

Differences in DNA Damage Produced by Incorporation of 5-Aza-2'-deoxycytidine or 5,6-Dihydro-5-azacytidine into DNA of Mammalian Cells

Joseph M. Covey,¹ Maurizio D'Incalci,² Eugene J. Tilchen, Daniel S. Zaharko, and Kurt W. Kohn

Laboratory of Molecular Pharmacology [J. M. C., M. D'I., E. J. T., K. W. K.], and Developmental Therapeutics Program, Division of Cancer Treatment [J. M. C., M. D'I., E. J. T., D. S. Z., K. W. K.], National Cancer Institute, NIH, Bethesda, Maryland 20892

ABSTRACT

The effects of 5-aza-2'-deoxycytidine (aza-dCyd) and 5,6-dihydro-5-azacytidine (H₂-aza-Cyd) on the integrity of DNA from several mammalian cell lines were compared using the alkaline elution technique. While both compounds have been shown to inhibit DNA methylation, a direct comparison of their effects on DNA structure has not previously been reported. Exposure of L1210 cells to H₂-aza-Cyd (1-100 µg/ml) and simultaneous labeling with [¹⁴C]thymidine for 24 h resulted in the production of single-strand breaks in DNA, which were significantly repaired when cells were incubated in drug-free medium for an additional 24 h. This differed from our previous findings for aza-dCyd, confirmed here in parallel experiments, which showed that this compound produces alkali-labile lesions that persist for 48 h. The DNA effects of both drugs were significantly reduced when cells were prelabeled with [¹⁴C]thymidine, indicating that production of DNA lesions requires incorporation of the anomalous base. Studies utilizing pulse-labeled DNA indicated that aza-dCyd has little effect on the rate of DNA elongation, whereas H₂-aza-Cyd produced a complete inhibition for at least 6 h after drug removal. The contrasting pattern of DNA damage induced by these compounds in L1210 was also observed in two human lymphoblastoid cell lines, one of which was derived from a patient with xeroderma pigmentosum. We had previously concluded that alkali-labile sites in DNA from aza-dCyd-treated cells probably arise due to the chemical instability of aza-dCyd. In contrast, incorporated H₂-aza-Cyd is chemically stable. The single-strand breaks produced in H₂-aza-Cyd treated cells were not of the alkali-labile type, and may represent an accumulation of DNA replication fragments and/or intermediates in an excision repair process. Thus, the DNA lesions produced by the two drugs have markedly different characteristics, and H₂-aza-Cyd should not be considered to be merely a stable pharmacological congener of aza-dCyd.

INTRODUCTION

aza-dCyd³ (1) has been shown to have cytotoxic and antineoplastic activities in a number of experimental tumor systems (2-5) and has undergone clinical trial (6). aza-dCyd is anabolized to the nucleoside triphosphate (7) and is incorporated into DNA (8-10) but not into RNA (9). The presence of azacytosine moieties in DNA (even a small percentage substitution of DNA cytosines) results in inactivation of DNA-cytosine-methyltransferase, an enzyme which catalyzes the transfer of a methyl group to the 5-position of cytosines in DNA (10-12). The hypomethylation of DNA produced by aza-dCyd and related compounds have been shown to result in activation of latent cellular genes (13).

Since azacytidine and aza-dCyd spontaneously decompose in aqueous solution (14) leading to difficulties in pharmaceutical formulation, the stable analogue H₂-aza-dCyd was synthesized

(15). This compound is phosphorylated by cytidine kinase from L1210 and HeLa cells, although less efficiently than is azacytidine (16, 17), yet is incorporated into L1210 nuclear RNA at levels equal to or exceeding those of azacytidine (18). Studies demonstrating effects of H₂-aza-dCyd on gene expression suggest that it is also incorporated into DNA and inhibits DNA methylation (19), although it may be less active than aza-dCyd.

While recent reports have demonstrated a correlation between inhibition of DNA methylation and aza-dCyd cytotoxicity (20), it is not certain that this is the unique mechanism of action for these compounds. We have shown that aza-dCyd treatment of L1210 leukemia cells *in vitro* results in alkali-labile sites in DNA as demonstrated by the alkaline elution technique (21). These DNA lesions increase during 24-48 h following drug removal and are then slowly removed over the next 48 h. It was proposed that this DNA damage results from the chemical instability of aza-dCyd incorporated into DNA.

In this paper we compare and contrast the DNA lesions produced by aza-dCyd and the stable analogue H₂-aza-Cyd in L1210 leukemia and in two human lymphoblastoid cell lines as measured by alkaline elution. We report here differences in both the nature of the lesions induced by these two drugs and in their persistence in DNA. It appears therefore that H₂-aza-Cyd should not be considered a pharmacological analogue of aza-dCyd.

MATERIALS AND METHODS

Cell Culture. Mouse L1210 leukemia cells were grown at 37°C in suspension culture in RPMI 1630 (HEM Research, Inc.) supplemented with 20% heat-inactivated horse serum (GIBCO, Grand Island, NY), 0.84 mM L-glutamine, penicillin (82 units/ml), streptomycin (82 µg/ml) (all from the Media Unit, NIH), and 50 µM mercaptoethanol (Sigma Chemical Co., St. Louis, MO). Stock cultures were maintained in exponential growth at a density between 0.5×10^5 and 1×10^6 cells/ml.

