethanol. Radioactivity retained on the filter disc was determined by liquid scintillation counting.

Effect of CdA and ara-C on Cell Type Distribution

Leukemia blasts obtained before the start of therapy and at 24, 48, and 72 hours after start of treatment were obtained to determine the percentage of leukemia cells (blasts plus promyelocytes) in the mononuclear cell preparations. Cells 1 to 4 × 10⁷ were suspended in 100 μL medium with 20% fetal calf serum and spread on slides by cytopsin. After staining with Giemsa dye, the differential morphology was analyzed using a total of 200 cells in each sample.

DNA Primer Extension Assay

A 17-base oligodeoxynucleotide obtained from Pharmacia LKB Biotechnology, Inc (Piscataway, NJ) was labeled with ³²P at the 5'-end, annealed to its complementary site on a defined sequence tem-
plate (Genosys Biotechnologies, Inc, Woodlands, TX), and precipitated with ethanol as described. This template/primer hybrid was used as a substrate for DNA pol α.

\[
5'[\text{32P}] \quad - \quad \text{CACACATGCACACA}
\]

The initial six nucleotides (sites 18 to 23) added to the primer were alternate dGMPs and dTMPs. In this way pol α had a "running start" before it encountered the T site and the G site (underlined) on the template, where CdATP and/or ara-CTP were incorporated.

The primer extension reaction mixture contained 20 mmol/L Tris-HCl (pH 7.4), 0.5 mmol/L MgCl₂, 0.25 mmol/L diithiothreitol, 40 μg/mL BSA, indicated concentrations of dNTPs and CdATP and/or ara-CTP along with the labeled, primer/template complexes, and DNA pol α. The enzyme was purified from exponentially growing CEM cells by Dr Peng Huang as described previously. The reactions were performed at 37°C for 10 minutes, stopped by addition of an equal volume of 50 mmol/L EDTA with 0.3% bromophenol blue in 90% deionized formamide, and analyzed by electrophoresis through a 10% polyacrylamide sequencing gel. A control reaction (with 5 μmol/L dNTPs) performed under similar conditions for up to 30 minutes showed that the reaction was linear with time (r value = 0.99), and by 10 minutes less than 25% of the primers were elongated beyond the incorporation sites.

Various concentrations of dATP (0.005, 0.01, 0.025, 0.05, 0.075, 0.1, 0.25, and 0.5 μmol/L) or CdATP (0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 μmol/L) were used to determine the kinetic parameters of nucleotide incorporation by pol α. Radioactivity associated with the band at the A site (site 24 on the extended primer) and in all the bands beyond this site (25 to 31) was quantitated using a Betascope 603 blot analyzer (Betagen, Waltham, MA). The radioactivity was normalized based on the average total radioactivity in each lane, and the background value (counts in the bands at the A site and beyond, in a reaction lacking dATP) was subtracted. These data (reaction velocity) were plotted against the concentration of dATP or CdATP (substrate). The apparent Kₘ values for incorporation were then calculated based on the Michaelis-Menten equation, using a computer-assisted program. The apparent Vₘₐₓ values obtained through these calculations were converted to percent product per minute based on the total radioactivity in each lane. To determine the inhibition of DNA primer elongation by analogs in the presence of CdATP and/or ara-CTP, the radioactivity above the pause site was quantitated and compared with the control lane (reaction containing all four dNTPs). The final values represent a mean ± SD of at least four sets of data points generated by two separate reactions each run twice by gel electrophoresis.

**Calculations and Statistical Analysis**

The levels of ara-CTP obtained by HPLC analysis were normalized based on the concentrations of endogenous nucleotides during sampling times in each individual. The rate of ara-CTP accumulation in leukemia cells was calculated by a linear regression analysis that included a 0-hour value. Generally, the ara-CTP accumulation rate was linear through 2 hours during therapy and to 4 hours when determined ex vivo. Pharmacologic data obtained during the first and second ara-C doses were compared using the two-tailed, paired t-test.

