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Specific Inhibition of DNA Biosynthesis Induced by 3′-Amino-2′,3′-dideoxycytidine†

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ABSTRACT: 3′-Amino-2′,3′-dideoxycytidine (3′-NH_2-dCyd) produced an S-phase-specific block in exponentially growing L1210 leukemia cells. The monophosphate and triphosphate forms of the drug were detected within a few hours of 3′-NH_2-dCyd treatment of intact cells. No significant change in the deoxynucleoside triphosphate levels was observed during the early stages of treatment. However, by 24 h a 2-fold increase in the amount of the deoxynucleoside triphosphates was seen. The triphosphate form of the drug competitively inhibited dCTP incorporation into calf thymus DNA using highly purified DNA polymerase α. The K_i was determined to be 9.6 μM with respect to dCTP. Incorporation of the analogue into DNA was not detected. On the other hand, sucrose gradient analysis suggested that incorporation of the analogue into actively synthesized DNA may account for the biological activity of this compound. Treatment with 3′-NH_2-dCyd induced single-strand breaks in actively synthesized DNA, but no double-strand breaks were observed in the presence of the analogue. The data indicate that 3′-amino-2′,3′-dideoxycytidine specifically interferes with DNA replication at the level of DNA polymerase by inhibiting chain elongation.

ara-C[1] is the prototype of deoxycytidine analogues used in the treatment of neoplastic disease. This agent is S-phase specific (Chu & Fischer, 1962), and the active metabolite, *ara*-CTP, exerts its action by inhibition of DNA biosynthesis (Momparler, 1969; Furth & Cohen, 1968; Chu & Fischer, 1968; Durham & Ives, 1969). *ara*-C has been shown by use of intact cells to be readily incorporated into DNA by a number of investigators (Graham & Whitmore, 1970; Manteuil et al., 1974; Kufe et al., 1980). Recently, the level of *ara*-CMP incorporation into DNA was correlated with the loss of clonogenic survival of both L1210 murine leukemic cells (Kufe et al., 1980) and HL-60 human promyelocytic leukemic

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[1] Abbreviations: *ara*-C, 1-β-D-arabinofuranosylcytosine; 3′-NH_2-dCyd, 3′-amino-2′,3′-dideoxycytidine; ddCyd, 2′,3′-dideoxycytidine; *ara*-CMP, 1-β-D-arabinofuranosylcytosine 5′-monophosphate; 3′-NH_2-dCMP, 3′-amino-2′,3′-dideoxycytidine 5′-monophosphate; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; dNTP, deoxynucleoside 5′-triphosphate; 5-aza-dCyd, 5-aza-2′-deoxycytidine; 3′-NH_2-dThd, 3′-amino-3′-deoxythymidine. For the triphosphates of the analogues, the M in the abbreviation is replaced by T.

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cells (Major et al., 1981). Therefore, it is believed that *ara*-CMP incorporation into DNA is necessary to produce cell death (Kufe et al., 1980; Major et al., 1981).

In addition, another antileukemic dCyd analogue, 5-aza-2′-deoxycytidine (5-aza-dCyd) (Vesely & Cihak, 1977; Momparler & Gonzales, 1978; Rivard et al., 1981), is known to be incorporated into DNA (Bouchard & Momparler, 1983), and it has been proposed that the lethal action of this drug is also due to incorporation into DNA (Vesely & Cihak, 1977; Momparler et al., 1979).

Recently, 3′-amino-2′,3′-dideoxycytidine (3′-NH_2-dCyd) was synthesized (Lin & Mancini, 1983) in an attempt to find a cytotoxic dCyd analogue that is resistant to deamination, since metabolic conversion of *ara*-C to the inactive *ara*-U by cytidine-deoxycytidine deaminase is responsible for the short biological half-life of *ara*-C (Camiener & Smith, 1965; Creasey et al., 1966; Chabner et al., 1974). Not only was 3′-NH_2-dCyd found to be resistant to deamination (Mancini & Lin, 1983), but also the compound exerted a specific inhibitory effect on DNA biosynthesis without affecting that of RNA or protein (Lin & Mancini, 1983; Mancini & Lin, 1983). However, the mechanism by which 3′-NH_2-dCyd exerts its specific DNA inhibitory action is unknown at this time. The present study investigates the mode of action of this new dCyd derivative to examine whether analogue incorporation into DNA is a prerequisite for cell lethality as has been observed for *ara*-C (Kufe et al., 1980; Major et al., 1981) and as has been proposed for 5-aza-dCyd (Vesely & Cihak, 1977; Momparler et al., 1979).

Materials and Methods

Materials. The synthesis of 3′-NH_2-dCyd has been reported (Lin & Mancini, 1983; Lin et al., 1983). [2,8-^{3}H]Deoxyadenosine (25 Ci/mmol), [*methyl*-^{3}H]thymidine (70 Ci/mmol), [2-^{14}C]thymidine (56 mCi/mmol), and [5,6-^{3}H]-*ara*-C (26 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). [5-^{3}H]Deoxycytidine 5′-triphosphate (30 Ci/mmol) was obtained from ICN Chemical & Radiochemical Division (Irvine, CA) while carrier-free [^{32}P]orthophosphate was supplied by New England Nuclear (Boston, MA). Pronase (76 600 proteolytic units/g) was obtained from Calbiochem-Behring (San Diego, CA) while deoxyribonuclease I (3300 Kunitz units/mg), ribonuclease A (79 Kunitz units/mg), nuclease P_1 (1600 units/mg), and nucleoside-5′-diphosphate kinase (1000 units/mg) were obtained from Sigma Chemical Co. (St. Louis, MO). The nucleosidemonophosphate kinase (0.5 unit/mg) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and Dowex AG 50W-X4 (hydrogen form) from Bio-Rad Laboratories (Richmond, CA).

