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Action of 2',2'-Difluoro-deoxycytidine on DNA Synthesis

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ABSTRACT

The action of the new deoxycytidine analogue 2',2'-difluorodeoxycytidine (dFdC) on DNA synthesis was investigated in whole cells and in vitro assay systems with purified DNA polymerases. DNA synthesis in human lymphoblastoid CEM cells was inhibited by dFdC in a concentration-dependent manner that could not be reversed by exogenous deoxyribonucleosides. The analogue was incorporated into cellular DNA; most of the incorporated dFdC 5'-monophosphate (dFdCMP) residues were in internucleotide linkage. In vitro DNA primer extension assays demonstrated that dFdC 5'-triphosphate (dFdCTP) competed with deoxycytidine triphosphate for incorporation into the C sites of the growing DNA strand. The ratios of the apparent $K_v$ values for the incorporation of dFdCTP and dCTP into a C site of M13mp19 DNA were 21.8 and 22.9 for DNA polymerases $\alpha$ and $\epsilon$, respectively. The apparent $K_v$ values of dFdCTP were 11.2 $\mu$M for DNA polymerase $\alpha$ and 14.4 $\mu$M for polymerase $\epsilon$. After dFdCMP incorporation, the primer was extended by one deoxynucleotide before a major pause in the polymerization process was observed. This was in contrast to the action of arabinosylcytosine 5'-triphosphate, which caused both DNA polymerases $\alpha$ and $\epsilon$ to pause at the site of incorporation. The 3'- to 5' exo-nuclease activity of DNA polymerase $\epsilon$ was essentially unable to excise nucleotides from DNA containing dFdCMP at either the 3'-end or an internal position, whereas arabinosylcytosine monophosphate was removed from the 3'-terminus at 37% the rate for deoxynucleotides. The cytotoxic activity of dFdC was strongly correlated with the amount of dFdCMP incorporated into cellular DNA. Our results demonstrate qualitative and quantitative differences in the molecular actions of dFdC and arabinosylcytosine on DNA metabolism, but are consistent with an important role for such incorporation in the toxicity of dFdC.

INTRODUCTION

dFdC (Gemcitabine) is a new analogue of deoxycytidine with geminal fluorine atoms at the 2'-carbon of the sugar moiety (1). This compound has potent cytotoxic activity against Chinese hamster ovary cells in cultures (2) and human leukemia cell lines (3, 4). Studies with animal tumor models have demonstrated that dFdC is active in vivo against a wide spectrum of murine solid tumors and human tumor xenografts (5, 6). Clinical studies show that this compound induced therapeutic responses in patients with adenocarcinomas of the colon and lung (7, 8). These biological activities were in contrast with those of ara-C, which is effective in hematological malignancies, particularly acute leukemia (9), but is essentially inactive against solid tumors.

The mechanisms responsible for the different antitumor activities of dFdC and ara-C have not been elucidated. Recent studies demonstrated that the cytotoxic action of dFdC, like ara-C, requires intracellular phosphorylation by deoxycytidine kinase (2, 10). The half-life of intracellular dFdCTP, however, is much longer than that of ara-CTP both in vitro (2, 3) and in vivo (11). Furthermore, dFdC, but not ara-C, induces a significant decrease in cellular dNTP pools, presumably by inhibiting ribonucleotide reductase (3, 4). The differences in the cellular metabolism of dFdC and ara-C may in part explain their different antitumor activities.

Inhibition of DNA synthesis is the most prominent activity of dFdC observed in cultured cells (2). It is likely that inhibition of ribonucleotide reductase and perturbation of deoxynucleotide pools contribute to this action (3, 4). Although inhibition of DNA synthesis has been strongly correlated with intracellular dFdCTP concentrations (2), little is known about the molecular action of dFdCTP on DNA replication. Therefore, we investigated the incorporation of dFdCMP into DNA and its action on DNA strand elongation. The action of dFdC on DNA metabolism was compared with that of ara-C to gain a better understanding of the biochemical basis for the different activities of these drugs. A preliminary report of this work has been published (12).

MATERIALS AND METHODS

Chemicals. dFdC and dFdCTP were synthesized as described previously (1, 13). [5-3H]dFdC was prepared by Amersham International, Inc. (Arlington Heights, IL). ara-C was purchased from Sigma Chemical Co. (St. Louis, MO). The 17-base M13 sequencing primer (5' GTAAAACGACGCTGGCAGT 3'), M13mp18(+) DNA, T4 polynucleotide kinase, and HPLC-purified dATP, dCTP, dGTP, and dTTP were obtained from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). M13mp19(+) DNA and Klenow enzyme were purchased from Bethesda Research Laboratories (Gaithersburg, MD). [methyl-3H]Thymidine, [5-3H]Juridine, and [y-32P]ATP were obtained from ICN Radiochemicals, Inc. (Irvine, CA). DNA polymerases $\alpha$ and $\epsilon$ were purified from human T-lymphoblastoid cells and characterized as previously described (14). The specific activities of the pol $\alpha$ and pol $\epsilon$ were 11,936 and 2,440 units/mg, respectively. One unit is the amount of enzyme required to catalyze the incorporation of 1 nmol of dTTP into acid-insoluble material in 1 h at 37°C.

