

2-Chloro-2'-deoxyadenosine, an Antileukemic Drug, Has an Early Effect on Cellular Mitochondrial Function

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SUMMARY

2-Chloro-2'-deoxyadenosine [CldAdo (cladribine)], a novel effective antileukemic agent, was examined for its effects on cellular mitochondrial function and DNA content after long term (≤ 7 -day) incubation of cultured CCRF-CEM human leukemia cells. Dideoxycytidine (ddC), which is known to have a delayed effect on mitochondrial DNA content, was used as a positive control to monitor mitochondrial dysfunction. CldAdo at 6–16 nM was toxic to cells within 24 hr, which is in contrast to 300 nM ddC, which had no effect on cell growth for the first 4 days of treatment. Cellular lactic acid production was used to monitor concomitant perturbations in oxidative phosphorylation during drug treatment. Unlike the delayed increase in lactate observed with ddC exposure, CldAdo-treated cells exhibited a 2–2.4-fold increase in lactate levels after 2 days of exposure to 16 nM

CldAdo. By days 4 and 7, however, lactate production returned to control levels. Shorter incubations with CldAdo revealed that lactate levels began to increase within 12 hr of drug exposure, paralleling cytotoxicity. We also examined mitochondrial DNA content during drug treatment by competitive polymerase chain reaction. ddC (300 nM) reduced mitochondrial DNA levels from ~ 1000 copies/untreated cell to ~ 130 copies/cell after 7 days of exposure. In contrast, cytotoxic doses of CldAdo had little or no effect on mitochondrial DNA content during the 1-week incubation. Thus, the early CldAdo-induced perturbation of mitochondrial function was not associated with a loss of mitochondrial DNA per cell. In addition, no evidence of DNA laddering, indicative of cellular apoptosis, was detected at these dosage levels and treatment times.

CldAdo (cladribine), an analog of deoxyadenosine, is a clinically important new drug for the treatment of adult and pediatric leukemias. CldAdo is very effective in producing complete remissions in patients with hairy cell leukemia (1) and has been approved by the Food and Drug Administration for use against this disease. Other clinical studies have investigated the therapeutic value of CldAdo in the treatment of a number of lymphoid and myeloid malignancies, including pediatric acute leukemias, adult chronic lymphocytic leukemia, low-grade lymphoma, and cutaneous T cell lymphoma (for a review, see Ref. 2). Toxic side effects from a single course of CldAdo treatment (0.1 mg/kg/day, continuous infusion for 7 days) are limited (primarily bone marrow suppression and immunosuppression) with no significant nausea, vomiting, skin rash, or hair loss (2). However, the immunosuppression observed with CldAdo has been of some concern. Bacterial infections occur and require vigorous treatment with antibiotics; recovery occurs between 6 and 12 months (3). Patients treated with sequential purine analog therapy have a heightened risk for toxicity. Subsequent treatment with CldAdo results in even greater myelosuppression and an inability to tolerate repeated courses (4).

To be effective as therapeutic agents, nucleoside analogs such as CldAdo must be phosphorylated within the cell by nucleoside kinases. Deoxycytidine kinase activity has been strongly linked to CldAdo-induced cell death (5). However, mitochondrial deoxyguanosine kinase was recently purified and identified as the CldAdo-phosphorylating enzyme in brain tissue (6). In addition, mitochondrial extracts from cultured CEM leukemia cells were found to contain significant CldAdo-phosphorylating activity that was not inhibited by excess 2'-deoxycytidine, indicating the involvement of deoxyguanosine kinase. Characterization of purified mitochondrial deoxyguanosine kinase revealed that CldAdo and dAdo were equally efficient substrates. CldAdo is a substrate for human DNA polymerase- γ , which is involved in mitochondrial DNA synthesis (7). *In vitro* DNA strand elongation by polymerase- γ is significantly reduced in the presence of CldATP but not absolutely arrested (7). However, the effects of CldAdo on mitochondrial DNA level and function have not been explored.

No studies have addressed whether interference with mitochondrial biogenesis and function is in part responsible for the short and long term myelosuppressive side effects observed with CldAdo. Several lines of evidence link impaired mitochondrial function to cell death and toxic side effects in patients undergoing treatment with other nucleoside ana-

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ABBREVIATIONS: CldAdo, 2-chloro-2'-deoxyadenosine; CldATP, 2-chloro-2'-deoxyadenosine-5'-triphosphate; ClAdo, 2-chloroadenine; AZT, azidothymidine; ddC, 2',3'-dideoxycytidine; PCR, polymerase chain reaction; dNTP, deoxyribonucleoside triphosphate; bp, base pair(s).