Cells. L1210 leukemia cells were grown in Fischer's medium supplemented with 10% horse serum (Grand Island Biological Co., Grand Island, NY). Only cells in the exponential phase of growth were used in this investigation. Cells were found to be free of mycoplasma contamination by a biological culture method (Barile, 1973).

Flow Cytometry. In an effort to reduce sample variability, dose–response curves were performed for each drug concentration chosen for study. The experiments were then set up so that for each condition the same cell density was achieved at the time of harvest [(2–3) × 10^5 cells/mL].

At harvest cells were fixed in 10% formalin and stored at 4 °C for 12–15 h prior to staining. Cells were stained in 0.02% acriflavin (Sigma Chemical Co., St. Louis, MO) as described (Gill & Jotz, 1974) and analyzed on the same day with a Becton Dickinson FACS IV flow cytometer (Becton Dickinson FACS Systems, Sunnyvale, CA). The DNA histograms were analyzed by employing the computer model of Gurley and Jett (1981).

Radiolabeled 3′-NH_2-dCyd. [^{3}H]-3′-NH_2-dCyd was prepared with the tritium-labeling service of New England Nuclear (Boston, MA). The initial purification of [^{3}H]-3′-NH_2-dCyd was carried out by HPLC with BP AN 6 cation-exchange resin (Benson Co., Reno, NV) and an isocratic buffer (0.3 M NH_4ClO_4, 10 mM HEPES, pH 7.1) at a flow of 1.25 mL/min at 60 °C. Fractions containing radiolabeled 3′-NH_2-dCyd were then applied to conventional cation-exchange chromatography (Dowex AG 50W, 1.5 × 18.5 cm column) and subjected to a pH gradient. The purified [^{3}H]-3′-NH_2-dCyd (specific activity 2.1 Ci/mmol) eluted as a single peak when rechromatographed on Dowex AG 50W and when subjected to HPLC using an isocratic elution at either pH 5.0, pH 7.1, or 7.7.

Nucleotide Preparation of 3′-NH_2-dCyd. 3′-NH_2-dCMP was prepared according to the Yoshikawa method (Yoshikawa et al., 1967), and 3′-NH_2-dCTP was synthesized essentially as described (Ruth & Cheng, 1981) except for the starting nucleoside. The concern was the possible reactivity of the 3′-amino group during chemical synthesis which might yield a mixture of the 5′-nucleotide and 3′-phosphoramidate nucleotides, especially since it is known that the phosphoramidate linkage is formed at the 5′-amino position of 5′-amino-5′-deoxyadenosine (Wilkes et al., 1973), 5′-amino-5′-deoxythymidine (Letsinger & Mungall, 1970), and 5-iodo-5′-amino-2′,5′-dideoxyuridine (Chen et al., 1976). Therefore, 3′-azido-2′,3′-dideoxycytidine (Lin & Mancini, 1983) was used as the starting nucleoside. After purification of the desired 3′-azido 5′-phosphate derivatives (mono- or triphosphate), the 3′-azido compound was converted to the corresponding 3′-amino analogue by hydrogenation under 50 psi for 2.5 h in the presence of 10% palladium on charcoal. The solution was filtered before being applied to DEAE-Sephadex column chromatography. The desired 3′-amino nucleotide was eluted in a gradient of triethylammonium bicarbonate, pH 7.5.

[^{3}H]-3′-NH_2-dCMP was prepared enzymatically with partially purified L1210 dCyd kinase (Lin et al., 1983) and purified [^{3}H]-3′-NH_2-dCyd. [^{3}H]-3′-NH_2-dCTP was prepared by incubation of [^{3}H]-3′-NH_2-dCMP in a reaction mixture containing 40 mM HEPES, pH 7.5, 6.9 mM phosphocreatine, 0.6 unit of creatine phosphokinase, 5 mM ATP, 5 mM $MgCl_2$, 2 mM dithiothreitol, 4 units of nucleoside-5′-diphosphate kinase (above reagents and enzymes obtained from Sigma Chemical Co., St. Louis, MO), and 1 unit of nucleosidemonophosphate kinase.

Sucrose Density Gradient Centrifugation. L1210 cells were labeled for 24 h with 0.5 μCi of [^{14}C]dThd followed by a 1.5-h chase in nonradioactive medium. After incubation with drug or saline, the DNA was pulse labeled with 0.05 mCi of [^{3}H]dThd for 7 min. The cells were harvested and immediately layered on top of the alkaline sucrose gradients as described (Yamada et al., 1985). Radioactivity was determined by dual-channel liquid scintillation counting, and the distribution of ^{3}H and ^{14}C in each sample was adjusted for quench. High molecular weight DNA was defined as fractions with greater than 25 dpm of ^{14}C-labeled DNA.

Neutral sucrose gradient centrifugation was performed as described (Mancini et al., 1983a). Cells were prelabeled with 0.5 μCi of [^{14}C]dThd for 12 h and treated with drug or saline for 7.5 h before harvest.

HPLC Analysis of L1210 DNA. The harvesting of cells, Pronase and ribonuclease A treatment, and isolation of the

Table I: Metabolism of [^{3}H]-3′-NH_2-dCyd in L1210 Cells

assay mixture[a]	NMPK	NDPK	% total cpm dNR/dNMP (R_f 0.88)	dNDP (R_f 0.71)	dNTP (R_f 0.35)	total cpm
[^{3}H]-3′-NH_2-dCMP	−	−	86.3	6.12	7.54	3968
	+	−	39.1	54.4	6.65	3529
	+	+	42.0	8.98	49.0	3764
[^{3}H]-3′-NH_2-dCyd (N = 4)[b]			54.9 (1.87)[c]	31.1 (1.48)[c]	14.0 (0.688)[c]	

[a] Marker preparation: [^{3}H]-3′-NH_2-dCMP was prepared enzymatically, and the assay conditions are described under Materials and Methods except that NMPK (1 unit) and NDPK (4 units) were added where indicated. Reaction aliquots were spotted on PEI–cellulose and developed in 2 N glacial acetic acid/0.5 M LiCl. By use of appropriate markers, areas were cut, and the amount of radioactivity was determined. [b] [^{3}H]-3′-NH_2-dCyd metabolism. L1210 cells (4×10^5 cells/mL) were subjected in duplicate cultures to either 2.5 or 10 μM [^{3}H]-3′-NH_2-dCyd (2.1 Ci/mmol) for 5.5 h before being harvested with two saline washes, extracted with 0.5 N PCA, and neutralized with 2 N KOH. The supernatant was spotted on TLC and developed as described above. [c] Mean (SE).