Cell Culture. Human T-lymphoblastoid CEM-CEM cells were maintained in exponential growth in RPMI 1640 suspension culture medium supplemented with 5% fetal bovine serum. The cellular clonogenicity assays were carried out as previously described (14).

Determination of DNA Synthesis Activity in Whole Cells. CEM cells in exponential growth phase were incubated with various concentrations of dFdC for a desired period and labeled with [3H]thymidine for 30 min to determine DNA synthesis activity (14).

Determination of dFdCMP Incorporated in DNA. Cells were incubated with various concentrations of [3H]dFdC for 4 h and washed twice with cold phosphate-buffered saline, and 106 cells/ml were lysed in 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.5% sodium dodecyl sulfate, and Pronase (1.25 mg/ml). The cell lysate was extracted with a phenol mixture (50 g of phenol:7 ml of m-cresol:80 mg of 8-hydroxyquinoline:5.5 ml of H2O), and the nucleic acids were precipitated with 2 volumes of ethanol. Each pellet was dissolved in 5 mM EDTA, and formamide was added to 50% (v/v) for a final volume of 1 ml. The
solution was heated to 80°C for 5 min, brought to 4.5 ml with 5 mM EDTA, mixed with 4.5 ml of saturated Cs2SO4, and centrifuged in a Ti75 rotor, first at 50,000 rpm for 16 h and then at 40,000 rpm for 1 h. The resulting gradients were aspirated from the top with a DensiFlow IIIC apparatus (Buchler) equipped in series with an absorbance monitor to locate the DNA and RNA bands. Fractions of 0.5 ml were collected, the specific gravities of each were determined, and after extensive dialysis the radioactivity associated with DNA and RNA peaks was determined by liquid scintillation counting. To determine the positions of the dFdCMP incorporated in the DNA strand, DNA isolated from cells labeled with [3H]dFdC and [3H]thymidine was degraded to its internal nucleoside-3' monophosphates and 3' terminal nucleotides by sequential digestion with micrococcal nuclease and spleen phosphodiesterase (14). The digests were separated by reverse phase HPLC, and the radioactivity associated with the resulting 3'-monophosphates and nucleotides was quantitated by liquid scintillation counting.

DNA Primer Extension Assay. The 17-base M13 sequencing primer was labeled with 32P at its 5'-end and annealed to its complementary site on either M13mp19(+) or M13mp18(+) DNA template. The DNA primer extension assay and sequence analysis were carried out as described previously (14). The reaction mixture (10 μl) contained 20 μg of bovine serum albumin/ml, 2.1 mM 32P-labeled primer/template (5 μg/ml), 0.15 unit of pol α or pol ε, and various concentrations of dNTP and dFdCTP or ara-CTP as indicated in the figure legends. The reactions were incubated at 37°C for 30 min and analyzed by 10% polyacrylamide sequencing gel. The radioactivity of each DNA band in the sequencing gel was quantitated with a Betascope 603 blot analyzer (Betergan Corporation, Waltham, MA). To determine the kinetic parameters of dFdCTP incorporation, the primed M13mp18(+) DNA,

(5') 32P-GTAAAAAGACCGGCCAGT123site
(3') . . . CATTITGGCTGCCGTCAGGCAGTACGGA . . .

where the numbers indicate the number of nucleotide sites from the 3'-end of the primer, was used as the template for DNA synthesis in the presence of 100 μM dGTP and various concentrations of dFdCTP. The relative velocity of incorporation was determined by dividing the radioactivity in the target site (Site 2, Nucleotide 6284) by the radioactivity in the Band one nucleotide shorter (Site 1) (15, 16). The apparent Km and Vmax values were then calculated based on the Michaelis-Menten equation, using a computer-assisted program (17).

