CONCISE REPORT

Deoxycytidine Preferentially Protects Normal Versus Leukemic Myeloid Progenitor Cells From Cytosine Arabinoside-Mediated Cytotoxicity

By Kapil Bhalla, William MacLaughlin, John Cole, Zalmen Arlin, Michael Baker, Gary Graham, and Steven Grant

We examined the ability of high concentrations of the naturally occurring nucleoside deoxycytidine (dCyd) to reverse the cytotoxicity of high (eg, ≥10⁻⁴ mol/L) concentrations of 1-B-D arabinofuranosylcytosine (Ara-C) toward normal (CFU-GM) and leukemic myeloid progenitor cells (L-CFU). Leukemic myeloblasts from patients with acute nonlymphocytic leukemia (ANLL) and normal human bone marrow mononuclear cells were cultured in soft agar in the continuous presence of 10⁻⁴ to 5 × 10⁻³ mol/L of Ara-C together with dCyd (10⁻⁴ to 5 × 10⁻³ mol/L). Administration of 10⁻⁴ mol/L of Ara-C alone eradicated colony formation in all samples tested. Coadministration of 10⁻⁴ mol/L of dCyd restored 72.2% of control colony formation for CFU-GM, but only 10.9% for L-CFU. When higher concentrations of Ara-C (eg, 5 × 10⁻³ mol/L) were administered, dCyd-mediated protection toward CFU-GM decreased, but remained significantly greater than that observed for L-CFU. Incubation with 10⁻³ mol/L of dCyd reduced the 4-hour intracellular accumulation of the triphosphate derivative of Ara-C (Ara-CTP) in both normal and leukemic cells by >98%; under identical conditions, a significant expansion of the intracellular of the triphosphate derivative of dCyd (dCTP) pools was observed in normal bone marrow mononuclear cells but not in leukemic blasts. This finding was associated with a greater reduction in Ara-C DNA incorporation in normal elements. These in vitro studies suggest that dCyd may preferentially protect normal v leukemic myeloid progenitor cells from the lethal actions of high-dose Ara-C.

The deoxycytidine (dCyd) analog 1-B-D arabinofuranosylcytosine (Ara-C) is an effective antileukemic drug in humans. Both dCyd and Ara-C (and their metabolites) are competitive substrates for a variety of intracellular enzymes including dCyd kinase; cytidine deaminase, and dCMP deaminase. In addition, dCyd and Ara-C are ultimately converted to their triphosphate derivatives (dCTP and Ara-CTP), which compete for binding to the enzyme DNA polymerase and for incorporation into DNA. The latter process has recently been highly correlated with Ara-C-mediated lethality toward leukemic cells in vitro. Because of its ability to antagonize Ara-C metabolism at various levels, dCyd has been associated with reversal of Ara-C toxicity both in vitroand in vivo. Intermittent high-dose infusions of Ara-C have achieved responses in up to 50% of patients with acute nonlymphocytic leukemia (ANLL) in relapse. More recently, high-dose Ara-C was administered as a continuous infusion for 96 hours to patients with refractory ANLL, and steady-state plasma levels in excess of 10⁻¹ mol/L were achieved. Although significant oncolytic responses have been observed, the dose-limiting toxicity has been myelosuppression. Studies in intact animals showed that dCyd can ameliorate the host toxicity of otherwise lethal doses of Ara-C, leading to a net gain in therapeutic index for the combination. More recently, our group reported that dCyd (10⁻⁴ to 10⁻³ mol/L) selectively enhances the in vitro growth of normal CFU-GM, but not leukemic myeloid progenitor cells (L-CFU). These studies raise the possibility that high concentrations of dCyd may preferentially protect normal marrow elements from Ara-C-induced lethality, thereby improving antileukemic efficacy. The aim of the present study was to compare the ability of high concentrations of dCyd to reverse the growth inhibitory effects of continuously administered high-dose Ara-C toward normal v L-CFU in culture. An additional aim was to establish the biochemical basis for potential selectivity, including examination of the intracellular accumulation of Ara-CTP and dCTP and the incorporation of Ara-C into DNA of normal and L-CFU.

MATERIALS AND METHODS

Drugs and chemicals. Deoxycytidine and Ara-C were purchased from Sigma Chemicals (St Louis). Drugs were stored as dry powders at −20°C and reconstituted in sterile media prior to use. [³H] Ara-C (26 Ci/mmol) and [³H] dCyd (16 Ci/mmol) were purchased from Amersham Radiochemicals (Arlington Heights, IL).

