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Inhibition of Ribonucleotide Reduction in CCRF-CEM Cells by 2′,2′-Difluorodeoxycytidine

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SUMMARY

The new deoxycytidine analogue 2′,2′-difluorodeoxycytidine (dFdC) is a specific inhibitor of DNA synthesis that has marked cytotoxicity and therapeutic activity. A 2-hr incubation with 0.1–10 μM dFdC decreased cellular viability 78–97%. This treatment reduced deoxynucleoside triphosphate pools, similar to the action of the ribonucleotide reductase inhibitor hydroxyurea. The most pronounced decrease occurred in the dCTP pool, quantitatively followed by the decrease of dATP, dGTP, and dTTP. In contrast, inhibition of DNA synthesis by arabinosylcytosine did not affect the dCTP level, whereas dATP, dGTP, and dTTP pools increased, but less than 2-fold. The incorporation of [5-^{3}H]cytidine into the dCTP pool, a measure of ribonucleotide reductase activity in whole cells, was reduced to 3% of controls by 0.1 μM dFdC, but to only 40% by 0.1 μM ara-C. Each drug decreased incorporation of [5-^{3}H]cytidine into DNA to a similar extent (>94%), suggesting limitation by a reaction proximal to this step. The cellular concentration of dFdC 5′-diphosphate was 0.3 μM at 50% inhibition of the *in situ* activity of ribonucleotide reductase. Direct assays of partially purified ribonucleoside diphosphate reductase (EC 1.17.4.1) demonstrated 50% inhibition by 4 μM dFdC 5′-diphosphate; dFdC 5′-triphosphate was much less inhibitory. We conclude that dFdC 5′-diphosphate acts as an inhibitor of ribonucleoside diphosphate reductase.

Nucleoside antimetabolites have been developed into some of the most effective anticancer and antiviral agents. dFdC is a new dCyd analogue (1) that is cytotoxic in cell lines (2, 3) and has therapeutic activity in several murine tumor models (4). Phase I clinical trials are proceeding in solid tumors (5) and relapsed leukemias (6). The cytotoxic action of dFdC, which is associated with a specific inhibition of DNA synthesis, requires intracellular phosphorylation and the cellular accumulation of the 5′-diphosphate dFdCDP and the 5′-triphosphate dFdCTP (2). The initial step in this pathway is catalyzed by dCyd kinase (2, 7), an enzyme that is regulated by dCTP-mediated feedback inhibition (8, 9). Low concentrations of dFdC (>0.01 μM) induced a significant depletion of cellular dNTP pools in K562 cells, a human leukemia cell line, with the major effect being on dCTP (3). The dFdC-induced dCTP depletion may be of importance, because this is likely to release feedback inhibition of dCyd kinase, which, in turn, may enhance dFdC phosphorylation. Furthermore, dFdCTP incorporation into DNA may be increased as competition with dCTP is diminished (10).

To understand the mechanisms underlying the dFdC-mediated dNTP perturbation, the pharmacodynamics of dFdC were compared with those of hydroxyurea and ara-C. Hydroxyurea acts as a specific inhibitor of ribonucleotide reductase (11), whereas ara-C nucleotides do not affect the enzyme (12) but may modulate dNTP metabolism as a secondary effect of the inhibition of DNA synthesis (13). The results indicate that the perturbation in the dNTP pools in dFdC-treated cells is a result of direct inhibition of ribonucleotide reduction.

Experimental Procedures

Materials. dFdC was synthesized as described (1). Radiolabeled cytosine was used for the synthesis of [2-^{14}C]dFdC (specific activity, 194 μCi/mg). Ara-C was purchased from Sigma Chemical Co., Inc. (St. Louis, MO). [2-^{3}H]Adenosine (40 Ci/mmol), [5-^{3}H]Cyd (26 Ci/mmol), [8-^{3}H]guanosine (8 Ci/mmol), [*methyl*-^{3}H]thymidine (52 Ci/mmol), [6-^{3}H]uridine (27.5 Ci/mmol), and [U-^{14}C]CDP (517 Ci/mol) were purchased from ICN Radiochemicals, Inc. (Irvine, CA). ATP-agarose type

