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ORIGINAL ARTICLE

Leo J. Ostruszka · Donna S. Shewach

The role of DNA synthesis inhibition in the cytotoxicity of 2′,2′-difluoro-2′-deoxycytidine

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Abstract *Purpose*: Cytotoxicity from the anticancer drug 2′,2′-difluoro-2′-deoxycytidine (dFdCyd) has been correlated with its incorporation into DNA. However, cytotoxicity may also result from inhibition of DNA synthesis, due to either (1) dFdCyd diphosphate-mediated inhibition of ribonucleotide reductase, or (2) direct inhibition of DNA polymerases by the 5′-triphosphate of dFdCyd (dFdCTP). To elucidate the role of DNA synthesis inhibition in the cytotoxicity of dFdCyd, we compared dFdCyd to hydroxyurea (HU), a ribonucleotide reductase inhibitor, and aphidicolin, an inhibitor of DNA polymerases, in the U251 and D54 human glioblastoma cell lines. *Methods*: Sensitivity to dFdCyd, HU, and aphidicolin were determined using a colony formation assay. The effects of these drugs on DNA synthesis were measured by dual parameter flow cytometry, while the effects on nucleotide pool levels were analyzed by high-performance liquid chromatography. *Results*: HU and aphidicolin elicited substantially less cytotoxicity than the multi-log killing with dFdCyd. When used at equitoxic concentrations (24-h IC_{50} values), dFdCyd and HU decreased purine dNTP pools primarily, but dFdCyd was less effective than HU. dFdCyd had decreased dATP by about 80% after 4–12 h, and required 8–24 h to decrease DNA synthesis by 50%. In contrast, HU rapidly depleted dATP by >98% within 2 h, which resulted in >90% inhibition of DNA synthesis. Aphidicolin at a concentration similar to its K_i values for DNA polymerases (1 μ*M*) decreased DNA synthesis by >70% within 2 h. However, this decreased cell survival by only 10% (U251 cells) and 40% (D54 cells). *Conclusions*: These results demonstrate that HU and aphidicolin produced a more rapid and profound inhibition of DNA synthesis than dFdCyd, but resulted in significantly less cytotoxicity. This suggests that inhibition of DNA synthesis accounted for less than one log of the multi-log cytotoxicity observed with dFdCyd, whereas incorporation of dFdCTP into DNA is a more lethal event.

Keywords dFdCyd · DNA synthesis · dNTP · Ribonucleotide reductase

L. J. Ostruszka · D. S. Shewach (✉)
Department of Pharmacology,
University of Michigan Medical Center,
1310 E. Catherine St., Ann Arbor, MI 48109-0504, USA
E-mail: dshewach@umich.edu
Tel.: +1-734-7635810
Fax: +1-734-7633438

Introduction

Many anticancer nucleoside analogs, such as cytosine arabinoside, cladribine, 2′,2′-difluoro-2′-deoxycytidine (dFdCyd), and fludarabine, share a common mechanism of cytotoxicity that targets DNA synthesis [35]. After phosphorylation to a 5′-triphosphate derivative, nucleoside analogs can interfere with DNA synthesis by: (1) direct inhibition of DNA polymerases, and (2) slowing of DNA elongation or chain termination after insertion into DNA [5, 10, 15, 26, 32, 38, 50]. For some nucleoside analogs, the incorporation of their 5′-triphosphates into DNA has been correlated with their cytotoxicity [21, 23, 41, 51].

In addition to direct effects on DNA synthesis, some nucleoside derivatives have been shown to inhibit ribonucleotide reductase, but the role this plays in overall cytotoxicity is not well understood [4, 14, 16, 28, 29, 33, 34, 46, 50]. Ribonucleotide reductase is responsible for the de novo biosynthesis of deoxyribonucleoside triphosphates (dNTPs) necessary for DNA synthesis in mammalian cells [22]. Inhibition of ribonucleotide reductase results in selective decreases in endogenous dNTPs, depending on the cell line [16, 40, 46]. An important result of the decrease in endogenous dNTP pools is that it can lessen competition against the nucleotide analog for incorporation into DNA, thus leading to greater cytotoxicity. This process is known as ''self-potentiation,'' and is likely a key factor in the cytotoxicity of several clinically active nucleoside analogs such as fludarabine, cladribine, and dFdCyd [6, 14, 17, 36].