DNA by isopycnic centrifugation have been reported (Mancini et al., 1983a). The digestions of DNA with deoxyribonuclease I and nuclease P_1 were performed essentially as described (Kuo et al., 1980). The digests were immediately analyzed by HPLC using a Whatman Partisil (SAX 10/25) anion-exchange column with 10 mM NaH_2PO_4, pH 5.5, at a flow of 1 mL/min. UV absorbance was monitored at 254 and 280 nm. Fractions were collected, and the amount of ^{32}P and ^3H was determined by double-channel liquid scintillation spectrometry with a Beckman LS 7500 counter.

DNA Polymerase. DNA polymerase α was purified from L1210 cells essentially as described by Fisher and Korn (1977) up to and including the DNA–cellulose chromatography step. The specific activity of the purified enzyme preparation was 21 000 units/mg.

Calf thymus DNA was activated by treatment with DNase I until maximum incorporation of [^{3}H]dCTP into acid-insoluble material was achieved during a 40-min incubation with *Escherichia coli* DNA polymerase I.

The DNA polymerase assay mixture in a volume of 0.1 mL contained 1 μmol of HEPES, pH 7.5, 30 μg of activated calf thymus DNA, 25 μg of bovine serum albumin (free of deoxyribonuclease, ribonuclease, and protease activity; Boehringer Mannheim Biochemicals, Indianapolis, IN), 0.4 μmol of $MgCl_2$, 50 nmol of dithiothreitol, 10 nmol each of dATP, dGTP, dTTP, and [5-^{3}H]dCTP (30 Ci/mmol), and the enzyme preparation. During the kinetic analysis the assay mixture was the same except that the [^{3}H]dCTP concentration was varied.

Antitumor Studies in Vivo. Transplantation of L1210 cells was carried out by withdrawing peritoneal fluid from donor CDF_1 mice bearing 7-day growths. Approximately 10^5 cells were injected intraperitoneally (ip) into each animal. Beginning 24 h after tumor implantation, 3′-NH_2-dCyd (20 mg/kg) or saline was administered ip twice daily for 9 consecutive days.

Results

Cell Cycle Effects Induced by 3′-NH_2-dCyd. As reported earlier, 3′-NH_2-dCyd was found to induce an inhibitory effect specifically on DNA biosynthesis following exposure to cultured L1210 cells (Mancini & Lin, 1983). This suggests that 3′-NH_2-dCyd may have a specific effect on the S phase of the cell cycle. To investigate this, L1210 cells were treated with varying concentrations of 3′-NH_2-dCyd for 26 h, after which time the cells were harvested, fixed, acriflavin stained, and analyzed by flow cytometry as described under Materials and Methods. The preliminary findings of this experimental approach have been reported (Mancini et al., 1983b). Some representative DNA histograms are illustrated in Figure 1. As can be seen, treatment with 3′-NH_2-dCyd resulted in a concentration-dependent decrease in the G_1 fraction with a concomitant increase in the S fraction with little change in G_2 + M.

Table II: dNTP Levels in L1210 Cells following Exposure to 3′-NH_2-dCyd[a]

3′-NH_2-dCyd	hours	pmol/10^6 cells dATP	dGTP	dCTP	dTTP
−	3.5	38	27	36	21
+	3.5	45	28	23	27
−	12	42	30	47	53
+	12	65	33	56	58
−	24	58	45	72	85
+	24	127	81	112	149
−	48	64	38	56	66
+	48	139[b]	74[b]	126[b]	153[b]

[a] L1210 cells (5.8×10^5 cells/mL) were treated with either 2.5 μM 3′-NH_2-dCyd or saline. At the indicated time, cells were harvested as described in Table I. The dNTP level was determined according to the procedure by Solter and Handschumacher, (1969). Standard curves (which were linear throughout the varied range of sample dNTP) were determined for each of the dNTP's at the same time that the sample supernatant was analyzed. Each determination is the average of duplicate samples. [b] Significantly different from the same condition without 3′-NH_2-dCyd using two-tailed t test ($p < 0.02$).

The addition of dCyd was previously reported not only to reverse the cytotoxic effects of 3′-NH_2-dCyd but also to prevent lethality if rescue was initiated within 9.5 h following treatment (Mancini & Lin, 1983). Rescue with dCyd for 14 h following 10 h of drug treatment led to a shift in the cell cycle distribution similar to that of cells not exposed to 3′-NH_2-dCyd (data not shown).

3′-NH_2-dCyd-Induced Changes in the dNTP Levels. Since the results indicate that the DNA biosynthetic phase of the cell cycle is specifically altered, we examined the effects of this agent on the deoxynucleoside triphosphate (dNTP) pools. The initial investigation focused on whether or not 3′-NH_2-dCyd is anabolized to the nucleotide level in L1210 cells (Table I). Incubation of radiolabeled 3′-NH_2-dCMP with nucleoside monophosphate kinase and nucleoside diphosphate kinase in vitro led to the formation of the nucleoside triphosphate form of 3′-NH_2-dCyd. Furthermore, treatment of L1210 cells in culture with [^{3}H]-3′-NH_2-dCyd for 5.5 h led to the formation of both nucleoside monophosphate and nucleoside triphosphate forms of the drug within the cell.