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ABBREVIATIONS: dFdC, 2′,2′-difluorodeoxycytidine (gemcitabine); dFdCDP and dFdCTP, the di- and triphosphates of 2′,2′-difluorodeoxycytidine; ara-C, 1-β-D-arabinofuranosylcytosine; dNTP, deoxynucleoside triphosphate; dCyd, deoxycytidine; Cyd, cytidine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

3 was purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). *Crotalus adamanteus* venom, hydroxyurea, and Dowex-1 were obtained from Sigma. All other reagents were of the highest purity available. dFdCDP and dFdCTP were synthesized chemically by established procedures (14). The products were isolated by preparative high pressure liquid chromatography using a Magnum-20 SAX column (Whatman, Inc. Clifton, NJ) and isocratic elution with 0.50 M $NH_4H_2PO_4$, pH 3.7. Nucleotides were recovered from the eluate by adsorption to activated charcoal.

Cell culture. The T lymphoblastic cell line CCRF-CEM was obtained from American Type Culture Collection (Rockville, MD). The cells were maintained in suspension culture in exponential growth in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 5% heat-inactivated fetal calf serum (GIBCO), at 37° in a humidified atmosphere containing 5% CO_2. Cell number and cell volume were determined by a Coulter counter equipped with a model C-1000 particle analyzer (Coulter Electronics, Hialeah, FL). The average volume of CCRF-CEM cells was 0.943 pl/cell.

The effect of dFdC on the viability of cells was determined by a clonogenic assay. Cells were incubated with various concentrations of dFdC for 2 hr and washed, and dilutions of 200 or 2000 cells were plated in Iscove's medium supplemented with 5% glutamine (GIBCO), containing final concentrations of 0.3% agar and 30% fetal calf serum. After 10 days, colonies of greater than 40 cells were counted with the aid of an inverted microscope.

Nucleotide extraction and analysis. Nucleotides were extracted from cells with 0.4 N $HClO_4$, as previously described (2). The neutralized $HClO_4$-soluble extracts were analyzed by high pressure liquid chromatography, using a Partisil-10 SAX (Whatman, Inc.) anion exchange column (250 × 4 mm). Mono-, di-, and triphosphates of dFdC were separated by a linear gradient run over 40 min at 2 ml/min, starting at 100% buffer A (0.005 M $NH_4H_2PO_4$, pH 2.8) and concluding at 100% buffer B (0.75 M $NH_4H_2PO_4$, pH 3.5) (2).

Determination of dNTP. Degradation of nucleoside triphosphates in the $HClO_4$-soluble extract from 1–3 × 10^7 cells was achieved by periodate oxidation (15). The dNTPs were separated on a Partisil-10 SAX column at a flow rate of 3 ml/min. After an isocratic elution with 75% buffer A and 25% buffer B (20 min), a linear gradient was run for the next 23 min to 79% buffer B.

***In situ* activity of ribonucleotide reduction.** Ribonucleotide reduction was estimated by the amount of radioactivity incorporated into the dCTP, dTTP, dATP, or dGTP pool and into DNA after a 10-min pulse with 0.5 μCi/ml [^{3}H]Cyd, [^{3}H]uridine, [^{3}H]adenosine, or [^{3}H]guanosine, respectively. Incorporation into dNTP pools was measured as described above, after quantitative removal of ribonucleotides with periodate. The method described by Jackson (16) was used to measure incorporation into DNA. Ribonucleotide reduction *in situ* was taken as the sum of these two values. The release of ^{3}H from the 5-position of [5-^{3}H]Cyd into ^3H_2O through the *in situ* activity of thymidylate synthase was measured after a 30-min incubation, according to the method of Nicander and Reichard (17). [^{3}H]Thymidine incorporation into DNA, conducted as a 10-min pulse of 0.5 μCi/ml at various times after drug addition, was quantitated as previously described (2). When cells were coincubated with [^{14}C]dFdC (to determine cellular metabolites) and [^{3}H]Cyd (to measure *in situ* reduction), the activity of each isotope was quantitated simultaneously, using a Beckman model LS-5801 liquid scintillation counter. Radioactivity associated with mono- and diphosphates of deoxynucleotides was less than 10% of that in the respective triphosphate fractions.