Other anticancer drugs, such as hydroxyurea (HU), 5-fluoro-2′-deoxyuridine, methotrexate, and *N*-phosphonacetyl-L-aspartate (PALA), elicit cytotoxicity primarily through the alteration of dNTP pools [1, 9, 12, 37]. As expected, these drugs also inhibit DNA synthesis. The role of decreased dNTP pools has become more interesting with the finding that nucleoside analogs and other drugs that inhibit ribonucleotide reductase can enhance the effects of ionizing radiation [13, 27, 39, 45, 46, 47, 48, 49]. The clinically important drugs HU and dFdCyd have the ability to inhibit ribonucleotide reductase, either directly as a free radical scavenger [22], or mechanistically as the 5′-diphosphate derivative of dFdCyd (dFdCDP) [2]. Previous studies in this and other laboratories have associated ribonucleotide reductase inhibition with radiosensitization by both agents [46, 47]. In contrast, the cytotoxicity by dFdCyd has been correlated with its incorporation into DNA [21]. However, incorporation into DNA and inhibition of ribonucleotide reductase are interrelated processes, since the level of the 5′-triphosphate of dFdCyd (dFdCTP) determines the extent of DNA incorporation, and the amount of dFdCDP is related to the amount of dFdCTP [15, 21]. This makes it difficult to distinguish the impact of each pathway individually on cytotoxicity.

In solid tumor cells, it is particularly important to evaluate the role of ribonucleotide reductase inhibition in the action of dFdCyd since non-cytotoxic concentrations can produce profound decreases in dATP [25, 46]. Therefore, we compared the effects of dFdCyd on DNA synthesis with the ribonucleotide reductase inhibitor HU, and with aphidicolin, which directly inhibits replicative DNA polymerases without affecting the dNTP pools [43]. The results demonstrated that perturbation of dNTP pools or direct polymerase inhibition contributes primarily to the inhibition of DNA synthesis without a major effect on cytotoxicity. A preliminary account of a portion of these results has been reported in abstract form [30].

Materials and methods

Chemicals

dFdCyd and dFdCTP were synthesized and generously provided by Eli Lilly and Co. (Indianapolis, Ind.). RNase A was purchased from Boehringer Mannheim (Indianapolis, Ind.). HU, propidium iodide, and nucleotide standards were all purchased from Sigma Chemical Company (St. Louis, Mo.).

Cell culture

The human glioblastoma cell lines U251 and D54 were cultured in RPMI 1640 medium supplemented with 10% calf serum (GIBCO, Grand Island, N.Y.) and L-glutamine (Fisher Scientific, Pittsburgh, Pa.). Cells were maintained in exponential growth in a humidified atmosphere containing 5% CO_2 at 37°C.

Cytotoxicity assays

Cytotoxicity was measured using a standard colony formation assay. Cell culture flasks (25 cm^2) were plated with between 3×10^5 and 6×10^5 cells a minimum of 36 h prior to addition of drug. Exponentially growing cells were incubated with drug for 24 h. At the conclusion of the drug incubation period, cells were washed with Dulbecco's PBS (phosphate-buffered saline), trypsinized, and counted using a Coulter (Hialeah, Fl.) electronic particle counter. Approximately 100 viable cells were plated into each 35-mm diameter well of a six-well culture dish and allowed to grow in the absence of drug for 10–14 days. At that time, the resulting colonies were fixed using a methanol/glacial acetic acid solution (3:1 v/v) and stained with 0.4% crystal violet. Colonies of more than 30 cells were counted and survival was determined as a fraction of plating efficiency of untreated control cells.