logs. The dose-limiting toxicity of both dideoxycytidine and dideoxyinosine is peripheral neuropathy (8). *In vitro*, 2',3'-dideoxycytidine-5'-triphosphate is a potent inhibitor of DNA polymerase- γ (9). *In vivo*, ddC inhibits mitochondrial DNA synthesis in proliferating cells (CEM leukemia and Molt-4F cells) and nonproliferating cells (differentiated rat pheochromocytoma) (10, 11) and depletes cellular mitochondrial DNA levels at very low drug concentrations (10, 11). Major toxicities of azidothymidine, also used in AIDS therapy, include severe bone marrow suppression and both skeletal and cardiac myopathy (12). AZT has likewise been shown to inhibit not only mitochondrial DNA replication in isolated mitochondria (13) but also protein glycosylation in the Golgi complex (14). These observations suggest that mitochondria and other cellular organelles may be important targets for the adverse effects of several nucleoside analogs. CldAdo is unique in its ability to kill both dividing and nondividing cells. Because ribonucleotide reductase is virtually undetectable and DNA synthesis is turned off in resting cells, the mechanism of CldAdo-induced cell death must be different in such cells, possibly via an effect on mitochondria. If incorporated into mitochondrial DNA of nonproliferating cells, CldAdo could likewise exert some of its effects on mitochondrial DNA synthesis, causing cell death, myelosuppression, or both.

In the current study, we assessed the effects of CldAdo on mitochondrial DNA content and function after continuous long term (≤ 7 -day) treatment of proliferating cells with low-to-moderate concentrations of CldAdo. We also examined possible cellular induction of apoptosis at the same dosage levels and incubation times of CldAdo.

Experimental Procedures

Materials. A lactate analysis kit was purchased from Sigma Chemical (St. Louis, MO). The PCR Mimic Construction Kit was from Clontech Laboratories (Palo Alto, CA). Spinner minimum essential medium was from Sigma Chemical (St. Louis, MO) or Atlanta Biologicals (Norcross, GA). Dideoxycytidine was from Pharmacia (Piscataway, NJ). *Taq* DNA polymerase and ultrapure dNTPs were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Oligonucleotide primers Mimic 1, Mimic 2, Mito 06, and Mito 187 were synthesized by Cruachem (Sterling, VA). CldAdo was a generous donation from Ortho-Biotech (Raritan, NJ). CCRF-CEM human acute lymphoblastic leukemia cells were from American Type Culture Collection (Rockville, MD).

Cell culture. CEM cells were maintained as a suspension culture in Spinner minimum essential medium, 10% fetal bovine serum, and antibiotics at 37° in a humidified atmosphere of 95% air/5% CO₂. To compare the effects of continuous CldAdo or ddC exposure on mitochondrial DNA function and content, the drug treatment schedules reported by Chen and Cheng (10) were used. CEM cells at a concentration of 2×10^5 cells/ml were treated with various concentrations of CldAdo. On days 2, 4, and 7, cell growth was assessed by hemacytometer and/or Coulter counter. Cells were then spun down and reseeded at 2×10^5 cells/ml in 2 ml of fresh medium without or with drug at days 2 and 4. As a positive control, cells were also incubated with 300 nM ddC for the same time periods.

DNA extraction. Cellular DNA was extracted using a simplified procedure described by Grimaldi *et al.* (15). On day 2, 4, or 7 of drug treatment, cells were rinsed twice with phosphate-buffered saline, centrifuged briefly, and resuspended in 340 μ l of lysis buffer (400 mM Tris-HCl, pH 8.0, 60 mM EDTA, 150 mM NaCl, and 1% w/v sodium dodecyl sulfate) and 100 μ l of 5 M sodium perchlorate. Cells were vortexed and then incubated at 37° for 20 min with shaking, followed by 65° incubation for 20 min with mild agitation. The mixture was

extracted once with cold chloroform through rotation at room temperature for 20 min. Tubes were spun in a microcentrifuge to separate the two layers. The DNA layer was removed and ethanol-precipitated in 0.3 M sodium acetate. The DNA was rinsed in 80% ethanol, dried, and resuspended in H₂O to give an equivalence of 5000 cells/ μ l.

Mitochondrial mimic DNA synthesis. Mitochondrial mimic DNA was generated by two rounds of PCR amplification using "neutral" double-stranded DNA from a PCR Mimic kit and two composite primers, Mimic 1 and Mimic 2, which had the following respective sequences: Mimic 1 primer, 5'-CAG GTC TAT CAC CCT ATT AA-CGC AAG TGA AAT CTC CTC CG; and Mimic 2 primer, 5'-CGC CTG TAA TAT TGA ACG TA-TTT CAT CTC CCT GTA TAA CA. Twenty bases at the 5' end of each primer (underlined) were mitochondrial DNA-specific sequences. The remaining 20 bases at the 3' end represented sequences that annealed upstream and downstream, respectively, to the neutral DNA fragment. During the first round of PCR, neutral DNA (2 ng) was amplified in 50 μ l of 1 \times PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, and 0.01% gelatin), 200 μ M final concentration of four dNTPs each, 20 pmol each of primers Mimic 1 and Mimic 2, and 2 units of *Taq* DNA polymerase. Amplification was conducted for 21 cycles for 45 sec at 94°, 45 sec at 60°, and 90 sec at 72°. From this reaction mixture, 2 μ l was diluted 100-fold, and 2 μ l of the diluted DNA product were then used in a second PCR amplification under similar conditions as above, except upstream and downstream mitochondrial DNA-specific primers, Mito 06 and Mito 187, with the following sequences, were substituted at 20 pmol each: Mito 06, 5'-CAG GTC TAT CAC CCT ATT AA-3'; and Mito 187, 5'-CGC CTG TAA TAT TGA ACG TA-3' (16). After 46 cycles, the 240-bp amplified mimic DNA was purified on Chroma spin 100 columns, and the DNA yield was determined by UV absorbance.