Culture of normal human bone marrow granulocyte macrophage progenitors. Human bone marrow myeloid progenitors were cultured by a previously described double-layer soft agar cloning method. Samples were obtained with informed consent from patients undergoing routine diagnostic bone marrow aspirations for non-neoplastic hematologic disorders that did not involve the myeloid series or from normal volunteers. These studies were sanctioned by the Investigational Review Board of Columbia University. Mononuclear cells were isolated by the method of Boyum, washed twice, and suspended in a mixture consisting of McCoy's 5a medium, 20% fetal calf serum (FCS), and 0.3% Bacto agar (Difco,
EFFECT OF Ara-C PLUS dCyd ON CFU-GM v L-CFU

Detroit). Aliquots (0.5 mL) of the mixture were pipetted into 18-mm 12-well plates (Costar, Cambridge, MA) containing a bottom layer consisting of McCoy's 5a medium, 20% FCS, and 0.5% agar. To each well, which contained 10⁵ cells/well, was added 0.1 mL of GCT-conditioned medium (GIBCO, Grand Island, NY) as a source of colony-stimulating factor, along with the designated concentrations of dCyd and Ara-C. After the agar was allowed to harden, the plates were placed in a fully humidified, 37°C, 5% CO₂ incubator (Detroit). Aliquots (0.5 mL) of the mixture were pipetted into 18-mm 12-well plates (Costar, Cambridge, MA) containing a bottom layer consisting of McCoy's 5a medium, 20% FCS, and 0.5% agar. To each well, which contained 10⁵ cells/well, was added 0.1 mL of GCT-conditioned medium (GIBCO, Grand Island, NY) as a source of colony-stimulating factor, along with the designated concentrations of dCyd and Ara-C. After the agar was allowed to harden, the plates were placed in a fully humidified, 37°C, 5% CO₂ incubator for 7 days. At the end of this incubation period, colonies, consisting of groups of 50-100 cells with granulocytic or macrophage-like appearance, were scored with an Olympus model CK inverted microscope. The effect of Ara-C, (with or without dCyd) on CFU-GM growth was expressed as the percentage of colony formation by drug-treated cells relative to untreated controls.

Culture of leukemia blast progenitors. Leukemic blasts were cultured by a minor modification of previously described technique. T lymphocyte-depleted mononuclear cells were obtained from bone marrow or peripheral blood samples from patients with acute nonlymphocytic leukemia, and plated at 2 x 10⁵ cells/mL, using the bilayer soft agar method previously described for normal CFU-GM. All samples used were comprised of 80% blasts. Phytohemagglutinin (PHA) or leucocyte conditioned medium (10% vol/vol) was used as a source of colony-stimulating activity. Plates were incubated in a 37°C, 5% CO₂, fully humidified incubator for 7 days, after which colonies, consisting of 20 blast-like cells, were scored with an inverted microscope. The effect of Ara-C and dCyd on L-CFU formation was evaluated in the same manner used for CFU-GM.

Intracellular Ara-CTP and dCTP levels. Normal bone marrow mononuclear cells and leukemic blasts were isolated as described above and suspended in McCoy's 5a medium containing 10% FCS at a cell density of 10⁵ cells/mL. The suspension was placed in 25-cm² tissue culture flasks (Corning, Corning, NY) to which were added the indicated concentrations of Ara-C and dCyd. The plates were incubated for 10 hours in 37°C, 5% CO₂, fully humidified incubator for 5 hours, after which the suspension was centrifuged at 400 g for 8 minutes at 4°C; neutralized HC10₄-soluble extracts of the cell pellet were obtained as described previously. Immediately prior to analysis, the acid-soluble extracts were treated with periodate oxidation to remove endogenous ribonucleotides, and Ara-C and dCTP were quantitated by a high-pressure liquid chromatography method using Waters HPLC system including a 440 dual-wavelength absorbance detector (Waters, Milford, MA). In brief, a Radial Pak SAX anion exchange column (Waters) was used with a 300 mmol/L NH₄H₂PO₄ buffer (pH 3.5) plus 2% methanol as the mobile phase at a 2.5 mL/min flow rate. Separation of the two metabolic products was greater than 1.5 minutes. Values were expressed as pmol/10⁶ cells ± SEM.