Two human lymphoblastoid cell lines were also used in these studies (Institute for Medical Research, Camden, NJ). One line (GM 3714) was derived from a clinically normal female, while the other (GM 2345A) was obtained from an 8-year-old female with xeroderma pigmentosum (complementation group A). Both lines were maintained *in vitro* at 37°C in RPMI 1630 medium (GIBCO) supplemented with 15% heat-inactivated fetal calf serum.

Labeling. In most experiments cells were labeled for 24 h concurrent with drug treatment using [2-¹⁴C]thymidine (specific activity, 52-58 mCi/mmol; New England Nuclear) at a concentration of 0.02 µCi/ml. For some experiments, L1210 cells were labeled for 24 h ([2-¹⁴C]thymidine, 0.02 µCi/ml), washed 2 times with fresh medium, and then treated with H₂-aza-Cyd or aza-dCyd for 24 h.

Drug Treatment. aza-dCyd was provided by Dr. D. DeVos, Pharmachemie B. V. (Haarlem, Holland), and by the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD), which also provided H₂-aza-Cyd. aza-dCyd is unstable in aqueous solution and decomposes in a temperature- and pH-dependent manner (14) to form several noncytotoxic products (4). In RPMI 1630 medium at 37°C aza-dCyd decomposed in a first-order fashion with a $t_{1/2}$ of 17.5 h. H₂-aza-Cyd has been shown to be stable for weeks in aqueous solution at room temperature (15). For *in vitro* experiments, both compounds were

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¹ To whom requests for reprints should be addressed, at Laboratory of Molecular Pharmacology, Developmental Therapeutics Program, National Cancer Institute, NIH, Building 37, Room 5A19, Bethesda, MD 20892.

² On leave from Istituto di Ricerche Farmacologiche "Mario Negri," Milan Italy.

³ The abbreviations used are: aza-dCyd, 5-aza-2'-deoxycytidine; H₂-aza-Cyd, 5,6-dihydro-5-azacytidine; ara-C, 1-β-D-arabinofuranosylcytosine; XP, xeroderma pigmentosum.

dissolved in sterile phosphate-buffered saline (0.15 M NaCl-10.6 mM KH₂PO₄-5.6 mM Na₂HPO₄) and then immediately added to cell suspensions ($\sim 5.0 \times 10^5$ cells/ml) to give a final drug concentration between 0.1 and 100 μ g/ml. After drug treatment, the cells were washed twice by centrifugation and resuspended in fresh medium. Cell density was determined using a Coulter Counter (Model ZBI, Coulter Electronics, Hialeah, FL), and aliquots of cells were removed for alkaline elution analysis. In some experiments, remaining cells were diluted as necessary to maintain cell density below 5×10^5 cells/ml and incubated in drug-free medium for an additional 24 h. At the end of this period, appropriate aliquots were again removed for alkaline elution.

Alkaline Elution. The method of alkaline elution has been reviewed in detail (22). In brief, approximately 10^6 cells were resuspended in cold phosphate-buffered saline and layered on polycarbonate filters, 0.8 μ m pore size, 25 mm diameter (Nuclepore Corp., Pleasanton, CA). Cells were then lysed with a solution containing 2% sodium dodecyl sulfate-0.02 M disodium EDTA-0.1 M glycine, pH 10.0 (lysis solution), which was allowed to flow through the filter by gravity. After connecting the outlet of the filter holders to the pumping system, 2 ml of proteinase K, 0.5 mg/ml (EM Labs, Darmstadt, West Germany), dissolved in the lysis solution, were added to a reservoir over the polycarbonate filters and pumped for approximately 1 h at 2 ml/h. DNA was eluted from the filters by pumping 0.02 M EDTA solution adjusted to pH 12.1 or 12.6 with tetrapropylammonium hydroxide (RSA Corp., Elmsford, NY) containing 0.1% sodium dodecyl sulfate through the filters at approximately 2 ml/h. Fractions were collected at 3-h intervals with fractions and filters processed as previously described (22). In most experiments, L1210 cells labeled overnight with [*methyl*-³H]thymidine (specific activity, 20 Ci/mmol; 0.1 μ Ci/ml cells; New England Nuclear) and irradiated with 300 rads of γ -radiation were added to suspensions of [¹⁴C]-labeled experimental cells to serve as internal elution standards.

Elongation Experiments. Exponentially growing L1210 cells ($5\text{--}6 \times 10^5$ cells/ml) were pulse labeled for 15 min with [*methyl*-³H]thymidine (1.0 μ Ci/ml cells), washed twice by centrifugation, and resuspended in warm RPMI 1630 medium. aza-dCyd, H₂-aza-Cyd, or ara-C was added and the cells were incubated at 37°C for 1 h. Cells were then washed, resuspended, and maintained at 37°C. Aliquots were removed at various times and analyzed by alkaline elution as described above, except that [¹⁴C]thymidine (0.02 μ Ci/ml)-labeled cells were used as internal standard.

Flow Cytometry. L1210 cells were treated with aza-dCyd or H₂-aza-Cyd as described above. The analysis of DNA by flow cytometry was performed at the end of 24 h treatment and after 24 h postincubation in drug-free medium. Before analysis, cells were washed and suspended in Hanks' solution. Cells were stained by adding 3 ml propidium iodide (Calbiochem-Behring, San Diego, CA) (propidium iodide, 50 μ g/ml in 0.1% sodium citrate plus 30 μ l Nonidet P-40) to 100 μ l cell suspension and were stored at 4°C for 30 min before DNA analysis. Absence of aggregates and the suitability of the preparation were checked by fluorescence microscopy before the samples were run. DNA analysis was performed using a 30L cytofluorograph (Ortho Instruments, Westwood, MA). The fluorescence pulses were detected in a spectral range between 580 and 780 nm (to exclude the overlapping region of excitation and emission spectra of unbound propidium iodide) and then integrated. Each cytofluorometric assay was performed with 5×10^4 cells. The percentage of cells in each cycle phase was calculated by the method of Baisch *et al.* (23).