The dFdCUMP or ara-CMP from DNA. Oligomers with either dFdCMP or ara-CMP incorporated at the 3'-terminus or with dFdCTP at one position penultimate to the 3'-terminus were annealed to the complementary M13 DNA strand and used as the substrate for excision by polymerase θ. The 3'-dFdCMP or ara-CMP-terminated oligomer was constructed by incubating the 32P-labeled 17-base primer/M13mp18 DNA hybrid with 100 μM dGTP, 30 μM of either dFdCTP or ara-CTP, and pol α at 37°C for 30 min. The dGTP molecule was incorporated into Site 1, which was further extended by incorporation of dFdCTP or ara-CTP into Site 2. The reaction products were separated by 15% polyacrylamide sequencing gel. The 19-base oligomers containing either dFdCMP or ara-CMP at their 3'-ends were recovered from the gel and annealed to the M13mp18(+) DNA as previously described (14). Likewise, oligomer with dFdCMP incorporated at the penultimate position was prepared by incubating the 32P-labeled 17-base primer/M13mp19 DNA hybrid

(5') 32P-GTAAAAAGACCGGCCAGT1234567site
(3') . . . CATTITGGCTGCCGTCAGGCAGTACGGA . . .

with 100 μM each of dATP, dGTP, and dTTP, 30 μM dFdCTP, and 0.15 units of pol α/10-μl reaction at 37°C for 30 min. A dFdCMP molecule and a dGMP molecule were incorporated into Sites 6 and 7, respectively. This incorporation resulted in a pause of the polymerization at Site 7. After separation of the reaction products in a DNA sequencing gel, the 24-base oligomer containing dFdCMP at its 3'-penultimate position was recovered and annealed to the M13mp19(+) DNA. The 17-base primer was also isolated from the gel and hybridized to the M13 DNA template. These DNA hybrids were used as the substrates of the polymerase θ 3'-&gt;5' excision activity as previously described (14).

RESULTS

Effect on DNA and RNA Synthesis. The action of dFdC on the metabolism of nucleic acids was evaluated first in whole CEM cells by measuring the incorporation of [3H]thymidine into DNA and [3H]uridine into RNA. As shown in Fig. 1, when CEM cells were incubated with various concentrations of dFdC for 2 h, DNA synthesis was inhibited in a concentration-dependent manner. About 15 nm dFdC and 100 nm dFdC were required to reduce DNA synthesis to 50% and 10% of control activity, respectively. In contrast, a 2-h incubation with dFdC did not inhibit the RNA synthesis activity, as determined by [3H]uridine incorporation (Fig. 1).

The diphosphate of dFdC has been shown to inhibit ribonucleotide reductase; incubation of CEM cells with 0.1 μM dFdC reduced dCTP to 20% of controls and dATP and dGTP to 60% of controls by 2 h (4). To evaluate the impact of this action on DNA synthesis, cells were incubated first with 0.10 μM dFdC for 2 h and then with 0.1 to 30 μM deoxycytidine for 20 min to replete dCTP. Incubation with 0.3 μM deoxycytidine fully restored cellular dCTP to the control level but did not enable the cells to synthesize DNA at the normal rate. No significant recovery of DNA synthesis was observed when cells were treated with higher concentrations (up to 30 μM) of deoxycytidine. In a parallel experiment, when dCyd, deoxyadenosine, deoxyguanosine, and thymidine were added simultaneously over the same concentration range, the inhibitory action of dFdC on DNA synthesis was not significantly reversed. For example, addition of 30 μM of each natural deoxynucleoside to cells treated with dFdC restored DNA synthesis activity to only 15% of the control (data not shown).

Incorporation of dFdCMP into DNA. Several experimental systems were used to further investigate the mechanisms underlying the inhibitory action of dFdC on DNA synthesis. First, CEM cells were incubated with 1 μM [3H]dFdC for 4 h and lysed, and the cellular DNA was separated from RNA by banding on a Cs2SO4 gradient. Fig. 2A shows the UV absorbance profile at 254 nm of such a density gradient separation.
DNA is lower in density (specific gravity, 1.55 g/ml) than RNA and was located in Fractions 6 to 10, whereas RNA (specific gravity, 1.71 g/ml) was detected in Fractions 14 to 16. Fig. 2B illustrates that most of the incorporated radioactivity was associated with the DNA fraction. The DNA in Fractions 6 to 10 was dialyzed, precipitated with ethanol, redissolved, and degraded to nucleosides by digestion with P1 nuclease and alkaline phosphatase. HPLC analysis demonstrated that greater than 90% of the radioactivity coeluted with authentic dFdC. Radioactivity associated with RNA was below the limit of detection, indicating that less than 0.0042 pmol of dFdCMP/μg of RNA had been incorporated during the incubation.

