Differential Incorporation of Ara-C, Gemcitabine, and Fludarabine Into Replicating and Repairing DNA in Proliferating Human Leukemia Cells

By Hiromichi Iwasaki, Peng Huang, Michael J. Keating, and William Plunkett

The major actions of nucleoside analogs such as arabinosylcytosine (ara-C) and fludarabine occur after their incorporation into DNA, during either replication or repair synthesis. The metabolic salvage and DNA incorporation of the normal nucleoside, deoxycytidine, is functionally compartmentalized toward repair synthesis in a process regulated by ribonucleotide reductase. The aim of this study was to investigate the metabolic pathways by which nucleoside analogs that do (fludarabine, gemcitabine) or do not (ara-C) affect ribonucleotide reductase are incorporated into DNA in proliferating human leukemia cells. Using alkaline density-gradient centrifugation to separate repaired DNA from replicating DNA and unreplicated parental DNA strands, approximately 60% of ara-C nucleotide in DNA was incorporated by repair synthesis in CCRF-CEM cells; the remainder was incorporated by replication. In contrast, fludarabine and gemcitabine, nucleosides that inhibit ribonucleotide reductase and decreased deoxynucleotide pools, were incorporated mainly within replicating DNA. Hydroxyurea also depleted deoxynucleotide pools and increased the incorporation of ara-C into DNA by replicative synthesis. Stimulation of DNA repair activity by UV irradiation selectively enhanced the incorporation of all nucleosides tested through repair synthesis. These findings suggest that the pathways by which therapeutically useful nucleoside analogs are incorporated into DNA are affected by cellular dNTP pools from de novo synthesis and by the relative activities of DNA repair and replication. The antitumor activity of these drugs may be enhanced by combination with either ribonucleotide reductase inhibitors to increase their incorporation into replicating DNA or with agents that induce DNA damage and evoke the DNA repair process.

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This functional compartmentation of DNA precursors raised a question regarding the metabolic route used by cells to incorporate the triphosphates of therapeutic nucleoside analogs into DNA that are generated by salvage pathways. A preference of the DNA replication machinery for triphosphates arising from the ribonucleotide reductase pathway would seem to limit the use of analogs generated by the salvage route. On the other hand, the nucleotides of analogs such as fludarabine\(^{36,37}\) and gemcitabine\(^{38,39}\) inhibit ribonucleotide reductase as well as DNA polymerization. It is likely that this causes an imbalance in dNTP pools that can shift the deoxynucleotide:analog nucleotide value to one that favors drug incorporation into replicating DNA. Inhibition of ribonucleotide reductase by the analogs is, therefore, able to modulate the route of entry of the analog into DNA. To gain a better understanding of these possibilities, the present study was directed at elucidating the metabolic route of several nucleoside analogs into DNA and at strategies that might be used to enhance their incorporation.

**MATERIALS AND METHODS**

Chemicals and reagents. \([\text{5-H}]\text{dCyd} (20 \text{ Ci/mmole}), [\text{methyl-}\text{3-H}]\text{Thd} (50 \text{ Ci/mmole}), [\text{6-H}]\text{BrdUrd} (20 \text{ Ci/mmole}), \text{and} [\text{5-H]}\text{ara-C} (25 \text{ Ci/mmole}),\) were obtained from ICN Biomedicals Inc (Irvine, CA), [\text{5-H}]\text{2',2'-difluorodeoxycytidine} (dFdC) (18.6 \text{ Ci/mmole}) was kindly supplied by Dr L.W. Hertel (Lilly Research Laboratories, Indianapolis, IN), and [\text{8-}\text{3-H}]-\text{D-arabino furanosyl-2'-fluorodeoxirine} (F-ara-A) (11.8 \text{ Ci/mmole}) was obtained from Moravek Biochemical (Brea, CA). BrdUrd, FdUrd, dFdC, and hydroxyurea were obtained from Sigma Chemical Co (St Louis, MO). Proteinase K and RNase (DNase-free) were purchased from Boehringer Mannheim Co (Indianapolis, IN), and DNA polymerase I was purchased from United States Biochemical Co (Cleveland, OH).