Table 1. Effect of dCyd on the Inhibitory Activity of Ara-C Toward L-CFU and CFU-GM Growth

<table>
<thead>
<tr>
<th>Condition</th>
<th>L-CFU (n = 10)</th>
<th>CFU-GM (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ara-C 10⁻⁴ mol/L</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ara-C 10⁻⁴ mol/L + dCyd 10⁻⁴ mol/L</td>
<td>12.9 ± 7.0</td>
<td>3.2 ± 1.1</td>
</tr>
<tr>
<td>Ara-C 10⁻⁴ mol/L + dCyd 10⁻⁵ mol/L</td>
<td>10.9 ± 3.7</td>
<td>72.2 ± 9.1*</td>
</tr>
<tr>
<td>Ara-C 5 x 10⁻⁴ mol/L</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ara-C 5 x 10⁻⁴ mol/L + dCyd 10⁻⁷ mol/L</td>
<td>3.8 ± 2.8</td>
<td>9.4 ± 2.2</td>
</tr>
<tr>
<td>Ara-C 5 x 10⁻⁴ mol/L + dCyd 5 x 10⁻⁵ mol/L</td>
<td>7.8 ± 3.0</td>
<td>42.4 ± 5.0*</td>
</tr>
</tbody>
</table>

Normal cells and L-CFU were cultured in soft agar in the presence of Ara-C (10⁻⁴ mol/L) and 5 x 10⁻⁵ mol/L with or without dCyd (10⁻⁴ to 5 x 10⁻⁵). After 7-day incubation, colonies, consisting of ≥50 (CFU-GM) or ≥20 (L-CFU) cells, were scored with an inverted microscope. Values are the percentage for each condition relative to control colony formation; n, number of samples studied.

*Significantly different (P < .05) from L-CFU growth under identical conditions.

RESULTS

The effect of dCyd on the growth inhibitory effects of continuously administered high-dose Ara-C (10⁻⁵ or 5 x 10⁻⁵ mol/L) toward normal and leukemic myeloid progenitors is shown in Table 1. Exposure of cells to Ara-C alone resulted in complete inhibition of colony formation in all samples (not shown). Coadministration of 10⁻³ mol/L of dCyd together with 10⁻⁵ mol/L of Ara-C restored 72.2% ± 9.1% of control CFU-GM colony formation. In contrast, under identical conditions, L-CFU growth remained substantially inhibited at 10.9% ± 3.7% of control (P < .01). When CFU-GM were cultured in the presence of 5 x 10⁻⁵ mol/L of Ara-C in conjunction with a hundred-fold excess of dCyd (5 x 10⁻³ mol/L), the degree of protection declined (eg, 42.4% ± 5.0% of control). Restoration of colony formation remained significantly greater than that observed for L-CFU under the same conditions, however (7.8% ± 3.0% of control). Culture of cells with dCyd in excess by only tenfold resulted in very little protection of either normal or leukemic cells from Ara-C-mediated toxicity.

Intracellular Ara-CTP and dCTP levels. Normal bone marrow mononuclear cells and leukemic blasts were isolated as described above and suspended in McCoy's 5a medium containing 10% FCS at a cell density of 10⁵ cells/mL. The suspension was placed in 25-cm² tissue culture flasks (Corning, Corning, NY) to which were added the indicated concentrations of Ara-C and dCyd. The plates were incubated in a 37°C, 5% CO₂, fully humidified incubator for 5 hours, after which the suspension was centrifuged at 400 g for 8 minutes at 4°C; neutralized HC10₄-soluble extracts of the cell pellet were obtained as described previously. Immediately prior to analysis, the acid-soluble extracts were treated with periodate oxidation to remove endogenous ribonucleotides, and Ara-C and dCTP were quantitated by a high-pressure liquid chromatography method using Waters HPLC system including a 440 dual-wavelength absorbance detector (Waters, Milford, MA). In brief, a Radial Pak SAX anion exchange column (Waters) was used with a 300 mmol/L NH₄H₂PO₄ buffer (pH 3.5) plus 2% methanol as the mobile phase at a 2.5 mL/min flow rate. Separation of the two metabolic products was greater than 1.5 minutes. Values were expressed as pmol/10⁶ cells ± SEM.

Ara-C incorporation into nucleic acids. After cells were isolated, washed, and suspended as described above, they were placed in T-flasks containing 10⁻² mol/L of [³H] Ara-C ± 10⁻³ mol/L of dCyd. The flasks were then incubated at 37°C for 4 hours, after which the suspension was transferred to 50-mL tubes and centrifuged at 400 g for 8 minutes at 4°C. The cell pellet was washed twice with cold phosphate-buffere din saline (PBS) to remove the radiolabeled drug. DNA was isolated and purified by pronase digestion, phenol extraction, and ethanol precipitation as previously described. After centrifugation at 9,000 g at 4°C for 30 minutes, the DNA was resuspended in Tris-HCl buffer, quantitated spectro photometrically, and aliquots were removed and placed in scintillation vials to determine the radioactivity. Values for each condition are expressed as pmol of Ara-C/μg of DNA.

