Arabinosylguanine-induced Apoptosis of T-Lymphoblastic Cells: Incorporation into DNA Is a Necessary Step

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ABSTRACT

9-β-D-Arabinosylguanine (ara-G) is a recently introduced and effective treatment for T-cell acute lymphoblastic leukemia, but how ara-G and ara-G triphosphate (ara-GTP) kill cells is not known. We hypothesized that, in cycling T-lymphoblastoid cells, ara-G may act directly by incorporation into DNA, which may lead to apoptosis. Hence, blocking the incorporation of ara-G monophosphate (ara-GMP) into DNA may prevent apoptosis. To test this hypothesis, we performed experiments in a T-lymphoblastic leukemia cell line (CCRF-CEM) after synchronization with a double aphidicolin block. Intracellular accumulation of ara-GTP was neither cell cycle dependent nor affected by aphidicolin (53 ± 5 μM/h without aphidicolin, 50 ± 5 μM/h with aphidicolin). Cells at the G1-S boundary accumulated 75 ± 7 μM ara-GTP with minimal incorporation into DNA (5 ± 2 pmol ara-GMP/mg DNA) and had little biochemical or morphological evidence of apoptosis. In marked contrast, cells in S phase had significantly more ara-G incorporated into DNA (24 ± 4 pmol ara-GMP/mg DNA), although the cytosolic concentration of ara-GTP (85 ± 7 μM) was similar to that in the G1-S-enriched population. In the S-phase cells, there was a corresponding increase in apoptosis (measured as high molecular weight DNA fragmentation and morphological changes), and the incorporation of ara-GTP into DNA resulted in a >95% inhibition of DNA synthesis. There was a direct linear relationship between the number of cells in S phase and both the total number of ara-GMP molecules in DNA and the inhibition of DNA synthesis. Blocking of ara-GTP incorporation into S-phase DNA abolished biochemical and morphological features of apoptosis, even in the presence of cytotoxic level of intracellular ara-GTP. Taken together, these data demonstrate that the incorporation of ara-GTP into DNA is the critical event that mediates the induction of apoptosis in CCRF-CEM cells.

INTRODUCTION

Nucleoside analogues belong to one of the most clinically useful and most often used class of drugs for the treatment of cancer and viral diseases. Numerous pyrimidine and purine nucleoside analogues have demonstrated clinical utility in the management of human malignancies. Our recent and first Phase I trial of a water-soluble prodrug of ara-G,2-amino-6-methoxypurine arabinoside (506U78; Ref. 1), demonstrated its efficacy against hematological malignancies (2, 3).

Metabolically, nucleoside analogues, including ara-G, must be phosphorylated to their triphosphate forms to exert cytotoxicity (4, 5). Nucleoside analogue triphosphates then compete with native deoxynucleotides for incorporation into DNA. Once incorporated, the fraudulent nucleoside monophosphate stops DNA synthesis and causes cell death, usually by apoptosis. This incorporation into DNA and subsequent apoptosis is essential for the cytotoxic actions of clinically tested and effective nucleoside analogues, such as gemcitabine (6), cytarabine (7), fludarabine (8, 9), and cladribine (10). However, how ara-G and ara-GTP kill cells remains speculative.

In contrast to other nucleoside analogues but similar to deoxyguanosine, ara-G appears to be selectively toxic to immature T cells (4, 11–13). This has been demonstrated in cell lines (4, 12, 13), freshly isolated leukemia cells (14), and leukemia cells from patients treated with 506U78 (2), the prodrug of ara-G (1). B-Lymphoblastoid and myeloid lineage cells, on the other hand, did not respond to ara-G during in vitro incubations (13, 14) or in the clinic with 506U78 therapy (2, 3). The exact mechanism for this differential sensitivity is not known, but it has been postulated that immature T-cells have biochemical properties that result in increased accumulation and decreased elimination of ara-GTP (4, 12, 13).