Analysis of dNTP pools

Cells were incubated with drug for 1–24 h, harvested by trypsinization, and counted. The nucleotides were extracted with ice-cold 0.4 *N* perchloric acid and neutralized with 10 *N* potassium hydroxyide. The majority of the ribonucleotides were removed from the deoxyribonucleotides by elution over a boronate affinity column as described previously [44]. Deoxyribonucleotides were separated and quantitated by strong anion exchange high-performance liquid chromatography (HPLC) using a Waters Alliance (Milford, Mass.) gradient system equipped with a model 996 photodiode array detector and controlled by Millennium 2010 software. Before injection, each sample was centrifuged at 16,000 *g* for 2 min and acidified to pH 2.8. Samples were then injected onto a 5-μm Partisphere 4.6×250 mm SAX column (Whatman Scientific, Hillsboro, Ore.) and eluted with a linear gradient of ammonium phosphate buffer ranging from 0.15 *M* (pH 2.8) to 0.6 *M* (pH 2.8–3.8) at a flow rate of 2 ml/min. Nucleotides were identified and quantitated by comparison to a known amount of authentic standards using their characteristic absorbance spectra over the range 200–350 nm.

DNA synthesis

Flow cytometric analysis was performed as previously described [31]. Briefly, at the conclusion of the drug incubation, cells were pulse-labeled with 30 μ*M* bromodeoxyuridine (BrdUrd) for 15 min, and then harvested by trypsinization, counted, and washed with PBS. Cells were then fixed in cold 70% ethanol at a concentration of 1×10^7 cells/ml, with samples not to exceed a total of 3×10^7 cells. Fixed cells were stored at 4°C for up to 10 days. Within 6 h prior to flow cytometric analysis, fixed cells were washed with PBS and resuspended in 1 ml PBS containing 0.5 mg/ml RNase A and incubated for 30 min at 37°C. Cells were then washed with PBS, resuspended in 1 ml 0.1 *N* hydrochloric acid containing 0.7% Triton X-100, and incubated for 10 min on ice. This was followed by another PBS wash, resuspension in 1 ml sterile HPLC grade water, and incubation at 95°C for 15 min. The samples were immediately transferred to an ice-water bath for an additional 15 min. Cells were then washed with PBS containing 0.5% Tween 20. PBS (100 μl) containing 0.5% Tween 20 and 5% calf serum was added to each cell pellet followed by the addition of 100 μl anti-BrdUrd mouse IgG1 antibody (1:100 dilution, BD PharMingen, San Diego, Calif.) and incubation for 30 min at room temperature. After centrifugation, 150 μl FITC-conjugated goat anti-mouse IgG antibody (1:20–35 dilution, Sigma Chemical Company, St. Louis, Mo.) was added to the pellet, mixed gently, and incubated for 30 min at room temperature. Samples were centrifuged and resuspended in 0.5 ml 18 μg/ml propidium iodide. Trout erythrocyte nuclei (Biosure, Grass Valley, Calif.) were added as an internal standard. Treated cells were placed in the dark a minimum of 30 min prior to cell cycle analysis using a Coulter EPICS Elite ESP flow cytometer. Cell cycle data were further analyzed using

WinMDI software (version 2.8.8) provided by Joseph Trotter of the Scripps Research Institute. Percent DNA synthesis was determined by the change in the mean fluorescence intensity of S-phase-specific BrdUrd incorporation in drug-treated in relation to control cells. The Quadstat gate to separate BrdUrd-negative cells and BrdUrd-positive cells was based on a negative fluorescent antibody control.

Results

Cytotoxicity

dFdCyd was significantly more potent than HU, with 24-h IC_{50} values 7,500–120,000 times lower in the U251 and D54 human glioblastoma cell lines (25–80 n*M* and 0.6–3 m*M* for dFdCyd and HU, respectively; Fig. 1). Using concentrations of HU threefold greater than the 24-h IC_{50} decreased cell survival to about 33% in U251 and about 23% in D54 cells, and a tenfold increase killed about 1.5 logs of D54 and 76% of U251 cells. In contrast, increasing the dFdCyd concentration three- and tenfold over the 24-h IC_{50} produced 1–3 logs of cell death in U251 and D54 cells. A similar difference in potency was also observed between dFdCyd and HU in HT-29 human colon carcinoma cells (data not shown).

