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 occurs 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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Following their incorporation into DNA, the nucleotides of arabinosylcytosine (ara-C) and other therapeutically useful nucleoside analogs inhibit further DNA synthesis, a condition that in many cases is strongly correlated with cell death. Such incorporation is generally thought to occur during replicative DNA synthesis by cells in the S phase of the cell cycle. Sustained high cellular concentrations of analog triphosphate relative to that of the competing deoxynucleotide are thought to favor drug incorporation in replicating DNA, initiating the sequelae of leukemia cell death that are associated with therapeutic response. The importance of these events to single-drug, high-dose ara-C therapy was reinforced by studies that showed higher remission induction rates in patients whose disease showed a larger growth fraction. It is a common observation that the extent of cytoreduction of hematologic malignancies by nucleoside analog-containing regimens exceeds the fraction of the malignant population in cycle. Moreover, it has been somewhat surprising that the purine nucleoside analogs used alone have provided effective therapy for indolent disease. This suggests a pathway for incorporation into the DNA of cells that are not in cycle and have not been otherwise induced into DNA repair. The low level of maintenance DNA repair that has been reported to occur in lymphoid cells provides one possible mechanism for such DNA incorporation.

Several investigations have shown the incorporation of nucleotide analogs into the DNA of quiescent cells that had been induced to undergo DNA repair. In these investigations, UV irradiation was used to initiate nucleotide excision repair, the best characterized of the repair mechanisms requiring DNA resynthesis. In these studies, drug incorporation was attributed to the successful competition of the nucleotide analog with the homologous deoxynucleotide during the DNA resynthesis step. The observation of synergistic cell killing of quiescent cells induced to undergo DNA repair suggested that this might form the mechanistic basis for combination chemotherapies in indolent diseases. Clinical trials evaluating nucleoside analogs in combination with DNA damaging drugs have shown effectiveness against low growth fraction tumors such as chronic lymphocytic leukemia and low-grade non-Hodgkin’s lymphoma. Recent studies have shown two functionally distinct metabolic pathways that supply the cell with deoxynucleotides for DNA replication and repair. In studies using proliferating cells, the de novo pathway was shown to direct ribonucleotides, converted to deoxynucleotides by ribonucleotide reductase, through a small volume, high-through-put pool into replicating DNA. This pathway contributed less than 5% of the dCTP incorporated into repairing DNA. In contrast, the salvage pathway initiated by deoxycytidine kinase accounted for the majority of deoxycytidine nucleotide in repairing DNA, while only a small portion of the dCTP derived from this pool served as a substrate for replication. These findings generated a model characterized by one dNTP pool being derived from the de novo pathway that was directed to replicating DNA, presumably at focal sites associated with the nuclear envelope. Only a small fraction of unused dNTPs that escaped the channeling mechanism was seen as being available for repair synthesis. A second more generalized pool at large in the cell, derived from salvage synthesis, was thought to be available for use in the more global function of DNA repair. However, the mechanisms channeling the de novo pool appeared to effectively exclude salvaged nucleotides from replicating DNA synthesis caused by a high concentration gradient of dNTPs derived from de novo synthesis at the replication sites.

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NUCLEOTIDES IN REPAIRING OR REPLICATING DNA

This functional compartmentation of DNA precursor pools 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, gemcitabine, and cladribine 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. [5-3H]dCyd (20 Ci/mmol), [methyl-3H]nucleoside analogs (ara-C, dFdC, and F-ara-A) (11.8 Ci/mmol) was obtained from Moravek Biochemical (Brea, CA). BrdUrd, FdUrd, dThd, 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% CO2. 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 centrifuged 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. Freshly obtained peripheral blood (PB) samples were drawn into sterile heparinized tubes and isolated by Ficoll-Hypaque density-gradient centrifugation procedures. 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% CO2.

Measurement of incorporation of [1H]dThd or [3H]BrdUrd into DNA. To determine the effect of nucleoside analogs on the incorporation of BrdUrd or dThd, CCRF-CEM cells (3 x 10^6 in 10 mL) in exponential growth phase were incubated for 60 minutes with either [1H]BrdUrd (0.2 uCi/mL) or [3H]dThd (0.2 uCi/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 HClO4, filtered onto Whatman 25-mm glass fiber filters, washed with 0.4 N HClO4 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. 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% sodium dodecyl sulfate, 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 H2O, digested with RNase (DNase-free, 10 μg/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 H2O, 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 [5-3H]dCyd (0.2 μCi/mL), [3H]dThd (0.2 μCi/mL), or [3H]nucleoside analogs (ara-C, dFdC, and F-ara-A) in the presence of 1.0 μmol/L BrdUrd and 10 mmol/L FdUrd for 13 hours. The cellular DNA was purified as described above, sheared by eight passages through a 20-gauge needle, which produced DNA fragments with an average length 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). 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 H2O 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 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 MgCl2, 7.5 μg bovine serum albumin, and synthetic oligonucleotides of defined sequences as templates annealed to a primer, [3H]dATP or [3H]dUTP, and either standard dNTPs or the extract from 1 x 106 CCRF-CEM cells. After incubation for 1 hour, the samples were applied to filter discs. The discs were then washed with 5% NaHPO4 three times and the amount of incorporated normal nucleotide was quantitated by liquid scintillation counting before calculating the pmols of each deoxynucleotide in the extract.

