Modulatory Activity of 2',2'-Difluorodeoxycytidine on the Phosphorylation and Cytotoxicity of Arabinosyl Nucleosides

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ABSTRACT

This investigation analyzed the metabolism of 2',2'-difluorodeoxycytidine (dFdC) in K562 human leukemia cells and evaluated it as a biochemical modulator for the phosphorylation of several arabinosyl nucleosides. The rate of accumulation of dFdC triphosphate was linear up to 3 h and maximal during incubation with 10 μM dFdC (92 μM/h). Deoxynucleotides analyzed at this time showed a decrease in dCTP, dATP, and dGTP levels, indicating an inhibitory role of dFdC nucleotides in ribonucleotide reduction. We evaluated the hypothesis that dFdC-mediated deoxycytidine triphosphate perturbation enhances the phosphorylation of substrates that use deoxycytidine kinase or deoxyguanosine kinase, because these enzymes are inhibited by dCTP or dGTP, respectively. When the activity of these nucleoside kinases was rate limiting to triphosphate formation, the accumulation of triphosphates of deoxycytidine, 1β-D-arabinofuranosylcytosine, and 1β-D-arabinofuranosylguanine was potentiated in cells pretreated with dFdC. In contrast, the phosphorylation of 9-D-arabinofuranosyladenine was not affected, since it is mainly phosphorylated by adenosine kinase, which is not influenced by deoxyribonucleoside triphosphates. Treatment of cells with dFdC followed by 1β-D-arabinofuranosylcytosine resulted in greater cytotoxicity than sum effects of each drug alone. The data indicate that an enhanced cytotoxicity could be obtained by administering dFdC as a modulator followed by 1β-D-arabinofuranosylcytosine or 1β-D-arabinofuranosylguanine in optimal sequence, suggesting that these results should be considered in the design of combination clinical protocols.

INTRODUCTION

Attempts to extend the success achieved with ara-C in the treatment of adult acute leukemia (1, 2) failed to demonstrate therapeutic effectiveness in other hematological malignancies (3, 4). This led to the synthesis of arabinosyl purines and fluorinated analogues of dCyd. Two of these, 2'-fluorodeoxycytidine, with a fluorine atom in either the arabinose or the ribose configuration, exhibited limited antiviral and poor anti-tumor activity in experimental systems (5, 6).

Another congener, 2',2'-difluorodeoxycytidine, in which hydrogens at the 2'-carbon were replaced by geminal fluorine atoms (7) showed remarkable activity against murine solid tumors, as well as leukemias (8, 9). Comparison of ara-C and dFdC pharmacokinetics in Chinese hamster ovary cells showed dFdC to be more rapidly anabolized and the more potent cytotoxic agent (10).

These findings generated interest in the metabolism of dFdC in human leukemia cell lines. K562 cells, derived from a patient with chronic myelogenous leukemia, were selected to compare the metabolism of dFdC with previously studied arabinosyl analogues (11). In addition, this cell line contains low levels of dCyd deaminase (12), thus reducing the interference of the deaminated products of dFdC in metabolism and cytotoxicity.

Previous studies have shown that antimetabolites that inhibit ribonucleotide reductase potentiate the metabolism of arabinosyl nucleosides (11, 13, 14). The activities of several nucleoside kinases are known to be regulated by dNTPs (15–19), thus inhibition of ribonucleotide reductase and consequent lowering of dNTP pools can modulate the activity of a nucleoside kinase. Consequently, inhibition of ribonucleotide reductase by antimetabolites has been a target for combinations with arabinosyl nucleosides (20, 21).

The placement of the two fluorine atoms at the 2'-carbon suggests that a dFdC nucleotide might act as an alternative inhibitory substrate of ribonucleotide reductase (22). Incubation of cells with dFdC does, in fact, cause a decrease in the endogenous dNTP pools (23). This background generated the present evaluation of dFdC as a biochemical modulator of arabinosyl nucleoside metabolism and cytotoxicity (24).