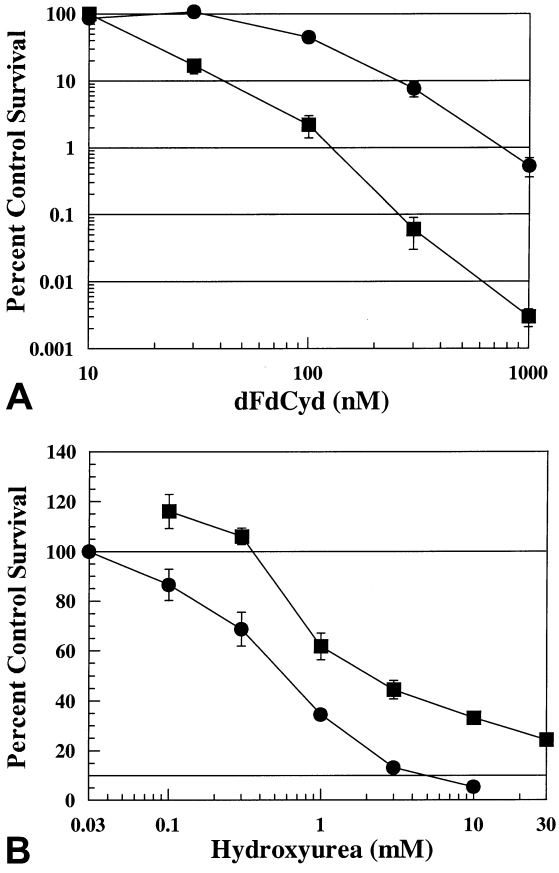

Fig. 1A, B Comparison of cytotoxicity of dFdCyd (**A**) and HU (**B**) in human glioblastoma cells. U251 (■) or D54 (●) cells were treated with drug for 24 h and assayed for clonogenic survival. The values shown are the means ± SE of triplicate determinations from a single experiment. Experiments were repeated at least three times

Effects on purine dNTP pools

In solid tumor cells treated with ribonucleotide reductase inhibitors, dATP is depleted more rapidly and to a greater extent compared to the other dNTPs [25, 46]. Similarly, in the studies presented here both dFdCyd and HU produced decreases primarily in the dATP pool. As shown in Table 1, dFdCyd (24-h IC_{50}) decreased dATP levels within 2 h after drug addition by $< 50\%$ in U251 and D54 cells, and dATP continued to decrease throughout the incubation period reaching its lowest value by 24 h. Significantly higher concentrations of dFdCyd (0.4 μ*M* in U251, 4.0 μ*M* in D54) were required to deplete dATP in both cell lines to less than 5% of control levels within 4 h (data not shown). In contrast, the 24-h IC_{50} doses of HU

Table 1 Perturbation of dNTP pools by inhibitors of ribonucleotide reductase in U251 and D54 cells. Exponentially growing cells were treated with either 25 n*M* or 80 n*M* dFdCyd (24-h IC_{50} values) or 0.6 m*M* or 3 m*M* HU (24-h IC_{50} values). Nucleotides were extracted with perchloric acid, eluted over a boronate affinity column to reduce ribonucleotides, and separated and quantitated using HPLC. Data represent the means ± SD of one to four determinations. 100% levels of dNTPs in U251 cells are (nmol/10^7 cells): dATP 0.171, dGTP 0.099, dCTP 0.412, and dTTP 0.748; and in D54 cells are dATP 0.341, dGTP 0.105, dCTP 0.479, and dTTP 1.384

Inhibitor	Time (h)	dATP (%)		dGTP (%)		dCTP (%)		dTTP (%)	
		U251	D54	U251	D54	U251	D54	U251	D54
dFdCyd	0	100	100	100	100	100	100	100	100
	2	53.6 ± 9.6	87.2 ± 7.0	87.4 ± 17.9	118.5 ± 42.2	73.3 ± 12.5	102.9 ± 5.3	74.9 ± 19.7	90.6 ± 14.9
	4	19.2 ± 11.1	60.5 ± 11.5	61.4 ± 11.1	75.2 ± 16.3	64.6 ± 1.0	105.7	86.2 ± 17.2	79.8 ± 16.1
	12	6.9 ± 5.9	21.6 ± 9.0	54.1 ± 20.9	84.5 ± 10.7	117.6 ± 18.0	88.7 ± 7.0	85.8 ± 15.6	68.6 ± 6.8
	24	0.7 ± 0.6	15.0 ± 10.3	44.5 ± 38.8	85.7 ± 25.4	149.4 ± 83.4	130.6 ± 1.1	120.5 ± 61.4	79.8 ± 21.0
HU	0	100	100	100	100	100	100	100	100
	2	0.9 ± 1.3	1.7 ± 2.0	23.3 ± 8.4	39.9 ± 16.9	93.3 ± 1.5	74.7 ± 3.1	130.8 ± 40.2	109.4 ± 14.4
	4	1.6 ± 1.8	1.7 ± 2.1	24.4 ± 10.0	40.4 ± 14.3	132.3 ± 2.8	84.8 ± 7.7	140.3 ± 5.1	111.7 ± 13.3
	12	0.7 ± 0.9	1.2 ± 2.1	25.5 ± 2.7	82.9 ± 23.1	186.7 ± 3.1	142.2 ± 12.4	152.7 ± 35.2	163.1 ± 23.9
	24	0.7 ± 0.9	8.4 ± 5.5	38.0 ± 6.7	142.4 ± 43.1	177.4 ± 10.4	261.6 ± 31.5	175.7 ± 31.4	213.8 ± 62.8