Purification and assay of ribonucleoside diphosphate reductase. CEM cells (5 × 10^9) in exponential growth were suspended and disrupted in 30 ml of extraction buffer (50 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 2 mM dithiothreitol, 15% glycerol), using a Parr cell disruption bomb (1800 psi for 30 min at 4°). Cell homogenates were centrifuged at 100,000 × *g* for 120 min. The supernatant was collected, streptomycin sulfate was added to a final concentration of 1%, and the resulting precipitate was removed by centrifugation. The enzyme was precipitated by bringing the supernatant to 80% $(NH_4)_2SO_4$ and, after centrifugation, the resulting pellet was dialyzed against extraction buffer for 4 hr with one change of buffer. Ribonucleoside diphosphate kinase was depleted 1300-fold, to a specific activity of 0.21 nmol of CDP phosphorylated to CTP/min/mg, using an ATP-agarose column as described (18). These procedures raised the apparent specific activity of CDP reductase to 0.71 pmol/min/mg.

The ribonucleoside diphosphate reductase assay mixture had a final volume of 150 μl, containing 200 mM HEPES, pH 7.4, 6 mM ATP, 6 mM $MgCl_2$, 3 mM dithiothreitol, 1 mM NaF, 3 μM $FeCl_3$, and 150 μM [^{14}C]CDP (0.1 μCi). The reactions, conducted in duplicate, were started by the addition of 1.0 mg of enzyme preparation and were stopped after a 15-min incubation at 37° by boiling of the sample for 3 min at 100°. After the addition of 0.5 mg of *C. adamanteus* venom to each sample and incubation at 37° for 60 mins, [^{14}C]dCyd was isolated using a Dowex-1 borate column and quantitated as described (19).

Results

The effect of dFdC on the viability of CEM cells is shown in Fig. 1. Nearly 80% of the population was killed by a 2-hr incubation with 0.10 μM dFdC; lethality was nearly complete at 10 μM. Although the cytotoxic action of dFdC is thought to be exerted on S phase cells, a greater proportion of the population lost clonogenic capacity than would be in S phase (20) during the 2-hr incubation. Thus, the active nucleotides of dFdC and/or their effects on normal metabolites and processes must be retained long enough for most of the population to progress into S phase. These findings are consistent with the observations that the active nucleotides of dFdC are eliminated slowly, thus allowing cells to cycle into the sensitive S phase (2, 3).

The effect of dFdC incubation on the cellular dNTPs was investigated in CEM cells exposed for 2 hr to dFdC concentrations in the range of 0.01–10 μM. In four separate experiments performed in untreated cells, dCTP was the smallest dNTP pool (28 ± 2 μM), when compared with dTTP (64 ± 6.5 μM), dATP (76 ± 8 μM), and dGTP (51 ± 6.5 μM). A 2-hr incubation with dFdC induced a decrease in all dNTP pools (Fig. 2). This effect was apparent at 0.01 μM dFdC and was not substantially increased at higher dFdC concentrations. Although 0.1 μM dFdC reduced the dCTP pool to 21% of controls, a further

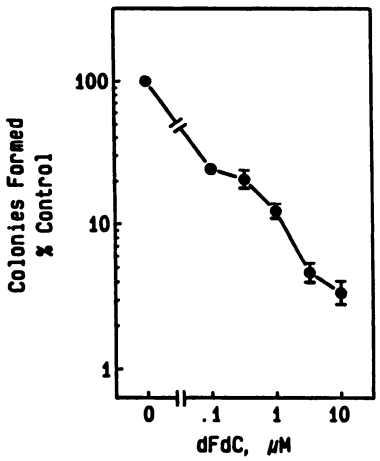

Fig. 1. Effect of dFdC on clonogenicity of CEM cells. Expontentially growing cells were incubated for 2 hr with the indicated concentrations of dFdC, washed into fresh medium, and plated to form colonies. Values are the mean ± standard deviation of six samples. Control colonogenicity was 38 ± 5%.