Determination of cellular mitochondrial DNA content. Cellular mitochondrial DNA levels were measured using competitive PCR described by Zhang *et al.* (16) with the following modifications. Reaction mixtures contained 2 μ l of cellular DNA (equivalent to 10,000 cells) with or without varying amounts of mitochondrial mimic DNA (from 6×10^8 to 6×10^5 molecules) in a final volume of 50 μ l that also contained 1 \times PCR buffer, 200 μ M concentration each of four dNTPs, 20 pmol each of primers Mito 06 and Mito 187, and 2 units of *Taq* DNA polymerase. DNA was amplified for 30 cycles with the same parameters used during mimic DNA amplification; PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide for viewing. Amplification of cellular mitochondrial DNA with primers Mito 06 and Mito 187 produced a 182-bp DNA fragment compared with the 240-bp mimic amplified product. The point of equivalent band intensities indicated a 1:1 molar ratio of mimic DNA and cellular mitochondrial DNA concentrations. Mitochondrial DNA levels were then expressed as copies per cell.

Lactate determination in CEM cells. CEM cells (2×10^5 cells/ml) were incubated as above with CldAdo or ddC (as a positive control) for 0–7 days. Cells were counted on days 2, 4, and 7 and then spun down; medium was saved and filtered through a 0.2- μ M filter; and lactate concentration in the medium was determined with the Lactic Acid Assay Kit. Cells were reseeded at 2×10^5 cells/ml in fresh medium with or without drug on days 2 and 4.

DNA fragmentation assay. Cellular induction of apoptosis was examined with the use of a DNA laddering assay. Control and CldAdo-treated CEM cells (5×10^5) were washed with phosphate-buffered saline and spun down gently. Pelleted cells were resuspended in 20 μ l of hypotonic lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.2% Triton X-100, pH 7.5). Lysates were centrifuged at $13,000 \times g$ for 10 min to separate the intact chromatin from small fragmented DNA in the supernatant. The supernatant was transferred to a separate tube, and large DNA in the pellet was resuspended in 100 μ l of lysis buffer. Proteinase K (0.5 mg/ml) was added to both supernatant and pelleted DNA, and samples were incubated at 50° for 1 hr. DNA was extracted twice with phenol/chloroform,

precipitated in ethanol, and air dried. DNA was then resuspended in 10 mM Tris-HCl, 1 mM EDTA, and 100 mM NaCl plus DNase-free RNase (80 $\mu\text{g}/\text{ml}$) and incubated for 1 hr at 50°. Glycerol loading buffer was added, and samples were heated at 65° for 10 min. DNA fragmentation was analyzed by electrophoresis of the DNA samples on a 1.75% agarose gel that was subsequently stained with ethidium bromide.

Results

Fig. 1 illustrates a cell-survival curve for various concentrations of CldAdo; points shown on the graph represent the mean of three determinations with standard deviations. As a positive control, separate cultures were incubated with 300 nM ddC. Dideoxycytidine has been shown to have dramatic, delayed effects on cellular mitochondrial DNA content and lactate levels. At each concentration, CldAdo induced cell death by day 2; treatment with 16 nM CldAdo killed approximately half of the cells. Longer exposures led to greater cytotoxicity. Cell survival at day 7 with 6 nM CldAdo was only $55 \pm 3\%$ that of control cells, and 16 nM CldAdo decreased survival to $\sim 25 \pm 3\%$ compared with untreated cells. Similar CldAdo toxicity data have been published previously for CEM cells (17). In contrast, 300 nM ddC caused very little CEM cell killing at days 2 and 4; only after 7 days of incubation with ddC did the cell number decrease by $\sim 45\%$.

