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ACCELERATED COMMUNICATION

Effect of Anti-Human Immunodeficiency Virus Nucleoside Analogs on Mitochondrial DNA and Its Implication for Delayed Toxicity

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SUMMARY

The anti-human immunodeficiency virus (-HIV) nucleoside analogs azidothymidine (AZT), dideoxycytidine (ddC), dideoxyinosine (ddI), dideoxydidehydrothymidine (D4T), and dideoxydidehydrocytidine (D4C) and the anticancer drug cytosine arabinoside (AraC) were compared for their effects on the mitochondrial DNA (mtDNA) content in a human lymphoblastoid cell line, CEM. The potency of these compounds in reducing mtDNA content was in the order of ddC > D4C > D4T > AZT > ddI. AraC did not have a significant effect on mtDNA content. All of the compounds tested, except AraC, stimulated lactic acid production at concentrations that inhibited mtDNA synthesis. The action of ddC and ddI occurred at concentrations that did not affect cell growth significantly in 4 days but retarded cell growth by day 6. D4T and D4C decreased mtDNA content by 50% at doses lower than those that inhibited cell growth by 50% in 4 days (ID_{50}). However, AZT required a dose higher than the ID_{50} to exert similar effects on mtDNA content. The decrease of mtDNA content caused by ddC also occurred in nerve growth factor-treated PC12 cells, which differentiate to neuron-like cells upon treatment with nerve growth factor. The preferential inhibition of mtDNA, compared with cell growth, by some of these anti-HIV nucleoside analogs correlates well with their ability to cause drug-limiting delayed toxicity, such as peripheral neuropathy, in patients. These data suggest that the selective mitochondrial toxicity could be responsible for the delayed toxicity caused by these anti-HIV analogs.

HIV is well accepted as being the causative agent of AIDS. Because AIDS is a fatal disease with increasing incidence and prevalence, much effort has been devoted to the development of an effective treatment against HIV. HIV is a retrovirus that requires viral reverse transcriptase for replication. This enzyme activity offers a target for development of selective anti-HIV drugs. Several nucleoside analogs have been shown to have anti-HIV activities both *in vitro* and *in vivo* (1–3), with this selectivity being attributed to the unique interaction between their active metabolites and the viral reverse transcriptase (4–6). Among these drugs, AZT has been widely used to treat patients with AIDS or AIDS-related complex and has been shown to improve the immunological function of patients with AIDS (7). ddC is a deoxycytidine analog that has been reported to be more potent than AZT against HIV in cell culture (8) and shown to have beneficial effects on patients with AIDS in clinical trials (1). ddI was also reported to have anti-HIV activity (3) and a favorable toxicity profile in clinical trials (2). Other dideoxynucleoside analogs, for example D4T and D4C, also have potent anti-HIV activity *in vitro* (9, 10). D4T was found to have an equivalent anti-HIV activity but less cytotoxicity than AZT in cell culture (11) and is currently under clinical trials.

It has been recognized that the continuous usage of these compounds is essential for the suppression of HIV replication in patients with AIDS. A complication of this continuous usage is the toxicity of these compounds. Bone marrow suppression, which generally occured within 4 weeks, was one of the major toxic effects in AIDS patient who received AZT therapy (12), whereas patients who took continuous doses of ddC developed a painful peripheral neuropathy after 8 to 12 weeks (13). A similar delayed toxicity to the peripheral nerve system was also reported in patients treated with ddI and D4T (2). It is unclear what mechanisms are responsible for the delayed-type toxicity of ddC, ddI, and D4T.