**RESULTS**

**Effect of CdA on Cellular Pharmacokinetics of ara-CTP**

**During therapy.** The pharmacokinetics of ara-CTP in circulating leukemia blasts of a typical patient after the first two doses of ara-C are presented in Fig 1. The accumulation of ara-CTP was linear during the 2-hour ara-C infusions and was increased after CdA infusion. After these times, semilogarithmic plots showed that the ara-CTP was eliminated in a monophasic pattern with similar half-lives suggesting that CdA had an effect only during the anabolism of ara-C to ara-CTP. Using a pharmacokinetic approach, we evaluated the ability of CdA to influence ara-CTP pharmacokinetics in leukemia blasts by comparing the peak cellular ara-CTP concentrations and the rates of ara-CTP accumulation during and after the first and second ara-C infusions in nine patients.

Analysis of the rates of ara-CTP accumulation after the first ara-C infusion showed heterogeneity among patients; the accumulation rates ranged between 60 and 310 μmol/L/h (Table 1). Similar differences were observed when ara-C was administered 24 hours after the continuous infusion of CdA. Moreover, when ara-CTP values after each ara-C infusion were compared within each patient, seven of the nine patients had an increase in the rate of ara-CTP accumulation. The median effect was a 1.4-fold (range, 0.9 to 2.0-fold) increase (P = .017) in the rate of ara-CTP accumulation after CdA infusion. The augmentation of the ara-CTP accumulation rate increased the peak concentration of ara-CTP, which was achieved within 1 hour after the ara-C infusion ended. The peak ara-CTP during the first dose ranged from 150 to 770 μmol/L, whereas during the second dose, ara-CTP peaks were higher, ranging from 220 to 800 μmol/L. As indicated by the ratio of the peak values after and before CdA, the peak ara-CTP concentration was elevated by a median of 1.4-fold after CdA infusion (P = .014, Table 1).

**During ex vivo incubations.** Leukemia blasts were obtained from each patient before therapy (before start of the first ara-C dose) and 24 hours after CdA infusion (before the start of the second ara-C infusion). These cells were incubated in vitro with 10 μmol/L ara-C; the concentration achieved in plasma during the clinical infusion of ara-C (0.5 g/m²/h). The kinetics of ara-CTP accumulation in AML
blasts before and after CdA infusion were then compared. The accumulation of ara-CTP was linear for 2 to 4 hours during ara-C incubation. The effect of CdA on ara-CTP accumulation during ex vivo incubation was less than that measured during therapy. Generally the modulatory effect of CdA on ara-CTP accumulation during ex vivo incubation was less than that measured during therapy.

Pharmacodynamics of ara-CTP and CdATP

DNA synthesis during therapy. To determine the effect of CdA and ara-C infusions alone and in combination on DNA synthesis, blasts were isolated at different times during therapy and incubated ex vivo with [3H]dThd. Compared to the control values (0 hours, Fig 2), 2 hours after ara-C infusion, there was a 78% inhibition of DNA synthesis. However, the DNA synthetic capacity recovered to between 30% and greater than 100% of control value (mean = 73%) at 24 hours. A 24-hour continuous infusion of CdA lowered DNA synthesis in blasts from each patient by greater than 50% (bar at 48 hours). Infusion of the second dose of ara-C along with CdA maximally inhibited DNA synthesis, dropping it to less than 20% of the pretreatment value. This inhibitory effect was maintained during the continual CdA infusion for another 24 hours (72 hours after start of therapy), just before the infusion of the third dose of ara-C (Fig 2).

Cellular dNTP pool during therapy. CdA triphosphate is a potent inhibitor of ribonucleotide reductase. To determine the effect of CdA infusion on ribonucleotide reductase, levels of deoxynucleotides were measured in the circulating blasts during therapy. The pretreatment levels of dNTPs varied among individuals. The concentration of dCTP in the circulating blasts was lowest in each individual (mean 3.6 μmol/L, range 2 to 5 μmol/L), whereas the dGTP pool size ranged between 2 and 8 μmol/L. Levels of the dATP pool ranged between 4 and 30 μmol/L, while dTTP pool sizes were between 8 and 16 μmol/L. Although the absolute starting levels of dNTPs varied among individuals, the perturbation pattern with ara-C and CdA infusions were similar among patients; a representative profile of the effects of this therapy on the dNTP pools of a single patient is presented in Fig 3. At 24 hours, after the first ara-C infusion, there was an increase of between 10% and 70% (n = 9) in the levels of all deoxynucleotides. This was expected because ara-C is known to block cells in S-phase. Compared with the G1 phase subpopulation, cells in S-phase have higher levels of deoxynucleotides and hence would be reflected in the pool of leukemia blasts analyzed for dNTP concentrations. Continuous infusion of CdA (Fig 3, bar at 48 hours) decreased each dNTP concentration compared with the respective levels at 24 hours. Nevertheless, at this time (48 hours) only the dATP (n = 9) and dGTP (n = 7) pools decreased below the pretreatment values. The present study, designed to evaluate pharmacokinetic effect of CdA on ara-C, did not permit us to analyze the perturbation of dNTP pool after only CdA infusion.