RESULTS

Representative elution profiles (at pH 12.1 and 12.6) of DNA from L1210 cells treated for 24 h with H₂-aza-Cyd (5–100 μ g/ml) are shown in Fig. 1. The data in Fig. 1, A and B were obtained at the end of drug treatment, whereas those in Fig. 1, C and D were obtained following an additional 24 h incubation of the cells in drug-free medium. The increased elution rate of DNA from H₂-aza-Cyd-treated cells is indicative of single-strand breaks in the DNA. Since cells were exposed to [¹⁴C]thymidine and H₂-aza-Cyd simultaneously, the observed single-

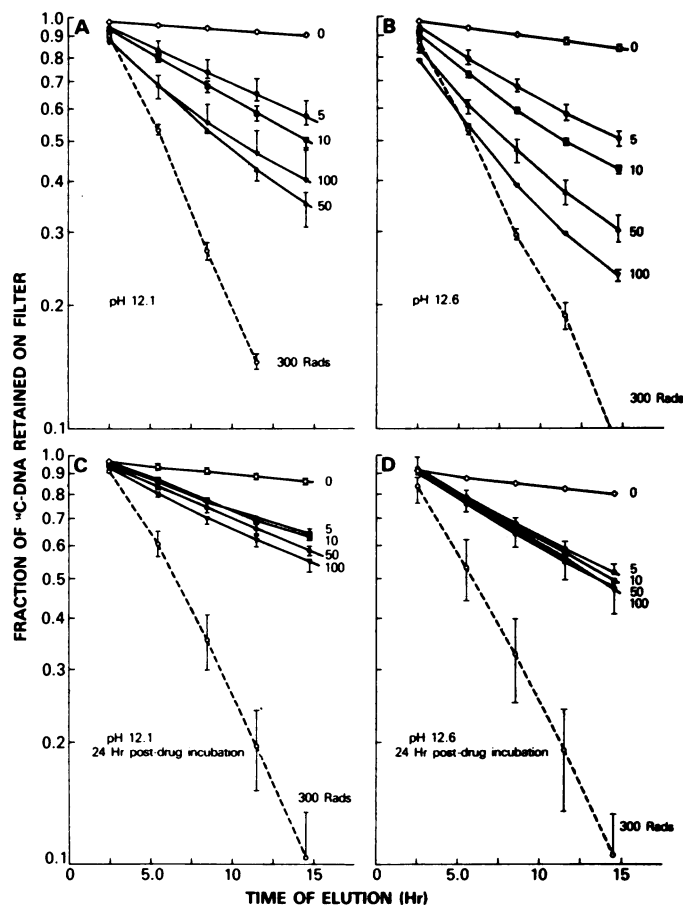


Fig. 1. Alkaline elution profiles of [¹⁴C]DNA from L1210 cells treated for 24 h with H₂-aza-Cyd at the indicated concentrations (μ g/ml). Elutions were carried out at pH 12.1 (A and C) or pH 12.6 (B and D) either immediately after drug washout (A and B) or following 24 h postincubation in drug-free medium (C and D). Labeling with [¹⁴C]thymidine (0.02 μ Ci/ml) and treatment with H₂-aza-Cyd were simultaneous. Points, mean of two to three values; bars, range; ----, elution of [¹⁴C]DNA from untreated L1210 after exposure to 300 rads in the cold.

strand breaks must occur in regions of DNA which were synthesized while drug was available for incorporation. Production of single-strand breaks was concentration dependent up to 100 μ g/ml. Treatment with H₂-aza-Cyd concentrations above this level caused increasing cell lysis during drug washout and resulted in elution curves with markedly reduced retention in the first fraction (data not shown). Incubation of cells in drug-free medium for 24 h resulted in some repair of the single-strand breaks, as evidenced by the reduced elution rates shown in Fig. 1, C and D. Repair was incomplete, with damage produced by the higher H₂-aza-Cyd concentrations diminishing to near the level initially produced by 5 μ g/ml. Single-strand breaks produced by the latter drug concentration were only slightly reduced after 24 h drug-free incubation. This pattern of repair is in contrast to the results obtained with aza-dCyd treated cells (Fig. 2); 24 h postdrug incubation resulted in either little change or an increased elution rate, with no significant repair at any drug concentration over this time period.

In addition, the elution profiles demonstrated a fundamental difference in the nature of DNA damage produced by these two agents. The marked increase in elution rate with increasing pH following aza-dCyd treatment, coupled with the characteristic convex shape of the elution curves (21, 22), demonstrates the formation of alkali-labile sites in the DNA. In contrast, the elution curves obtained after H₂-aza-Cyd treatment were essentially linear and showed only a minimal pH dependency.