Both cellular dFdCMP and the amount of its incorporation into DNA increased in a concentration-dependent manner when 0.001 to 10 μM dFdC was added to the cell culture (Fig. 3). Although cellular dFdCAMP accumulation rates appeared to be saturated at 1 μM, incorporation of the analogue into DNA increased in proportion to the concentrations of exogenous dFdC. No evidence of saturation was observed at dFdC concentrations up to 10 μM. The location of the incorporated dFdCMP in DNA was determined using DNA isolated from cells incubated with [3H]dFdC and [14C]thymidine. After digestion with micrococcal nuclease and spleen phosphodiesterase, the products were separated by HPLC, and the radioactivity associated with the respective internal nucleotides or 3'-terminal nucleotides was quantitated by liquid scintillation counting. Less than 10% of the incorporated dFdCMP was located at terminal positions. About 93% of dFdCMP was incorporated at internal positions. A longer incubation period (24 h) did not significantly alter the terminal/interal incorporation ratio. The incorporation ratio of dFdC was similar to that of thymidine, which served as a control. The observation that greater than 90% of the dFdCMP was incorporated at internal positions suggests that the incorporated dFdCMP residues can be extended by DNA polymerases.

**Action of dFdC on DNA Strand Elongation in Vitro.** A DNA primer extension system was used to further characterize the action of dFdC and to compare it with that of ara-CTP on DNA synthesis. A 17-base M13 sequencing primer was labeled with 32P at the 5'-end and annealed to the complementary site of the M13 DNA. The ability of pol α and pol ε purified from CEM cells to extend the primer was then evaluated in the presence and absence of dFdCTP or ara-CTP. Fig. 4 compares the incorporation of the analogues into the extending DNA primer and their effect on DNA strand elongation by pol α and pol ε. Site 0 indicates the position of the 17-base primer. Lane 1 was the reaction with pol α plus 30 μM each of dATP, dGTP, and dTTP without either dCTP or dFdCTP. The intense band at Site 5 indicates that most of the extending primer stopped before the first C site at Site 6, since no dCTP was present in the reaction. The faint band at the Site 6 reflects that a small amount of mismatched dNTP was misincorporated into the first C site. When various concentrations of dFdC were included in the reaction without dCTP (Lanes 2 to 5), the density of the intense band at Site 5 decreased in inverse proportion to the dFdCTP concentration, indicating that dFdC can serve as an alternative substrate for incorporation into the C site (Site 6); thus, less primer accumulated at Site 5. Quantitation of the radioactivity with a Betascope 603 blot analyzer demonstrated that, during the 60-min counting period, there were 1485 counts in the band at Site 5 of Lane 4, which contained 30 μM each of dFdC, dATP, dGTP, and dTTP, and 786 counts at the same site of Lane 10, which contained 30 μM each of dCTP, dATP, dGTP, and dTTP (control). The data suggest that pol α used dFdC less efficiently than did dCTP as substrate for incorporation, because more primer accumulated at Site 5, waiting for further extension in the presence of 30 μM dFdCTP.

In Fig. 4, Lanes 2 to 5, there were only a faint band at the first C site (Site 6) but a substantial amount of longer oligomers beyond this site. This result indicates that the incorporated dFdCMP residue could be further extended by addition of dGTP at Site 7. This is in agreement with the finding that greater than 90% of the incorporated dFdCMP residues were located at the internal position of cellular DNA strands. However, the incorporated dFdCMP at Site 6 caused a pause band at Site 7. This is of particular interest because it indicates that pol α was able to extend the incorporated dFdCMP by one nucleotide (dGMP at Site 7) but then had difficulty extending it further. This pause in polymerization was not permanent; the DNA primer was extended beyond this site as indicated by the bands of greater molecular weight DNA. Similar behavior was...
observed when the enzyme encountered the second C site (Site 10). The enzyme paused at Site 9 before dFdCMP was incorporated into the second C site. The incorporated dFdCMP was extended further by one nucleotide (dTTP at Site 11), but again there was a pause band at the site 1 nucleotide after the second C site (Fig. 4, Lanes 2 to 5, Site 11).

In contrast, when ara-CTP was incorporated into the first and second C sites, it strongly impaired the polymerization process at the sites of its incorporation, as demonstrated by the intense bands at Sites 6 and 10 (Fig. 4, Lanes 6 to 9). The enzyme was unable to efficiently extend the primer with an incorporated ara-CTP at its 3'-end, although the α-OH at 3'-carbon of ara-CTP allowed further extension beyond the first C site. The pause band at Site 9 (the second pre-C site) but not at Site 5 (the first pre-C site) indicates that the efficiency of incorporation was affected by the sequence of the DNA template. Furthermore, no pause band was observed at the post-C sites (Sites 7 and 11). These results demonstrate that dFdCTP and ara-CTP inhibit DNA synthesis by different mechanisms of action at the molecular level.