At the same time points, we measured lactate concentration (expressed as $\text{mg}/10^6$ cells) in the medium as a marker of mitochondrial dysfunction (Fig. 2). With the positive control of 300 nM ddC, cellular lactate was slightly elevated at day 2 (0.53 mg/ml compared with control levels of 0.44, a 1.2-fold increase) and then increased with longer exposures to the drug. At day 4, lactate levels in ddC-treated cells had increased ~ 1.9 fold to 0.69 $\text{mg}/10^6$ cells, whereas control levels were at 0.36 $\text{mg}/10^6$ cells; after 7 days, cellular lactate reached 0.7 $\text{mg}/10^6$ cells compared with 0.29 $\text{mg}/10^6$ control cells, a 2.4-fold difference. Cellular CldAdo treatment resulted in a very different lactate production profile. A large

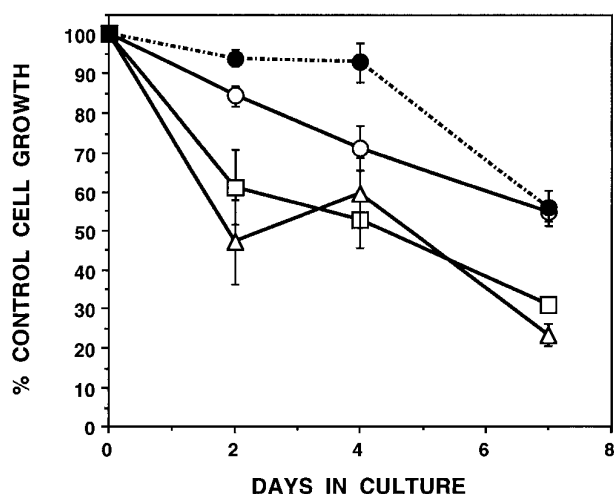


Fig. 1. Cytotoxicity after continuous exposure to CldAdo or ddC. CEM leukemia cells at a concentration of 2×10^5 cells/ml were treated with various concentrations of CldAdo or 300 nM ddC. On days 2, 4, and 7, cell growth was assessed by hemacytometer and/or Coulter counter. On days 2 and 4, cells were then reseeded at 2×10^5 cells/ml in 2 ml of fresh medium with or without drug. \circ , 6 nM CldAdo; \square , 12 nM CldAdo; \triangle , 16 nM CldAdo; \bullet , 300 nM ddC. Values are the mean of three determinations with standard deviations.

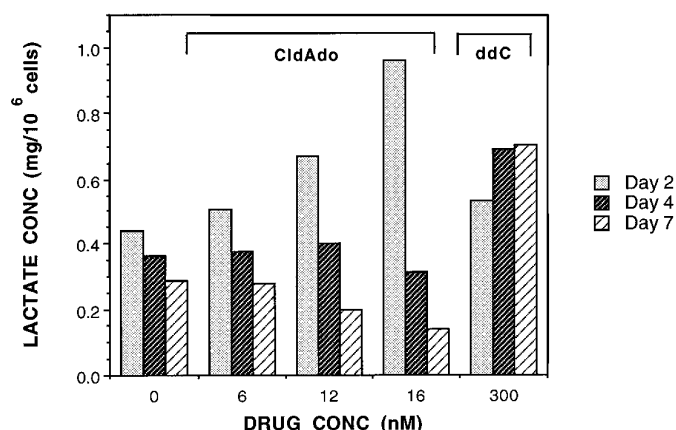


Fig. 2. Lactic acid production after continuous exposure to CldAdo or ddC. CEM cells were incubated as described in the legend to Fig. 1 with either 6, 12, or 16 nM CldAdo or 300 nM ddC. Culture medium was collected on days 2, 4, and 7, was filtered, frozen, and subsequently assayed for lactic acid content. Lactate concentration at each time interval was reported as $\text{mg}/10^6$ cells.

spike in lactate concentration that was dose dependent was observed at day 2. With 6 nM CldAdo, the lactate concentration was elevated slightly to 0.51 $\text{mg}/10^6$ cells compared with control levels of 0.44 $\text{mg}/10^6$ cells. With 16 nM CldAdo, lactate levels increased 2.2-fold to 0.97 $\text{mg}/10^6$ cells compared with control levels. However, by days 4 and 7, lactate production returned to control levels at all CldAdo concentrations used or decreased slightly. These experiments were repeated three times, and identical trends were observed.

To more accurately establish the time of onset of lactate production, we measured cytotoxicity and lactic acid in medium from CldAdo-treated cells within 24 hr of drug exposure. CldAdo-induced cytotoxicity was undetectable for the first 6 hr of drug treatment after 6, 12, or 16 nM CldAdo (Table 1) but was elevated slightly at all three concentrations by 12 hr. Within 24 hr after 16 nM CldAdo exposure, cell survival had decreased to $\sim 53\%$ compared with control cells. At all three CldAdo doses used, little change occurred in lactic acid production compared with control levels after 3 hr (data not shown) or 6 hr (Table 1). However, after a 12-hr incubation with CldAdo, lactate amounts began to increase compared with control amounts, even with 6 nM CldAdo. By 24 hr, the ratio of lactate in CldAdo-treated cells and control

TABLE 1
Cytotoxicity and lactate production in CEM cells after short-term CldAdo treatment

Cells were seeded at an initial cell concentration of 2×10^5 cells/ml in 1 ml of medium with or without CldAdo; control cell number was set at 100% at each time point. After a 24-hr incubation, untreated cells were at a level of 4.14×10^5 cells/ml. Cell survival after CldAdo treatment was reported relative to control levels. Percentages shown are the average of two determinations. Lactate concentration ($\text{mg}/10^6$ cells) in the culture medium was assessed at each time point shown. Control lactate amounts were set at 1.0; measurements with CldAdo were calculated relative to control cells and reported as the ratio of [lactate] + CldAdo/[lactate] control medium. The average value of two experiments is shown.