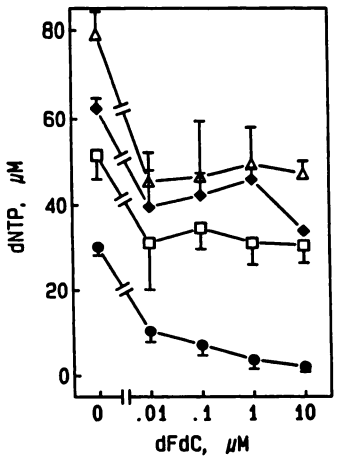

Fig. 2. Effect of dFdC on dNTP pools in CEM cells. Cells were incubated with the indicated concentrations of dFdC for 2 hr and dNTPs were extracted with $HClO_4$ and quantitated as described in Experimental Procedures. The data are the mean ± standard deviation of three separate experiments. ●, dCTP; ♦, dTTP; △, dATP; □, dGTP.

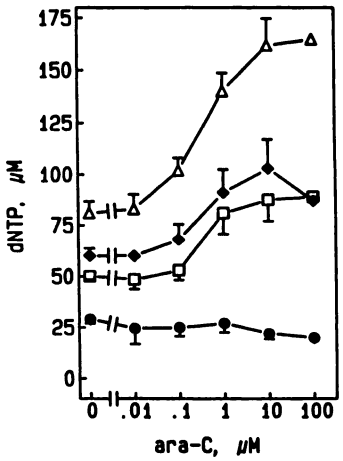

Fig. 3. Effect of ara-C on dNTP pools. CEM cells were treated with the indicated concentrations of ara-C for 2 hr and dNTPs were extracted with $HClO_4$ and quantitated as described in Experimental Procedures. The data are the mean ± standard deviation of three separate experiments. ●, dCTP; ♦, dTTP; △, dATP; □, dGTP.

reduction to only 6% of control levels was achieved by 10 μM dFdC (Fig. 2). At this concentration, however, dTTP, dATP, and dGTP were not reduced by more than 50%. The intracellular dFdCTP concentrations were 1.6, 48, 330, and 410 μM after a 2-hr exposure to 0.01, 0.1, 1.0, and 10 μM dFdC, respectively. Although not measured in these experiments, other experience with CEM cells (see below) and Chinese hamster ovary cells (2) indicates that dFdCDP concentrations are 2–3% of the dFdCTP levels. Incubations with 0.1 μM dFdC resulted in greater than 90% inhibition of [^{3}H]thymidine incorporation into DNA by 45 min (data not shown). Similar concentrations of 2′,2′-difluorodeoxyuridine, the dFdC deamination product, had no discernible effect on any dNTP pool (data not shown).

dNTP pools were studied after incubation with ara-C to better understand the effect of a primary inhibitor of DNA synthesis on dNTP metabolism in CEM cells (Fig. 3). Ara-C affected dNTP metabolism in a strikingly different manner than did dFdC. Cells exposed to 0.01–100 μM ara-C showed no significant change of the dCTP pool size. The cellular concentrations of dATP, dTTP, and dGTP, however, rose slightly in response to higher ara-C levels, increasing by 76, 66, and 57% above control values, respectively, after a 2-hr exposure to 10 μM ara-C. The ara-C triphosphate concentrations were 1.7, 22, 216, 364, and 297 μM in cells incubated with 0.01, 0.1, 1.0, 10, and 100 μM ara-C, respectively. Inhibition of thymidine incorporation was similar for 0.1 μM ara-C and dFdC (data not shown). These results indicate that inhibition of DNA synthesis alone is unlikely to cause the dNTP pool depletion observed in dFdC-treated cells.

Parallel cultures of CEM cells were incubated for 2 hr with hydroxyurea or dFdC to compare the cellular pharmacodynamics of a pure ribonucleotide reductase inhibitor with the action of dFdC on dNTP pools in CEM cells. The effects of 0.1 μM dFdC (Fig. 4A) and of 5 mM hydroxyurea (Fig. 4B) on dNTP pools were qualitatively and quantitatively similar. Both drugs induced a pronounced dCTP decrease that was rapid during the initial 45 min, followed by a slower plateauing phase of dCTP depletion. The major effect of each drug on dATP and dGTP pools was seen within the first 30 min of incubation; dTTP was the least affected of all dNTPs.