The incorporation of dFdCMP into C sites, the appearance of pause bands at the sites 1 nucleotide after sites of dFdCMP incorporation, and termination of primer extension at sites where ara-CTP was incorporated were also observed when pol ε was used to catalyze the reaction (Fig. 4, Lanes 11 to 20).

Less misincorporation was observed in the C reaction with pol ε (Lane 11).

The interaction between dFdCTP and dCTP could be visualized using DNA sequencing gel analysis of the M13 primer extension reactions. When various concentrations of dCTP were added to the reaction, dCTP competed with either dFdCTP or ara-CTP for incorporation into the C sites to support the synthesis of longer DNA strands by pol α (Fig. 5A) and pol ε (Fig. 5B). In reactions that included dFdCTP (Lanes 1 to 7), the pause bands caused by the incorporated dFdCMP at both the pre-C and post-C sites diminished with increasing concentrations of dCTP, consistent with competition between dCTP and dFdCTP. dCTP, 10 μM, was able to effectively compete with 100 μM dFdCTP to support the synthesis of full-length DNA strands (Fig. 5, Lane 5). In the case of ara-CTP, 100 μM dCTP was required to achieve a similar level of competition with 100 μM ara-CTP (Lane 13).

Kinetic Parameters of dFdCMP Incorporation. A filter disk assay was used to kinetically characterize the competition of dFdCTP and dCTP. Double reciprocal (Lineweaver-Burk) plots that intersected at the y-coordinate (Fig. 6) further confirmed that dFdCTP was a competitive inhibitor of pol α and pol ε with respect to dCTP. Using a computer-assisted program based on the Michaelis-Menten kinetics (17), the apparent Kₐ values of 11.2 ± 2.0 μM and 14.4 ± 0.5 μM were calculated for pol α and pol ε, respectively (Table 1).

To quantitatively evaluate the kinetics of dFdCTP incorporation, the primed M13mp18(+) DNA was used as the template for DNA synthesis by both pol α (Fig. 7A) and pol ε (Fig. 7B) with 100 μM dGTP and various concentrations of dFdCTP. Pol α was able to incorporate dGMP into Site 1, and a small amount of dGMP was misincorporated into Site 2 (Fig. 7A, Lane 1). When 1 to 100 μM dFdCTP was added to the reaction, pol α incorporated the analogue into Site 2, pairing with dGMP in the opposite strand (Fig. 7A, Lanes 2 to 8). This incorporation was concentration dependent. It is of interest to note that pol α was unable to efficiently extend the 3'-dFdCMP-containing primer with a second dFdCMP molecule; only a faint band appeared at Site 3 at higher dFdCTP concentrations (Lanes 6 to 8). Thus, it was difficult for the enzyme to incorporate 2 consecutive dFdCMP molecules. Similar behavior was observed in the reaction with pol ε (Fig. 7B). Less dFdCMP was incorporated into the second C site (compare Lanes 6 to 8 of Fig. 7, A and B). Again, pol ε showed little misincorporation of dGMP into the first C site (Fig. 7B, Lane 1).

The relative velocity of incorporation was determined by dividing the radioactivity in each Site 2 band by the radioactivity in the Site 1 band as previously described (14, 15), and the apparent Km and Vmax values for dFdCTP incorporation were calculated as described in “Materials and Methods.” In a parallel experiment, dCTP was used instead of dFdCTP under identical experimental conditions, and the reaction products were analyzed on a sequencing gel (figure not shown). The apparent kinetic parameters are compared in Table 1. dFdCTP was incorporated into DNA by pol α and pol ε with the apparent Km values of 26.7 μM and 45.8 μM, respectively. The substrate efficiency, as indicated by the apparent Vmax/Km values, was 0.17 for pol α and 0.1 for pol ε. When dCTP was used as the substrate, these values were 6.8 and 3.5 for pol α and pol ε, respectively. Thus, dFdCTP was a much poorer substrate than dCTP for both enzymes.

Excision of the Incorporated dFdCMP from DNA. The excision of incorporated dFdCMP from DNA was evaluated in two
Fig. 5. Competition between dCTP and dFdCTP or ara-CTP for incorporation into DNA catalyzed by pol α (A) and pol ε (B). A DNA primer extension assay was carried out in the presence of ±P-primer/template (5 μg/ml), 0.15 unit of enzyme, 30 μM each of dATP, dGTP, dTTP, and various concentrations of dCTP, dFdCTP, and ara-CTP as indicated below. Lane 1, C reaction (30 μM each of dATP, dGTP, and dTTP); Lanes 2 to 7, C reactions plus 100 μM dFdCTP and 0, 1, 3, 10, 30, and 100 μM dCTP, respectively; Lanes 8 to 13, C reactions plus 100 μM ara-CTP and 0, 1, 3, 10, 30, and 100 μM dCTP, respectively; Lane 14, reaction containing 30 μM each of dATP, dCTP, dGTP, and dTTP.