We hypothesized that, in immature T-lymphoblasts, the mechanism of action of ara-G, like other purine and pyrimidine analogues, is directly related to its incorporation into DNA. Specifically, the incorporation of ara-GTP may result in chain termination and the cessation of DNA synthesis, which may, in turn, trigger the signaling pathways involved in the execution of apoptosis. Hence, if the incorporation of ara-GMP into DNA were prevented, the apoptosis program would not be triggered, despite the presence of intracellular ara-GTP. Our study tested this hypothesis in an experimental system in which T cells are allowed to accumulate intracellular ara-GTP but incorporation into DNA is prohibited.

MATERIALS AND METHODS

Chemicals and Reagents. ara-G was purchased initially from Calbiochem (La Jolla, CA) and subsequently from R. I. Chemicals, Inc. (Orange, CA). [8,3H]ara-G and [14C]thymidine were purchased from Moravek Biochemicals (Brea, CA). For HPLC standards, we used ara-GTP that was chemically synthesized by Sierry Biosource (Tucson, AZ). Aphidicolin was obtained from Sigma Chemical Co. (St. Louis, MO). Proteinase K and DNase-free RNase were purchased from Boehringer Mannheim Co. (Indianapolis, IN). All other chemicals were reagent grade.

Cell Culture. CCRF-CEM cells were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc., Grand Island, NY) and maintained at 37°C in 5% CO2 in a fully humidified incubator. The cell doubling time was 24 h under these conditions. Cells were routinely tested for Mycoplasma by using a commercially available kit and following the manufacturer’s recommendations (Gen-Probe Inc., San Diego, CA).

Cell Synchronization and Experimental Plan. The cells were synchronized by double aphidicolin block, as described previously (9). Briefly, CCRF-CEM cells were incubated with 2 μM aphidicolin for 24 h, washed, and incubated in fresh aphidicolin-free medium for 12 h and then incubated with 2 μM aphidicolin for an additional 24 h. This 60-h double-aphidicolin treatment blocked the cells at the G1-S boundary (Fig. 1). These synchronized cells were then washed and resuspended in either fresh medium without aphidicolin for cell cycle progression or fresh medium containing 2 μM aphidicolin for continued inhibition of DNA synthesis. The latter cultures that were incubated continuously with aphidicolin served as controls, and the first time point was designated Pre. The first time point for the other culture, which was released from aphidicolin block was designated 0 h. As shown in Fig. 1, at 3, 6, 9, 12, 18, and 24 h, aliquots of cells were taken from both these cultures. These cells were routinely tested for Mycoplasma by using a commercially available kit and following the manufacturer’s recommendations (Gen-Probe Inc., San Diego, CA).

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3 The abbreviations used are: ara-G, 9-β-D-arabinosylguanine; ara-GTP, ara-G triphosphate; ara-GMP, ara-G monophosphate; HPLC, high-performance liquid chromatography; dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase.
were analyzed for various end points without any further treatment or after a 3-h incubation with 100 μM ara-G (Fig. 1). Preliminary experiments demonstrated that this concentration of aphidicolin used for synchronization of cells was not cytotoxic, even after 48 h of continuous incubation (9).

Flow Cytometry. To determine the distribution of cells within the cell cycle, we pelleted (1500 rpm for 5 min at 4°C) aliquots of cells (1 × 10^6) of each culture. Synchronized cells were split into two and incubated either in the presence or absence of aphidicolin. At designated times, aliquots of cells were removed and incubated with either ara-G (experimental) or no drug (control) prior to analysis for ara-GTP accumulation, DNA synthesis, or apoptosis.

**ENDPOINTS**
- ara-GTP Accumulation
- DNA Synthesis
- DNA Fragmentation
- Apoptotic Morphology

To determine the distribution of cells within the cell cycle, we pelleted (1500 rpm for 5 min at 4°C) aliquots of cells (1 × 10^6); the cells were washed twice in ice-cold PBS (8.1 g of NaCl, 1.14 g of Na2HPO4, 0.22 g of KCl, and 0.25 g of KH2PO4 per liter), fixed in ice-cold 70% ethanol, and stored at 4°C until analysis. Before analysis by flow cytometry, the fixed cells were pelleted, washed in PBS, and resuspended in ice-cold flow buffer (PBS containing 0.5% Tween 20, 15 μg/ml propidium iodide, and 5 μg/ml DNase-free RNase). The stained cells were analyzed with an Epics Profile II flow cytometer (Coulter Electronics, Inc., Hialeah, FL).