It was shown in this laboratory that ddC caused mitochon-

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ABBREVIATIONS: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency virus; AZT, azidothymidine; ddC, dideoxycytidine; ddI, dideoxyinosine; D4C, 2′,3′-dideoxy-2′,3′-didehydrocytidine; D4T, 2′,3′-dideoxy-2′,3′-didehydrothymidine; mtDNA, mitochondrial DNA; NGF, nerve growth factor; PCR, polymerase chain reaction; kb, kilobases; $mtID_{50}$, mitochondrial 50% inhibitory dose; AraC, cytosine arabinoside; Top I, topoisomerase I; ID_{50}, 50% inhibitory dose.

drial toxicity, which was characterized by profound inhibitory effects on mtDNA synthesis, and caused delayed cytotoxicity in cell culture at a clinically relevant dose (14). The mitochondrial toxicity of ddC is likely due to the preferential inhibition of DNA polymerase γ, compared with α or β, by its active metabolite ddC triphosphate (15, 16). Because mitochondria provide efficient energy metabolism and are essential for cell and tissue functions, anti-HIV drugs that affect mitochondria could produce undesired side effects during the course of chemotherapy. Thus, we hypothesized that the drug-limiting delayed-type toxicity exhibited by some of these anti-HIV nucleoside analogs could be due to their preferential effect on mtDNA of affected tissue. To test this hypothesis, the effects of AZT, ddC, D4T, ddI, D4C, and AraC on the mtDNA of CEM cells were investigated.

Experimental Procedures

Materials. ddC was purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). D4T and ddI were provided by Bristol-Myers Squibb Co. (Wallingford, CT). AZT was obtained from Sigma Chemical Co. (St. Louis, MO). D4C was a gift from C. K. Chu, College of Pharmacy, University of Georgia (Athens, GA). AraC was supplied by Upjohn Co. (Kalamazoo, MI). The lactic acid assay kit, random primer labeling kit, and NGF (2.5 S) were from Boehringer Mannheim (Indianapolis, IN). The DNA amplification reagent kit was purchased from Perkin Elmer Cetus (Norwalk, CT).

Cell cultures. CEM cells, a human T lymphoblastoid cell line, were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum. The CEM cells were obtained from Dr. William Beck, St. Jude Children's Research Hospital (Memphis, TN). PC12 cells, a rat pheochromocytoma cell line, were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 5% heat-inactivated horse serum. The PC12 cells were obtained from Diane Rosin, Department of Pharmacology Yale University (New Haven, CT). The cells were treated with NGF (40 ng/ml) for 2 weeks before drug treatments. NGF was refreshed every 2 days.

Lactic acid production. CEM cells were treated with drugs for 4 days. The cell number was determined and the culture medium was collected as previously described (14). The lactic acid content in the media was measured by using the Boehringer lactic acid assay kit, following the supplier's instructions. Briefly, 50 μl of the culture medium were used for lactic acid determination. The amount of lactic acid in the medium was expressed as mg of lactic acid production/10^6 cells.

Measurement of mtDNA content. Total cellular DNA from CEM cells or PC12 cells was prepared as previously described (14). The DNA (10 μg) was digested with *Bam*HI and analyzed on a 0.8% agarose gel. DNA in the gel was transferred to nitrocellulose paper. The mtDNA of CEM cells on the nitrocellulose paper was detected by using a ^{32}P-labeled human mtDNA fragment (14). The signals for the Top I control (14) were used to standardize the amount of total cellular DNA loaded. To quantitate the relative amount of mtDNA, the mtDNA bands or Top I bands on the nitrocellulose paper were cut out, and the radioactivities of the mtDNA bands were determined by scintillation counting. The probe used to estimate the mtDNA of PC12 cells is a 292-base pair fragment of the rat mitochondrial cytochrome oxidase II gene (17). The rat mtDNA fragment was prepared by amplifying the sequence with PCR. The two primers used for the PCR were 5′-AGACGCTACATCACCTATTATAGA-3′ and 5′-GCTTCAGTATCATTGGTGACCTAT-3′. The PCR was performed by following the supplier's protocol. The restriction enzyme *Bam*HI cuts the mtDNA of PC12 into two fragments, of 10.8 and 4.95 kb. The cytochrome oxidase II gene is located in the 10.8-kb fragment. A slot blot procedure was used as an alternative method for the determination of mtDNA content. The total cellular DNA was extracted as described (14) and blotted on nitrocellulose paper by using a Minifold slot blot apparatus (Schleicher & Schuell, Keene, NH). The mtDNA on the nitrocellulose paper was detected by using the 292-base pair DNA probe.