The differential effect on ribonucleotide reduction of dFdC

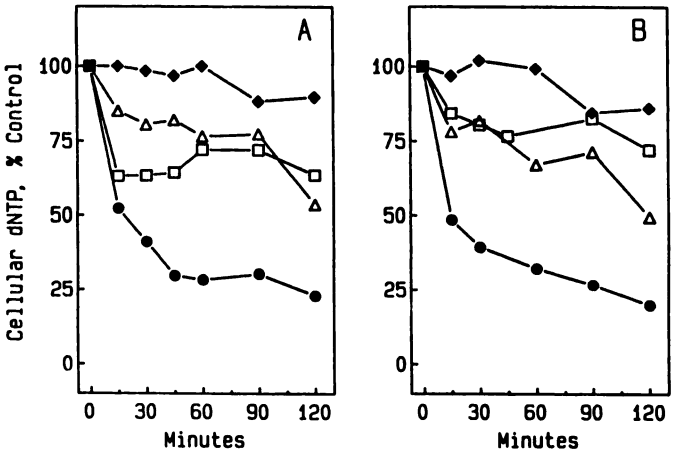

Fig. 4. Effect of dFdC and hydroxyurea on dNTP pools in CEM cells. Cells were exposed to 0.1 μM dFdC (A) or 5 mM hydroxyurea (B) over the indicated intervals and dNTPs were extracted with $HClO_4$ and quantitated as described in Experimental Procedures. The data are representative of duplicate experiments. ●, dCTP; ♦, dTTP; △, dATP; □, dGTP.

and hydroxyurea, relative to ara-C, was characterized further by pulse treatment with [^{3}H]Cyd at the end of a 2-hr drug exposure (Table 1). The incorporation of [^{3}H]Cyd into the dNTP pool and into DNA was followed as a measure of ribonucleotide reduction activity *in situ*. Nearly complete inhibition of [^{3}H]Cyd incorporation into dCTP and into DNA (96.6% and 96.8%, respectively) was achieved by 0.10 μM dFdC. Although 0.10 μM ara-C reduced the incorporation of [^{3}H]Cyd into DNA to a similar extent (93.5%), incorporation into the dCTP pool was only inhibited to 60% of controls. The total amount of radioactivity incorporated into the combined compartments of dCTP and DNA was 8.3-fold less after 0.1 μM dFdC than after 0.1 μM ara-C. Not only was dFdC more potent than ara-C at decreasing the metabolism of [^{3}H]Cyd to dCTP, but the pattern of inhibition was different. Higher concentrations (1.0 μM) of ara-C did not substantially augment this action, whereas dFdC-induced inhibition became nearly complete. After exposure of cells to 5 mM hydroxyurea, no radioactivity was detected in the dCTP pool, and [^{3}H]Cyd incorporation into DNA was reduced to 8.9% of controls. The release of ^{3}H was almost completely

TABLE 1

Effect of dFdC, ara-C, and hydroxyurea on the *in situ* activity of ribonucleotide reductase

CEM cells were incubated for 2 hr with the indicated concentrations of dFdC, ara-C, or hydroxyurea. At the end of the incubation time, cells were pulsed with [5-^{3}H]Cyd for 10 min and the amount of ^{3}H incorporated into the dCTP pool and into DNA was quantitated, as described in Experimental Procedures. Incorporation of ^{3}H into ^3H_2O was determined by a 30-min exposure to [5-^{3}H]Cyd, which was started 30 min before the end of drug incubation. Control incorporation was: dCTP, 11,240 dpm/5 × 10^7 cells; DNA, 15,940 dpm/5 × 10^7 cells; ^3H_2O, 16,843 dpm/5 × 10^4 cells. The results are the average ± coefficient of variation of three experiments.

	[5-^{3}H]Cyd incorporation		
	dCTP	DNA	^3H_2O release
	% of control		
Control	100 ± 5	100 ± 1	100 ± 12
dFdC, 0.01 μM	42.3 ± 7.1	39.0 ± 2	3.9 ± 2.2
dFdC, 0.1 μM	3.4 ± 3.6	3.2 ± 0.6	0.6 ± 0.7
dFdC, 1.0 μM	0.7 ± 0.9	4.7 ± 1.2	0
Ara-C, 0.01 μM	60.9 ± 4.1	22.0 ± 0.7	6.6 ± 0.9
Ara-C, 0.1 μM	40.3 ± 6.2	6.5 ± 2.9	4.0 ± 3.2
Ara-C, 1.0 μM	33.3 ± 2.4	2.6 ± 0.3	0
Hydroxyurea, 5 mM	0	8.9 ± 0.1	0