MATERIALS AND METHODS

Chemicals and Cell Line. dFdC was supplied by Lilly Research Laboratories (Indianapolis, IN). The dFdC triphosphate was synthesized by Dr. Alina Sen as described previously (25). ara-C, ara-CTP, and ara-ATP were purchased from Sigma Chemical Co. (St. Louis, MO). ara-G and ara-GTP were purchased from Calbiochem-Behring Corporation (La Jolla, CA). F-ara-A was provided by Dr. V. L. Narayanan, Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). F-ara-ATP was synthesized chemically (25). ara-A (Pfanstiehl Laboratory, Waukegan, IL) and deoxycoformycin (Parke Davis, Detroit, MI) were obtained through the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). All other chemicals were of the highest grade available.

For enzyme assays, [5,6-3H]dCdC (26 Ci/μmol) was purchased from ICN Biochemicals (Costa Mesa, CA), [5,6-3H]ara-C (20 Ci/μmol) was obtained from Moravek Biochemicals (Brea, CA), and [5,14C]dFdC (66 mCi/μmol) was supplied by Lilly Research Laboratories (Indianapolis, IN). All nucleosides were purified by reverse-phase HPLC to >99% purity.

The K562 cell line was obtained from the American Type Culture Collection (Rockville, MD) and used throughout the study (26). Maintenance of the cultures, Mycoplasma analysis, and doubling time of this cell line have been described previously (14).

Enzyme Assays. Cells were harvested and stored at −70°C. Frozen cells were thawed in a buffer containing the protease inhibitors phenyl-methylsulfonyl fluoride and o-phenanthroline (200 μM each) and then subjected to bomb cavitation by Parr bomb (Parr Instrument, Moline, IL) after 30 min at 1000 psi of nitrogen. Kinases were separated and eluted by passing the cell extract through a DE-52 column with a linear gradient (300 ml) of 0–600 mM KCl. Fractions (3 ml) were collected, and every third fraction was assayed for kinase activity for phosphorylation of dCdC, ara-C, and dFdC.

The phosphorylation activity of these substrates was determined with the anion-exchange filter disk method described by Saunders and Lai (27). All the nucleosides were used at a final concentration of 25 μM (1
control cells was 29 ± 1% (SD). The dCyd kinase activity is shown in Fig. A. These extracts of K562 cells by DEAE-cellulose chromatography. As reported before, separate activity peaks (kinases) were observed for the phosphorylation of adenosine, dAdo, dGuo, and dCyd reported before, separate activity peaks (kinases) were observed responsible for the phosphorylation of dFdC, we fractionated the combined drugs was the product of survival fractions of cells treated with the indicated concentrations of dFdC, and cellular dFdC was measured after 3 h as described in “Materials and Methods.” In B, cells were incubated with 10 μM dFdC. At the indicated times, the intracellular concentration of dFdCTP was determined. The curve was plotted by using nonlinear regression-

**RESULTS**

**Kinase Activity in Cell Extracts.** To identify the activity responsible for the phosphorylation of dFdC, we fractionated extracts of K562 cells by DEAE-cellulose chromatography. As reported before, separate activity peaks (kinases) were observed for the phosphorylation of adenosine, dAdo, dGuo, and dCyd (14). The dCyd kinase activity is shown in Fig. 1A. These fractions were used to specify the kinase responsible for the phosphorylation of dFdC. The peak (fractions 51–66) responsible for the phosphorylation of dFdC also phosphorylated dFdC, indicating that dCyd kinase is the enzyme that converts dFdC to 2',2'-difluorodeoxycytidine 5'-monophosphate (Fig. 1B). When all fractions were assayed for the activity against ara-C as a substrate, fractions 51–66 phosphorylated ara-C (data not shown).

**Accumulation of dFdCTP.** K562 cells were incubated with various concentrations of dFdC for 3 h. Investigation of the relationship between the exogenous nucleoside concentration and cellular triphosphate accumulation indicated that the increase of intracellular dFdCTP was proportional to the dFdC concentration only up to 3 μM (Fig. 2A). Ten μM dFdC increased the triphosphate concentration slightly; however, any further increase in the concentration of dFdC resulted in less

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Fig. 1. DEAE-cellulose chromatography of K562 cell kinases. Cell extract was applied to a column of DEAE-cellulose. Elution was with a 300-mL linear gradient of KCl. Each third fraction was assayed for phosphorylation of dCyd to dCMP (A) or dFdC to dFdCMP (B).