The effects of aza-dCyd and H₂-aza-Cyd on L1210 DNA

DNA DAMAGE PRODUCED BY Aza-dCyd AND H₂-aza-Cyd

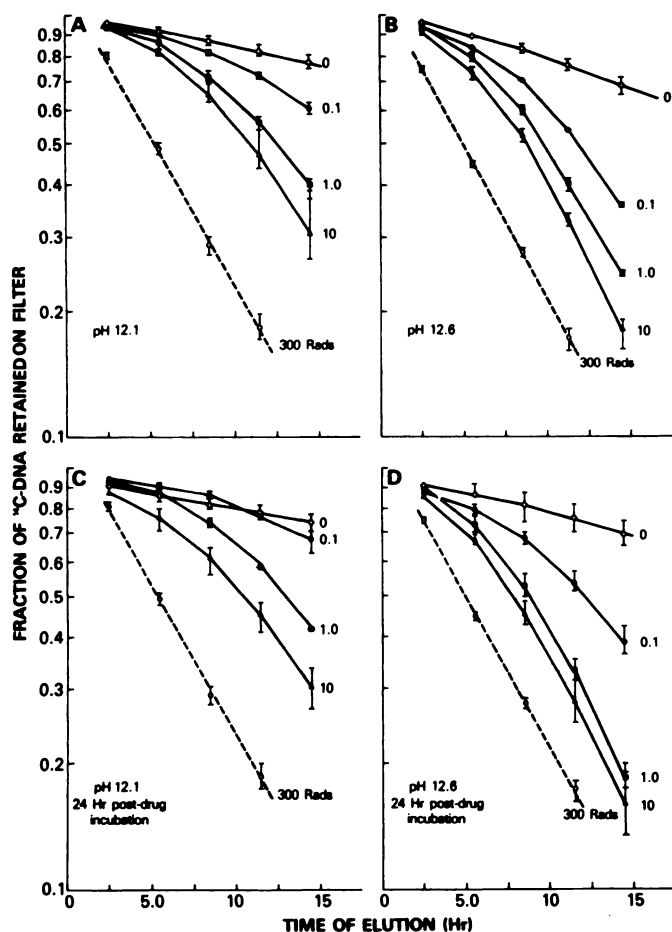


Fig. 2. Alkaline elution profiles of [¹⁴C]DNA from L1210 cells treated for 24 h with aza-dCyd at the indicated concentrations (µg/ml). Elutions were carried out at pH 12.1 (A and C) and pH 12.6 (B and D) either immediately after drug washout (A and B) or following 24 h postincubation in drug-free medium (C and D). Labeling with 0.02 µCi/ml [¹⁴C]thymidine and treatment with aza-dCyd were simultaneous. Points, mean of three values; bars, range; —, elution of [³H]DNA from untreated L1210 after irradiation with 300 rads in the cold; 0.1 µCi [³H]-thymidine for 24 h was used to label these cells; points, mean of 25 values; bars, SE.

depicted in Figs. 1 and 2 as well as data from additional experiments are summarized in Fig. 3, which shows the relationship between drug concentration and the DNA elution rate (plotted as the negative logarithm of the fraction of DNA retained on the filter at a fixed point of internal standard elution). Both drugs caused a dose-dependent increase in DNA elution rate following a 24-h exposure with simultaneous labeling. aza-dCyd-induced lesions demonstrated a large pH-dependent increase (Fig. 3B), whereas there was much less pH dependence in the case of H₂-aza-Cyd-treated cells (Fig. 3A). Repair of H₂-aza-Cyd-induced lesions was evident, while 24 h of postincubation following aza-dCyd treatment resulted in a clear increase in DNA damage for concentrations above 0.1 µg/ml. We previously reported (21) that aza-dCyd lesions persist for at least 48 h after drug removal with no evidence of repair. In contrast, the present data indicate that many of the H₂-aza-Cyd lesions are cleared from the DNA within 24 h, probably by a repair process. When cells were labeled with [¹⁴C]thymidine before aza-dCyd or H₂-aza-Cyd exposure, only a small increase in DNA elution rate was produced by either compound (Fig. 3). This suggests that significant DNA damage is limited to DNA strand segments synthesized in the presence of drug.

The observed reductions in DNA strand length could result from strand cleavage at sites of drug incorporation as a conse-

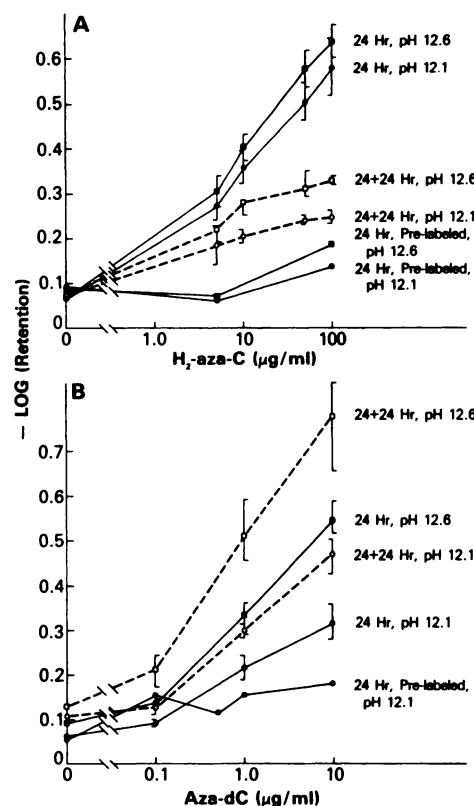


Fig. 3. Effect of elution pH and treatment protocol on DNA elution kinetics from L1210 cells treated with H₂-aza-Cyd (H₂-aza-C) (A) or aza-dCyd (Aza-dC) (B). -Log (retention) is proportional to DNA strand length, where (retention) represents the fraction of [¹⁴C]DNA retained on the filter at the time when internal standard ([³H]DNA) retention is equal to 0.2. The duration of drug treatment and simultaneous labeling with [¹⁴C]thymidine was 24 h and the elutions were carried out at pH 12.1 (○, ●) or pH 12.6 (□, ■) either immediately after drug washout (○, ●) or after 24 h postincubation in drug-free medium (□, ■). ○, □, -log retention of [¹⁴C]DNA from cells that were pre-labeled with [¹⁴C]-thymidine, then exposed to drug for 24 h and eluted immediately. Points, mean of two to six values; bars, range, except in A (○, ■) for which SE is shown.