**Measurement of Intracellular Nucleoside Triphosphates by HPLC.** To quantitate ara-GTP accumulation, we incubated exponentially growing CEM cells with 100 μM ara-G for the indicated times. This concentration was chosen because our recent Phase I trial indicated that at the maximally tolerated dose of the ara-G prodrug 506U78, the median peak level of ara-G in plasma was 10 μM (2). After incubation with ara-G, aliquots of cells (5 × 10^6–1 × 10^7) were removed at various times. Table 1 shows data from cells that were synchronized as described above (Fig. 1) and then incubated with 100 μM [8,14C]ara-G for 3 h. After the incubation, the cells were pelleted, washed twice with ice-cold PBS, resuspended in lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM EDTA, 0.5% SDS, and 2 mg/ml proteinase K), and incubated at 65°C overnight. The nucleic acids were extracted with one volume of phenol: chloroform:isoamyl alcohol (25:24:1, v/v/v) and then precipitated in three volumes of 100% ice-cold ethanol. The nucleic acid pellet was washed twice with 70% ethanol, allowed to air-dry, and then resuspended in water. Contaminating RNA was digested with DNase-free RNase (2 μg/ml) for 2 h at 37°C. The DNA was extracted, precipitated with ethanol, washed, and finally resuspended in water as described above. The concentration (μg/ml) and purity (A260 nm/A280 nm ratio) of the DNA in solution was determined with an Ultra spec 3000 spectrophotometer (Pharmacia Biotech, Cambridge, England). In all cases, the A260 nm/A280 nm value was greater than 1.9. The amount of ara-GMP incorporated into DNA was determined by scintillation counting (Packard Instrument Co., Meriden, CT).

**High Molecular Weight DNA Fragmentation.** Cells were prelabeled with [14C]thymidine as described above. At various times after release from aphidicolin, [14C]thymidine-prelabeled cells were incubated with 100 μM ara-G for 3 h. The cells were then checked in 6.0% agarose plugs (75 mM NaCl, 5 mM EDTA, and 5 mM Tris-HCl [pH 7.8]). The agarose plugs were allowed to solidify at room temperature for 5 min and then incubated overnight at 45°C in plug lysis buffer (1% Sarkosyl, 50 mM EDTA, 50 mM Tris-HCl [pH 7.8], and 0.2 mg/ml proteinase K). The agarose plugs were analyzed by pulsed field gel electrophoresis (CHEF-DRII; Bio-Rad Laboratories, Richmond, CA) at 200 V with an initial switch time of 50 s and a final switch time of 100 s for 16 h at 7°C in 0.5× TBE. The gel was stained with ethidium bromide and photographed. The radioactivity associated with the −50-kb fragments was quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or Betascope 603 analyzer after the gel was dried at 60°C under vacuum as described previously (15). The results were normalized with respect to total radioactivity in each lane and expressed as a percentage of radioactivity associated with the high molecular weight DNA fragments in aphidicolin-blocked cells (Pre).

### Table 1 Relationships among cell cycle distribution, intracellular ara-GTP accumulation, and nuclear incorporation of ara-GMP

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
<th>ara-GTP (μM)</th>
<th>ara-GMP (pmol/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>83 ± 4</td>
<td>4 ± 5</td>
<td>8 ± 4</td>
<td>75 ± 7</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>0</td>
<td>78 ± 4</td>
<td>10 ± 3</td>
<td>9 ± 4</td>
<td>109 ± 7</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>6</td>
<td>14 ± 5</td>
<td>80 ± 7</td>
<td>7 ± 2</td>
<td>85 ± 7</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>12</td>
<td>27 ± 2</td>
<td>20 ± 7</td>
<td>57 ± 4</td>
<td>123 ± 7</td>
<td>8 ± 2</td>
</tr>
</tbody>
</table>
| 24      | 65 ± 10 | 40 ± 7 | 9 ± 6 | 114 ± 7 | ND 

* Cells were synchronized by 60-h double aphidicolin block as described in “Materials and Methods,” and cultures were washed and split in two. The first culture (Pre) was incubated in the presence of aphidicolin (to prevent DNA synthesis) and 100 μM ara-G for 3 h. The second culture was incubated in the absence of aphidicolin, which allowed cells to progress through cell cycle. At designated times after release (0, 6, 12, and 24 h), they were incubated with 100 μM ara-G for 3 h. Hence, all cultures received ara-G for 3 h.