Results and Discussion

Cellular and mitochondrial toxicity of anti-HIV compounds. All of the tested anti-HIV drugs were able to decrease the mtDNA content of CEM cells within 4 days of exposure to drugs, as shown in Fig. 1. The mtDNA content in the cells treated with 0.2 μM ddC, 10 μM D4C, or 20 μM D4T was 3, 5, and 2% of control, respectively. This includes a correction for the total cellular DNA loaded (see Experimental Procedures). The preferential effect of these compounds on mtDNA, compared with nuclear DNA, is clearly demonstrated.

The drug concentrations that decreased mtDNA content by 50% in 4 days ($mtID_{50}$) are shown in Table 1. The potency in decreasing the mtDNA content in CEM cells was in the order of ddC > D4C > D4T > AZT > ddI (Table 1 and Fig. 1). AraC, an antitumor compound, had no inhibitory effect on mtDNA at the concentrations used, in spite of the cell growth being inhibited. All of these compounds exhibited different degrees of acute cytotoxicity. The ID_{50} values, which are the drug concentrations required to inhibit 50% of cell growth in 4 days in this study, are shown in Table 1. ddI was much less toxic to CEM cells than the other anti-HIV compounds examined. The ID_{50} value of D4T for CEM cells was 6-fold higher than that of AZT in this study (Table 1). This is consistent with the reports of others (11). Although ddI is not a potent inhibitor of mtDNA synthesis, the concentration of this drug required to exert

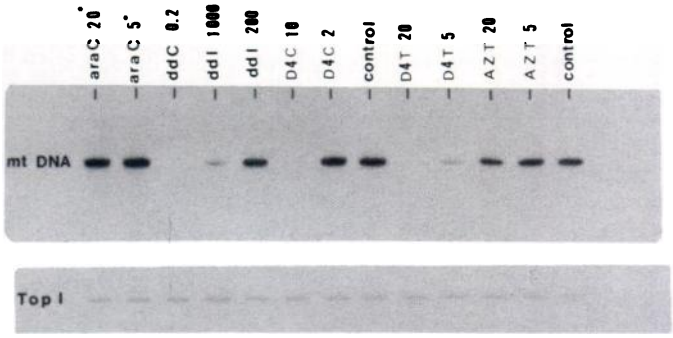

Fig. 1. Decrease of mtDNA content after anti-HIV compound treatment. *Upper*, CEM cells (3×10^4 cells/ml) were treated with 5 μM AZT, 20 μM AZT, 5 μM D4T, 20 μM D4T, 2 μM D4C, 10 μM D4C, 200 μM ddI, 1 mM ddI, 0.2 μM ddC, 5 nM AraC, or 20 nM AraC for 4 days or were not given drug treatment, as indicated. The method used to detect mtDNA levels was described in Experimental Procedures. *Lower*, the same nitrocellulose paper was probed with ^{32}P-labeled Top I cDNA fragment, to normalize the amount of total cellular DNA loaded in the agarose gel. The Top I cDNA fragment is a 1.8-kb 3′ end sequence of Top I cDNA, which was cloned by B. S. Zhou in this laboratory (14). *, Concentrations expressed in nM.