CldAdo	Cell survival			Lactate concentration		
	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr
None	100	100	100	1.00	1.00	1.00
6 nM	96	88	79	0.97	1.15	1.11
12 nM	98	81	62	1.03	1.24	1.58
16 nM	96	74	53	1.03	1.35	1.83

cells had increased to 1.58 and 1.83 after 12 and 16 nM doses, respectively. Thus, enhanced lactate expression seemed to follow CldAdo-induced cytotoxicity profiles.

In addition, we assessed cellular mitochondrial DNA levels through competitive PCR after CldAdo or ddC treatment for the 1-week period. With this technique, a competitor "mimic" DNA molecule is first synthesized that contains complementary primer sequences identical to those of a cellular target DNA fragment (i.e., mitochondria-encoded DNA) but differs in size from the cellular sequence. A range of known mimic DNA concentrations is then included as an internal standard along with a fixed amount of cellular DNA during PCR amplification reactions. Both DNA species compete for the gene-specific primer set, and coamplification yields two products of differing sizes that are separated by gel electrophoresis. The point of equivalent band intensities on an ethidium bromide-stained gel is where mimic DNA equals cellular target DNA; this represents a 1:1 molar ratio. Shown in Fig. 3 are competitive PCR results from several time points. All reactions had 10,000 cells of DNA and varying amounts of the internal standard mitochondrial mimic DNA. In the experiment shown, the point of equivalence between mimic DNA and cellular mitochondrial DNA in control cells at day 2 was between *lane 2* (1.2×10^7 copies of mimic per reaction) and *lane 3* (9×10^6 copies); at day 4, bands of equal intensity were observed in *lane 2*, and at day 7, equivalent bands occurred between *lanes 2* and *3*. With 300 nM ddC treatment, bands of equal intensity were observed in *lane 3* at days 2 and 4, but these shifted dramatically to the right by day 7, to *lane 5* (1.2×10^6 copies of mimic per reaction), a 90% decrease in copy number. The ddC effects on mitochondrial DNA levels were in accordance with other published trends and provided a good control for our CldAdo experiments. In contrast to ddC, however, with 16 nM CldAdo exposure, we detected little or no change in the equivalence points between mimic and cellular DNA compared with control levels at any time examined up to day 7. After continuous CldAdo treatment, bands of equal intensity were observed between *lanes 2* and

3 on both day 2 and 4 and in *lane 3* (9×10^6 copies/reaction) on day 7.

The competitive PCR experiments were repeated at least two or three times, and the copy number of mitochondrial DNA under each condition was quantified based on the point of equal band intensities (Table 2). In control cells, mitochondrial DNA levels displayed little variability in copy number during the 7-day experiments and ranged from 900 to 1200 copies/cell, which was in fairly close agreement with other published determinations for cultured leukemia cells. With 300 nM ddC treatment, the average mitochondrial DNA level at day 2 was ~ 950 copies/cell. We observed a 30% reduction in this level to 650 copies at day 4, and by day 7, mitochondrial DNA content had fallen dramatically to 130 ± 23 copies/cell compared with the control (no drug) level of 980 copies/cell. Furthermore, CldAdo treatment resulted in similar mitochondrial levels to those of control (untreated) cells during days 2 and 4, and there was only a slight, insignificant 10% decrease from the control copy number of 980 ± 76 /cell at day 7 to 930 ± 60 copies/CldAdo-treated cell. Thus, the strong inhibitory effect on mitochondrial DNA content that has been found with other nucleoside analogs was not seen after 7 days of cellular incubation with CldAdo.

We then assessed whether apoptosis exhibited by the formation of DNA oligomers could be detected in treated CEM cells at various times of cytotoxic drug exposures (Fig. 4). A comparison of DNA electrophoretic migration patterns on an agarose gel from either the pellet (high-molecular-weight chromatin) or supernatant (soluble low-molecular-weight DNA) of lysed cells revealed no significant differences between control untreated cells and cells exposed to 16 nM CldAdo for 0.5, 2, or 4 days. At day 7, when cell survival in CldAdo-treated cultures was only 25% of the control level, there seemed to be a higher degree of DNA degradation in the supernatant from drug-treated cells, but no characteristic DNA laddering was observed with drug treatment at any of the time points examined.