Cell culture and isolation of leukemic lymphocytes from patients. Human T-lymphoblastic cell line CCRF-CEM was obtained from the American Type Culture Collection (Rockville, MD) and maintained in exponential growth phase in suspension culture in RPMI 1640 medium (Life Technologies, Inc, Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Life Technologies, Inc) at 37°C in humidified atmosphere containing 5% CO\(_2\). Cell number and mean volume were determined by a Coulter counter equipped with a model C-1000 particle size analyzer (Coulter Electronics, Hialeah, FL). During exponential growth, the population doubling time was 22 hours. Cell cultures were periodically certified to be free of mycoplasma by the American Type Culture Collection. Leukemic lymphocytes were isolated from whole blood of three previously untreated B-cell chronic lymphocytic leukemia (CLL) patients diagnosed according to the National Cancer Institute criteria for the diagnosis of CLL.\(^{40}\) Freshly obtained peripheral blood (PB) samples were drawn into sterile heparinized tubes and isolated by Ficoll-Hypaque density-gradient centrifugation procedures.\(^{41}\) Cells were washed once with phosphate-buffered saline (PBS) and then suspended in RPMI 1640 medium containing 10% FBS. The cultures were kept at 37°C in humidified atmosphere containing 5% CO\(_2\).

**Measurement of incorporation of \([\text{3-H}]\text{Thd}\) or \([\text{3-H}]\text{BrdUrd}\) into DNA.** To determine the effect of nucleoside analogs on the incorporation of BrdUrd or Thd, CCRF-CEM cells (3 \times 10\(^6\) in 10 mL) in exponential growth phase were incubated for 60 minutes with either \([\text{3-H}]\text{BrdUrd} (0.2 \mu\text{Ci/mL})\) or \([\text{3-H}]\text{Thd} (0.2 \mu\text{Ci/mL})\) in the presence either ara-C, dFdC, or F-ara-A. The radioactivity incorporated into cellular DNA was precipitated with ice-cold 0.4 N HClO\(_3\), filtered onto Whatman 25-mm glass fiber filters, washed with 0.4 N HClO\(_3\), three times and 100% ethanol once, dried, and quantitated in a toluene-based scintillation fluid on a liquid scintillation counter.

**Purification of cellular DNA.** Cellular DNA was purified as described previously.\(^{41}\) Briefly, after the cells were washed with cold PBS, the samples were incubated in digestion buffer (10 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, 25 mmol/L EDTA, 0.5% saponin, and 0.1 mg proteinase K/mL) at 50°C for 12 hours, the cell lysates were extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous phase was transferred to a fresh tube, added to 150 mmol/L sodium acetate, and precipitated with 3 volume of ethanol at −20°C for 12 hours. The DNA pellet was dissolved in H\(_2\)O, digested with RNase (DNase-free, 10 \mu\text{g/mL}) at 37°C for 2 hours, and extracted with an equal volume of phenol/chloroform/isoamyl alcohol. The DNA was precipitated with ethanol again, dissolved in H\(_2\)O, and quantitated by UV spectrometry at 260 nm (1 mg/mL = 20 absorbance units).

**Separation of repaired DNA from replicated and parental DNA.** CCRF-CEM cells were labeled with [\text{5-H}]\text{dCyd} (0.2 \mu\text{Ci/mL}), [\text{3-H}]\text{ara-C} or 0.2 \mu\text{Ci/mL}, or [\text{5-H}]-\text{Nucleoside analogs} (ara-C, dFdC, and F-ara-A) in the presence of 1.0 \mu\text{Ci/mL} BrdUrd and 10 mmol/L FdUrd for 13 hours. The cellular DNA was purified as described above, sheared by eight passes through a 20-gauge needle, which produced DNA fragments with an average width of 15 kb. The purified DNA was then subjected to alkaline CsCl density-gradient centrifugation, which separated DNA into parental DNA (low density), newly replicated DNA (high density), and repaired DNA (intermediate density).\(^{42,43}\) Each centrifugation tube contained a total volume of 10 mL. Alkaline CsCl density-gradient centrifugation was performed with a Beckman Ti75 rotor (Palo Alto, CA) at 37,000 rpm at 25°C for 90 hours. Each gradient was aspirated from the top with a Densiflow IIC apparatus (Buchler Instruments, Inc, Fort Lee, NJ), and 0.2-mL fractions were diluted with H\(_2\)O for measurements of UV absorbance at 260 nm and of radioactivity by liquid scintillation counting.