Fig. 2. Accumulation of dFdCTP by K562 cells. A. Cells were incubated with the indicated concentrations of dFdC, and cellular dFdCTP was measured after 3 h as described in "Materials and Methods." In B, cells were incubated with 10 μM dFdC. At the indicated times, intracellular concentration of dFdCTP was determined. The curve was plotted by using nonlinear regression-

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**Statistical Considerations.** All the analyses are presented as means of 2–4 separate experiments. The SDs were <15% of the mean values.

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**Modulatory Activity of dFdC on Arabinosyl Nucleosides**

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dFdCTP in cells. Because 10 μM dFdC was saturating the accumulation of dFdCTP, this concentration was used for subsequent experiments. To determine whether longer incubation would result in a greater cellular concentration of dFdCTP, cells were incubated with 10 μM dFdC for up to 5 h and were analyzed every hour. The cellular concentration of dFdCTP was linear through 3 h and increased only slightly thereafter (Fig. 2B). As suggested from the concentration dependence curve (Fig. 2A) a similar rate of dFdCTP accumulation was observed in cells incubated with 3 μM dFdC (data not shown). Cells treated with 30 and 100 μM dFdC had lesser rates of dFdCTP accumulation with maxima close to 3 h (data not shown).

Effect of dFdC Incubation on dNTP Pools. The effect of dFdC on dNTP pools was examined after incubating K562 cells with 10 μM dFdC for 3 h. Compared with the endogenous dNTP levels in untreated cells, the results indicated that, after dFdC treatment, there was more than 50% reduction in dATP and dGTP and a 75% reduction in the dCTP pool. The dTTP pool was not altered (Table 1). Similar incubations with ara-C, nucleotides of which do not inhibit ribonucleotide reduction, showed a tendency toward increase in dNTPs (Table 1). Ribonucleotide pools remained unaffected after treatment with either dFdC or ara-C (data not shown). These results indicate that dFdC incubation may create a favorable intracellular milieu, by decreasing the dNTP pools, for the phosphorylation of substrates which require dCyd kinase and dGuo kinase.

Table 1 Effect of dFdC and ara-C on deoxynucleotide pools

<table>
<thead>
<tr>
<th>Condition</th>
<th>dCTP</th>
<th>dTTP</th>
<th>dATP</th>
<th>dGTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>dFdC</td>
<td>107</td>
<td>37</td>
<td>43</td>
<td>114</td>
</tr>
<tr>
<td>ara-C</td>
<td>142</td>
<td>155</td>
<td>131</td>
<td>114</td>
</tr>
</tbody>
</table>

Values are expressed as a percentage of the dNTP concentrations in untreated cells: dCTP, 3.5 ± 1.3 μM; dTTP, 9.9 ± 2.7 μM; dATP, 8.6 ± 1.4 μM; and dGTP, 3.3 ± 0.4 μM. Results are the means ± SD of 4 separate experiments.

Effect of dFdC as a Biochemical Modulator. To evaluate the hypothesis developed in the previous paragraph, we studied the phosphorylation of ara-C, the natural substrate for ara-C kinase, in untreated and dFdC-treated whole cells. Cells were treated with 10 μM dFdC, washed, and incubated with 100 μM [3H]dCyd. The results show that dFdC-treated cells accumulate ara-CTP faster than untreated control cells (Fig. 3). These results, which are consistent with the hypothesis that modulation of cellular dNTPs by dFdC affects dCyd kinase activity, suggest that the metabolism of other substrates of this enzyme would be influenced after dFdC treatment. Because ara-C is also dependent on the activity of dCyd kinase for its initial phosphorylation, the effect of dFdC preincubation on the metabolism of ara-C was determined. As observed for dCTP, the accumulation of ara-CTP was significantly enhanced by dFdC pretreatment (Fig. 4). The peak ara-CTP in control cells was at 100 μM; however, dFdC-treated cells accumulated nearly 300 μM ara-CTP.