rapidly decreased dATP in both cell lines, achieving values of less than 2% of control within 2 h of drug addition in both cell lines. The dGTP pool was also decreased by both drugs in the two cell lines, although to a lesser extent compared to dATP. Upon closer inspection, it was noted that the rate and extent of dGTP depletion was different between the two agents. dFdCyd required 4–12 h to deplete dGTP to its lowest level (54% of control in U251 cells and 75% in D54 cells). In contrast, HU depleted dGTP to 23% of control in U251 cells and to 40% in D54 cells within 2 h of drug addition. However, by 12 h, dGTP in D54 cells had recovered to 83% of control, and by 24 h had increased further to 142% of control. In U251 cells, the recovery of dGTP in the presence of HU was minimal with only 38% of control levels present after 24 h. These data also highlight the fact that, although IC_{50} doses were used for both drugs, the effects on the purine dNTP pools were more marked in the U251 than in the D54 cells.

Effects on pyrimidine dNTP pools

After treatment with either dFdCyd or HU, the pyrimidine dNTP pools exhibited a markedly different pattern from the purine dNTP pools. U251 and D54 cells showed little or no changes in dCTP during the first 4 h after addition of dFdCyd or HU (Table 1). However, by the end of 24 h, dCTP levels in both cell lines had increased to 131%–262% of their initial values. Treatment with dFdCyd only produced marginal changes in the dTTP pool in the two cell lines during the 24 h incubation (Table 1). In contrast, HU treatment increased dTTP levels within 2 h, and dTTP continued to increase achieving final levels of 176% and 214% of control values in U251 and D54 cells, respectively, after 24 h. It should be noted that the cell numbers did not decrease during drug treatment. This indicated that the increase in pyrimidines observed was not the result of salvage from lysed cells.

Accumulation of dFdCTP

During a 24-h incubation with equitoxic doses of dFdCyd (24-h IC_{50}), accumulation of dFdCTP was similar in the two cell lines (Fig. 2). dFdCTP levels increased during the first 12 h, when they appeared to reach a plateau with concentrations of 0.06 nmol/10^7 cells in U251 cells and 0.05 nmol/10^7 cells in D54 cells. We have previously reported that D54 cells incorporate 0.57 nmol/10^7 cells of dFdCMP and U251 cells incorporate 0.40 nmol/10^7 cells of dFdCMP under these conditions [31]. Thus, although the IC_{50} doses for these cell lines differed by more than threefold, the amount of dFdCTP and the level of its incorporation into DNA were similar under equitoxic conditions.

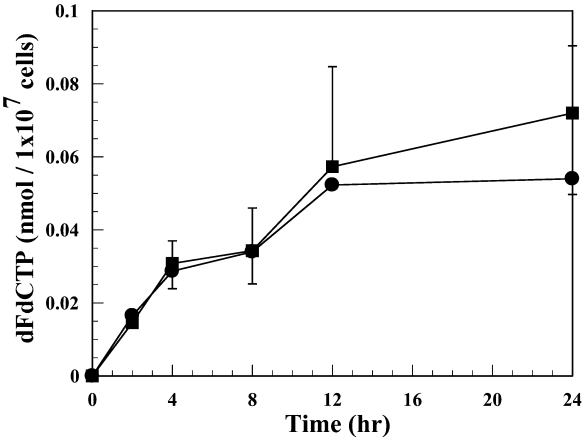

Fig. 2 Accumulation of dFdCTP in U251 and D54 human glioblastoma cells. Exponentially growing U251 (■) or D54 (●) cells were treated with 25 nM and 80 nM dFdCyd (24-h IC_{50} values for U251 and D54, respectively). Nucleotides were extracted with perchloric acid and separated using HPLC. The values shown are the means ± SE from two to four determinations (*error bars* are shown only in the up direction or down direction for U251 and D54 cells, respectively)