* Values represent mean ± SD of two experiments conducted in duplicate.

## Incubation of ara-G INTO DNA AND APOPTOSIS

**Inhibition of DNA Synthesis by ara-G.** Prior to synchronization with double aphidicolin block, cellular DNA was prelabeled four times over 48 h with [3H]thymidine (0.02 μCi/ml every 12 h) before double aphidicolin block. The labeled cells were treated as described above (Fig. 1). [3H]Thymidine (2 μCi/ml) was added to these cultures, and incubation was continued for an additional 0.5 h in a MultiScreen assay system (Millipore Corp., Bedford, MA). The cells were then collected on MultiScreen-GV filters under vacuum and washed four times with ice-cold 8% trichloroacetic acid, four times with ice-cold water, and four times with ice-cold 100% ethanol. The radioactivity in the acid-insoluble material retained on the filters was measured by scintillation counting using energy windows that minimized the spillover of the 3H signal into the 14C signal. The values were normalized to the total 14C retained on the filters and were expressed as percentages of control values from cells that were not treated with ara-G.

**Determination of ara-GMP Incorporation into DNA.** The cells were synchronized as described above (Fig. 1) and then incubated with 100 μM [8,14C]ara-G for 3 h. After the incubation, the cells were pelleted, washed twice with ice-cold PBS, resuspended in lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM EDTA, 0.5% SDS, and 2 mg/ml proteinase K), and incubated at 65°C overnight. The nucleic acids were extracted with one volume of phenol: chloroform:isoamyl alcohol (25:24:1, v/v/v) and then precipitated in three volumes of 100% ice-cold ethanol. The nucleic acid pellet was washed twice with 70% ethanol, allowed to air-dry, and then resuspended in water. Contaminating RNA was digested with DNase-free RNase (2 μg/ml) for 2 h at 37°C. The DNA was extracted, precipitated with ethanol, washed, and finally resuspended in water as described above. The concentration (μg/ml) and purity (A260 nm/A280 nm ratio) of the DNA in solution was determined with an Ultra spec 3000 spectrophotometer (Pharmacia Biotech, Cambridge, England). In all cases, the A260 nm/A280 nm value was greater than 1.9. The amount of ara-GMP incorporated into DNA was determined by scintillation counting (Packard Instrument Co., Meriden, CT).
INCORPORATION OF ara-G INTO DNA AND APOPTOSIS

RESULTS

Cell Cycle Progression after Release from Double Aphidicolin Block. To determine the cell cycle distribution of the culture at various times after release from double aphidicolin block, we stained cells with propidium iodide and analyzed them by flow cytometry. The data show that, immediately before release from the double aphidicolin block, most (80 ± 5%) of the cells were at the G1-S boundary, and the rest were distributed evenly between the S and G2-M phases (Fig. 2). Six h after release from the double aphidicolin block, most of cells (80 ± 5%) were in S phase. The S-phase distribution decreased to 20 ± 10% between 12 and 18 h after release from aphidicolin. Subsequently, the number of cells in S phase gradually increased in the period from 18 to 24 h as the cells entered the next cell cycle. The G1 distribution was the opposite of that of S-phase distribution, having a maximum value immediately before release, a decrease during the first 6 h, and an increase from 12 to 18 h. Because the normal population doubling time of CCRF-CEM cells is 22–24 h, the G1 distribution was synchronously through the cell cycle.

Influence of Aphidicolin and Cell Cycle on ara-GTP Accumulation. To determine whether the rate of ara-GTP accumulation was affected by aphidicolin, we incubated exponentially growing cells with 100 μM ara-G, alone or in combination with 2 μM aphidicolin for up to 12 h (Fig. 3). The rates of accumulation of ara-GTP remained linear for 12 h in the absence (53 ± 5 μM/h; \( r^2 = 0.84 \)) or presence (50 ± 5 μM/h; \( r^2 = 0.88 \)) of aphidicolin (\( P = 1.0 \)). These results demonstrate that aphidicolin did not affect the intracellular accumulation of ara-GTP.