TABLE 2

Effect of dFdC and hydroxyurea on ribonucleotide reduction *in situ*

CEM cells were treated for 2 hr with either dFdC or hydroxyurea before pulse incubation for 10 min with ^{3}H-ribonucleoside. Accumulation of radioactivity into dNTPs and DNA was quantitated as described in Experimental Procedures. Control incorporation into each dNTP and DNA (dpm/3 × 10^7 cells), respectively, was: uridine, 18,780 (into dTTP) and 24,561; adenosine, 21,724 (into dATP) and 24,626; and guanosine, 1,222 (into dGTP) and 4,312. Results are the average of two experiments.

Nucleoside	Treatment	Incorporation	
		dNTP	DNA
		% of control	
[6-^{3}H]Uridine	0.1 μM dFdC	5	16
	1.0 μM dFdC	2	19
	5 mM Hydroxyurea	14	17
[2-^{3}H]Adenosine	0.1 μM dFdC	43	9
	1.0 μM dFdC	21	6
	5 mM Hydroxyurea	18	9
[8-^{3}H]Guanosine	0.1 μM dFdC	19	5
	1.0 μM dFdC	20	4
	5 mM Hydroxyurea	31	5

inhibited by all three drugs, suggesting that flux through ribonucleotide reduction, dCMP deaminase, and thymidylate synthase was particularly sensitive to inhibition of DNA synthesis. Thus, the effects of dFdC and the ribonucleoside diphosphate reductase inhibitor hydroxyurea were similar, in that Cyd incorporation into dCTP was blocked. In contrast, ara-C, an inhibitor of DNA synthesis, permitted significant accumulation of [^{3}H]Cyd in dCTP.

This approach was extended to evaluate the effects of dFdC on the reduction of other ribonucleosides (Table 2). The accumulation of radioactivity from [^{3}H]uridine into dTTP was as severely inhibited as Cyd accumulation in dCTP (Table 1), although somewhat less inhibition was evident in DNA. Incorporation of adenosine and guanosine into dATP and dGTP, respectively, was also inhibited, but not to the extent of Cyd and uridine reduction. Nevertheless, incorporation of the purines into DNA was curtailed by >90%. Hydroxyurea had an effect similar to that of dFdC on the reduction of each ribonucleoside and incorporation into DNA.

The kinetics of *in situ* inhibition of ribonucleotide reduction were studied in cells exposed to 0.1 μM [^{14}C]dFdC over 120 min (Fig. 5). During this incubation interval, dFdCDP accumulated to 1.3 μM, whereas dFdCTP reached 44 μM (not shown). Analogous to the kinetics of the cellular dCTP concentration (Fig. 4A), a rapid initial decline of [^{3}H]Cyd incorporation into dCTP and DNA was noted. The dFdC-induced inhibition of [^{3}H]Cyd incorporation into DNA occurred more slowly than the inhibition of [^{3}H]Cyd incorporation into the dCTP pool, suggesting that inhibition of DNA synthesis in dFdC-treated cells may be partially mediated by dCTP depletion (not shown). When the total incorporation of [^{3}H]Cyd into dCTP and DNA was analyzed as an estimate of *in situ* ribonucleotide reduction, a 50% inhibition occurred after about 20 min at cellular dFdCDP concentration of 0.3 μM (Fig. 5).

The activity of partially purified ribonucleoside diphosphate reductase was inhibited by 50% in the presence of 4 μM dFdCDP (Fig. 6A). The inhibition was not augmented by preincubation of dFdCDP with the enzyme preparation for 10 min before addition of CDP (not shown). Parallel studies demonstrated that dFdCTP was much less inhibitory to CDP reduction at this concentration (Fig. 6A). Inhibition of CDP reduction by