Fig. 6. Kinetic analysis of DNA primer extension by pol α and pol ε. Reaction mixtures contained 10 μg of unlabeled primed M13mp19(+)-DNA/ml, 100 μM each of dATP, dGTP, and dTTP, and the indicated concentrations of [3H]ara-CTP and dFdCTP. The mixtures (30 μl) were incubated with 0.1 unit of either pol α or pol ε at 37°C for 20 min, spotted on GF/A filters, washed 3 times with 0.4 M HClO4, rinsed with ethanol, and quantitated by liquid scintillation counting.

Figure 6: Graph showing the incorporation of dCMP and dCTP by pol α and pol ε at various concentrations of dCTP and dFdCTP. The y-axis represents the dCMP incorporated, pmol/l, and the x-axis represents the dCTP concentration, μM. Two sets of data are shown, one for pol α and one for pol ε, with different concentrations indicated by different lines.

In test tubes, the ability of purified pol ε to remove incorporated dFdCMP was examined in vitro. Three DNA hybrids with 5'- 32P-labeled oligomers containing normal dNMP, dFdCMP, and ara-CMP at the 3'-terminal position and dFdCMP at 3'-penultimate position were constructed as described in "Materials and Methods." The DNA hybrids were incubated with pol ε to test the ability of the 3'→5' exonuclease activity of this enzyme to excise the target nucleotides. As illustrated in Fig. 8A, DNA pol ε was unable to efficiently excise nucleotides from the primers containing dFdCMP at either the 3'-end or in the penultimate position. In contrast, pol ε was able to remove a substantial amount of ara-CMP from the 3'-end of the primer and to excise the subsequent nucleotides. When the primer containing normal deoxynucleotides was used as the substrate, it was almost completely degraded in 40 min.

The radioactivity in each band was quantitated, and the excision activity was plotted against the digestion time (Fig. 8B). After subtracting zero-time background from the product, 57,661 counts (about 58% of the input) were removed from 105 counts of the normal oligomer within 10 min. In the case of dFdCMP-terminated oligomer, 3,848 counts (<4% of the input) were excised; about 2% (1,951 counts) of the input radioactivity was removed from the oligomer containing dFdCMP at the penultimate position. In contrast, 21,537 counts (21.5% of the input) were excised from the ara-CMP-terminated primer.

In the second experiment, CEM cells were labeled with [3H]dFdC, and DNA was isolated from the cells and dialyzed extensively to remove free [3H]nucleoside and nucleotides. The [3H]dFdCMP-cellular DNA was incubated with pol ε for 60 min, and the reaction products were analyzed by HPLC equipped with a radioactive flow detector for the release of [3H] dFdCMP. No detectable free [3H]dFdCMP (less 30 dpm) was released from the input of 1150 dpm of [3H]DNA in two separate experiments. Thus, it appears that pol ε was unable to remove a significant amount of incorporated dFdCMP from either synthetic oligomers or native cellular DNA.

Cytotoxicity and Incorporation. The cytotoxic activity of dFdC was evaluated by exposing CEM cells to various concentrations of the analogue for 4 h, washing the cells in drug-free medium,
Table 1  Apparent kinetic parameters of dFdCTP and dCTP

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>pol α</th>
<th>pol ε</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td>dFdCTP</td>
<td>26.7 ± 4.3$^2$</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>dCTP</td>
<td>1.2 ± 0.05</td>
<td>7.9 ± 0.5</td>
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* $V_{max}$, maximum relative velocity.

* Mean ± SE of two separate experiments.

DISCUSSION

This study demonstrated that dFdCMP was incorporated into DNA but not into RNA in whole cells. Experiments to determine the molecular mechanism of this action showed that purified DNA polymerases α and ε incorporated dFdCMP into the C sites of the elongating DNA strand, but paused at the site 1 nucleotide after the incorporation site. In contrast, ara-CMP largely terminated DNA strand elongation at the incorporation site. Furthermore, the exonuclease activity of pol ε was unable to excise dFdCMP, whereas ara-CMP was removed from DNA at 37% of the initial excision rate of deoxynucleotides. These results provide evidence for differences in the action of these drugs at the molecular level.