Since the nucleotides of 3′-NH_2-dCyd are observed within a brief exposure period, the effect on dNTP pools was examined. The results shown in Table II revealed no significant changes in the levels of dNTP until after a 24-h exposure. At 24 and 48 h the dNTP levels were increased by approximately 2-fold.

Effect of 3′-NH_2-dCyd on Nucleotide Incorporation into L1210 DNA. Thus far, the data indicate that a specific in-

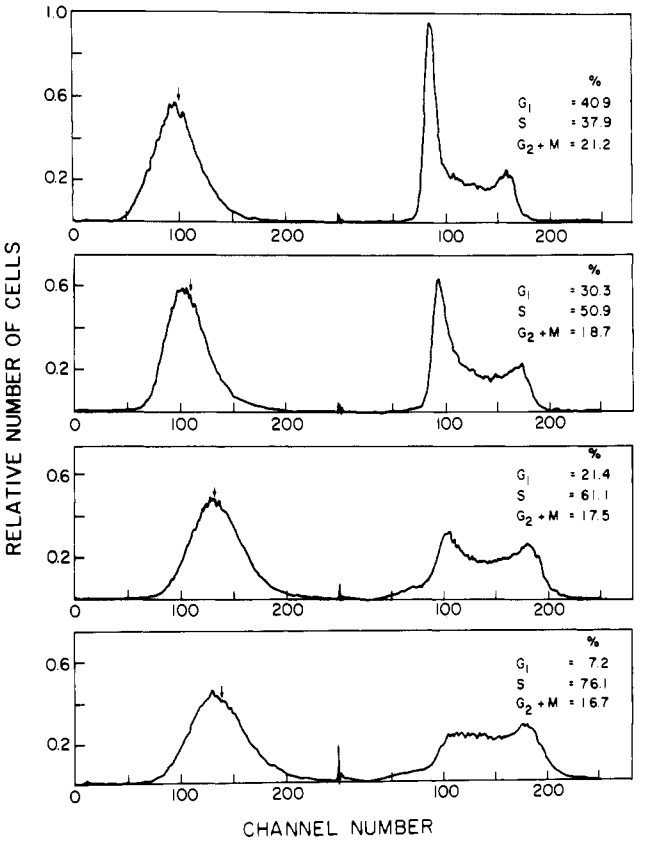

FIGURE 1: DNA histograms of untreated and 3′-NH_2-dCyd-treated L1210 cells. The left panel illustrates light scattering which is indicative of the size distribution of the cell population. The arrow indicates the mean channel number. The right panel depicts the DNA histograms of acriflavin-stained cells as determined by flow cytometry (see Materials and Methods for details). From top to bottom the 3′-NH_2-dCyd concentration is 0, 0.4, 2.5, and 10 μM. The drug exposure time is 24 h. The coefficients of variation for the G_1 peak assignment are 5.79, 5.31, 4.85, and 5.10, respectively.

hibition of DNA biosynthesis occurs when cells are exposed to 3′-NH_2-dCyd (Figure 1; Lin & Mancini, 1983). Furthermore, since 3′-NH_2-dCTP is formed (Table I), the analogue may or may not be incorporated into DNA or may alter the incorporation into DNA of the natural occurring bases. To examine this, the DNA from 3′-NH_2-dCyd-treated cells was isolated and enzymatically digested to the 5′-monophosphates and analyzed by HPLC as described under Materials and Methods.

Initial attempts to detect incorporation utilizing [^{3}H]-3′-NH_2-dCyd were negative (data not shown). To increase the sensitivity of detection, cells were incubated with [^{32}P]orthophosphate in phosphate-free medium prior to incubation with drug. This was done to increase the probability of 3′-NH_2-dCyd getting labeled with ^{32}P in the α position as it is metabolized in the cell. The ^{32}P atom will associate with the drug if it is incorporated into the DNA as well as when the DNA is digested to nucleoside monophosphates. No ^{32}P was detected at the retention time of 3′-NH_2-dCMP (Table III). To further increase the sensitivity of detection, fractions encompassing the retention time of 3′-NH_2-dCMP were pooled, lyophilized, resuspended, and reinjected. Even with smaller fractions collected along with increased UV sensitivity, no incorporated drug was detected.

Since it is known that *ara*-C is readily incorporated into the DNA of cultured cells (Kufe et al., 1980; Major et al., 1981), this experiment was repeated where cells were incubated with 0.3 μM [^{3}H]-*ara*-C for 6 h. Under the conditions of the assay,

Table III: HPLC Analysis of L1210 DNA following Treatment with 3′-NH_2-dCyd or [^{3}H]-*ara*-C[a]

		^{32}P dpm/10^6 cells per hour (×10^2)			
			3′-NH_2-dCyd		*ara*-C,
	RT (min)	control	25 μM	35 μM	0.3 μM
3′-NH_2-dCMP	11.0		0	0	0
ara-CMP	26.4		0	0	a
dTMP	15.6	9.3	4.0	4.5	2.1
dUMP	20.4	6.4	2.0	1.9	0.8
dCMP	25.2	17.3	8.8	5.3	2.5
dAMP	30.0	6.1	5.1	4.1	4.0
dGMP	42.0	18.6	9.7	5.0	2.7
total ^{3}H dpm[b]		100	107	130	89
total ^{32}P dpm[b]		100	46.8	35.3	39.2

[a] L1210 cells were prelabeled with 0.05 mCi/mL [^{3}H]dThd for 18 h. The cells were washed and resuspended in phosphate-free medium supplemented with 0.015 mCi/mL carrier-free [^{32}P]orthophosphate. After 1.5 h, drug was added and incubated for 6 h prior to harvest. *ara*-CMP elutes with dCMP, and thus relative amounts of ^{32}P cannot be determined. However, 1071 dpm of tritium was detected at the appropriate retention time. At a specific activity of 3.3 Ci/mmol, this corresponds to approximately 0.75 pmol of incorporated *ara*-C. [b] Percentage of total tritium and ^{32}P recovered with respect to control.