**Determination of dNTPs.** CCRF-CEM cells incubated with 0.6 mmol/L ara-C, 0.8 mmol/L dFdC, 120 mmol/L F-ara-A, or 0.2 mmol/L hydroxyurea for 13 hours were extracted by 60% methanol. The DNA polymerase assay\(^{44}\) was used to quantitate dNTPs in the cell extracts. The reaction in a mixture contained 100 mmol/L HEPES buffer (pH 7.3), 10 mmol/L MgCl\(_2\), 7.5 \mu\text{g bovine serum albumin, and synthetic oligonucleotides of defined sequences as templates annealed to a primer, [\text{3-H}]\text{dATP} or [\text{3-H}]\text{dTTP, and either standard dNTPs or the extract from 1 \times 10^6 CCRF-CEM cells. After incubation for 1 hour, the samples were applied to filter discs. The discs were then washed with 5% \text{Na}_2\text{HPO}_4 three times and the amount of incorporated normal nucleotide was quantitated by liquid scintillation counting before calculating the pmoles of each deoxynucleotide in the extract.**

**Effects of irradiation on incorporation of [\text{5-H}]\text{dCyd} and triitated nucleoside analogs into DNA.** CCRF-CEM cells (1.5 \times 10^6) were suspended in 3 mL of fresh medium and transferred to 100 \times 20-mm sterile plastic Petri dishes (Falcon; Becton Dickinson Labware, Oxnard, CA) with the lid removed. The dishes were exposed to 10 J/m\(^2\) of UV\(^{22,23}\) light emitted from a mineral light lamp (UVGL-25; UVP Inc, San Gabriel, CA).\(^{22,23}\) Fresh medium with or without drug was then added immediately to the cells to a total of 30 mL, and the samples were incubated in the presence of 1.5 \times 10^{-2} \mu\text{Ci/mL} of [\text{5-H}]\text{ara-C} or 0.2 \mu\text{Ci/mL} of [\text{5-H}]\text{dCyd} for 4 or 13 hours. For comparison, cells irradiated in parallel were incubated with 1.5 \times 10^{-2} \mu\text{Ci/mL} of [\text{3-H}]\text{FdC} or 1.5 \mu\text{Ci/mL} of [\text{3-H}]\text{ara-A} for 13 hours. All culture media contained 1.0 mmol/L BrdUrd and 10 mmol/L FdUrd. The incorporation of [\text{5-H}]\text{dCyd}, [\text{5-H}]\text{ara-C}, [\text{5-H}]\text{dFdC}, or [\text{5-H}]\text{ara-A} into DNA was determined by alkaline CsCl density-gradient centrifugation as described before.

**RESULTS**

Nucleoside analogs were incorporated into DNA through two distinct pathways. To determine the metabolic path-
way(s) responsible for the incorporation of nucleoside analogs into cellular DNA, we first compared the pattern of ara-C incorporation into DNA with that of thymidine and deoxycytidine, two well-characterized precursors of DNA replication and repair synthesis, respectively. The BrdUrd density labeling of cellular DNA and alkaline CsCl density-gradient centrifugation techniques were used to separate the repaired DNA fragments (intermediate density) from the replicated DNA strand (high density) and from the parental DNA strand (low density). As shown in Fig 1A, the parental DNA containing no BrdUrd was banded as a low-density DNA peak detected by UV absorbance at 260 nm (fractions 12 to 30), whereas the replicated DNA containing BrdUrd was banded in the high-density region (fractions 38 to 56). [3 H]dThd was incorporated into the DNA fractions that banded in the high-density region. In contrast, when cells were incubated with [5-3 H]dCyd, most of the radioactivity was incorporated into a peak of intermediate density (fractions 20 to 33); a smaller portion of the [5-3 H]dCyd was incorporated into the high-density DNA (Fig 1B). These results agree with the observation that [3 H]dThd serves predominantly as a precursor for DNA replication, whereas [5-3 H]dCyd is mainly used for DNA repair synthesis. The quantity of [5-3 H]dCyd incorporation into DNA was low relative to dThd. This probably represents a background level of DNA repair activity in CCRF-CEM cells, that may further be stimulated by the action of BrdUrd during the density labeling process.

When CCRF-CEM cells were labeled with [3 H]ara-C and its incorporation into DNA was examined by the techniques described, a unique incorporation profile was observed (Fig 1C). Approximately 60% of the incorporated [3 H]ara-C was located in the fractions of intermediate density; the remaining 40% of the incorporated [3 H]ara-C was detected in the DNA fractions of high density. The latter incorporation represented the incorporation of ara-C by replication synthesis, and possibly by repair synthesis in the newly replicated DNA strand. These results suggest that ara-C was incorporated into DNA by both repair and replication synthesis, with the former being the major pathway. When the incorporation of [3 H]dFdC (gemcitabine) and [3 H]F-ara-A (fludarabine) were evaluated in parallel experiments, these two analogs were found to be mainly incorporated into DNA through replication synthesis (Fig 1D and E). Only a small portion of dFdC or F-ara-A was incorporated into the repairing DNA, which appeared as a small shoulder located at the region corresponding to intermediate density in each gradient. These patterns of incorporation suggest that the biochemical pathways by which different nucleoside analogs are incorporated into DNA vary, probably depending on the biochemical properties of the analogs. The biochemical mechanism responsible for this difference was further investigated as described later.