Inhibition of DNA synthesis

In order to determine whether these equitoxic conditions produced similar effects on DNA synthesis, we compared the ability of each drug to inhibit DNA synthesis as measured by BrdUrd incorporation and flow cytometric analysis. S-phase cells were identified by their ability to incorporate BrdUrd, and the mean fluorescence intensity of drug-treated cells compared to control cells yielded the percent control DNA synthesis values. During a 24-h IC_{50} incubation with dFdCyd (25 n*M* and 80 n*M* for U251 and D54, respectively), neither cell line showed a decrease in DNA synthesis within the first 2 h (Fig. 3A). After 4 h, DNA synthesis had decreased to 61% in U251 cells, whereas D54 cells showed no inhibition. DNA synthesis decreased in both cell lines between 8 and 24 h, at which time it achieved its lowest levels (18% in U251 cells and 48% in D54 cells). Measurement of DNA synthesis using [^{3}H]thymidine incorporation produced similar results (data not shown).

In contrast to dFdCyd, HU (24-h IC_{50}) produced a more rapid inhibition of DNA synthesis. Within 2 h of the addition of HU, DNA synthesis was reduced to $<2\%$ of control activity in U251 cells and to $<9\%$ of control in D54 cells (Fig. 3B). In U251 cells, DNA synthesis was inhibited by $>95\%$ throughout the entire 24-h HU exposure. In D54 cells, the level of DNA synthesis had begun to recover by 8 h and had returned to 30% of control by 24 h. These effects on DNA synthesis appear to reflect the effects on the purine dNTP pools, which were depleted faster and to a greater extent with HU than with dFdCyd.