To determine whether the accumulation of intracellular ara-GTP was cell cycle dependent, we incubated cells with 100 μM ara-G for 3 h at 0, 6, 12, and 24 h after release from double aphidicolin block (Table 1). We chose sample times where there would be minimal (Pre, 0, and 12 h) and maximal (6 and 24 h) numbers of cells in S phase (Fig. 2 and Table 1) to serve as positive controls. The intracellular level of ara-GTP ranged from 75 to 123 μM and was not significantly different from the median value of 109 ± 9 μM (\( P = 1.0 \)). These data demonstrate that the accumulation of ara-GTP was not related to the cell cycle phase.

Incorporation of ara-GTP into DNA. Synchronized cells were incubated with 100 μM ara-G for 3 h in the presence of 2 μM aphidicolin (Pre; Table 1). Because similar levels of intracellular ara-GTP accumulated in different phases of the cell cycle, in these same cultures, we analyzed the incorporation of ara-GMP into DNA (Table 1). We hypothesized that ara-GMP would be incorporated into DNA only during periods of DNA synthesis and that the greatest incorporation into DNA would occur in the cultures that had the most cells in S phase. After double aphidicolin block, ~80% of the cells were at G1-S, and 9% of the synchronized population was in S phase. The level of DNA incorporation in this population maintained in the continuous presence of aphidicolin (Pre) was 5 ± 2 pmol ara-GMP/mg DNA after a 3-h incubation with 100 μM ara-G. Immediately after release from aphidicolin (time 0), there was little change in the population cell cycle distribution (8% S phase) or in the quantity of ara-GMP incorporated into DNA (8 ± 4 pmol ara-GMP/mg DNA) after a 3-h incubation with 100 μM ara-G. In contrast, when 80% of the cells had entered S phase (6 h), the level was 24 ± 4 pmol ara-GMP/mg DNA. Twelve h after release from aphidicolin, 20% of the cells remained in S phase, and the amount of ara-GMP incorporated into DNA was 3 ± 1 pmol ara-GMP/mg DNA. The quantity of
Incorporation of ara-G into DNA and Apoptosis

Inhibition of DNA Synthesis by ara-G. We hypothesized that incorporation of ara-G into DNA would inhibit DNA synthesis and subsequently stimulate apoptosis. To test this postulate, we first measured the DNA synthesis in cells before and after a 3-h incubation with 100 μM ara-G, and at various times after release from double aphidicolin block (Fig. 4). The cells began actively synthesizing DNA immediately after release from aphidicolin. The level of DNA synthesis peaked by 6 h, returned to baseline values by 9 h, and remained at baseline levels until 18 h, and began to rise 24 h after release from aphidicolin (Fig. 4). The changes in DNA synthesis levels (compared with the prerelease value) were significant at 3, 6, and 24 h (P < 0.001). In contrast, in cells maintained in the presence of aphidicolin, there was no significant change (P = 1.0) in the levels of DNA synthesis in the absence (Fig. 4, □) or presence (Fig. 4, ▽) of ara-G at any time tested.

To test the effect of ara-G on DNA synthesis, we quantitated thymidine incorporation before (Fig. 4, □) and after (data not shown) a 3-h incubation with 100 μM ara-G at 0, 6, 12, and 24 h after release of cells from double aphidicolin block. Incubation of cells with ara-G in the absence of aphidicolin (released) reduced thymidine incorporation to background levels at all times tested. This was significantly lower than in the ara-G-untreated aphidicolin-released cells (Fig. 4, □) at 6 and 24 h (P = 0.001).

To determine whether the inhibition of DNA synthesis by ara-G was directly dependent on the percentage of cells in S phase, we analyzed and plotted the data in Figs. 2 and 4 (Fig. 5A). There was a direct linear relationship between ara-GTP-mediated inhibition of DNA synthesis and number of cells in S phase (r = 0.94, P = 0.0001). Similarly, a strong linear relationship (r = 0.92, P = 0.0002) was observed between the number of molecules of ara-GMP incorporated into DNA and the percentage of S-phase cells in the population (Fig. 5B).