Because ara-C, dFdC, and F-ara-A are analogs of deoxynucleosides and have an inhibitory effects on DNA synthesis, they may affect the incorporation of BrdUrd into DNA and potentially compromise the density labeling assay. To ensure the validity of our assay system, we examined the effect of various concentrations of the three analogs on the incorporation of [3 H]BrdUrd into DNA, using [3 H]dThd as a reference.
NUCLEOTIDES IN REPAIRING OR REPLICATING DNA

Table 1. Effect of Nucleoside Analogs on the Incorporation of [3H]BrdUrd and [3H]dThd

<table>
<thead>
<tr>
<th>Analog</th>
<th>Dose (nmol/L)</th>
<th>[3H]BrdUrd</th>
<th>[3H]dThd</th>
<th>% of Control (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ara-C</td>
<td>60</td>
<td>27.3 ± 3.7</td>
<td>93.2 ± 7.4</td>
<td>24.3 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>72.7 ± 5.6</td>
<td>104.6 ± 6.8</td>
<td>68.1 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>102.2 ± 3.8</td>
<td>107.7 ± 7.2</td>
<td>102.5 ± 8.7</td>
</tr>
<tr>
<td>dFdC</td>
<td>80</td>
<td>26.0 ± 4.5</td>
<td>55.7 ± 4.6</td>
<td>29.9 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>87.3 ± 3.7</td>
<td>101.6 ± 6.3</td>
<td>95.3 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>107.3 ± 4.9</td>
<td>102.1 ± 8.4</td>
<td>100.3 ± 8.2</td>
</tr>
<tr>
<td>F-ara-A</td>
<td>120</td>
<td>98.6 ± 6.4</td>
<td>103.8 ± 7.2</td>
<td>100.2 ± 8.9</td>
</tr>
</tbody>
</table>

CCRF-CEM cells were treated with nucleoside analogs at various concentrations and simultaneously incubated with 1.0 μmol/L [3H]BrdUrd (0.02 μCi/mL), [3H]dThd (0.2 μCi/mL), or [3H]dThd (0.2 μCi/mL) combined with unlabeled 1.0 μmol/L BrdUrd for 60 minutes. The results are triplicate determinations. Radioactivity was incorporated into DNA in control cells not treated with nucleoside analogs as follows: [3H]BrdUrd, 3,355 ± 156 dpm/106 cells; [3H]dThd, 202,008 ± 22,304 dpm/106; [3H]dThd combined with nonradioactive BrdUrd (1.0 μmol/L), 16,619 ± 789 dpm/106 cells.

a control. As shown in Table 1, low concentrations of analogs (0.6 nmol/L ara-C, 0.8 nmol/L dFdC, or 120 nmol/L F-ara-A) did not affect the incorporation of either BrdUrd or dThd into DNA, whereas higher concentrations of the analogs showed significant inhibitory activity. Thus, these low concentrations of analogs were used in our studies to avoid inhibition of BrdUrd incorporation. The presence of 1.0 μmol/L nonradioactive BrdUrd did not significantly increase the inhibitory effect of the analogs on [3H]dThd incorporation.

The pathways of analog incorporation were affected by cellular dNTP pools derived by de novo synthesis. The observations that ara-C was incorporated into DNA by both repair and replication synthesis and that dFdC and F-ara-A were incorporated mainly through replication led us to investigate the biochemical mechanism responsible for this striking difference. Based on the facts that nucleotides of F-ara-A and dFdC, but not ara-C, inhibit ribonucleotide reductase activity and decrease cellular dNTP pools, we hypothesized that the dNTPs derived from the de novo pathway might affect the distribution of analog incorporation between repaired and replicated DNA fractions. Because dCTP and perhaps other cellular dNTPs are functionally compartmentalized, with the dNTPs from de novo synthesis by ribonucleotide reductase being the precursors of replicating DNA, we reasoned that a decrease of competing normal dNTPs associated with inhibition of ribonucleotide reductase should favor the incorporation of analog nucleotides into the replicating DNA strand. To test this hypothesis, we incubated CCRF-CEM cells with 0.6 nmol/L [3H]ara-C alone or in the presence of 0.2 nmol/L hydroxyurea, an inhibitor of ribonucleotide reductase that decreases dNTP pools in cultured cells, and examined the incorporation patterns of ara-C under these conditions. As illustrated in Fig 2, addition of hydroxyurea substantially increased the incorporation of ara-C into the replicated DNA; the radioactivity in the repair fractions became a minor portion and appeared as a shoulder on the curve similar to the incorporation of [3H]dFdC (Fig 1D) and [3H]F-ara-A (Fig 1E). In separate experiments, addition of unlabeled dFdC mimicked the actions of hydroxyurea by shifting the incorporation of [3H]ara-C from the repairing into the region of replicated DNA (Fig 3). The specific radioactivity of the high-density DNA from cells incubated with [3H]ara-C and either 8 nmol/L or 80 nmol/L dFdC was 1.6-fold and 2.4-fold greater, respectively, than that from cells treated with [3H]ara-C alone. These results suggested that hydroxyurea and dFdC, acting as ribonucleotide reductase inhibitors, could affect ara-C metabolism in cells to potentially enhance the cytotoxic mechanism of ara-C.