Effect of ara-C and CdA on Cell Type Distribution

Mononuclear cell populations isolated before and after ara-C and CdA infusions were evaluated for possible

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**Table 1. ara-CTP Pharmacokinetics in AML Blasts During Therapy**

<table>
<thead>
<tr>
<th></th>
<th>ara-CTP Rate, μmol/L/h</th>
<th>ara-CTP Peak, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>Pre CdA*</td>
<td>Post CdA</td>
</tr>
<tr>
<td>1</td>
<td>240</td>
<td>120</td>
</tr>
<tr>
<td>2</td>
<td>140</td>
<td>190</td>
</tr>
<tr>
<td>3</td>
<td>120</td>
<td>310</td>
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<td>4</td>
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<td>5</td>
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<td>6</td>
<td>150</td>
<td>250</td>
</tr>
<tr>
<td>7</td>
<td>150</td>
<td>250</td>
</tr>
<tr>
<td>8</td>
<td>180</td>
<td>250</td>
</tr>
<tr>
<td>9</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Median</td>
<td>150</td>
<td>210</td>
</tr>
</tbody>
</table>

* Sample taken before treatment started.
† Sample taken 48 hours after start of therapy, ie, 24 hours after CdA infusion.

**Table 2. Pharmacokinetics of ara-CTP in AML Blasts Ex Vivo**

<table>
<thead>
<tr>
<th></th>
<th>ara-CTP Rate, μmol/L/h</th>
<th>ara-CTP Peak, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>Pre CdA*</td>
<td>Post CdA</td>
</tr>
<tr>
<td>1</td>
<td>210</td>
<td>180</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
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<td>nd</td>
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<td>9</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td>Median</td>
<td>85</td>
<td>110</td>
</tr>
</tbody>
</table>

* Sample taken before treatment started.
† Sample taken 48 hours after start of therapy, ie, 24 hours after CdA infusion.

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changes in cell type distribution that might be related to the therapy. Cytospin preparations from seven patients were analyzed and scored for cell differential morphologies. Before therapy, the median blast percentage in the isolated mononuclear cell fraction was 94% (range 72% to 99%). The remaining 1% to 9% values represented lymphs in majority of patients; in one patient, 10% of total cells were lymphs and 18% were other types. The blast percentage values were 94% and 95%, 24 and 48 hours after the first ara-C infusion, respectively. These data suggest that the cell type distribution remained similar after ara-C and CdA infusions, hence the pharmacokinetic and pharmacodynamic studies represent comparison in similar population of cells.

Molecular Interactions of ara-CTP and CdATP

Action of CdATP on DNA strand elongation in vitro. To determine the affinity of human DNA polymerases to insert CdA monophosphate (CdAMP) in the growing DNA chain and the effect of this incorporation on further DNA synthesis, we used a DNA primer extension assay that employed DNA pol α purified from human leukemia cells. The incorporation of CdATP was studied in the absence of dATP (lane 2). The faint band at the A site after CdATP insertion as evidenced by the band at the A site with increasing concentrations of CdATP (lanes 8 through 11), primers were elongated by pol α to make full length products (30- and 31-mer). These data suggested that CdATP was utilized as an alternative substrate and that DNA pol α efficiently extended a DNA primer that had a CdAMP residue at its 3'-end.