0.75 pmol of incorporated drug was detected. Further proof of the label being associated with *ara*-CMP was obtained upon TLC separation in 1-butanol–acetic acid–H_2O (2:1:1) (not shown).

The data in Table III show a large decrease in the total amount of ^{32}P incorporated into L1210 DNA induced by treatment with both 3′-NH_2-dCyd and *ara*-C as expected for inhibitors of DNA biosynthesis.

3′-NH_2-dCTP Inhibition of L1210 DNA Polymerase α. Since no 3′-NH_2-dCMP incorporation into DNA was detected, this implied that the inhibition of DNA biosynthesis observed with this agent might be the result of a specific effect on DNA polymerase. To test this hypothesis, 3′-NH_2-dCTP was prepared and analyzed for its inhibitory effect on purified DNA polymerase α (see Materials and Methods for details). The results (not shown) revealed that 3′-NH_2-dCTP competitively inhibited [^{3}H]dCTP incorporation into DNA. The K_i (for 3′-NH_2-dCTP) was determined to be 9.6 μM. The correlation coefficient of the Lineweaver–Burk replot (slope versus 3′-NH_2-dCyd concentration) was 0.9905 (N = 7).

Sucrose Density Gradient Centrifugation. In order to better understand the mechanism by which 3′-NH_2-dCyd exerts its specific inhibition of DNA synthesis, the effect of drug treatment on the integrity of cellular DNA was examined through sucrose density gradient centrifugation. The cultures utilized in these experiments were asynchronous; therefore, the distribution of labeled DNA fragments in the gradient represents the various stages of DNA synthesis existing in the cells. This is best represented by the sedimentation profile of control cells pulse labeled for 7 min with [^{3}H]dThd (Figure 2A). However, when cells were treated for 9.5 h with equiinhibitory concentrations of either *ara*-C (0.15 μM) or 3′-NH_2-dCyd (20 μM), most of the tritium-labeled DNA remained at or near the top of the gradient (Figure 2E,I). In separate experiments (Figure 2B–D,F–H,J–L), both the drug and label were removed after the tritium pulse to examine the effects of these analogues on the elongation of DNA. Elongation of control DNA proceeded rapidly; after 45 min almost 80% of the tritium-labeled DNA sedimented with the high molecular weight DNA. After 1.5 h, this had reached maximum (90% of total tritium) (not shown). In contrast, *ara*-C inhibited the elongation of the tritium-labeled fragments. After 45 min only 25% of the labeled DNA chased to high molecular