Thus, as we expected in the functional de novo pathway of dNTP synthesis mediated by ribonucleotide reductase was a determinant of the metabolic route for analog incorporation into DNA. To provide additional evidence for this notion, the dNTP pools of CCRF-CEM cells were quantified after incubation with ara-C, dFdC, F-ara-A, or hydroxyurea. Consistent with previous observations,36.48 dFdC and F-ara-A each decreased the levels of all four dNTPs, whereas ara-C did not affect cellular dNTP pools32,49-51 (Table 2). Furthermore, treatment with 0.2 mmol/L hydroxyurea also decreased the cellular dCTP pool to a level similar to that seen in cells incubated with dFdC or F-ara-A. Together, these results are consistent with the hypothesis that the cellular concentrations of dNTPs derived from de novo synthesis affect the relative amount of analog incorporation by the replication pathway.

Induction of DNA damage enhanced the incorporation of ara-C through repair synthesis. Because a major portion of ara-C was incorporated into DNA through repair synthesis, we tested the hypothesis that stimulation of DNA repair activity may increase ara-C incorporation into repaired DNA. UV irradiation was used to induce DNA damage and, thus, stimulate repair activity. CCRF-CEM cells were first exposed to UV irradiation (10 J/m2) and then the cells were...
DISCUSSION

The functional compartmentation of dCTP was first suggested by Nicander and Reichard and subsequently shown by Xu et al. Exogenous dCyd can serve as a precursor of dCTP and dTTP through the salvage pathways, whereas dCTP used in DNA replication is derived from the de novo pathway mediated by ribonucleotide reductase. The present study showed that the metabolic pathways that provide the two functionally distinct dCTP pools have important implications for the cellular disposition of nucleoside analogs and for the design of therapeutic strategies.

Using a DNA density labeling technique, it was possible to discern and quantitate the incorporation of nucleotides into either replicating or repairing DNA in a population of proliferating cells. Metabolized by the same salvage pathway enzymes as dCyd, ara-C was incorporated into replicating DNA to a greater extent than into the replicating DNA of proliferating cells. Inhibition of ribonucleotide reductase with either hydroxyurea (Fig 2) or dFdC (Fig 3) and the consequent decrease in cellular dNTP pools (Table 2) shifted ara-C incorporation predominantly into replicating DNA. These experiments lead to the conclusion that when ribonucleotide reductase was functional, the dCTP generated by the de novo pathway competed successfully with ara-CTP for incorporation into replicating DNA to largely exclude the analog. When the de novo supply of dCTP was blocked at

labeled with [3H]ara-C in the presence of BrdUrd. Figure 4 shows representative profiles of radioactivity associated with the DNA fractions separated by alkaline CsCl density-gradient centrifugation. In control cells, peaks corresponding to repairing and replicating DNA were again observed in samples incubated with [3H]ara-C for 4 hours or 13 hours without UV irradiation. UV irradiation (10 J/m²) selectively enhanced the amount of [3H]ara-C incorporation into the intermediate-density peak (fractions 23 to 38) by 36% at 4 hours and 65% after 13 hours. In contrast, incorporation of [3H]ara-C into the high-density region (fractions 39 to 50) was decreased by 47% and 32% at 4 and 13 hours, respectively. This may reflect the inhibition of DNA replication activity by UV light as observed previously.