Greater than 90% of the incorporated dFdCMP residues in cellular DNA were in internucleotide linkage. This is in agreement with the DNA primer extension experiments, in which the incorporated dFdCMP was extended by addition of other dNTPs (Fig. 4). A similar pattern was observed in experiments with 2′,2′-difluorodeoxyguanosine 5′-triphosphate (data not shown), suggesting that this behavior may be specific to the difluoro modification of the carbohydrate. We speculate that the difluoro molecules incorporated at the 3′-penultimate position may disrupt the normal enzyme/primer/template interaction and hamper the polymerization process. In contrast, ara-CMP was incorporated into the C site and inhibited the polymerization process, leaving most of the analogue at the 3′-end of the DNA strand (Fig. 4). This type of DNA chain termination and determining the cellular clonogenicity after 12 days. As shown in Fig. 9, about 40 nM dFc was required to cause 50% loss of clonogenicity. When the relative survival was plotted against log pmol of dFdCMP incorporated into DNA, a linear relationship between the two parameters was revealed ($r = -0.954$, $P < 0.0001$). Thus, it appears that the amount of dFdCMP incorporated into cellular DNA is an important determinant of cytotoxicity.

Fig. 7. Kinetic incorporation of dFdCMP into primed M13mp18(+) DNA by pol α (A) and pol ε (B). Lanes 1 to 8, reactions containing $32^P$-primer/template (5 µg/ml), 0.1 unit of enzyme, and 100 µM dGTP plus 0, 1, 1.5, 2, 4, 10, 30, and 100 µM dFCTP, respectively.

Fig. 8. Excision of dFdCMP and ara-CMP from DNA by pol α. The M13 DNAs hybridized with oligomers with either dFdCMP or ara-CMP incorporated at the 3′-end or with dFdCMP in the penultimate positions were constructed as described in "Material and Methods." The normal 17-base primer was prepared by the same procedure and used as the control. The ability of polymerase ε (1 unit/100-µl reaction) to excise deoxynucleotides from the DNA substrates was evaluated at various time intervals. The reaction products were analyzed in a 20% DNA sequencing gel (A). Lanes 1 to 5, DNA hybrid containing dFdCMP at the 3′-end of the primer (19 bases) incubated with pol ε for 0, 5, 10, 20, and 40 min, respectively; Lanes 6 to 10, DNA hybrid containing dFdCMP at the 3′-penultimate position of the primer (24 bases) incubated with pol ε for 0, 5, 10, 20, and 40 min, respectively; Lanes 11 to 15, DNA hybrid containing the normal primer (17 bases) incubated with pol ε for 0, 5, 10, 20, and 40 min, respectively; Lanes 16 to 20, DNA hybrid containing ara-CMP at the 3′-end of the primer (19 bases) incubated with pol ε for 0, 5, 10, 20, and 40 min, respectively. The radioactivity in each band was quantitated by a Betascope 603 blot analyzer, and the excision activity was expressed as the percentage of total input radioactivity as illustrated in B. B: normal primer; ♦, primer-3′-ara-CMP; ■, primer-3′-dFdCMP; A, primer-3′-penultimate dFdCMP.

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activity was also observed for ara-C in experiments with pol α (18) and pol β (18, 19) and for other arabinosyl compounds (14, 20, 21).

Studies with HL-60 cells and acute myelogenous leukemia cells incubated with $^{3}H$ara-C in vitro demonstrated that greater than 95% of ara-CMP was located at the internucleotide linkage positions (22, 23). Ligation of the 3′-ara-CMP-terminated DNA fragment with an adjacent DNA strand by DNA ligase may explain the different ara-CMP positions observed in the DNA primer extension assay and in cell culture. Mikita and Beardsley (24) demonstrated that ara-CMP-terminated fragments were ligated with another DNA fragment in vitro, although the reaction rate was slower than the ligation of normal DNA fragments. Thus, it is possible that dFdC and ara-C were internally incorporated into cellular DNA by different mechanisms.

Pol ε, a highly processive enzyme that possesses 3′→5′ exonuclease activity, is thought to be involved in both DNA replication (25, 26) and repair (27, 28). This enzyme effectively excised ara-CMP, but not dFdCMP, from the 3′-terminus of DNA primers. Furthermore, when dFdCMP was in the 3′-penultimate position, pol ε was unable to remove the terminal deoxynucleotidylate. Thus, it is likely that incorporation of dFdCMP into DNA results in a steric change in the template-primer interaction that renders the analogue and at least one deoxynucleotidylate on the 3′-side poor substrates for excision.