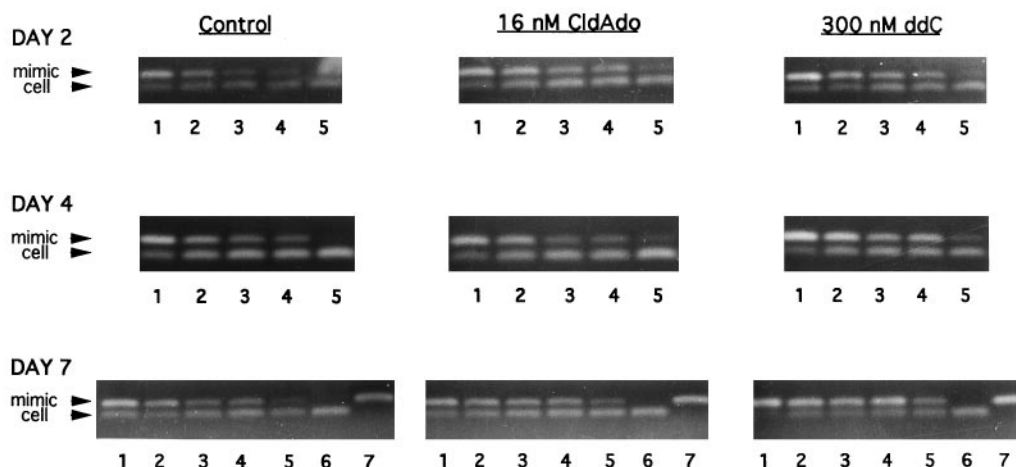


Fig. 3. Cellular mitochondrial DNA levels in CldAdo- and ddC-treated CEM cells. Cells were incubated as described in the legend to Fig. 1 with either 16 nM CldAdo or 300 nM ddC. Total cellular DNA was extracted after 2, 4, and 7 days. Mitochondrial DNA levels were measured by competitive PCR in a mixture that contained 2 μ l of cellular DNA (equivalent to 10,000 cells) with or without varying amounts of mitochondrial mimic DNA and mitochondrial DNA-specific primers, Mito 06 and Mito 187. DNA was amplified for 30 cycles; PCR products were electrophoresed on a 1.75% agarose gel and stained with ethidium bromide. *Top bands*, amplified competitor mimic; *bottom bands*, amplified cellular mitochondrial DNA. The concentration of mitochondrial mimic DNA in each reaction was as follows: *lane 1*, 6×10^7 copies; *lane 2*, 1.2×10^7 ; *lane 3*, 9×10^6 ; *lane 4*, 6×10^6 ; and *lane 5*, 1.2×10^6 copies/PCR reaction; *lane 6*, cellular DNA only; *lane 7*, only mitochondrial mimic DNA at 1.2×10^7 copies.

TABLE 2

Effect of CldAdo and ddC on mitochondrial DNA copy number

Mitochondrial DNA content was assessed by competitive PCR (as shown in Fig. 3) and is reported as copies per cell. Numbers shown are the average of two determinations for days 2 and 4; on day 7, the mean value of three determinations with and without standard deviation is reported.

Time of incubation	Control	16 nM CldAdo	300 nM ddC
Day 2	1000	1000	950
Day 4	1100	1000	650
Day 7	980 \pm 76	930 \pm 60	130 \pm 23

Discussion

Despite fairly extensive investigations, the exact cause of patient toxicity from various nucleoside analogs is not understood. The bone marrow suppression observed after clinical CldAdo treatment regimens resembles to some extent AZT-induced patient toxicity. With AZT, neutropenia and anemia are prevalent early side effects that are followed at later times and longer treatment regimens by cardiac and skeletal myopathies, which have not been observed with short term CldAdo therapy. In contrast to CldAdo and AZT, the nucleoside analogs 2',3'-dideoxyinosine and ddC do not result in myelosuppression but instead produce painful peripheral neuropathy. Mitochondrial toxicity in the absence of cell killing has been strongly correlated to AZT-induced side effects (11). Others, however, have attributed the toxic side effects of AZT to an accumulation of AZT-monophosphate, which has been demonstrated to inhibit potently cellular protein glycosylation and nucleotide-sugar import into the Golgi complex (14). In addition, AZT has been shown to hinder preferentially globin gene expression in erythroid cells (18), which would lead to anemia.

Although we have not conducted such an exhaustive study of CldAdo-induced cellular effects, we did not observe in CldAdo-treated cells the progressive, delayed (5–7-day exposure) elevated lactate production and concomitant reduction in mitochondrial DNA content that have been detected in both AZT- and ddC-treated cells. However, we did detect an unusual early increase in lactic acid synthesis in CldAdo-treated cells that occurred within a 12-hr incubation with CldAdo and was sustained for 48 hr. With longer exposures, however, the viable cells did not continue to exhibit this increase. This is the first report of an effect on cellular mitochondrial function by CldAdo. Furthermore, mitochondrial DNA content was not significantly affected at days 2, 4, and 7 of CldAdo exposure, which is in contrast to the dramatic reduction in mitochondrial copy number at day 7 that was induced by ddC incubation. Thus, the observed elevated lactate levels in CldAdo-treated cells were not linked to an apparent loss of mitochondrial DNA and must be produced by other mechanisms.