The quantitative effect of UV irradiation on the specific radioactivity (disintegrations per minute/UV absorbance unit) in DNA from cells incubated with [3H]ara-C and [3H]-dCyd was determined and summarized in Table 3. The specific radioactivity associated with the DNA fractions of intermediate density from [3H]ara-C-labeled and UV-treated cells was significantly greater than that of unirradiated cells. In contrast, UV irradiation selectively decreased the incorporation of [3H]ara-C into the high-density peak fractions. These results are consistent with the role of UV light in causing DNA damage and inhibiting DNA replication. A similar effect of UV light on DNA repair and replication was observed (Table 3) when [3H]dCyd was used instead of [3H]ara-C in a parallel experiment. Thus, stimulation of DNA repair increased the relative amount of each nucleoside incorporation into repairing DNA, as reflected by similar changes in the repair/replication value (Table 3).

Similar experiments were performed to investigate the effect of UV irradiation on the incorporation of [3H]dFdC and [3H]F-ara-A into DNA. As shown in Fig 5, 10 J/m² UV irradiation selectively increased the relative amount of [3H]dFdC and [3H]F-ara-A incorporation into the intermediate-density peak (fractions 20 to 38) by 4-fold and 1.5-fold, respectively. In contrast, the incorporation of [3H]dFdC and [3H]F-ara-A into the high-density peak (fractions 39 to 50) was decreased by 54% and 60%, respectively. These results indicate that the enhancement of analog incorporation into repairing DNA by UV irradiation was not restricted to ara-C. Rather, it appeared to be a general phenomenon for all analogs tested.
Table 2. Effect of Nucleoside Analogs on Deoxynucleotides in CCRF-CEM Cells

<table>
<thead>
<tr>
<th>Agents</th>
<th>dATP (µmol/L)</th>
<th>dCTP (µmol/L)</th>
<th>dGTP (µmol/L)</th>
<th>dTTP (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>78.0 ± 6.8</td>
<td>13.6 ± 1.3</td>
<td>14.8 ± 1.5</td>
<td>58.0 ± 12.3</td>
</tr>
<tr>
<td>ara-C (0.6 nmol/L)</td>
<td>61.6 ± 3.3</td>
<td>11.5 ± 1.8</td>
<td>12.7 ± 5.6</td>
<td>46.4 ± 4.0</td>
</tr>
<tr>
<td>dFdC (0.8 nmol/L)</td>
<td>33.8 ± 5.5*</td>
<td>8.3 ± 0.6*</td>
<td>9.2 ± 1.6*</td>
<td>24.2 ± 6.7*</td>
</tr>
<tr>
<td>F-ara-A (120 nmol/L)</td>
<td>27.1 ± 4.4*</td>
<td>7.3 ± 0.6*</td>
<td>9.7 ± 1.9*</td>
<td>28.1 ± 7.4*</td>
</tr>
<tr>
<td>Hydroxyurea (0.2 mmol/L)</td>
<td>4.7 ± 0.5*</td>
<td>7.6 ± 1.0*</td>
<td>5.7 ± 1.5*</td>
<td>11.4 ± 4.2*</td>
</tr>
</tbody>
</table>

CCRF-CEM cells were incubated for 13 hours with 0.6 nmol/L ara-C, 0.8 nmol/L dFdC, 120 nmol/L F-ara-A, or 0.2 mmol/L hydroxyurea. Intracellular dNTP concentrations were determined as indicated in Materials and Methods.

Values are mean ± SD (n = 3) (*P < .01 by Student’s t-test).

Table 3. Specific Activity of [3H]ara-C or [3H]dCyd in DNA of Growing CCRF-CEM Cells

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>UV (J/m²)</th>
<th>Repair (dpm/UV absorbance unit)</th>
<th>Replication (dpm/UV absorbance unit)</th>
<th>Repair/Replication*</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]ara-C</td>
<td>0</td>
<td>11,159</td>
<td>7,599</td>
<td>1.47</td>
</tr>
<tr>
<td>[3H]ara-C</td>
<td>10</td>
<td>15,014</td>
<td>4,939</td>
<td>3.04</td>
</tr>
<tr>
<td>[3H]dCyd</td>
<td>0</td>
<td>336,847</td>
<td>185,361</td>
<td>1.82</td>
</tr>
<tr>
<td>[3H]dCyd</td>
<td>10</td>
<td>416,356</td>
<td>154,642</td>
<td>2.69</td>
</tr>
</tbody>
</table>

Each experiment was performed with exponentially growing CCRF-CEM cells labeled for 13 hours 1.5 × 10⁻² µCi/mL of [3H]ara-C (0.6 nmol/L) and 0.2 µCi/mL of [3H]dCyd (4 nmol/L) with or without 10 J/m² UV irradiation. The peak of incorporation of [3H]ara-C or [3H]dCyd into DNA was measured using alkaline CsCl density-gradient ultracentrifugation. The intermediate-density peak between fractions 28 and 39 was regarded as repair, and the high-density peak between fractions 40 and 53 as replication. The total dpm was divided by the sum of UV absorption at 260 nm in each set of fractions. DNA-specific activity in fractions located at the repair or replication peak was indicated in this table.