Incorporation of ara-GTP into DNA Is Necessary for Induction of Apoptosis. The results presented above indicated that, whereas intracellular ara-GTP accumulation was not cell cycle dependent, the incorporation of ara-GTP into DNA occurred in a cell cycle-specific manner. To test whether incorporation of ara-GTP into DNA was the critical event that triggered apoptosis, we quantitated biochemical (Fig. 6) and morphological (Fig. 7) hallmarks of apoptosis in cells at various times after release from double aphidicolin block prior to and 3 h after incubation with 100 μM ara-G. The formation of high molecular weight DNA fragmentation was chosen as a biochemical end point for apoptosis. High molecular weight DNA fragmentation has been established as an early event in apoptosis (16–18). It has been previously shown that morphological features of apoptosis are not always associated with internucleosomal DNA fragmentation (DNA laddering; Refs. 16–18). In addition, formation of high molecular weight DNA fragmentation has been demonstrated to be a critical event in nucleoside analogue-induced apoptosis in CCRF-CEM cells (19, 20).

Prior to release from double aphidicolin block, the presence of 100 μM ara-G for 3 h did not result in an increase in the formation of ~50-kb fragments (Fig. 6, Pre) or in the number of cells with apoptotic morphology (Fig. 7, Pre). Immediately after release from the double aphidicolin block, there was a rapid increase in the formation of ara-G-induced 50-kb DNA fragments (Fig. 6A, 0). Quantitation of the radioactivity in the gels demonstrated that the formation of 50-kb fragments peaked at 3 h (4.0 ± 0.8), returned to baseline levels by 9 h, and began to rise again as the cells entered the next cell cycle (Fig. 6B). The increase in DNA fragmentation was significant at 3, 6, and 24 h (P = 0.01). In contrast to cells released from aphidicolin block, the cells that remained incubated with aphidicolin despite the presence of ara-G (Fig. 6B, ▽) had basal levels of high molecular weight DNA fragmentation. This level was similar to that seen in cells incubated with aphidicolin alone (Fig. 6B, △). These data suggested that, although cells had accumulated potentially lethal concentrations of ara-GTP (Table 1), the prevention of ara-GTP incorporation into...
DNA by aphidicolin block was sufficient to prevent high molecular weight DNA fragmentation, an early event in apoptosis.

The changes in cellular morphology were consistent with the high molecular weight DNA fragmentation data (Fig. 7). Immediately after release from aphidicolin, the percentage of ara-G-treated cells with condensed chromatin, pyknotic nuclei, and membrane blebbing increased. The percentage of apoptotic cells peaked at 3 h (70 ± 5%), returned to baseline by 9 h, and began to increase again as the cells entered the next cell cycle (Fig. 7). These data clearly demonstrate that the cells most sensitive to the effects of ara-GTP were those actively synthesizing DNA. In contrast, the cells that contained ara-GTP but remained blocked by aphidicolin did not show morphological characteristics of apoptosis.

DISCUSSION

A previous study demonstrated that ara-G permeates T-lymphoblastoid cells via nitrobenzylthioinosine-sensitive and -insensitive equilibrative nucleoside transporters (21). Experience with other pyrimidine analogues has shown that, once it is inside the cell, the rate-limiting step in the accumulation of the cytotoxic analogue triphosphate is the phosphorylation of the nucleoside analogue to its respective monophosphate. Although this is also true for purine analogues such as fludarabine, for other clinically used purine analogues such as cladribine and clofarabine, the analogue monophosphate is the maximally accumulated intracellular species. In any event, for ara-G, this critical first step is catalyzed by cytosolic dCK (1, 13, 22, 23). This enzyme also phosphorylates other analogues such as gemcitabine (24, 25), cytarabine (22, 26), fludarabine (22, 27), and cladribine (23). Unlike these other nucleoside analogues, a second enzyme, mitochondrial dGK, also phosphorylates ara-G (23, 28, 29). Whereas thymidine kinase has been demonstrated to be cell cycle dependent, with maximum activity in S-phase (30), the ara-G-phosphorylating enzyme dCK is not cell cycle regulated (26, 31, 32). Similar work investigating the cell cycle-dependent regulation has not been done for the other ara-G-phosphorylating enzyme, dGK. However, because dGK is a mitochondrial protein (28, 29) and mitochondrial replication is not synchronous with the cell cycle, we do not predict that dGK will be cell cycle related. Therefore, one could postulate that the intracellular accumulation of ara-GTP would be independent of the cell cycle. Our data on ara-GTP accumulation (Table 1) are consistent with this postulate. Our observations are also consistent with the cell cycle-independent accumulation of other arabinosyl analogues, such as cytarabine and fludarabine, which are substrates for these enzymes (32).