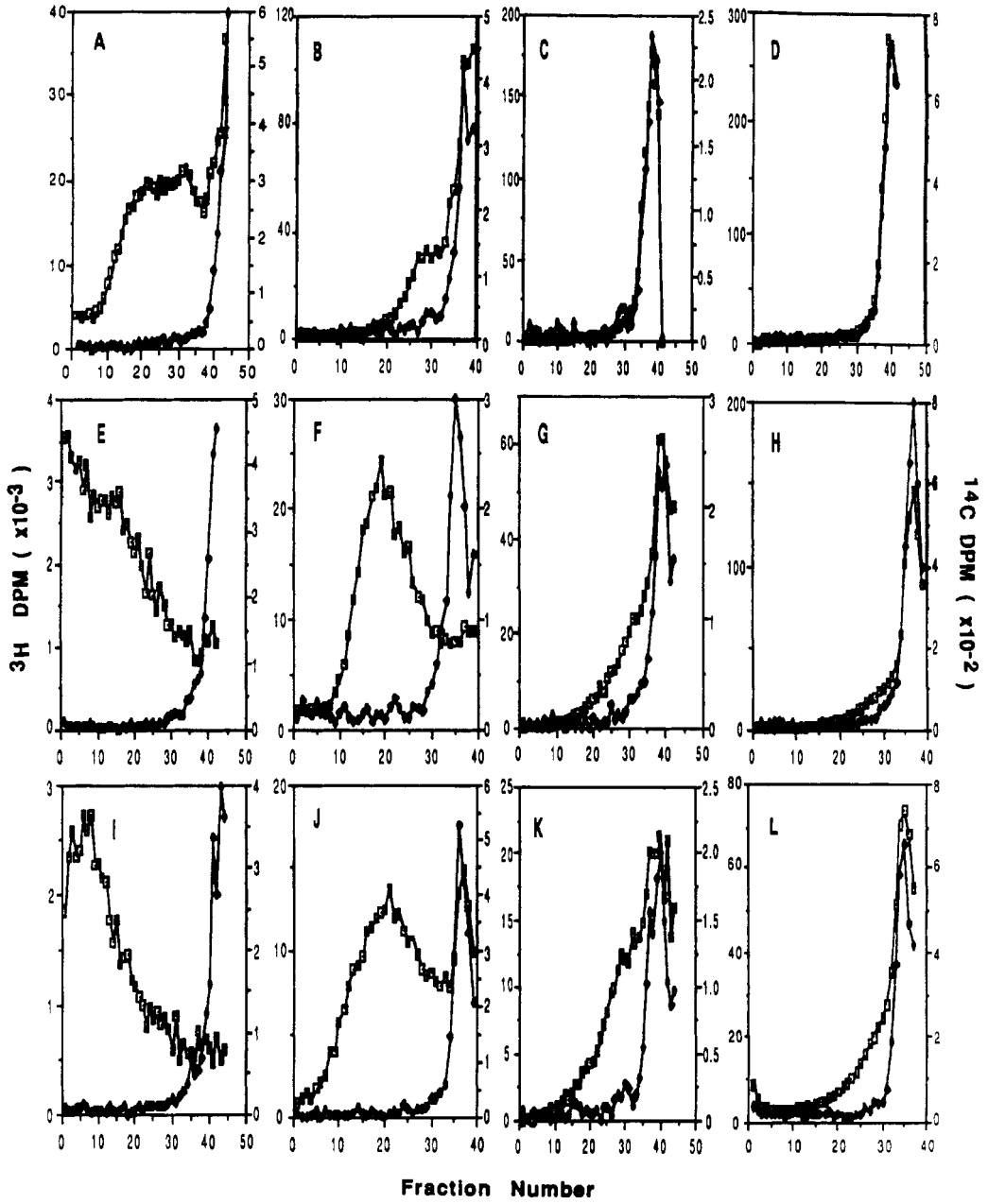

FIGURE 2: Alkaline sucrose gradient profiles of L1210 prelabeled 24 h with [^{14}C]dThd (●), chased 1.5 h in nonradioactive medium, and then treated with saline (A–D), 0.15 μM *ara*-C (E–H), or 20 μM 3′-NH_2-dCyd (I–L) for 9.5 h. Following drug exposure, the cultures were pulse labeled with [^{3}H]dThd (□) for 7 min. The cells were immediately harvested (A, E, and I) or washed, resuspended in nonradioactive, drug-free medium, and allowed to incubate for 45 min (B, F, and J), 120 min (C, G, and K), or 180 min (D, H, and L) prior to harvest. Each curve presents the average of two experiments.

weight DNA, but 80% of the tritium chased to high molecular weight DNA after 2-h incubation in the absence of drug, and this increased to 85% after 3-h chase.

Treatment with 3′-NH_2-dCyd also resulted in an inhibition of elongation (Figure 2J–L). In comparison with *ara*-C, a slightly greater proportion of the tritium-labeled fragments chased to high molecular weight DNA after 45 min (~32%). However, following a 2-h chase in the absence of drug, the rate of elongation had slowed. The percentage of the total tritium that sedimented as smaller fragments was 70% after 2 h and 75% after a 3-h chase. The appearance of single-strand breaks as detected in the alkaline sucrose gradients could be the result of the production of strand breaks encompassing both strands of the DNA. However, the results of neutral sucrose gradient centrifugation (Figure 3) show that treatment of L1210 cells with either 5 or 20 μM 3′-NH_2-dCyd for 7.5 h produced no double-strand breaks in the DNA.

Antitumor Studies of 3′-NH_2-dCyd in Vivo. The antitumor effect of 3′-NH_2-dCyd was investigated with mice bearing L1210 leukemia. Mice in the control group died on days 8, 9, and 10. Mice treated with 20 mg/kg of 3′-NH_2-dCyd twice a day (ip) for 9 consecutive days yielded on 80% "cure" (four out of five mice survived 60 days) (Figure 4).

Discussion

The majority of 3′-amino-2′,3′-dideoxy nucleoside derivatives have been developed and investigated for biological activity only recently. Lin and Prusoff (1978) reported that although 3′-amino-3′-deoxythymidine (3′-NH_2-dThd) only modestly inhibited the replication of HSV-1, it was a very effective inhibitor of cultured L1210 and sarcoma 180 cells. A similar pattern of effectiveness was observed with 3′-NH_2-dCyd for these two cell lines (Lin & Mancini, 1983). Krenitsky et al. (1983) reported the synthesis and biological activity of 3′-

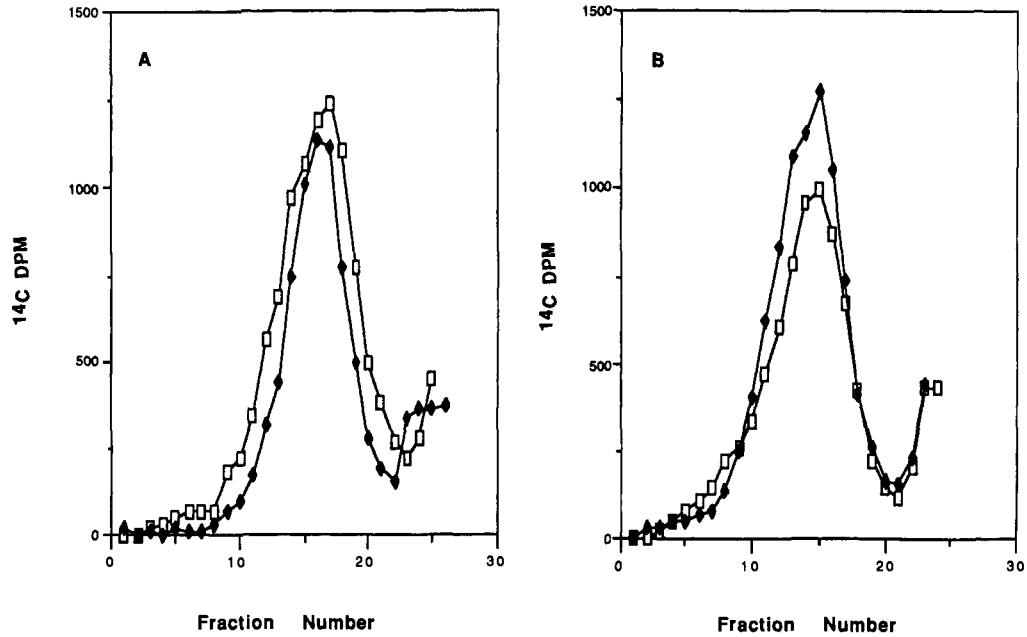

FIGURE 3: Neutral sucrose gradients. L1210 cells were labeled 12 h with 0.5 μCi of [^{14}C]dThd, treated with saline (□) or 5 (A) or 20 μM (B) 3′-NH_2-dCyd (●) for 7.5 h, and immediately harvested. The nuclear fraction was lysed and separated on a neutral sucrose gradient as described under Materials and Methods.