Kinetic studies with DNA polymerase α. The data obtained from the gel described above (Fig 4) and analysis of other reactions using different concentrations of CdATP, ara-CTP, dATP, and dCTP were used to calculate the kinetic parameters for utilization of these substrates by DNA pol α. In each case, the natural nucleotide, dATP or dCTP, was a preferred substrate for incorporation opposite the T or the G site of the template, respectively (Table 3). Similar to the incorporation of natural substrates, DNA pol α incorporated both CdATP and ara-CTP efficiently. The relative efficiency of incorporation of these analogs into DNA was between CdATP could serve as an alternate substrate for incorporation into the A-site. Although some primer extension halted at the A site after CdATP insertion as evidenced by the band at the A site with increasing concentrations of CdATP (lanes 8 through 11), primers were elongated by pol α to make full length products (30- and 31-mer). These data suggested that CdATP was utilized as an alternative substrate and that DNA pol α efficiently extended a DNA primer that had a CdAMP residue at its 3'-end.

Table 3. Kinetics of CdATP and ara-CTP Incorporation by DNA Polymerase α

<table>
<thead>
<tr>
<th>Substrate</th>
<th>App K_m μmol/L</th>
<th>App V_max μmol/L/min</th>
<th>V_max/K_m</th>
<th>Relative Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>dATP</td>
<td>0.015 ± 0.003</td>
<td>0.26 ± 0.04</td>
<td>17.3</td>
<td>1</td>
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<tr>
<td>CdATP</td>
<td>0.028 ± 0.017</td>
<td>0.30 ± 0.16</td>
<td>10.7</td>
<td>5.60</td>
</tr>
<tr>
<td>dCTP</td>
<td>0.044 ± 0.003</td>
<td>0.83 ± 0.05</td>
<td>18.9</td>
<td>1</td>
</tr>
<tr>
<td>ara-CTP</td>
<td>0.053 ± 0.027</td>
<td>0.46 ± 0.05</td>
<td>8.7</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Abbreviation: App, apparent.
40% and 60% of the natural substrate, suggesting that DNA pol α incorporated these analogs with high affinity into the growing DNA chain.

**Incorporation of CdATP and ara-CTP in sequence.** Because the DNA primers with a CdAMP residue at the 3'-end could be extended by DNA pol α, we decided to determine whether the enzyme could incorporate an ara-CMP molecule after CdAMP. This would be relevant to the in vivo condition, in which both CdATP and ara-CTP are present in the circulating blasts. Reactions were performed in the absence of dATP and dCTP, as well as the analog triphosphates, had a major stop site before the A site (Fig 5, lane 2). Again, the faint band at the A site represents a small amount of noncognate dNTP incorporated into this site. When 10 μmol/L CdATP was added to the reaction mixture, pol α incorporated CdAMP at the A site, resulting in efficient elimination of the band at the pre-A site (Fig 5, lane 3). Because dCTP was not present, the primer extension stopped at the A site (lane 3). Increasing concentrations of ara-CTP inversely decreased the density of the band at the A site (lanes 4 through 10), indicating that DNA pol α extended a primer terminated by CdAMP by incorporating a second nucleotide analog, ara-CTP. Quantity of the radioactivity at the A-site and the C-site showed that at 10 μmol/L CdATP alone, greater than 93% of the radioactivity was at the A site and less than 7% at the C-site. Addition of 10 μmol/L or 50 μmol/L ara-CTP resulted in 60% (lane 8) or 85% (lane 10) of the radioactivity in the C-band, respectively.

**Effect of CdATP on ara-CTP-induced inhibition of primer extension.** Primer extension reactions containing different concentrations of ara-CTP were run in the presence of either dATP or CdATP (Fig 6), and the products formed after these reactions were compared. A reaction containing 4 dNTPs (5 μmol/L each) resulted in full-length product formation without a major pause site (lane 2). In a reaction lacking dATP, pol α stopped at the pre-A site (lane 3). Addition of 10 μmol/L CdATP extended the primer to its full length (lane 4), which was comparable to the complete reaction (lane 2). Incorporation of ara-CTP was compared in reactions containing all three dNTPs (dCTP, dTTP, dGTP) with either dATP (lanes 5, 7, and 9) or CdATP (lanes 6, 8, and 10). DNA pol α incorporated ara-CTP at the C site after dAMP (lanes 5, 7, and 9) or CdATP (lanes 6, 8, and 10) in a dose-dependent fashion, resulting in a dose-dependent decrease in the full-length product formation (Table 4). In reaction mixtures containing CdATP and ara-CTP similar or slightly greater percent product was formed than in reactions containing similar concentrations of dATP and ara-CTP. This may be due to the fact that DNA pol α incorporated ara-CMP more easily in a primer terminated