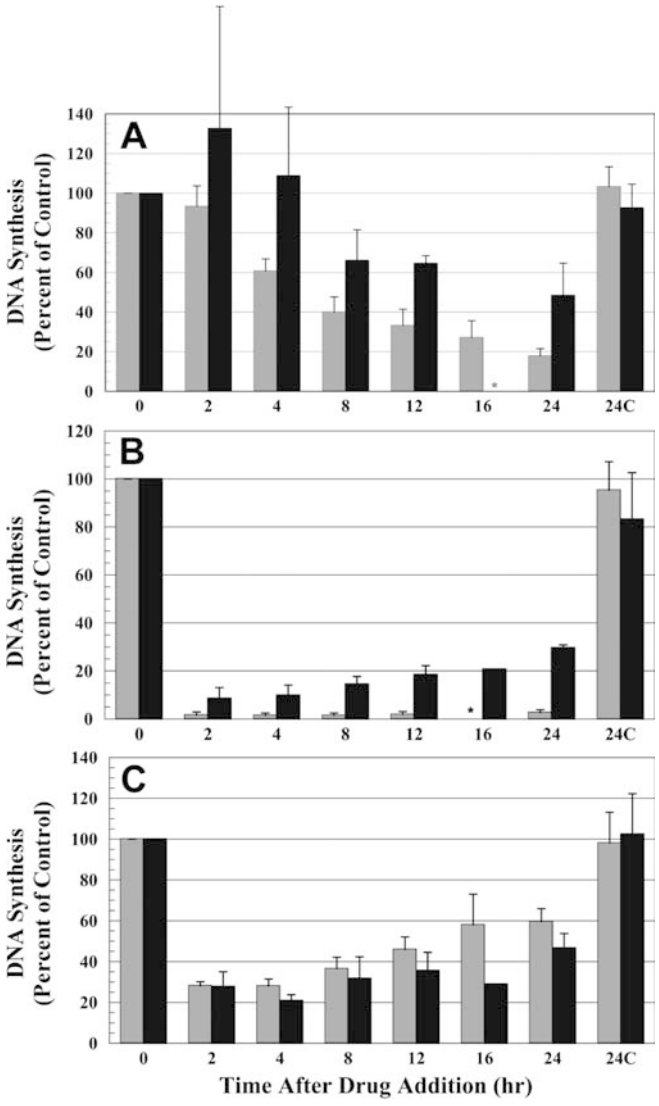

Fig. 3 Effects of dFdCyd (**A**), HU (**B**), and aphidicolin (**C**) on DNA synthesis. Exponentially growing U251 cells (*gray bars*) or D54 cells (*black bars*) were treated with dFdCyd or HU at their respective IC_{50} values, or 1 μ*M* aphidicolin. At the indicated times, cells were incubated with 30 μ*M* BrdUrd for 15 min prior to harvest. Cells were prepared as described in Materials and methods and the BrdUrd content was measured in terms of FITC-conjugated BrdUrd fluorescence. DNA synthesis inhibition was determined by the change in mean fluorescence in BrdUrd-incorporating cells. The data are presented as means ± SE of three determinations (*not done)

Effects of aphidicolin

Aphidicolin inhibits replicative DNA polymerases without any effect on ribonucleotide reductase [43]. Thus, aphidicolin was used to evaluate the effect of DNA polymerase inhibition alone in the absence of fraudulent nucleotide incorporation or ribonucleotide reductase inhibition. Both cell lines were treated with 1 μ*M* aphidicolin for 24 h (U251 IC_{10}, D54 IC_{40}) and DNA synthesis was measured. DNA synthesis decreased rapidly in both cell lines. Within 1 h, DNA synthesis decreased to 40% and 25% of control activity in U251 and D54 cells, respectively (data not shown). DNA synthesis continued to decrease throughout the first 4 h with minimum levels of 28% in U251 and 21% in D54 cells (Fig. 3C). By 24 h, U251 cells had recovered to 60% of control DNA synthesis and D54 cells had recovered to 47% of control.

Discussion

Typically, the ability of a nucleoside analog to be incorporated into DNA has been associated with its ability to produce cytotoxicity [21, 23, 35]. Incorporation of analogs can lead to inhibition of DNA synthesis by several mechanisms such as chain termination, difficulty extending DNA due to an altered strand conformation, and repetitive attempts to remove the nucleotide from the DNA strand [10, 20, 21, 26, 32, 33, 35, 38, 42, 50]. However, nucleoside analogs such as dFdCyd, cladribine, and fludarabine all have additional effects on DNA synthesis due to their ability to inhibit ribonucleotide reductase [6, 16]. Although the effects that these agents have on dNTP pools and DNA polymerases are well documented, the relative contribution of these effects on DNA synthesis and cytotoxicity is not clear. In the study reported here, we attempted to separate the effects of dFdCTP accumulation and dNTP depletion on DNA synthesis and cytotoxicity by comparing dFdCyd to a pure ribonucleotide reductase inhibitor, HU, and a pure polymerase inhibitor, aphidicolin.

Both cell lines demonstrated marked differences in cytotoxicity and dNTP pool depletion with each drug. At equitoxic concentrations, HU displayed a more rapid and marked effect on dNTP pools than dFdCyd. The increased time required by dFdCyd to affect dNTP pools was most likely due to the requirement of dFdCyd to be phosphorylated in order to inhibit ribonucleotide reductase [2, 16]. In fact, the majority of the purine dNTP depletion occurred during the first 12 h, paralleling the time required to achieve peak dFdCTP levels. Considering that the peak dFdCTP levels in these studies did not exceed 2 μ*M* (see below), that dFdCDP levels are typically 5–10% of the dFdCTP concentration [15], and that 50% inhibition of ribonucleotide reductase activity in situ requires 0.3 μ*M* dFdCDP [16], the slow decrease in dNTPs with dFdCyd in these studies is reasonable.