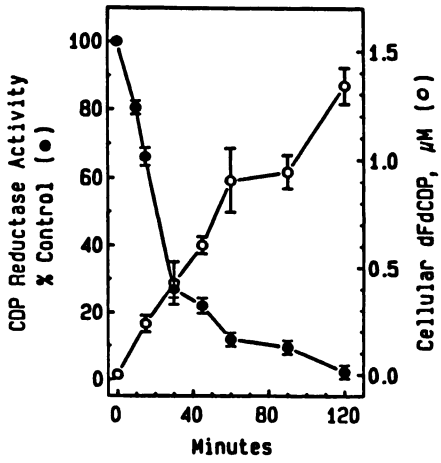

Fig. 5. Relationship between cellular dFdCDP concentrations and *in situ* CDP reductase activity. CEM cells were incubated with 0.10 μM [2-^{14}C] dFdC (2.2 Ci/ml) for the indicated times. Ten minutes before the end of each incubation, cells were pulsed for 10 min with [5-^{3}H]Cyd. *In situ* reductase activity was quantitated as the sum of incorporation of [5-^{3}H] Cyd into dCTP and DNA, as described in Experimental Procedures. Control incorporation was: dCTP, 7,026 ± 422 dpm/5 × 10^7 cells; DNA, 10,820 ± 650 dpm/5 × 10^7 cells. The results are the mean ± standard deviation of duplicate experiments.

hydroxyurea was inhibited with an IC_{50} of about 1 mM, but even 10 mM did not give complete inhibition (Fig. 6B).

Discussion

A significant reduction in cellular dCTP, dTTP, dATP, and dGTP pools was observed in CEM cells treated with cytotoxic concentrations of dFdC. This decrease occurred in a concentration-dependent manner (Fig. 2) and could not be affected by 2′,2′-difluorodeoxyuridine, which is readily produced in biologic systems by dFdC deamination (3). Because dFdC treatment is also associated with inhibition of DNA synthesis (2, 3, 10), we hypothesized that dNTP pool depletion may be induced either by a direct inhibition of ribonucleotide reduction or by an indirect effect that is secondary to the interruption of DNA synthesis. To distinguish between these alternatives, the effects of dFdC were compared with those of the ribonucleotide reductase inhibitor hydroxyurea and with the action of ara-C, the

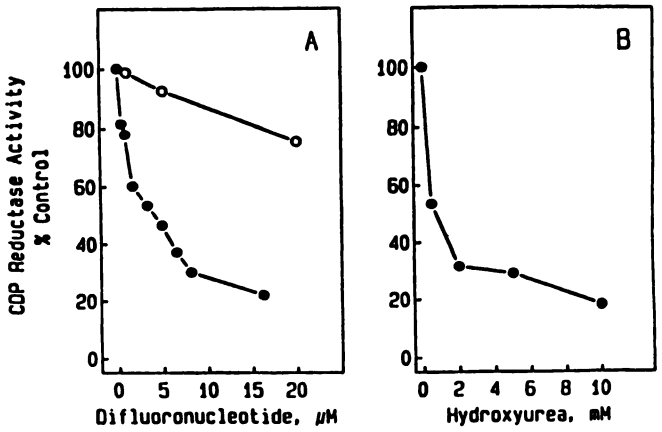

Fig. 6. Inhibition of CDP reduction by dFdCDP, dFdCTP, and hydroxyurea. Ribonucleotide reductase was partially purified from CEM cells. The effects of the indicated concentrations of dFdCDP (●) and dFdCTP (○) (A) or hydroxyurea (B) on CDP reduction were determined as described in Experimental Procedures.

major activity of which is directed at DNA synthesis by inhibition of DNA polymerization.

These studies demonstrated that ara-C (Fig. 3) had a markedly different effect on dNTP metabolism than did dFdC (Fig. 2) or hydroxyurea (Fig. 4). Both dFdC and hydroxyurea caused a major decrease in the dCTP pool and lesser but significant reductions in the dATP and dGTP pools. In contrast, ara-C essentially had no effect on the dCTP pool, and an increase in the dATP, dTTP, and dGTP pools was noted. This observation may be explained by a blockade of cells in the S phase of the cell cycle (4), which is associated with higher dNTP concentrations. It is likely that, when DNA synthesis is inhibited, *de novo* dNTP synthesis exceeds dNTP consumption, contributing to a net increase in the pools. Thus, the observed perturbations in dNTP pools during incubation with dFdC are not explained satisfactorily as an effect arising secondary to inhibition of DNA synthesis, which is observed in dFdC-treated cells (2, 3, 10).