* The ratio of the repair and replication values.

ribonucleotide reductase, the replication complex was able to use ara-CTP more efficiently.

Gemcitabine is metabolized by the same salvage enzymes as ara-C, and the phosphorylation of F-ara-A to the triphosphate differs by only the nucleoside monophosphate kinase. In contrast to ara-C, both dFdC and F-ara-A were incorporated predominantly into replicating DNA (Fig 1). At the concentrations used, nucleotides of each drug inhibited ribonucleotide reductase and caused a decrease in the dNTP pools (Table 2). Thus, these patterns of incorporation are consistent with our hypothesis that dNTPs generated by ribonucleotide reductase are directed into replicating DNA, a function that effectively excludes nucleotide analogs such as ara-C. A corollary is that inhibition of the reductase affords a better opportunity for analog triphosphates generated by the salvage pathway to compete with dNTPs for incorporation. These findings suggest that therapeutic strategies combining ara-C with inhibitors of ribonucleotide reductase will increase incorporation of the analog into replicating DNA.

In fact, such combinations have been evaluated, although their design was based on a rationale of biochemical modulation. The activity of deoxycytidine kinase, the rate limiting step in the accumulation of ara-CTP, appears to be regulated by dNTPs, particularly dCTP. Administration of fludarabine before ara-C to cells in culture, to primary human leukemia cells in vitro or ex vivo, or in therapeutic regimens resulted in decreased cellular dCTP levels. This was associated with a doubling of the rate of ara-CTP accumulation, increased incorporation of ara-C nucleotide into DNA, and greater DNA synthesis inhibition. Presumably, the dual effect of increased ara-CTP levels combined with a decrease of dCTP conferred a competitive advantage for drug incorpo-

![Figure 4. Effects of UV irradiation on [3H]ara-C incorporation into DNA. Exponentially growing CCRF-CEM cells were treated without (○), or with 10 J/m² UV light (●). Then cells were immediately labeled with 0.6 nmol/L [3H]ara-C (1.5 × 10⁻² µCi/mL) in the presence of 1.0 µmol/L BrdUrd and 10 nmol/L FdUrd for 4 hours (A) and 13 hours (B). DNA isolation and CsCl ultracentrifugation were performed as described in Materials and Methods. Fractions were collected for the measurement of radioactivity.](image)
Fractions were collected for the measurement of radioactivity. 

and 10 nmol/L FdUrd for 13 hours. DNA isolation and CsCl ultracentrifugation were performed as described in Materials and Methods. 

\[ [\text{H}] \text{F-ara-A (1.5} \text{Ci/mL}) \] or with 0.8 nmol/L \([\text{3H}]\text{dFdC (1.5} \text{Ci/mL}) \] (A) and 120 nmol/L \([\text{3H}]\text{F-ara-A (1.5} \text{Ci/mL}) \] (B) in the presence of 1.0 \text{mmol/L BrdUrd and 10 nmol/L FdUrd for 13 hours. DNA isolation and CsCl ultracentrifugation were performed as described in Materials and Methods. Fractions were collected for the measurement of radioactivity.}

Fig 5. Effect of UV irradiation on the incorporation of dFdC or F-ara-A. Exponentially growing CCRF-CEM cells were treated without (C) or with 10 J/m\(^2\) UV light (D). Then cells were immediately labeled with 0.8 nmol/L \([\text{3H}]\text{dFdC (1.5} \times 10^3} \text{Ci/mL}) \] (A) and 120 nmol/L \([\text{3H}]\text{F-ara-A (1.5} \times 10^3} \text{Ci/mL}) \] (B) in the presence of 1.0 \text{mmol/L BrdUrd and 10 nmol/L FdUrd for 13 hours. DNA isolation and CsCl ultracentrifugation were performed as described in Materials and Methods. Fractions were collected for the measurement of radioactivity.}

ination. Similar results were obtained in AML blasts during clinical trials that combined ara-C with 2-chlorodeoxyadenosine, another reductase inhibitor. The results of the present studies suggest that these combination strategies direct the analog into replicating DNA, an approach that would be most effective in malignancies with relatively large growth fractions.