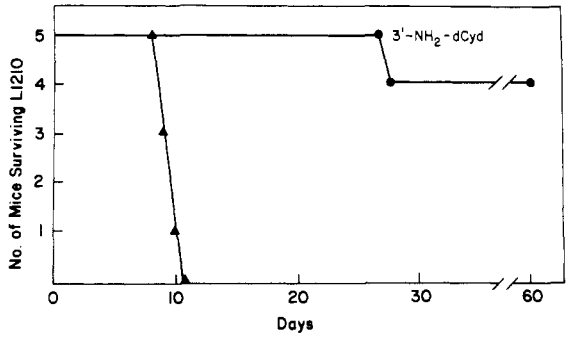

FIGURE 4: Effect of 3′-NH_2-dCyd administered twice per day at a dose of 20 mg/kg for a total of 9 days on the survival of mice bearing the L1210 leukemia. (▲) Saline control; (●) 3′-NH_2-dCyd. This experiment was repeated with three mice per group (not shown). The results were similar with 3′-NH_2-dCyd treatment yielding two 60-day survivors and the third mouse with a T/C × 100 of 361.

amino-dThd, -dUrd, and -dCyd and 5-halogenated derivatives of deoxyuridine. Only 3′-amino-5-fluoro-2′,3′-dideoxyuridine showed antiviral and antibacterial activity while both 3′-NH_2-dThd and 3′-NH_2-dCyd were the most potent inhibitors of mouse L cells. In addition, only 3′-NH_2-dCyd was found to be effective against the human D-98 cultured cells (Krenitsky et al., 1983). The 3′-amino-2′,3′-dideoxy derivatives of adenosine and guanosine did not exhibit appreciable antiviral properties, and the adenosine analogue was shown to be a weak inhibitor of L1210 replication as reported by DeClercq et al. (1980). Although the 3′-amino-2′,3′-dideoxy nucleoside derivatives do possess biological activity, the exact mechanism by which these agents produce the growth inhibitory effect is not known.

The results of this investigation indicate that 3′-NH_2-dCyd exerts its action during the S phase of the cell cycle. The block appears to be in late S since no change in G_2/M was noted while the population of cells in G_1 is depleted as cells progress into the DNA biosynthetic phase (Figure 1). The block is apparently reversible since the administration of dCyd within 10 h after treatment results in a DNA staining pattern which approaches that of the untreated cells. These results are consistent with our earlier findings that 3′-NH_2-dCyd-induced cytotoxic events can be reversed by dCyd (Mancini & Lin, 1983). It should be noted that in the presence of 3′-NH_2-dCyd the cell volume increases (Mancini & Lin, 1983). This is expected for a drug which specifically inhibits DNA biosynthesis, since RNA and protein synthesis continue uninterrupted (Lin & Mancini, 1983).

It was of interest to find that although the nucleotide derivatives of 3′-NH_2-dCyd are formed in intact cells (Table I), no significant alteration in dNTP pools was observed within 12 h of drug treatment (Table II). This finding is similar to that observed with *ara*-C. Graham and Whitmore (1970) found that a 4-h treatment of 20 μM *ara*-C did not significantly alter dCTP levels in mouse L cells, and Skoog and Nordenskjold (1971) reported that depletion of dNTP pools was not correlated with the inhibition of DNA synthesis produced by *ara*-C in mouse embryo cells. The results depicted in Table II reveal no effect on dNTP levels during the time when a 60% inhibition of DNA synthesis is observed (Lin & Mancini, 1983; Mancini & Lin, 1983). Therefore, it is reasonable to assume that the catalysis of ribonucleosidediphosphate reductase is not significantly altered during this time. However, inhibition of DNA polymerase could account for the observed decrease in DNA synthesis. It should be pointed out that following a 24-h exposure to 3′-NH_2-dCyd, the levels of all four dNTP's increased by about 2-fold (Table II). The simultaneous rise in the amount of each dNTP is consistent with either a nondiscriminant slowing or blockage of the DNA replicative process.

The K_i for 3′-NH_2-dCTP was determined to be 9.6 μM. Lower K_i values for *ara*-CTP (Furth & Cohen, 1968) and 5-aza-dCTP (Bouchard & Momparler, 1983) inhibition of calf thymus DNA polymerase have been observed [K_i(*ara*-CTP) = 1 μM; K_i(5-aza-dCTP) = 4.3 μM], but Graham and Whitmore (1970) determined the K_i for *ara*-CTP to be 8.7 ± 5.2 μM for DNA polymerase from mouse L cells. Therefore, although the magnitude of the inhibitory effect on DNA biosynthesis is similar for *ara*-C, 5-aza-dCyd, and 3′-NH_2-dCyd, the mechanism by which each analogue exerts its action may be different.

An unexpected finding in the present study was that although 3′-NH_2-dCTP is made in intact cells (Table I), no

evidence of 3′-NH_2-dCMP incorporation into L1210 DNA was found (Table III). This is not the case with *ara*-C, since *ara*-CMP was detected under our assay conditions. *ara*-C incorporation into DNA has been previously observed by other investigators (Graham & Whitmore, 1970; Manteuil et al., 1974; Kufe et al., 1980). *ara*-C is not an absolute chain terminator since its DNA incorporation was found to be internucleotide (Graham & Whitmore, 1970; Manteuil et al., 1974). Other evidence was provided by Fridland (1977), who observed that *ara*-C inhibits the synthesis of new DNA chains but not the elongation of preexisting ones. Early studies reported that the level of *ara*-CMP incorporation into mouse L cell DNA did not correlate with *ara*-C-induced cell lethality (Graham & Whitmore, 1970). However, recent studies found a highly significant correlation ($p < 0.0001$) between the levels of *ara*-CMP incorporation into L1210 DNA and the lethal cellular events (Kufe et al., 1984), but not with the inhibition of DNA polymerase.

In the present report, even though we avoided the use of alkaline conditions during the isolation of [^{3}H]-3′-NH_2-dCyd-treated DNA, no 3′-NH_2-dCMP was detected during HPLC analysis of the DNA digests either by UV absorbance at 254 and 280 nm or by detection of tritiated or phosphorus-32-labeled analogue (Table III). This suggests that either 3′-NH_2-dCyd is not incorporated into DNA or that the levels incorporated are beyond the limits of detection of this experimental approach, even though the approach used did detect 0.75 pmol of *ara*-C incorporated into L1210 DNA and was similar to that which resulted in detection of appreciable amounts of *ara*-CMP in DNA (Kufe et al., 1980, 1984; Major et al., 1981).