As found previously in vitro and in vivo [3, 7, 9, 11, 18, 24, 46], inhibition of ribonucleotide reductase in these studies resulted in a selective decrease in the purine dNTP pools with no effect on or an increase in the pyrimidine dNTP pools. Although both dCTP and dTTP were increased by dFdCyd and HU in these studies, it is unlikely that these effects played a major role in the toxicity of these drugs. It has been demonstrated that the increase in dTTP following treatment of 3T6 mouse fibroblasts or V79 Chinese hamster lung cells with HU is due to an increased uptake of

pyrimidine deoxyribonucleosides from the medium [3, 19], which may account for the increase in pyrimidine dNTP pools observed here. In the studies presented here, HU produced a greater increase in dCTP and dTTP than dFdCyd at their IC_{50} values, and this effect increased at higher HU (but not dFdCyd) concentrations (data not shown), yet HU was less toxic. Thus, the increased pyrimidine dNTP pools did not appear to contribute substantially to cytotoxicity.

The results presented here suggest that the level of DNA synthesis primarily reflected purine dNTP pool depletion. The rapid decrease in dATP and dGTP by HU was associated with an almost complete inhibition of DNA synthesis in U251 cells and a 90% reduction in D54 cells. The slower decline in the dATP and dGTP pools produced by dFdCyd paralleled the slow decrease in DNA synthesis. Furthermore, the increase in DNA synthesis observed between 8 and 24 h in D54 cells treated with HU may be due to the marked recovery of dGTP levels at 12–24 h.

It was unlikely that dFdCTP had a substantial effect on DNA polymerases because the peak dFdCTP concentrations of 0.06 and 0.05 nmol/10^7 cells in U251 and D54 cells, respectively (approximately 1.97 μM and 1.03 μM based on average cell volumes), were 45- to 110-fold lower than the apparent K_i values for either polymerase α or ϵ in CEM cells as reported by others [21]. In addition, the levels of dFdCTP in U251 and D54 cells were seven- and tenfold lower, respectively, than the endogenous dCTP present at the beginning of drug treatment, further indicating that this low level of dFdCTP would be a poor competitor with the higher levels of dCTP for DNA polymerases. While the incorporation of dFdCTP into DNA may also contribute to DNA synthesis inhibition, D54 cells incorporate more dFdCTP into DNA (0.57 nmol/10^7 cells) than U251 cells (0.40 nmol/10^7 cells) over 24 h under these conditions [31], yet the level of DNA synthesis at 24 h was more than twofold higher in D54 cells. Taken together, these results indicate that dNTP perturbation played a greater role in DNA synthesis inhibition than the effects of dFdCTP under the conditions utilized here. Heinemann et al. have shown that the recovery of DNA synthesis after washout of dFdCyd correlates with the amount of dFdCTP remaining in CHO cells [15]. However, dNTP pools, which are directly related to dFdCDP and, therefore, indirectly related to dFdCTP, were not measured in that study.

Similar to HU, aphidicolin inhibited DNA synthesis within 2 h of treatment, although the amount of DNA synthesis inhibition was less than that produced by the depletion of dNTPs in response to HU. The degree of inhibition was reasonable considering that the concentration of aphidicolin used was in the reported range of apparent K_i values for DNA polymerases α, δ, and ϵ of 0.2–2.2 μM [8, 43]. The relative level of aphidicolin with respect to the K_i values for DNA polymerases was greater than the comparative values for dFdCTP, yet aphidicolin produced a similar amount of inhibition as a 12–16 h exposure to dFdCyd. This suggests that DNA synthesis inhibition with dFdCyd is the result of the combined effects of dNTP pool depletion and dFdCTP incorporated into DNA, rather than direct inhibition of polymerases by dFdCTP. Despite a more rapid inhibition of DNA synthesis compared to dFdCyd, aphidicolin was only mildly cytotoxic, further supporting the conclusion that DNA synthesis inhibition alone is not a highly cytotoxic event in these cells.

Direct polymerase inhibition produced by aphidicolin and the rapid depletion of dATP and dGTP produced by HU resulted in a profound inhibition of DNA synthesis, yet these drugs produced a maximum of approximately one-log of cell death at high concentrations. dFdCyd has the ability to produce dNTP depletion as well as direct DNA synthesis inhibition, but at equitoxic concentrations had a lesser effect on DNA synthesis compared to aphidicolin and HU. However, dFdCyd was significantly more cytotoxic than either of the other two drugs. These results taken together suggest that a decrease in dNTP pools and the resulting decrease in DNA synthesis contributed less than one-log of cell death with dFdCyd, and the remaining multiple logs of cell death resulted from the incorporation of dFdCTP into DNA.

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