Statistical analysis. Significant differences between experiment groups were assessed using Student's t test for paired observations.
levels significantly different in either cell type. Coincubation with 10^{-7} mol/L of dCyd reduced the 4-hour intracellular accumulation of Ara-CTP in both normal and leukemic cells by >98% (0.14 and 0.11 pmol/10^6 cells, respectively). Under these conditions, however, a significant expansion of intracellular dCTP pools was observed only in normal bone marrow mononuclear cells (0.96 to 9.82 pmol/10^6 cells); dCTP pools in leukemic cells were expanded to a much smaller degree (0.39 to 1.13 pmol/10^6 cells).

The effect of dCyd on the 4-hour incorporation of [3H] Ara-C (10^{-5} mol/L) into normal and leukemic cell DNA is shown in Table 2. When cells were exposed to 10^{-3} mol/L of Ara-C, leukemic blasts incorporated more of Ara-C than did normal bone marrow mononuclear cells (0.148 ± 0.064 pmol of Ara-C/μg of DNA, respectively); however, this difference was not statistically significant (P > 0.5). Coadministration of 10^{-3} mol/L of dCyd reduced the Ara-C DNA incorporation to a greater extent in normal v leukemic bone marrow cells (0.002 ± 0.007 pmol of Ara-C/μg of DNA). The magnitude of this difference in Ara-C DNA incorporation did not parallel the significantly greater expansion (~eightfold) of dCTP pools in normal marrow cells under identical conditions, however.

DISCUSSION

Pharmacokinetic studies of Ara-C have consistently demonstrated that steady-state plasma levels of ≥ 10^{-4} mol/L are achieved during continuous-infusion high-dose Ara-C therapy. These Ara-C levels decrease the variability in DNA inhibition, saturate the capacity of the leukemic myeloblasts to accumulate Ara-CTP, and increase the incorporation of Ara-C into DNA. The present study demonstrates that high concentrations of dCyd (eg, ≥ 10^{-7} mol/L) preferentially protect normal v L-CFU from the in vitro cytotoxic effects of high-dose Ara-C (eg, 10^{-5} and 5 × 10^{-3} mol/L). Previous investigators have reported that dCyd is relatively ineffective in reversing the lethal actions of Ara-C toward murine leukemias in vivo and inefficient in antagonizing Ara-CTP formation in some human leukemic myeloblasts in vitro. Moreover, we showed that 10^{-4} to 10^{-3} mol/L dCyd administered alone preferentially stimulates the in vitro growth of normal but not L-CFU. Together, these results suggest that differences in the metabolism of high concentrations of dCyd may exist between normal and leukemic cells, and that these differences may account for the relative inability of dCyd to antagonize high-dose Ara-C–mediated lethality in leukemic elements.

In our studies, 10^{-3} mol/L of dCyd dramatically inhibited the formation of Ara-CTP in both normal and leukemic cells. Despite this antagonism, substantial inhibition of leukemic cell clonogenicity was preserved and in all cases exceeded that observed for normal myeloid progenitors. Following exposure to high concentrations of dCyd and Ara-C, normal bone marrow mononuclear cells exhibited a considerable expansion of dCTP pools, whereas leukemic myeloblasts did not. Expansion of intracellular dCTP pools can antagonize Ara-C metabolism and cytotoxicity through a variety of mechanisms, including inhibition of dCyd kinase, stimulation of dCMP deaminase, and inhibition of Ara-C incorporation into DNA. Data from our studies indicate that higher intracellular ratios of dCTP/Ara-CTP in normal v leukemic cells exposed to high concentrations of Ara-C and dCyd result in greater reduction in Ara-C DNA incorporation in normal cells. Although the magnitude of this reduction in Ara-C DNA incorporation did not parallel the eightfold greater expansion of dCTP pools in normal cells, it might be sufficient to account for the preferential reversal of Ara-C–mediated cytotoxicity in these elements.

Despite enhanced antileukemic effects of high-dose Ara-C in clinical trials, severe myelosuppression has been a dose-limiting toxicity. If, as suggested by our findings, high concentrations of dCyd selectively antagonize Ara-C–mediated toxicity toward normal myeloid elements, it is possible that continuous-infusion high-dose Ara-C could be administered with improved therapeutic efficacy.

REFERENCES


Deoxycytidine preferentially protects normal versus leukemic myeloid progenitor cells from cytosine arabinoside-mediated cytotoxicity

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