Inhibition of ribonucleotide reduction by hydroxyurea paralleled the dFdC-induced dNTP pool changes (Fig. 4). This observation is consistent with the hypothesis that nucleotides of dFdC directly induce an inhibition of ribonucleotide reduction and, thus, may cause the dNTP pool decrease. Pulse treatment of CEM cells with [^{3}H]Cyd showed that dFdC and ara-C similarly blocked the incorporation of radiolabel into DNA (Table 1). By contrast, 0.1 μM dFdC inhibited the incorporation of radiolabel into the dCTP pool by over 96%, whereas 0.1 μM ara-C reduced [^{3}H]Cyd conversion to dCTP by only 60%. This indicates an inhibitory action of dFdC proximal to DNA synthesis that was substantially less in ara-C-treated cells. The effect of hydroxyurea on the *in situ* activity of ribonucleotide reduction of all ribonucleosides was, however, similar to that of dFdC. These results are again consistent with a direct inhibition of ribonucleotide reduction by metabolites of dFdC.

Inhibition of ribonucleotide reduction *in situ* occurred at low intracellular concentrations of dFdC metabolites; the cellular dFdCDP concentration was 0.3 μM when the incorporation of [^{3}H]Cyd into dCTP was inhibited by 50% (Fig. 5). The results in Fig. 6 are consistent with the hypothesis that dFdCDP acts as an effective inhibitor of ribonucleoside diphosphate reductase. The potency of inhibition is similar to that noted by others in a preliminary report (21), although the IC_{50} of dFdCDP for the partially purified enzyme was greater than that determined *in situ* (Fig. 6A). It has been suggested that the efficient coupling of ribonucleotide reductase with DNA synthesis observed in intact cells is disrupted when cells are lysed (22). If so, this may provide an explanation for the apparent discrepancy in the potency of dFdCDP for the inhibition of ribonucleotide reductase when measured by these two approaches.

There is precedent for inhibition of ribonucleoside diphosphate reductase by dFdCDP, in that other nucleoside diphosphates substituted at the 2′-carbon with halogens or an azido group have been found to be effective inhibitors of the ribonucleoside diphosphate-reducing enzyme (23–25). These compounds are mechanism-based inactivators (26); however, it is not known whether the inhibition of ribonucleoside diphosphate reductase by dFdCDP is irreversible. Cellular elimination of dFdC nucleotides is relatively slow (2, 3) and, as a result, it has not been possible to determine whether the delayed repletion of cellular dNTP pools following washout of dFdC was due to irreversible inhibition of the enzyme or prolonged retention of dFdCDP. It should be noted that the mechanism of dFdCDP-mediated inhibition of ribonucleoside diphosphate reductase is likely to be different from that of the clinically useful purine nucleoside analogues arabinosyl-2-fluoroadenine (27) and 2-chlorodeoxyadenosine (28). The triphosphates of these drugs are thought to act as alternative inhibitors at the dATP binding site of ribonucleoside diphosphate reductase (29, 30), whereas no inhibitory activity has been demonstrated for the respective nucleoside diphosphates.

Our understanding of the metabolism of dFdC to nucleotides and their actions suggests that inhibition of ribonucleoside diphosphate reductase may, by several mechanisms, have a self-potentiating effect on the activity of the drug. First, the activity of dCyd kinase, the enzyme required for phosphorylation of dFdC, is tightly regulated by dCTP (8, 9). A decrease in the cellular dCTP pool is likely to lead to an increased rate of dFdC phosphorylation (7), as is the case for other nucleosides that require this enzyme (31, 32). Second, dFdC monophosphate is a substrate for dCMP deaminase and this enzyme may be critical for the elimination of dFdC nucleotides, particularly at low cellular concentrations (33). dCTP is required as an activator of dCMP deaminase; reduction of cellular dCTP levels would be expected to decrease enzyme activity, thus favoring maintenance of the dFdC nucleotide concentrations. Third, dFdCTP competes with dCTP for incorporation into DNA by DNA polymerase α and δ (10). A lowered dCTP concentration would favor incorporation of the analogue, an action that is associated with cytotoxicity. Thus, inhibition of ribonucleoside diphosphate reductase by dFdCDP may trigger several actions that would lead to self-potentiation of the activity of dFdC.

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