This approach, however, would not be expected to change the pattern of incorporation of either dFdC or F-ara-A, which are largely directed into replicating DNA. Nevertheless, it is possible that combination of either of these nucleosides with one another or with drugs such as hydroxyurea, all of which appear to act at different sites on ribonucleotide reductase, might block more completely the function of this enzyme. Such a multifaceted attack on the reductase could increase the amount of analog nucleotide incorporated in replicating DNA and also circumvent possible mechanisms of resistance.

Earlier studies showed that UV irradiation of CCRF-CEM cells caused a decrease in replicating DNA synthesis and an increase in repairing DNA. In quiescent cells, UV irradiation was associated with incorporation of nucleoside analogs into DNA, presumably during the resynthesis step. In the present experiments with proliferating cells, UV irradiation was shown to divert the incorporation of dFdC and F-ara-A from replicating DNA almost entirely to repairing DNA (Fig 5). Also, the portion ara-C incorporated into replicating DNA was blocked by irradiation, and the amount of drug associated with repairing DNA was increased (Fig 4). Because UV irradiation may induce the expression of p53 protein and trigger the G1 checkpoint, it is possible that UV irradiation might affect cell cycle progression and thus alter DNA replication activity in the cells. However, CCRF-CEM cells used in our study contain a mutant p53 gene that is constitutively expressed without blocking cell cycle progression. It is unlikely that the low dose (10 J) UV irradiation would significantly affect the CEM cell cycle distribution through the defective p53.

Because the cytotoxic action of nucleoside analogs was correlated with their incorporation into DNA, the enhanced incorporation of the analogs through the repair pathway by DNA-damaging agents may provide a biochemical basis for designing strategies to increase drug action by combining nucleoside analogs with agents that evoke DNA repair. This approach should be particularly useful for killing cancer cells without significant DNA replication activity such as leukemic lymphocytes in patients with CLL. In fact, we observed that incubation of UV-irradiated CEM cells or UV-irradiated CLL cells with ara-C was associated with greater than additive cell killing (data not shown). Other studies showed that the triphosphates of dFdC and F-ara-A were inhibitory to repair by cell extracts of cisplatin-adducted DNA, presumably after incorporation into the repair patch with the consequent DNA chain termination. Combination of F-ara-A with cisplatin generated synergistic cytotoxicity that was associated with a delay in the removal of cisplatin-induced DNA interstrand cross-links. Together, these results provide a mechanistic basis for the design of therapeutic strategies that combine nucleoside analogs with agents that damage DNA and induce a repair response. Several protocols incorporating these approaches are under evaluation for indolent hematologic malignancies.

The possibility of selectively directing nucleotide analogs into replicating or repairing DNA also raises the likelihood of mechanisms of resistance associated with each strategy. For instance, overexpression of ribonucleotide reductase would be expected to confer resistance to approaches aimed at enhancing drug incorporation into replicating DNA. On the other hand, a particularly active salvage pathway might sustain higher concentrations of generalized dNTP pools available for DNA repair, which could compete with analogs for incorporation. Deoxyxycytidine kinase is the rate-limiting step for the accumulation of many therapeutic nucleosides; this enzyme exhibits a wide spectrum of affinities for various nucleoside analogs, eg, the substrate efficiencies \((V_{max}/K_m)\) for dCyd, ara-C, F-ara-A, and dFdC are 11, 4.2, 0.1, and 19, respectively. It is reasonable to assume that analogs that compete effectively with dCyd for phosphorylation are likely to generate higher triphosphate concentrations; this would increase the chances of the drug being incorporated into DNA.

In conclusion, it appears that deoxynucleotides generated by the de novo pathway mediated by ribonucleotide reduc-
tase are compartmentalized into a low-volume, high-throughput pool that is directed into replicating DNA. Nucleotides, including analogs such as ara-CTP, arising by the salvage pathway have limited access to this route into DNA and are instead favored substrates for DNA repair. Inhibition of ribonucleotide reductase appears to decrease the concentration of dNTPs in the regions of DNA replication, permitting nucleotides from the generalized pool to serve as substrates for replication. Therefore, nucleotides of analogs such as dFdC and F-ara-A that themselves inhibit the reductase are preferred substrates for replicative DNA synthesis. This balance may be disrupted by DNA damage that induces DNA repair, processes once activated that favor incorporation of the analogs. These findings suggest a mechanistic basis for therapeutic strategies that can be applied to malignancies with different growth fractions.

REFERENCES


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Differential Incorporation of Ara-C, Gemcitabine, and Fludarabine Into Replicating and Repairing DNA in Proliferating Human Leukemia Cells

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