Effects of irradiation on incorporation of [5-3H]dCyd and tritiated nucleoside analogs into DNA. CCRF-CEM cells (1.5 x 10^6) were suspended in 3 mL of fresh medium and transferred to 100 x 20-mm sterile plastic Petri dishes (Falcon; Becton Dickinson Labware, Oxnard, CA) with the lid removed. The dishes were exposed to 10 J/m2 of UV light emitted from a mineral light lamp (UVG-125; UVP Inc, San Gabriel, CA). 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 x 10^-7 μCi/mL of [1H]ara-C or 0.2 μCi/mL of [5-3H]dCyd for 4 or 13 hours. For comparison, cells irradiated in parallel were incubated with 1.5 x 10^-7 μCi/mL of [3H]dFdC or 1.5 μCi/mL of [1H]ara-A for 13 hours. All culture media contained 1.0 μmol/L BrdUrd and 10 mmol/L FdUrd. The incorporation of [5-3H]dCyd, [1H]ara-C, [1H]dFdC, or [1H]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). [3H]dThd was incorporated into the DNA fractions that banded in the high-density region. In contrast, when cells were incubated with [5-3H]dCyd, most of the radioactivity was incorporated into a peak of intermediate density (fractions 20 to 33); a smaller portion of the [5-3H]dCyd was incorporated into the high-density DNA (Fig 1B). These results agree with the observation that [3H]dThd serves predominantly as a precursor for DNA replication, whereas [5-3H]dCyd is mainly used for DNA repair synthesis. The quantity of [5-3H]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 [3H]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 [3H]ara-C was located in the fractions of intermediate density; the remaining 40% of the incorporated [3H]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 [3H]dFdC (gemcitabine) and [3H]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 [3H]BrdUrd into DNA, using [3H]dThd as...
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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>[3H]dThd – Cold BrdUrd</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>82.4 ± 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>

Exponentially growing CCRF-CEM cells were treated without (●) or with 0.2 mmol/L hydroxyurea (○) 15 minutes before adding 0.6 mmol/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 13 hours as described in Fig 1. DNA isolation and CsCl ultracentrifugation were performed as described in Materials and Methods. Fractions of 0.2 mL were collected and diluted for the measurement of radioactivity.

Fig 2. Effect of hydroxyurea on [3H]ara-C incorporation into DNA. Exponentially growing CCRF-CEM cells were treated without (●) or with 0.2 mmol/L hydroxyurea (○) 15 minutes before adding 0.6 mmol/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 13 hours as described in Fig 1. DNA isolation and CsCl ultracentrifugation were performed as described in Materials and Methods. Fractions of 0.2 mL were collected and diluted for the measurement of radioactivity.

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 inhibit ribonucleotide reductase activity and decrease cellular dNTP pools, we hypothesized that the dNTPs derived from de novo synthesis 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 replication, 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 mmol/L [3H]ara-C alone or in the presence of 0.2 mmol/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 mmol/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, it appeared that the functioning of the 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, dFdC and F-ara-A each decreased the levels of all four dNTPs, whereas ara-C did not affect cellular dNTP pools (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/m^2) 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 repairing 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

creased 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]dFdC 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.

Fig 3. Changes of [3H]ara-C incorporation into DNA by dFdC. Exponentially growing CCRF-CEM cells were treated without (A), or with 8 nmol/L (B) and 80 nmol/L (C) nonradioactive dFdC, simultaneously

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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 de-

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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 nmol/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).

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

Gemcitabine is metabolized by the same salvage enzymes as ara-C,52 and the phosphorylation of F-ara-A to the triphosphate differs by only the nucleoside monophosphate kinase.7 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.53,54 Administration of 5-fludarabine before ara-C to cells in culture,55 to primary human leukemia cells in vitro or ex vivo,56 or in therapeutic regimens57 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.58 Presumably, the dual effect of increased ara-CTP levels combined with a decrease of dCTP conferred a competitive advantage for drug incorpo-

![Fig 4. Effects of UV irradiation on [3H]ara-C incorporation into DNA. Exponentially growing CCRF-CEM cells were treated without (C), 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)

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>DNA-Specific Activity (dpm/UV absorbance unit)</th>
<th>UV (J/m²)</th>
<th>Repair</th>
<th>Replication</th>
<th>Repair/Replication*</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]ara-C</td>
<td>11,159</td>
<td>0</td>
<td>7,599</td>
<td>1.47</td>
<td></td>
</tr>
<tr>
<td>[3H]ara-C</td>
<td>15,014</td>
<td>10</td>
<td>4,939</td>
<td>3.04</td>
<td></td>
</tr>
<tr>
<td>[3H]dCyd</td>
<td>336,847</td>
<td>0</td>
<td>185,361</td>
<td>1.82</td>
<td></td>
</tr>
<tr>
<td>[3H]dCyd</td>
<td>416,356</td>
<td>10</td>
<td>154,642</td>
<td>2.69</td>
<td></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.
Clinical trials that combined ara-C with 2-chlorodeoxyadenosine (2-CDA) demonstrated that ara-C incorporation into DNA was significantly inhibited by 2-CDA. 

**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/m² UV light (●). Then cells were immediately labeled with 0.8 nmol/L [3H]dFdC (1.5 × 10⁻³ μCi/mL) (A) and 120 nmol/L [3H]F-ara-A (1.5 μCi/mL) (B) in the presence of 1.0 μmol/L BrdUrd and 10 nmol/LFdUrd for 13 hours. DNA isolation and CsCl ultracentrifugation were performed as described in Materials and Methods. Fractions were collected for the measurement of radioactivity.

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 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.

In conclusion, it appears that deoxynucleotides generated 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.
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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