TABLE 1

Cytotoxicity and mitochondrial toxicity (as delayed cytotoxicity or depletion of mtDNA)

	ddC	ddI	D4C	D4T	AZT	AraC
ID_{50}[a]	10 μM	>1 mM	22 μM	48 μM	8 μM	
$mtID_{50}$[b]	0.022 μM	290 μM	2 μM	3 μM	19 μM	—[c]
$ID_{50}/mtID_{50}$	455	>3.5	11	16	0.42	—

[a] Concentration required to reduce the cell number by 50% after a 4-day incubation period.
[b] Concentration required to reduce the mtDNA content of CEM cells by 50% after a 4-day drug treatment.
[c] —, No significant effect.

mitochondrial effects did not affect cell growth until day 6 of drug exposure (Fig. 2). This delayed cytotoxicity of ddI is similar to that of ddC. On the other hand, 5 μM AZT inhibited CEM cell growth but did not affect the mtDNA content of the cells (Figs. 1 and 2). This result suggests that the limiting toxicity of AZT in CEM cells is not its effect on mitochondria. The ratio of $ID_{50}/mtID_{50}$ was used as an indicator of the selective effect of the anti-HIV compounds on mitochondria (Table 1). Among the tested compounds, ddC was the most selective antimitochondrial agent, with a $ID_{50}/mtID_{50}$ ratio greater than 200.

Lactic acid production. Mitochondria are the major site of ATP synthesis. Cells require ATP as an immediate energy source. There are no significant alterations in cellular ATP content and energy charge in MOLT-4F cells after ddC treatment for 5 days, in spite of the severe depletion of mtDNA content (14). This could be due to the enhanced glycolysis observed, as reflected by an increase in lactic acid production. In the present study, all of the compounds that affected mtDNA content increased lactic acid production in CEM cells, in a dose-dependent manner (Fig. 3). However, AraC, which did not decrease mtDNA content, did not have a significant effect on lactic acid production in CEM cells. These results reflect functional alterations of mitochondria under the influence of the anti-HIV drugs. However, the ability of the anti-HIV compounds to increase lactic acid production was not correlated with their potency in mtDNA depletion (Table 1 and Fig. 3). It is possible that the anti-HIV nucleoside analogs could increase lactic acid production by mechanisms other than the loss of mtDNA.

Mitochondrial toxicity in nonproliferative cells. The delayed cytotoxicity and selective mitochondrial toxicity of ddC, ddI, and D4T could be responsible for their delayed side effects, such as peripheral neuropathy, in clinical trials. However, it should be noted that the CEM cells used in this study are a proliferating cell line. Nonproliferating cells, for example, nerve cells, may differ in the metabolism of these compounds,

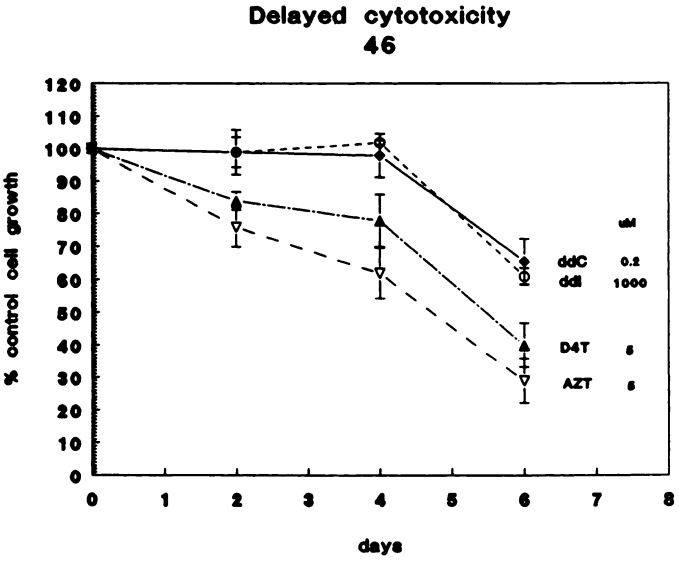

Fig. 2. Cytotoxicity profiles of anti-HIV compounds. CEM cells (3 × 10^3 cells/ml) were treated with 5 μM AZT (▽), 0.2 μM ddC (♦), 1 mM ddI (○), or 5 μM D4T (▲). The cell number was estimated every 2 days by using a Coulter counter. The trypan blue test showed no significant difference in viability between treated cells and untreated cells after 6 days.