The influence of dFdC preincubation on the phosphorylation of other arabinosyl nucleosides was also investigated. K562 cells were incubated with 10 μM dFdC to perturb the deoxynucleotide pools (Table 1). In contrast to the previous observations, the phosphorylation of F-ara-A (which also requires dCyd kinase that has low affinity for F-ara-A, K_m 200–500 μM) was inhibited 86% by dFdC preincubation (Fig. 4B). ara-G phosphorylation, which depends on dGuo kinase, was analyzed in control cells and dFdC-pretreated cells. The rate of accumulation of ara-GTP (1.8 μM/h) was potentiated by dFdC pretreatment (2.8 μM/h) (Fig. 4C). Accumulation of ara-ATP from exogenous ara-A, which is primarily phosphorylated by adenosine kinase, was not significantly affected (Fig. 4D).

Effect of dFdC on ara-C Metabolism and Action. Since the clinical response to ara-C is correlated with the intracellular accumulation of ara-CTP in patient cells, we focused our attention on the potentiation of ara-CTP accumulation in dFdC-pretreated cells. First, the effect of dFdC on the elimination of ara-CTP was investigated. Cells were incubated with ara-C to accumulate ara-CTP and washed into drug-free medium. One-half of this culture was incubated with no dFdC, whereas the other half was incubated with dFdC. Comparison of the elimination of ara-CTP in these two cultures indicated that the t_1/2 of ara-CTP elimination was similar: 1.6 h in control and 1.7 h in dFdC-treated cells (Fig. 5). This suggests that the potentiation of ara-CTP accumulation is due to an effect on the anabolism of ara-C, not on the dephosphorylation of ara-CTP.
MODULATORY ACTIVITY OF dFdC ON ARABINOSYL NUCLEOSIDES

To act as cytotoxic agents, dFdC and ara-C have to be converted to the corresponding nucleotides by cellular kinases. The paucity of mono- and diphosphates in cells relative to the triphosphates indicates that initial phosphorylation of these nucleosides is the rate-limiting step in triphosphate synthesis (10, 29). The finding that the cytotoxicity of both ara-C and dFdC may be reversed by dCyd suggests that these drugs may be activated by a common metabolic pathway. Furthermore, the dCyd kinase-deficient cells were resistant to dFdC, indicating that dFdC is phosphorylated by this enzyme (10). Fractionation of cell extracts on a DEAE-cellulose column demonstrated that dCyd kinase is responsible for the phosphorylation of dFdC to its monophosphate (Fig. 1); the same fractions were responsible for ara-C phosphorylation (14).

The rate of accumulation of triphosphate from exogenous nucleoside at a concentration that saturated the ability of the cells to accumulate triphosphate is indicative of the activity of the rate-limiting kinase in whole cells (29). dCyd kinase appears to be required for phosphorylation of ara-C and dFdC which was linear up to 2 h at a rate of 92 µM/h for dFdC (Fig. 2B) and of 35 µM/h for ara-C (11). The efficiency of phosphorylation (Vmax/Km) of dFdC was 5 times higher than that of ara-C in Chinese hamster ovary cells (10) and 3 times greater in K562 cells. This could contribute to the more rapid rate of dFdCTP accumulation. The rate of elimination is also a determinant of the rate of triphosphate accumulation. In K562 cells, the rate of degradation of ara-CTP was faster than the initial elimination of dFdCTP (0-6 h) (ara-CTP, t1/2, 1.6 h; dFdCTP, t1/2, 3.3 h) (11, 23). However, these rates are considerably slower than the triphosphate accumulation rates and therefore may not have a major effect.