quence of the chemical instability of the incorporated molecule (aza-dCyd) or from an accumulation of strand scission intermediates in an excision repair process (H₂-aza-Cyd; see below). It might also be proposed that incorporation of anomalous bases in the growing DNA strand produces an inhibition of further elongation (chain termination) and the resultant accumulation of small size DNA fragments. The latter mechanism has been proposed to explain the DNA damage observed in cells treated with ara-C (24).

To investigate the effects of aza-dCyd and H₂-aza-Cyd on DNA elongation, pulse-labeled cells were treated with drug for 1 h. After drug washout, DNA size was followed using alkaline elution. The results of two separate representative experiments are shown in Fig. 4. At the end of pulse labeling (*t*₋₁), control DNA was small but increased towards normal length over 5–6 h of incubation. Treatment of cells with aza-dCyd (1–25 µg/ml) resulted in only slight slowing of elongation during and immediately following drug exposure; by 4 h, treated and control DNA were nearly equivalent in size. In contrast, ara-C, a compound with known chain-terminating activity (25), produced marked inhibition of elongation. Somewhat surprisingly, H₂-aza-Cyd blocked further chain elongation completely and irreversibly for at least 6 h after drug removal. These data suggest that whereas aza-dCyd produces little or no chain termination, H₂-aza-Cyd may induce failure of DNA elongation and/or ligation.

The effects of aza-dCyd and H₂-aza-Cyd on L1210 cell cycle

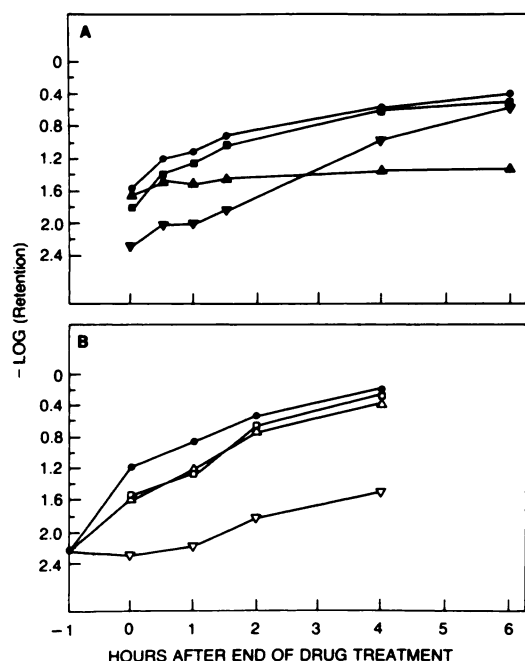


Fig. 4. Effect of cytidine analogues on DNA elongation. L1210 cells were pulse labeled for 15 min, washed twice, and then incubated for 1 h with either no drug (●) or A: aza-dCyd, 1 µg/ml (■); ara-C, 0.5 µg/ml (▼); H₂-aza-Cyd, 50 µg/ml (▲); B: aza-dCyd, 10 µg/ml (□); aza-dCyd, 25 µg/ml (△); ara-C, 12 µg/ml (▽). Cells were then washed and incubated at 37°C. Aliquots were removed at the indicated times and analyzed by alkaline elution. -Log (retention) is proportional to DNA strand length, where (retention) is the fraction of [³H]DNA remaining on the filter when internal standard retention ([¹⁴C]DNA) is equal to 0.2. The ordinate is such that average single-strand length increases with height. Points, mean of two determinations.

Table 1 Cell cycle distribution of L1210 cells treated with aza-dCyd

	Concentration (µg/ml)	% of cells in			
		G ₁	Early S phase	Mid-S phase	Late S phase + G ₂ -M
After 24 h treatment	0	45	12	13	30
	0.1	29	10	16	45
	1.0	31	6	19	44
	10.0	24	12	22	43
After an additional 24 h postdrug incubation	0	64	9	9	18
	0.1	38	16	13	33
	1.0	34	9	14	43
	10.0	32	10	16	42

Table 2 Cell cycle distribution of L1210 treated with H₂-aza-Cyd

	Concentration (µg/ml)	% of cells in			
		G ₁	Early S phase	Mid-S phase	Late S phase + G ₂ -M
After 24 h treatment	0	45	12	13	30
	10	45	11	15	29
	50	51	13	11	24
	100	39	25	15	22
After an additional 24 h postdrug incubation	0	64	9	9	18
	10	60	8	9	23
	50	70	9	9	12
	100	NE ^a	NE	NE	NE

^a NE, not evaluable.

progression were examined using flow cytometry. These results are tabulated in Tables 1 (aza-dCyd) and 2 (H₂-aza-Cyd). Following 24 h of aza-dCyd exposure, the percentage of cells in

late S phase and G₂ + M was increased compared to control, whereas the percentage in G₁ was reduced. After an additional 24 h incubation in drug-free medium, there appears to have been some renewed cell cycle progression at 0.1 µg/ml but not at the higher drug concentrations. In contrast, H₂-aza-Cyd treatment produced no cell cycle effects during 24 h of exposure. After a 24-h drug-free period, control and H₂-aza-Cyd treated cells showed an increase in G₁, probably because a plateau density was achieved. Again, a tendency for 100 µg/ml and higher levels of H₂-aza-Cyd to cause cell fragmentation was observed, yielding a nonevaluable DNA histogram.