An additional manipulation in our experimental setting was the presence of aphidicolin during ara-GTP accumulation. To rule out the possibility that aphidicolin affected the accumulation of ara-GTP, we compared ara-GTP concentrations in cells incubated with and without...
aphidicolin. Whereas aphidicolin reversibly inhibits DNA polymerases (33), blocks DNA synthesis (Fig. 3), and reduces thymidine kinase activity (34), it has no known interactions with either dCK (34) or dGK. As mentioned above, cell cycle perturbation by aphidicolin also did not affect ara-GTP accumulation. Consistent with these observations, our data (Fig. 3 and Table 1) indicate that the presence of aphidicolin did not influence the accumulation of ara-GTP.

This study demonstrated that ara-G exerts its cytotoxic action by inducing apoptosis and that incorporation of ara-G into DNA is required for this action. For example, by inhibiting ara-G incorporation into DNA with aphidicolin, we were able to prevent high molecular weight DNA fragmentation and morphological changes characteristic of apoptosis, despite the presence of intracellular free analogue triphosphate (Figs. 6 and 7). Therefore, the incorporation of ara-GMP into DNA appears to be the critical event in triggering apoptosis. Furthermore, these experiments appear to rule out alternative potentially toxic mechanisms of G-nucleotide action, which do not require DNA incorporation.

Several lines of evidence support this postulate. (a) Aphidicolin alone does not reduce clonogenic survival of CCRF-CEM cells, despite its ability to inhibit DNA synthesis (8). Therefore, it is clear that inhibition of DNA synthesis alone is not cytotoxic to CCRF-CEM cells. (b) When cells were coincubated with aphidicolin and ara-G, DNA synthesis was inhibited, and therefore, no analogue was incorporated into DNA. Under these experimental conditions, neither high molecular weight DNA fragmentation nor apoptotic morphology was observed. These results suggested that presence of toxic intracellular concentration of ara-GTP in the absence of ara-GMP incorporation into DNA was not sufficient to stimulate programmed cell death in CCRF-CEM cells. (c) In marked contrast to the previous two experimental conditions, when the cells were released from aphidicolin double block and then incubated with ara-G, they incorporated ara-GTP (Table 1). S-phase cells were most sensitive to the inhibition of DNA synthesis by ara-G and the induction of apoptosis. In this phase of the cell cycle, the cells actively synthesized DNA (Figs. 2 and 4) and when treated with ara-G, they incorporated ara-GMP during DNA replication (Table 1). This is further substantiated by a strong linear relationship (r = 0.94; Fig. 5) between the percentage of S-phase cells in the population and inhibition of DNA synthesis by ara-G. In addition, there was biochemical and morphological evidence that the maximum induction of apoptosis occurred when there was the maximum number of S-phase cells (Figs. 6 and 7). Taken together, these observations demonstrate that intracellular ara-GTP incorporates into DNA, inhibits DNA synthesis, and induces programmed cell death.

Similar scenarios have been proposed for other nucleoside analogues such as gemcitabine (6), cytarabine (7), fludarabine (8), and cladribine (10). Unlike these analogues, however, it appears that in CCRF-CEM cells the actions of ara-G are directed only to DNA. Fludarabine, for example, has been shown to inhibit RNA synthesis (35), and gemcitabine, fludarabine, and cladribine inhibit other targets, such as ribonucleotide reductase (36). Inhibition of this enzyme perturbs DNA precursor pools (37), which may subsequently inhibit DNA synthesis (6, 8). Even though cytarabine does not inhibit ribonucleotide reductase, other nonnuclear targets, such as the cell membrane, have been suggested for the biological effect of ara-CTP (38). Although we do not rule out the possibility that ara-G or its phosphorylated metabolites interact with nonnuclear targets, our data suggest that, in CCRF-CEM cells, the cytotoxic actions of ara-GTP are induced only after its incorporation into DNA.