<table>
<thead>
<tr>
<th>Reactions</th>
<th>ara-CTP μmol/L</th>
<th>% Product Mean ± SD</th>
<th>Product CdATP/ Product dATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CdATP</td>
<td>0</td>
<td>105 ± 13</td>
<td>1.0</td>
</tr>
<tr>
<td>dATP</td>
<td>2</td>
<td>76 ± 2</td>
<td>1.1</td>
</tr>
<tr>
<td>CdATP</td>
<td>3</td>
<td>84 ± 3</td>
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</tr>
<tr>
<td>dATP</td>
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<tr>
<td>CdATP</td>
<td>30</td>
<td>27 ± 6</td>
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with dAMP than in a primer with CdAMP at its end. Hence pol α extended a primer with CdAMP residue at the 3’ end by inserting dCMP molecules more often than ara-CMP. This is consistent with the fact that the ratios between the product formed with the CdATP versus dATP containing reactions increase with increasing concentrations of ara-CTP (Table 4).

DISCUSSION

The goal of this investigation was to evaluate the use of CdA, a potent inhibitor of ribonucleotide reductase, in a new biochemical modulation strategy to increase the concentrations of ara-CTP in leukemia cells over those that could be achieved by simply raising the dose rate of ara-C, and thus its plasma concentration. Consistent with our hypothesis, both the rate of ara-CTP formation and the peak ara-CTP concentration were increased in the circulating leukemia blasts of 7 of the 9 patients studied during therapy. This biochemical modulation strategy elevated the effective dose intensity of the active metabolite of ara-C by 40% in the tumor cell population. It is important to recognize that it is not possible to achieve this by simply increasing the ara-C dose rate,28–31 because the plasma ara-C concentrations produced by the present study design (15 μmol/L)30 already exceed those that saturate ara-C phosphorylation by deoxy- cytidine kinase (7 to 10 μmol/L) in human leukemia blasts.32

It may be possible to increase the intracellular exposure to ara-CTP even more by extending the duration of ara-C infusion at the same dose rate (0.5 g/m²/h). The fact that the rate of ara-CTP accumulation remained linear during the ara-C infusion (Fig 1) suggests that continued accumulation of ara-CTP would be likely if the plasma, and therefore cellular, concentration of ara-C had been maintained. In studies of a similar design, ara-CTP accumulation by leukemia blasts was enhanced by prior infusion of fludarabine, and extending the duration of ara-C infusion further increased triphosphate levels in leukemia cells.15–16 This augmentation was limited by the ability of cells to retain sufficient concentrations of fludarabine triphosphate to activate deoxycytidine kinase.33 Thus, the optimal schedule for enhancing ara-CTP accumulation was a 4-hour ara-C infusion (total daily dose, 2 g/m²) after fludarabine administration.

An advantage of the present study design is that CdATP, the primary effector of the modulation strategy, is likely to be present at steady-state concentrations throughout treatment.34,35 Therefore, we expect that the enhancement of ara-CTP accumulation showed in the present study will be increased as the duration of ara-C infusion is extended. Factors that will determine just how much ara-CTP leukemia cells can be induced to accumulate include their innate capacity to do so, which presumably is determined by the factors regulating the balance between the synthetic and degradative pathways, and the clinical tolerance for the treatment. Therefore, further phase I investigations of extended ara-C infusions will be important in determining the pharmacologic and clinical limits of this approach.

Clinically, the continuous infusion schedule of CdA as a single agent has been an effective treatment for indolent leukemias.36–38 For acute leukemias, a similar schedule resulted in a 59% response rate in both relapsed and newly diagnosed pediatric patients.39,40 Because the dose (8.9 mg/ m²/d) was tolerated well, it may be the appropriate dose for combinations with ara-C in these patients.