The results of the sucrose gradient analysis suggest a mechanism that involves incorporation of 3′-NH_2-dCyd into DNA being actively synthesized. For analogue comparison we selected concentrations of *ara*-C and 3′-NH_2-dCyd that inhibited DNA synthesis to approximately 25% of controls. The observed shift in the sedimentation profiles of the pulse-labeled DNA following 9.5 h of drug treatment is indicative of an extensive inhibition of DNA synthesis. This does not prove that 3′-NH_2-dCyd incorporation into DNA is required for inhibition though, since a drug such as methotrexate, which perturbs nucleotide pools, inhibits DNA synthesis and induces strand breaks without incorporation into DNA (Li & Kaminskas, 1984).

On the other hand, following a 45-min chase in the absence of drug, samples treated with either 3′-NH_2-dCyd or *ara*-C show a marked inhibition of elongation (Figure 2F,J). Aphidicolin, a purely competitive inhibitor of DNA polymerase α and δ, has also been shown to inhibit elongation, but its effects are reversible upon a 15-min incubation in the absence of the drug (Lonn & Lonn, 1983). 3′-NH_2-dCyd appeared to be a more potent inhibitor of elongation than *ara*-C even after a 3-h chase in the absence of the drug. *ara*-C has previously been shown to be incorporated into the DNA of cells in culture, and we have detected *ara*-CMP from DNA digests with our assay. Incorporated *ara*-C residues are poor primers for elongation, but elongation does proceed as shown by the predominance of internucleotide *ara*-C residues in DNA isolated from cells treated with *ara*-C (Major et al., 1982). 3′-NH_2-dCyd's greater inhibition of elongation is consistent with an incorporated analogue that is a relatively poor primer for elongation as compared to *ara*-C or one that must be excised prior to continued synthesis. These results suggest that 3′-NH_2-dCyd-induced inhibition of DNA synthesis cannot be explained solely by the inhibition of the polymerase.

Although the data suggest that 3′-NH_2-dCyd is probably not incorporated internucleotide, it does not rule out the possibility that this agent is incorporated into DNA of actively growing cells. *ara*-CMP is incorporated into internucleotide linkages, and this incorporation correlates with lethality (Kufe et al., 1980, 1984; Major et al., 1981). Also, the lethal event of 5-aza-dCyd is believed to be a result of analogue incorporation into DNA (Vesely & Cihak, 1977; Momparler et al., 1979). The lethality of a brief (2-h) exposure of murine leukemia cells to greater than 3 μM *ara*-C could not be rescued by the continued presence of 50 μM dCyd in the cloning medium (Chu & Fischer, 1967). However, L1210 cells exposed to 10 μM 3′-NH_2-dCyd for 9.5 h could be completely rescued from lethality by the presence of 25 μM dCyd for a period of 14.5 h before cloning (Mancini & Lin, 1983). These findings suggest that it is apparently more difficult to prevent *ara*-C-induced lethality compared to that of 3′-NH_2-dCyd. This is consistent with the hypothesis that it may be more difficult to rescue cells from an analogue-incorporated internucleotide than from one that is incorporated at the 3′-OH terminus or from an agent that is not incorporated into DNA.

A possible hypothesis to explain the observations of this investigation is that 3′-NH_2-dCyd is a chain terminator and that the low levels incorporated were beyond the limits of detection of the experimental methodology employed. Chidgeavadze et al. (1984) demonstrated that the 3′-amino-2′,3′-dideoxy nucleoside 5′-triphosphates of adenosine, guanosine, cytidine, and thymidine are effective terminators of DNA synthesis when included in polymerase reactions in vitro. However, the possibility that the 3′-NH_2-dNTP's are simply not substrates for the DNA polymerases was not ruled out, and it is not known if DNA chain termination occurs in vivo.

Compounds such as ddCyd and acyclovir, which, like 3′-NH_2-dCyd, are without a 3′-hydroxyl, have been shown to be incorporated into the DNA of mammalian cells (Starnes & Cheng, 1987; Furman et al., 1980). Cells incubated with acyclovir incorporated low levels of the analogue, and upon digestion of the DNA the drug was found solely at the chain terminus (Furman et al., 1980). In addition, once incorporated by mammalian polymerases, acyclovir has been shown to potently, but not irreversibly, inhibit elongation as shown by alkaline sucrose gradient centrifugation (Furman et al., 1980).

Recently, using intact cells it was reported that the 3′-amino derivative of thymidine was not incorporated into L1210 DNA (Chen et al., 1984). Although only one nonlethal drug concentration (0.3 μM) was used, the tritiated analogue was incubated with cells for 48 h. The harvest procedure used was similar to that of the present study except that the DNA was digested to the nucleoside level before HPLC analysis. No [^{3}H]-3′-amino-3′-deoxythymidine was detected in the DNA digests. This report (Chen et al., 1984) coupled with the present investigation suggests that the 3′-aminopyrimidine analogues are not incorporated into internucleotide linkages when intact cells are used.

In summary, 3′-NH_2-dCyd, like *ara*-C and 5-aza-dCyd, is a potent inhibitor of DNA biosynthesis. However, the present study suggests that the mechanism of 3′-NH_2-dCyd-induced cell death is different from that of the other dCyd analogues. While most likely related to interference of the replicative process, the inhibition exerted by 3′-NH_2-dCyd may be due to chain termination as was previously suggested (Chidgeavadze et al., 1984).

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Registry No. 3′-NH_2-dCyd, 84472-90-2; dATP, 1927-31-7; dGTP, 2564-35-4; dCTP, 2056-98-6; dTTP, 365-08-2; DNA polymerase, 9012-90-2.

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