To evaluate the hypothesis that dFdC nucleotides might inhibit ribonucleotide reduction (23, 30), the endogenous deoxynucleotides were analyzed after a 3-h incubation with 10 µM dFdC. The cellular dFdCDP after such an incubation was 11 µM which is higher than the IC50 of dFdCDP for inhibition of ribonucleotide reductase (22, 30). The results of the measurements of dNTP pools presented in Table 1 indicate that, unlike ara-C-treated cells, ribonucleotide reduction in dFdC-treated cells was inhibited, resulting in a decrease in the cellular concentrations of deoxynucleotides. The greatest effect was on dCTP, whereas dTTP was the least perturbed. The inhibitory role of dCPT on dCyd kinase has been well documented in whole cells (11, 29, 31) and in cell-free preparations (15-17, 32, 33). Thus it is likely that the dFdC-mediated depletion of dCTP induces a cellular milieu favorable to its own phosphorylation and that of other substrates that require dCyd kinase.

The function of dFdC as a potentiator of dCyd kinase was studied in cells treated with dFdC to affect dNTP pools. The accumulation of dCPT or ara-CTP from exogenous dCyd or ara-C, respectively, in dFdC-treated or untreated cells indicated that dFdC-mediated dNTP pool perturbation potentiated the phosphorylation of both dCyd and ara-C (Figs. 3 and 4A). In contrast, the phosphorylation of F-ara-A, also a substrate for dCyd kinase, was inhibited by dFdC treatment. A major difference between F-ara-A, dCyd, and ara-C as substrates for dCyd kinase is the relatively low affinity of the enzyme for ara-A (Km, 200-500 µM) (34-37) compared to dCyd (Km, 0.2-4 µM) (16, 17, 34-39) and ara-C (Km, 20-40 (16, 17, 36, 38, 39). For comparisons, the Km of dCyd kinase for dFdC was 3.6 in Chinese hamster ovary cells (10) and 3.1 µM in extracts of K562 cells. Cells loaded with dFdCPTP, when washed and incubated with F-ara-A, contain dFdC generated as a dephosphorylation product of dFdCTP. Because these nucleosides compete for phosphorylation by dCyd kinase, it is possible that the phosphorylation of dFdC is favored over that of F-ara-A, resulting in the observed lower rates of F-ara-ATP accumulation. Alternatively, dFdC and/or its nucleotides may act by as yet undefined mechanisms to inhibit F-ara-A phosphorylation.

DISCUSSION

Second, the effect of the higher levels of ara-CTP induced by dFdC pretreatment on cytotoxicity was determined. K562 cells were incubated with either no drug (O) or 10 µM dFdC ( ), washed, and incubated with 10 µM ara-C. After appropriate dilution, cells were plated on agar and analyzed for clonogenicity after 10 days. , the expected survival after a combination of dFdC and ara-C.

Fig. 5. Effect of dFdCPTP on the rate of ara-CTP elimination. The cells incubated for 3 h with 10 µM ara-C were washed into drug-free medium and were incubated with no drug (O) or with 10 µM dFdC ( ). The cellular concentration of ara-CTP was determined as described in "Materials and Methods."

Fig. 6. Effect of dFdC and ara-C on K562 clonogenicity. Cells were incubated with either no drug (O) or 10 µM dFdC ( ), washed, and incubated with 10 µM ara-C. After appropriate dilution, cells were plated on agar and analyzed for clonogenicity after 10 days. , the expected survival after a combination of dFdC and ara-C.

Effect of Arabinosyl Nucleosides on dFdC Metabolism. To investigate whether pretreatment with other nucleosides to perturb the dNTP pools would enhance phosphorylation of dFdC, K562 cells were preincubated with either F-ara-A or ara-A, after dFdC and plated in agar. Compared with untreated cells, a 3-h incubation with dFdC resulted in a 50% decrease in clonogenicity. A sequential combination of dFdC (3 h) and ara-C (1-5 h) exerted more than additive cytotoxicity (Fig. 6, compare dashed line with solid symbol line). A 5-h incubation with ara-C alone resulted in a 65% cell survival; however, preincubation with dFdC resulted in <20% cell survival (Fig. 6).