Inhibition of cellular ATP production may instead be associated with reduced levels of mitochondrial-encoded proteins involved in electron transport, such as subunits for cytochrome-*c* oxidase or ATP synthase. CldAdo could interfere either directly (by incorporation into mitochondrial DNA) or indirectly with mitochondrial transcription or mitochondrial mRNA levels. We have shown previously that incorporated ClAdo residues in template DNA dramatically inhibit DNA transcriptional processes (19). When ClAdo was present in place of Ade in both strands of a synthetic DNA molecule, the yield of full-length transcripts by RNA polymerase *in vitro*

was reduced by ~90% compared with control DNA. Transcription was also reduced to a slightly lesser degree when ClAdo substitutions occurred in only one of two strands (19). Furthermore, *in vitro* studies have demonstrated that CldATP is incorporated into DNA by mitochondrial DNA polymerase- γ but is not a chain terminator (7). Mitochondrial DNA doubles on the average once every cell cycle, and therefore, after a 12–48-hr incubation period in CldAdo, sufficient analog could be incorporated in mitochondrial DNA to decrease transcription by mitochondrial RNA polymerase. Most mitochondrial-encoded mRNA species, including cytochrome-*b* and ATPase-6, are relatively short lived; their half-lives range from 50 to 80 min (20). This would subsequently prevent sufficient production of crucial mitochondrial proteins.

On the other hand, high intracellular CldATP levels could indirectly repress transcription of mitochondrial genes. CldAdo uptake and accumulation as the triphosphate within a cell markedly inhibit ribonucleotide reduction of ADP to dADP within 4 hours of drug treatment, leading to an abnormally high ADP concentration (21). ADP at high levels is thought to cause a net influx of adenine nucleotides into mitochondria and an increase in the intramitochondrial ATP concentration (22). Recently, Enriquez *et al.* (22) demonstrated that high intramitochondrial ATP levels in turn inhibit mitochondrial RNA polymerase activity both *in organello* and *in vitro*. Inhibition of mitochondrial DNA transcription decreases the levels of 13 mRNA species and will eventually reduce the levels of a subset of mitochondrial proteins that are necessary for electron transport and oxidative phosphorylation. Alternatively, CldAdo may affect mitochondria through a mechanism similar to that of cellular treatment with type I interferon. Interferons cause impairment of mitochondrial function, a down-regulation of mitochondrial gene expression, and a reduction in the synthesis of several mitochondrially encoded proteins (23). At least part of the interferon effect is thought to occur via induction of a nuclear gene that modulates mitochondrial gene expression (24).

The large spike in lactate production at 12–48-hr incubation with CldAdo paralleled to some extent cell killing. This finding is of interest in light of reports that a number of diverse cytotoxic agents, such as interferon and benzo[*a*]pyrene, have unusual early effects on mitochondrial function (24–26). Furthermore, as noted above, AZT was thought to affect mitochondrial function only after relatively long exposures. However, recent studies demonstrated that in addition to drug-induced delayed mitochondrial toxicity, AZT causes rapid effects, after only a 7–11-hr incubation, on oxidative phosphorylation (27). In a similar manner to the lactate profile observed with CldAdo incubation, lactate levels after cellular AZT treatment reached a peak at 48 hr and then fell to control levels by day 4. Hobbs *et al.* (27) thus concluded that AZT may also be exerting an early effect on an unidentified metabolic step that is crucial to cell growth and oxidative phosphorylation.

Mitochondria provide energy metabolism, are essential for cell and tissue functions, and are the major site of ATP synthesis. Enhanced cellular lactate synthesis is suggestive of a disturbance in oxidative phosphorylation that, in turn, reflects impaired mitochondrial function. It should be noted, however, that although both elevated lactic acid production