In an attempt to further elucidate the nature and repair mechanisms of H₂-aza-Cyd- and aza-dCyd-induced DNA damage, studies were done using two human lymphoblastoid cell lines. One (GM2345A) was derived from a patient with XP, while the other (GM3714) was from a clinically normal subject. It was anticipated that comparison of the DNA damage produced in these two cell lines would indicate the degree of dependency of H₂-aza-Cyd- and aza-dCyd-associated DNA repair on the excision repair mechanism absent in XP cells (complimentation group A). Fig. 5 presents elution profiles for DNA from the normal (GM3714) cell line. Fig. 5, A and C show the elution of DNA from H₂-aza-Cyd-treated cells after 24 h exposure (A) and after an additional 24 h of drug-free incubation (C). Fig. 5, B and D present analogous data for aza-dCyd-treated cells. The characteristics of DNA damage pro-

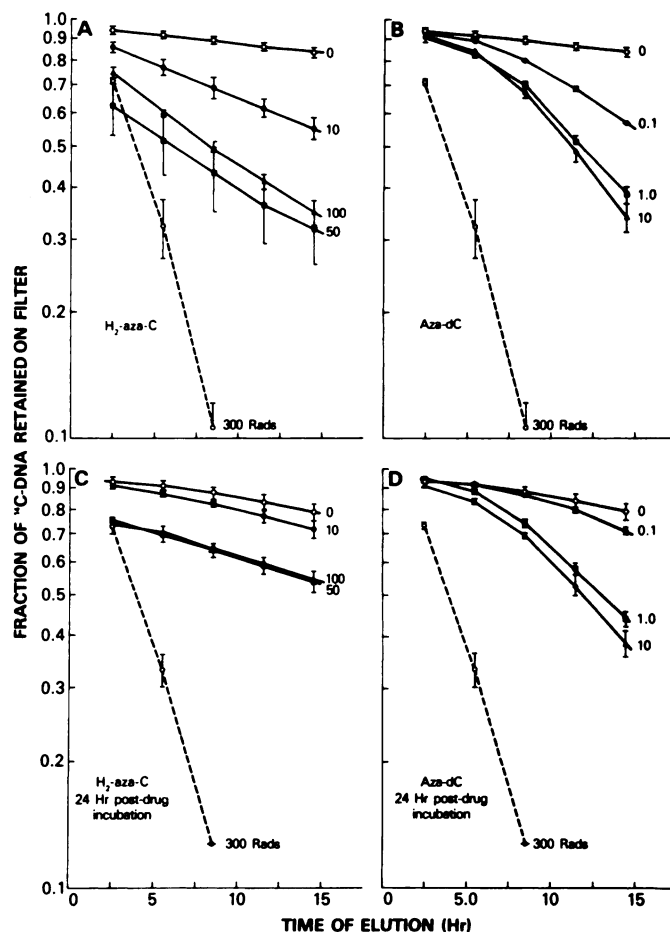


Fig. 5. Alkaline elution profiles of [¹⁴C]DNA from human lymphoblasts (GM 3714) treated with H₂-aza-Cyd (H₂-aza-C) (A and C) or aza-dCyd (Aza-dC) (B and D) at the indicated concentrations (µg/ml). Elutions were carried out at pH 12.6 either immediately after drug washout (A and B) or following 24 h postincubation in drug-free medium (C and D). Labeling and drug treatment were simultaneous. Points, mean of two values; bars, range; —, elution of [¹⁴C]DNA from untreated L1210 after exposure to 300 rads in the cold.

duced in this cell line was similar to that seen with L1210 cells (Figs. 1–3). H₂-aza-Cyd produced linear elution curves, whereas aza-dCyd curves were convex, again suggesting that the latter drug produces alkali-labile sites. It is noteworthy that H₂-aza-Cyd treatment resulted in a reduced DNA retention in the first fraction (Fig. 5A). This pattern is generally indicative of non-specific cell death and lysis, suggesting that these human cell lines are more sensitive than L1210 cells to the postulated lytic effect of H₂-aza-Cyd.

After 24 h of drug-free incubation, aza-dCyd-induced DNA lesions showed evidence of repair only at the lowest dose tested (0.1 µg/ml). In contrast, H₂-aza-Cyd-induced lesions were repaired even at drug doses up to 100 µg/ml. These results are summarized in Fig. 6A, which again relates drug concentration to a measure of DNA retention. The overall degree of DNA damage produced by H₂-aza-Cyd and aza-dCyd in GM 3714 was less than that produced in L1210 with identical concentrations and exposure periods. The present experiments are unable to distinguish whether this difference results from more extensive incorporation of the drugs into L1210 DNA (resulting from greater anabolic activity and/or cell cycle effects), differences in the kinetics of DNA repair in the two cell lines, or both.