Several lines of evidence indicate that dFdC diphosphate is a potent inhibitor of ribonucleotide reductase: dFdC induced a substantial decrease in cellular dNTP pools in K562 human leukemic cells (10) and human T-lymphoblastic CEM cells (4); partially purified ribonucleotide reductase was inhibited 50% by 4 μM dFdC diphosphate (4). The alterations in dNTP pools induced by dFdC incubation would certainly have an inhibitory effect on DNA synthesis. The inhibition of DNA synthesis by dFdC in whole cells could not be effectively reversed by the addition of deoxynucleosides that replenished cellular dNTPs. This observation is not a critical proof, however, that dFdC-induced depletion of dNTP pools is not important in the inhibition of DNA synthesis. It has been proposed that the dNTPs generated through the de novo pathways by ribonucleotide reductase are channeled to the DNA replication machinery and serve as preferred substrates for DNA polymerases (29, 30). Thus, it is possible that the dNTPs replenished through the salvage pathways were not efficient precursors for DNA synthesis.

![Figure 9. Cytotoxicity of dFdC in CEM cells and its correlation with the incorporation of dFdCMP into DNA. The cytotoxic activity of a 4-h incubation with the indicated concentrations of dFdC (A) was evaluated by clonogenic assays. Points, mean of three determinations; bars, SE. The relative cell survival was plotted against log pmol of dFdCMP incorporated in 1 mg of DNA (B).](image-url)

Although the kinetic study (apparent $V_{max}/K_{m}$) indicated that dFdCMP was incorporated into DNA at a lower rate than dCMP was, the incorporated dFdCMP molecules could not be removed in a significant amount from DNA by the 3′→5′ exonuclease activity of pol ε. The dFdCMP incorporation was significantly correlated with the loss of clonogenicity, suggesting its importance in causing cytotoxicity. The incorporation of dFdCMP into DNA may lead to perturbation of cellular DNA replication in a variety of ways. (a) dFdCMP was incorporated into the growing primer and caused a major pause of the DNA polymerases at the site 1 nucleotide after the incorporation site. Although the incorporated dFdCMP residues in DNA could be extended by the addition of other dNTPs, dFdCTP was not able to substitute dCTP to support the synthesis of full-length DNA (Fig. 4, Lanes 2 to 9). (b) The internally incorporated dFdCMP residues may cause distortion of the DNA configuration and impair its function as a template in subsequent DNA replication cycles. There was evidence that ara-CMP located at an internucleotide site in the template strand markedly slowed replication (24). It would be of interest to construct a primed DNA molecule with an internal dFdCMP in the template strand and examine its effect on DNA synthesis in vitro. (c) dFdCMP may mispair with nucleotides other than dGMP and result in the production of a mutated DNA daughter strand.

The incorporation of dFdCMP into DNA, however, may not solely account for the potent inhibitory activity of dFdC on DNA synthesis in whole cells. Cellular dFdCTP may directly inhibit DNA polymerases by competing with dCTP for the binding site on the enzyme. In fact, our kinetic analysis indicated that, with respect to dCTP, dFdCTP was a competitive inhibitor of pol α and pol ε with the apparent $K_{i}$ values of 11.2 μM and 14.4 μM, respectively. The cellular dFdCTP concentrations in leukemia cells from patients during dFdC therapy ranged from 64 μM to 362 μM (31, 32). These concentrations of dFdCTP also readily accumulate in cultured cells (10). On the other hand, the cellular dCTP is 3.5 μM in K562 cells (10) and about 1 μM in circulating leukemia cells. Thus, the ratio of cellular concentrations of dFdCTP to dCTP is in favor of the inhibitory action of dFdCTP on DNA synthesis.

Furthermore, inhibition of ribonucleotide reductase, which leads to the depletion of cellular dNTPs (4), may also significantly contribute to the action of dFdC on DNA synthesis. This may explain why this compound is more potent in inhibiting DNA synthesis in whole cells (Fig. 1) than in reactions with purified DNA polymerases (Fig. 5).

Thus, dFdC nucleotides have several sites of action in cells. (a) DNA polymerases are probably the major targets. dFdCTP is a weak competitor with dCTP for direct inhibition of DNA polymerases. Nevertheless, dFdCDTP: dCTP ratios that are achieved in cells in culture (3, 4, 10) and likely during therapy (7, 31, 32) suggest that competition might contribute to the inhibition of DNA synthesis. More importantly, incorporation of dFdCMP into the C sites of the DNA strands causes a major pause in polymerization and, once incorporated, dFdCMP is a poor substrate for editing activities, at least for the 3′→5′ exonuclease of pol ε. The internally incorporated dFdCMP may also perturb the next DNA replication cycle. (b) dFdCDP inhibits ribonucleotide reductase and causes a substantial decrease in DNA precursors. The reduction in cellular dCTP may also potentiate the incorporation of dFdC into DNA. (c) Reduction of dCTP may release deoxycytidine kinase from

* Unpublished data.
feedback inhibition by dCTP and thus increase dFdC phosphorylation. Thus, deoxycytidine kinase is also a target for the self-potentiation mechanism of dFdC-induced DNA inhibition.

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REFERENCES