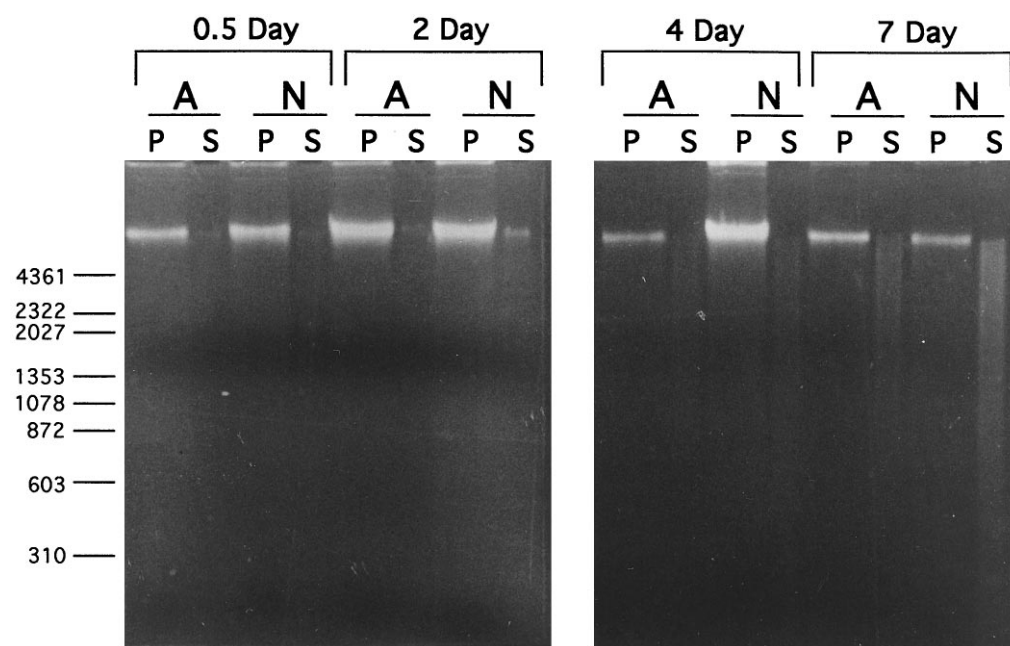


Fig. 4. Effect of low-dose CldAdo on the induction of cellular DNA fragmentation. CEM cells were incubated without (A) or with (N) 16 nM CldAdo for the times indicated and lysed in 0.2% Triton X-100. Soluble low-molecular-weight DNA fragments (S) were separated from high-molecular-weight DNA (P) through centrifugation. DNA samples were treated with proteinase K and RNase. Electrophoretic mobility was assessed on a 1.75% agarose gel followed by ethidium bromide staining. *Numbers on the left*, size of DNA fragments in base-pairs.

in cultured cells and the development of lactic acidosis in patients treated with other nucleoside analogs have been linked in several reports to mitochondrial abnormalities (11, 28), there are other mitochondria-independent mechanisms that can lead to altered lactate production, such as enhanced glucose uptake, that has been observed in tumor cells. Oxidative phosphorylation is a complex process that is connected with other cellular events whose impairment would lead to rapid inhibition of cell growth. ATP produced through the citric acid cycle is the preferred cellular immediate energy source. When this process is faulty or impaired, cells resort to glycolysis to make ATP, albeit inefficiently. Several studies have also suggested that an early marker of cellular apoptosis is a perturbation of this critical mitochondrial function (29). Mitochondrial dysfunction is first evident in distressed cells as a loss of mitochondrial transmembrane potential (30, 31), followed by an uncoupling of electron transport from ATP synthesis. This, in turn, can lead to enhanced formation of reactive oxygen species (29). In addition, levels of mitochondrial DNA have been demonstrated by others to remain approximately constant despite impaired mitochondrial function (32, 33), as we found in the current study.

More importantly, altered mitochondrial function and disturbed oxidative phosphorylation have been shown by several groups (31, 32) to occur before and independent of common signs of cellular apoptosis, such as chromatin condensation and DNA laddering. In a similar manner, the CldAdo-induced effects on mitochondria that we observed in this study were not associated with or at least preceded nuclear DNA fragmentation. At the low but cytotoxic doses used in our experiments (6–16 nM CldAdo), we did not observe any internucleosomal laddering effects after 0.5, 2, 4, or 7 days. Although CldAdo treatment has been shown previously to induce cellular apoptosis (34–36), all three studies detected DNA laddering characteristic of apoptosis at doses that were extremely toxic to the cells (1–30 μ M CldAdo). In addition, the relevance of such findings to clinical or *in vitro* cytotoxicity is disputable. In contrast, steady state plasma CldAdo concentrations achieved in pediatric patients with

acute leukemia are much lower, ranging from ~23 to 85 nM, with a mean of 37.7 nM (37). Likewise, in a separate study conducted in adult leukemia patients, Liliemark and Juliusson (38) reported a mean plasma CldAdo concentration of only 22.5 nM after a 24-hr infusion. *In vitro* cytotoxicity for several leukemia cell lines is also observed at nanomolar quantities (17). The mitochondrial effects that we have detected occur at much lower doses (in the nanomolar range) than the drug levels used previously in apoptosis experiments, and therefore, they more accurately reflect clinically relevant drug concentrations.

It is also of interest that comparative, deleterious effects on mitochondrial function have been noted for both interferon-treated cells (23, 24) and CldAdo-treated cells (current study). Beginning in 1980, interferon was the preferred drug treatment for hairy cell leukemia (2) until the introduction and subsequent approval in 1993 of CldAdo as the drug of choice for this form of leukemia. The effects on mitochondria may explain why two seemingly disparate drugs (interferon and CldAdo) can achieve a comparable antiproliferative consequence to some extent against hairy cell leukemia. This leads us to speculate that the highly successful outcome for hairy cell leukemia patients, in particular, after CldAdo therapy may be linked to an unusual sensitivity of leukemic cell mitochondria to the disturbance of oxidative phosphorylation by CldAdo. Our future experiments will address the mechanism by which CldAdo affects mitochondrial function.

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