Experiments were also simultaneously performed in the XP cell line (GM2345A). Elution curves for both H₂-aza-Cyd- and aza-dCyd-treated cells were essentially identical to those obtained with the normal cell line. The results are summarized in Fig. 6B. The quantitative similarity of DNA damage produced in the two cell lines is evident (Fig. 6, A versus B). DNA strand breaks produced by both drugs were repaired to a similar extent

in each cell line. While these results do not exclude the possibility that the H₂-aza-Cyd-induced lesions were removed by excision repair, it appears that the specific mechanism that is deficient in XP cells plays no role in the observed repair of aza-dCyd- and H₂-aza-Cyd-induced DNA damage.

DISCUSSION

In this report we have investigated the DNA alterations produced by incorporation of the cytidine analogues aza-dCyd and H₂-aza-Cyd into DNA of L1210 murine leukemia and two human lymphoblastoid cell lines. When cells are exposed to [¹⁴C]thymidine and H₂-aza-Cyd simultaneously for 24 h, a significant frequency of DNA single-strand breaks was observed in the labeled DNA by means of the alkaline elution technique. The lack of dependence of the frequency of these lesions on the pH of the eluting solution (Fig. 3A) suggests that they are true single-strand breaks and not a consequence of alkali-labile sites in the DNA. In contrast, the effect of aza-dCyd in simultaneously [¹⁴C]thymidine-labeled cells was to produce an alkaline elution profile characteristic of alkali-labile lesions: a marked pH dependence of the elution rate and elution curves which bend downward (22). The markedly reduced amount of DNA damage seen in prelabeled cells suggests that lesions are produced only in DNA which was synthesized in the presence of aza-dCyd or H₂-aza-Cyd and which has therefore incorporated the abnormal bases. Moreover, this observation eliminates the possibility that a significant fraction of the DNA damage seen in simultaneously labeled and treated cells resulted from non-specific toxicity and cell lysis, which would be expected to also damage preexisting DNA.

Glazer and Knode (9), using alkaline agarose gel electrophoresis, demonstrated that aza-dCyd exposure resulted in formation of low molecular weight fragments of newly synthesized DNA, whereas previously synthesized DNA remained intact. They postulated that aza-dCyd inhibited DNA elongation. Our studies suggest that aza-dCyd has little effect on DNA elongation (Fig. 4) and that the observed damage results from the presence of alkali-labile sites in the DNA which are converted to single-strand breaks under the conditions of elution or electrophoresis. These effects of aza-dCyd are in contrast to those demonstrated for ara-C. ara-C has been shown to possess a partial, concentration-dependent DNA chain-terminating activity (25) and to produce alkaline elution profiles showing no evidence of alkali-labile sites (24). This is consistent with our demonstration that ara-C markedly slows chain elongation but that the block is incomplete and gradual growth to full size DNA can occur. When these observations are considered together with the finding that ara-C potentially inhibits overall DNA synthesis (25), whereas aza-dCyd does not (2), it can be concluded that these two compounds have contrasting cellular effects and produce DNA damage through different mechanisms.

Following a 1-h treatment of L1210 with H₂-aza-Cyd (50 µg/ml), further DNA elongation was completely inhibited for at least 6 h (Fig. 4). This is somewhat surprising in light of the near normal elongation which occurred during the 1 h of exposure (*t*₀ in Fig. 4A). Moreover, it was demonstrated in Figs. 1 and 3 that rapidly eluting DNA present after 24 h of simultaneous H₂-aza-Cyd and label will increase in size if cells are incubated for an additional 24 h in the absence of drug. These observations are consistent with a model in which H₂-aza-Cyd incorporation does not produce chain termination within a single replicon but prevents subsequent ligation of replicons to

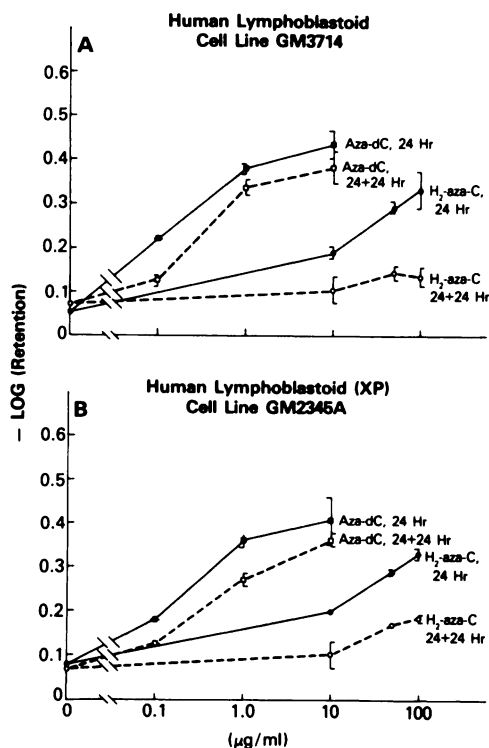


Fig. 6. Strand length [proportional to $-\log$ (retention)] of [¹⁴C]DNA from normal human lymphoblasts (A) or xeroderma pigmentosum lymphoblasts (B) as a function of H₂-aza-Cyd (H₂-aza-C) (●, ○) or aza-dCyd (Aza-dC) (■, □) concentration. (Retention), fraction of DNA retained on the filter after 15 h of elution (fifth fraction) considering the retention after collection of the first fraction to be 1.0. Duration of drug treatment and simultaneous labeling with [¹⁴C]thymidine was 24 h, with elutions carried out at pH 12.6 immediately after drug washout (●, ■) or following 24 h postincubation in drug-free medium (○, □). Points, the mean of two values; bars, range.

form large DNA strands. If the drug-free incubation time is sufficiently long, it appears that this block can be partially overcome.