In adult AML, based on the following observations, another alternative approach of a bolus infusion of CdA may be considered. Continuous infusion CdA has provided a maximum tolerated dose of 17 mg/m²/d for 5 days21 and similar doses infused for 7 days resulted in marrow hypoplasia in most of these patients without untoward toxicity.22 The mean plasma concentration of CdA achieved with these dose rates was 22 nmol/L.23 The fact that deoxycytidine kinase catalyzes the phosphorylation of both ara-C (Kₚ = 1 to 3 μmol/L)35,36,38 and CdA (Kₚ = 5 μmol/L)40 with similar efficacy suggested that activation of ara-C (present at 10 to 15 μmol/L) is favored over CdA when both drugs are present in plasma. Although a role of dGuo kinase for phosphorylation of CdA has been suggested, the affinity of this enzyme to CdA (Kₚ = 80 μmol/L)31 may not be high enough to phosphorylate CdA which is present in the nanomolar range in plasma during therapy. To increase CdA concentrations in plasma to facilitate its phosphorylation, CdA may be given as a bolus infusion, followed by administration of ara-C for a duration of 2 hours or more. As reported recently, a 2-hour infusion of CdA achieves a plasma peak of 40 to 200 nmol/L and an intracellular level of 5 to 30 μmol/L CdA nucleotides in AML cells.23 Because the CdA nucleotides are eliminated at a slow rate (tl/2 = 9 hours) from AML blasts,38 higher levels of CdA nucleotides are expected during the duration of ara-C infusion.

The fact that dATP serves as a global negative inhibitor of ribonucleotide reductase22 suggested that analogs of dATP may also behave as allosteric inhibitors of this protein. Comparison of deoxadenosine analogs for this capacity showed that CdATP is the most potent inhibitor of this enzyme available for clinical use.12,20 Because CdATP inhibits both CDP and ADP reduction in the in vitro systems, a decrease in the dCTP and dATP pools is expected and has been observed in human leukemia cells in culture.12,20,26 The lowering of the dCTP and dATP pools would increase the ratio of CdATP to dATP and ara-CTP to dCTP. Compared to deoxynucleotide concentrations in leukemia blasts, just before CdA infusion, there was a significant decrease in the dCTP, dATP, and dGTP levels after 24 hours of CdA infusion (Fig 3). The resulting increased ratios of analogs to competing deoxynucleotides should facilitate the incorporation of analogs into growing DNA strands.

Mechanistically, because of the modification in the sugar or base moiety, nucleotide analogs incorporated at the 3’-end of the DNA retard further extension of the primer by DNA polymerases. Although ara-CTP serves as a true representative for this model, incorporation of a single CdAMP molecule did not result in a major pause site and did not change the kinetics of further primer extension (present study17,24,25). Furthermore, and relevant to the present combination therapy, DNA pol α successfully inserted an ara-CTP molecule after CdAMP incorporation at the 3’-end of a primer (Fig 5). This tandem incorporation sequence of nucleotide analogs resulted in nearly complete inhibition of...
DNA primer extension (Fig 5). Additionally, although incorporation of a CdAMP molecule alone did not inhibit further extension of the DNA primer over a template by DNA polymerases, subsequent replication over CdAMP-containing templates was greatly inhibited.11

In general during CdA and ara-C therapy, both metabolic and mechanistic interactions influence the synthesis of DNA and hence the cytotoxicity. As shown in the present investigation and consistent in the patients studied, administration of CdA and ara-C resulted in maximum inhibition of DNA synthesis that was sustained until the next infusion of ara-C. Hence it could be predicted that the accumulation of higher concentrations of ara-CTP, greater ratios of analog triphosphates to normal dNTPs, perturbation of dNTP pools, and tandem incorporation of CdATP and ara-CTP in growing DNA strands, would affect the synthesis of DNA leading to cytotoxicity. These data provide a metabolic and mechanistic rationale for combining these drugs in an optimal schedule to improve the clinical outcome of this approach.

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Chlorodeoxyadenosine and arabinosylcytosine in patients with acute myelogenous leukemia: pharmacokinetic, pharmacodynamic, and molecular interactions

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