The function of dFdC as a potentiator of dCyd kinase was studied in cells treated with dFdC to affect dNTP pools. The accumulation of dCPT or ara-CTP from exogenous dCyd or ara-C, respectively, in dFdC-treated or untreated cells indicated that dFdC-mediated dNTP pool perturbation potentiated the phosphorylation of both dCyd and ara-C (Figs. 3 and 4A). In contrast, the phosphorylation of F-ara-A, also a substrate for dCyd kinase, was inhibited by dFdC treatment. A major difference between F-ara-A, dCyd, and ara-C as substrates for dCyd kinase is the relatively low affinity of the enzyme for ara-A (Km, 200-500 µM) (34-37) compared to dCyd (Km, 0.2-4 µM) (16, 17, 34-39) and ara-C (Km, 20-40 (16, 17, 36, 38, 39). For comparisons, the Km of dCyd kinase for dFdC was 3.6 in Chinese hamster ovary cells (10) and 3.1 µM in extracts of K562 cells. Cells loaded with dFdCPTP, when washed and incubated with F-ara-A, contain dFdC generated as a dephosphorylation product of dFdCTP. Because these nucleosides compete for phosphorylation by dCyd kinase, it is possible that the phosphorylation of dFdC is favored over that of F-ara-A, resulting in the observed lower rates of F-ara-ATP accumulation. Alternatively, dFdC and/or its nucleotides may act by as yet undefined mechanisms to inhibit F-ara-A phosphorylation.

* V. Heinemann and W. Plunkett, unpublished results.

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As with ara-C, the accumulation of ara-GTP was enhanced by dFdC pretreatment. Unlike other nucleosides studied, ara-G is phosphorylated by dGuo kinase (18, 40, 41) or by both dCyd kinase and dGuo kinase (42). In K562 cell extracts at a 25 μM substrate concentration, dGuo kinase was the only enzyme that phosphorylated ara-G (14). The activity of dGuo kinase is inhibited by dGTP (18, 19). After dFdC incubation, the level of endogenous dGTP was reduced by >50%, which may have released the feedback inhibition by dGTP on ara-G phosphorylation (Fig. 4C).

Accumulation of ara-ATP served as a negative control to evaluate the effect of dFdC. ara-A is phosphorylated by adenosine kinase (14, 37, 43), the activity of which is not affected by the observed perturbations in the dNTP levels. A slight stimulation of the rate of ara-ATP accumulation (Fig. 4D) is consistent with the suggestion that dCyd kinase is involved to a minor extent in ara-A phosphorylation (14, 37).

The effect of a decline in dNTP pools on the activity of dCyd kinase was observed again when F-ara-A and ara-A were used as modulators of dFdC metabolism. The phosphorylation of dFdC was potentiated by either F-ara-A or ara-A pretreatment. The triphosphates of both these arabinosyl nucleosides inhibit ribonucleotide reductase (34, 44), resulting in a decrease in the deoxynucleotide pools (45).

The results indicate that dFdC acts as a biochemical modulator for the intracellular accumulation of ara-CTP. This modulation seems to be due to a higher rate of phosphorylation rather than decreased catabolism of ara-CTP, as ara-CTP elimination was not affected in cells containing dFdCTP (Fig. 5). The mechanism for potentiation of ara-C anabolism is most likely due to a reduction in the cellular dCTP pools. This would have the dual effect of first decreasing the feedback inhibition of dCyd kinase by dCTP, hence a greater rate of ara-C phosphorylation and ara-CTP accumulation. Second, it would contribute to an increased incorporation of ara-C nucleotide into DNA, because ara-CTP and dCTP compete for DNA polymerase. This possibility is currently under investigation.

Sequential treatment with dFdC and ara-C generated higher cellular levels of ara-CTP. Because cellular exposure to ara-CTP is proportional to cytotoxicity (46), it is likely that the increased ara-CTP concentrations following dFdC treatment were responsible for the more additive effect on the cytotoxicity of K562 cells. In the clinical situation, higher levels of ara-CTP and synergistic cell killing can be associated with a positive response. Appropriate scheduling of dFdC should be considered in the design of combination protocols. As a modulator, dFdC may be used before ara-C or ara-G in a sequence-dependent manner. As an effector, dFdC might be used most effectively following either F-ara-A or ara-A.

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