H₂-aza-Cyd and aza-dCyd are related compounds differing in the sugar moiety, and in that the azapyrimidine ring of H₂-aza-Cyd is 5,6-saturated. This latter feature confers stability to the H₂-aza-Cyd molecule: aza-dCyd undergoes spontaneous ring opening in aqueous solution ($t_{1/2}$ = 17 h at 37°C, pH 7.4) ultimately forming 1- β -D-2'-deoxyribofuranosyl-3-guanyurea (4, 14), whereas H₂-aza-Cyd demonstrates no decomposition under similar conditions (15). We have previously proposed (21) that the formation of DNA alkali-labile sites in aza-dCyd-treated cells is related to the incorporation of the chemically unstable azacytosine base into DNA. It is uncertain if aza-dCyd incorporated into DNA undergoes spontaneous ring opening *in vivo*; however, azapyrimidine ring cleavage in alkaline solution may lead to opening of the deoxyribose ring and ultimately to strand scission in a manner analogous to the behavior of DNA base-free sites upon alkali treatment (21). In contrast, H₂-aza-Cyd is chemically stable and did not produce alkali-labile sites. Presumably, therefore, the dihydro-5-azacytosine ring was incorporated as such and not after reduction to azacytosine.

DNA damage produced by both aza-dCyd and H₂-aza-Cyd decreases when treated cells are incubated in drug-free medium, although the rate at which this process occurs is quite different. H₂-aza-Cyd lesions are partially repaired within 24 h in both L1210 and the human cells (Figs. 3A and 6), while lesions produced by aza-dCyd are only moderately decreased in the human cell lines (Fig. 6) and actually increase in L1210 (Fig. 3B) over the first 24 h. Previous studies (21) demonstrated that the frequency of aza-dCyd lesions in L1210 is reduced somewhat after 72 h of postdrug incubation.

If aza-dCyd-induced DNA damage arises by alkali-dependent strand cleavage, the lesions present in native DNA *in vivo* would simply be misincorporated intact aza-dCyd molecules or possibly their degradation products. Since the lesions observed by alkaline elution can decrease with time, some cellular repair process may recognize and remove, albeit slowly, aza-dCyd moieties from DNA. In contrast, H₂-aza-Cyd damage, part of which may result from an inhibition of DNA elongation and/or ligation, is repaired more rapidly and extensively. If incorporated H₂-aza-Cyd moieties are removed, elongation of DNA to normal size may then occur. It remains unclear as to why repair of H₂-aza-Cyd lesions proceeds to a limit (the level of damage initially produced by 5 μ g/ml) and goes no further.

Differences in the chemical conformation of aza-dCyd and H₂-aza-Cyd (planar *versus* nonplanar) may contribute to the contrasting efficiencies of their recognition in DNA by repair enzymes. The essentially identical repair observed in the normal human lymphoblastoid line and in the XP line indicates that the excision repair activity deficient in XP cells is not involved in the removal of lesions produced by either cytidine analogue.

Flow cytometry studies demonstrated that aza-dCyd blocked L1210 cells in late S phase or G₂-M, while H₂-aza-Cyd had no pronounced effect on cell cycle progression. This result for H₂-aza-Cyd conflicts with the findings of Traganos *et al.* (26). These investigators demonstrated a significant effect on H₂-aza-Cyd on L1210 cell kinetics. Continuous exposure to concentrations of 50 μ g/ml for 24 or 48 h depleted the percentage of cells in S phase, while higher concentrations (200–500 μ g/ml) blocked cells in S phase. Traganos *et al.* (26) did not investigate the cell cycle effects of aza-dCyd. The reasons for these disparate results is unknown, although it is possible that

differences in cell culture methods and/or analysis conditions were involved.

When the range of concentrations of aza-dCyd and H₂-aza-Cyd used for alkaline elution and flow cytometry studies are compared relative to effects on L1210 cell viability, a contrast between the two compounds is again observed. As measured by soft-agar colony forming ability of treated cells, 24 h exposure to aza-dCyd yields a 1-log cell kill at 0.1 μ g/ml and a maximum of 3 logs at 1–100 μ g/ml (4). H₂-aza-Cyd exposure at concentrations less than 5 μ g/ml produces little or no cytotoxicity, while 100 μ g/ml yields only a 1- to 1.5-log cell kill (27). Thus H₂-aza-Cyd appears both less potent and less efficacious than aza-dCyd *in vitro*, a pattern which is repeated in the results of therapy studies with L1210 bearing mice *in vivo* (5, 27). While these findings are consistent with the lower potency of H₂-aza-Cyd for producing DNA lesions and with the more extensive reversal of H₂-aza-Cyd-induced damage, the exact relationship between the cytotoxicity of these compounds and the DNA damage produced remains uncertain. It has been reported that aza-dCyd cytotoxicity is also correlated with the inhibition of DNA methylation (20). Moreover, Jones and Taylor (28) have shown H₂-aza-Cyd to be less active than aza-dCyd in inhibiting methylation. Since this process and the production of DNA lesions appear to require anabolism to the nucleotide triphosphate and incorporation of the drugs into DNA (10), differences in cytotoxicity between H₂-aza-Cyd and aza-dCyd may ultimately be related to the extent of their metabolic activation. Nevertheless, the contrasting nature of DNA damage produced by these two compounds suggests that the biochemical basis for their cytotoxic activity may be quite different.

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