Ara-GTP competes effectively with its native nucleotide, dGTP, for incorporation into DNA (39). In a DNA primer extension assay system designed to model in vitro DNA synthesis by using purified DNA polymerase , the values for incorporation into DNA for dGTP and ara-GTP were 0.02 and 0.2 , respectively (40). Although there is a 10-fold difference in values, ara-GTP is a good substrate for incorporation into DNA. In a calf thymus DNA assay system using either dATP ( values for incorporation into DNA) as substrates, the values of ara-GTP were 20 and 0.5 , respectively (39). These results suggest that ara-GTP competes effectively with dGTP for incorporation into DNA. In whole CCRF-CEM cells, the dGTP concentration is ~20 (26), whereas the ara-GTP concentration was ~100 (Table 1). On the basis of the above kinetic values, we would expect efficient incorporation of ara-GTP into DNA and inhibition of DNA synthesis in CCRF-CEM cells, a conclusion that is consistent with our data (Fig. 3 and Table 1). Once incorporated, ara-GTP behaves as a frank chain terminator (40). This was shown using an in vitro DNA primer extension assay and is due to the inability of DNA polymerase to extend a primer with a 3'-terminal ara-GMP. This suggests that, in whole cells, ara-GTP incorporation may result in chain termination and inhibition of DNA synthesis.

Experiments are underway to test this hypothesis by determining whether ara-G incorporation into DNA is internal or terminal in CCRF-CEM cells.

The cytotoxicity to leukemia cell lines and primary leukemia cells during in vitro incubation of other clinically relevant nucleoside analogues, such as cytarabine, has been related to the amount of analogue monophosphate incorporated into DNA (41). An attempt was made to relate cytoreductiveness by formation of ara-C(DNA) during therapy of patients with acute myelogenous leukemia (42), but such a relationship could not be shown because of technical limitations. Our data from a recently published Phase I trial (3) demonstrated that the clinical response to 506U78 (the ara-G prodruk) is directly related to the intracellular accumulation of ara-GTP in circulating leukemia cells (2). To evaluate whether the response to ara-G seen in the Phase I trial is directly related to the ara-GMP accumulation in DNA, it will be necessary to develop the technical capability to quantitate ara-GMP incorporation into the DNA of leukemia cells isolated from patients treated with ara-G prodruk. Presently, this goal has not been achieved; however, in an in vitro leukemia-cell model system, we found there was a direct relationship between the intracellular accumulation of ara-GTP and the amount of ara-GMP incorporated into DNA (43). If a similar relationship exists in circulating leukemia cells during 506U78 therapy, then increased ara-GTP accumulation indirectly predicts incorporation of ara-GMP in DNA.

Phase I investigations of 506U78 in indolent leukemias established the clinical utility of ara-G in B-CLL diseases that were refractory to other purine analogues (44). Because of the quiescent nature of these leukemia cells, other mechanisms of action, different from that proposed in the present study, may exist. Studies are underway to identify factor(s) that are involved in drug-induced apoptosis in these noncycling leukemia lymphocytes.

In conclusion, our data demonstrated that the accumulation of ara-GTP was cell cycle independent and was not affected by aphidicolin. During S phase, cells maximally incorporated ara-GMP molecules into DNA and were sensitive to ara-G-induced apoptosis. Furthermore, despite the presence of ara-GTP, cells in other phases of the cell cycle did not undergo apoptosis. Because our data demonstrated that the incorporation of ara-GTP into DNA is the critical event that mediates cytotoxicity, developing strategies to increase the incorporation of ara-GTP into DNA might increase the clinical utility of this promising new guanine nucleoside analogue.
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REFERENCES

Arabinosylguanine-induced Apoptosis of T-Lymphoblastic Cells: Incorporation into DNA Is a Necessary Step

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