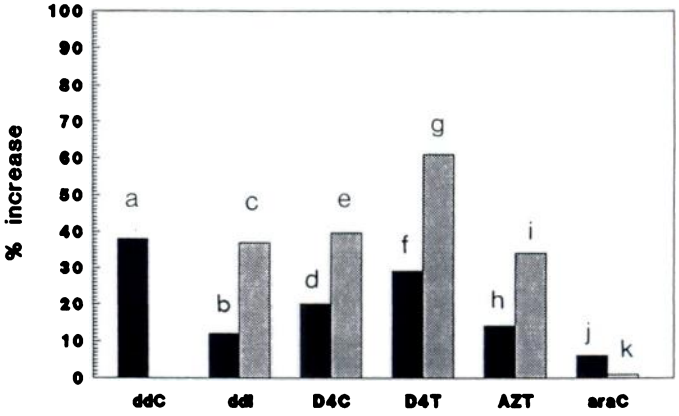

Fig. 3. Increase of lactic acid production. CEM cells (6 × 10^3 cells/ml) were treated with 0.2 μM ddC (*a*), 200 μM ddI (*b*), 1 mM ddI (*c*), 5 μM D4C (*d*), 20 μM D4C (*e*), 5 μM D4T (*f*), 20 μM D4T (*g*), 5 μM AZT (*h*), 20 μM AZT (*i*), 5 nM AraC (*j*), or 20 nM AraC (*k*).

because the enzyme activities that are required to transform the nucleoside analogs to their active metabolites are sometimes cell cycle specific. Furthermore, the growth of mitochondria in nonproliferating cells may be different from that in proliferating cells. Thus, the mitochondrial effect of these anti-HIV analogs in CEM cells may not be the same in nonproliferating cells. To address this question, a rat pheochromocytoma cell line, PC12, was chosen for the study. It is known that, upon treatment with NGF, PC12 cell growth is arrested (18) and the cells differentiate into neuron-like cells. It is interesting to note that the mtDNA content of the PC12 cells increased after differentiation (Fig. 4A, *lanes a* and *b*). The differentiated PC12 cells were exposed to various concentrations of ddC for 8 days. There was a dose-dependent decrease in mtDNA content of the PC12 cells after ddC treatment (Fig. 4A, *lanes c, d, e,* and *f*). The mtDNA of the PC12 cells was affected at 0.5 μM ddC and was severely depleted at 50 μM ddC. ddI also showed mitochondrial toxicity in PC12 cells (Fig. 4B). Much higher concentrations of ddC were required to deplete mtDNA from PC12 cells, compared with CEM cells. This phenomenon was not observed in the case of ddI. There was no significant difference in viability of the differentiated PC12 cells before or after ddC or ddI treatment, under the experimental conditions used. The viability of the PC12 cells was determined by the trypan blue exclusion method.

Although the concentration of ddC required to deplete mtDNA from PC12 cells was higher than that for CEM cells, these results imply the potential of ddC to decrease the mtDNA content in nonproliferating neuron-like cells. This result also suggests that mtDNA is in a state of constant turnover in the differentiated neuron-like PC12 cells. mtDNA turnover in rat tissues, including brain, was reported by Gross *et al.* (19). In addition to the turnover rate of mtDNA, the metabolism of the nucleoside analogs might also play an important role in their mitochondrial toxicity. For instance, the mitochondrial toxicity of ddC is highly cell type dependent.[1] The effect of ddC on the neuronal characteristics of differentiated PC12 cells is currently under investigation.

It is customary to assess the potential toxicity of antiviral nucleoside analogs by determining their cytotoxicity against different cell lines. This approach usually provides a guideline

[1]Unpublished data.

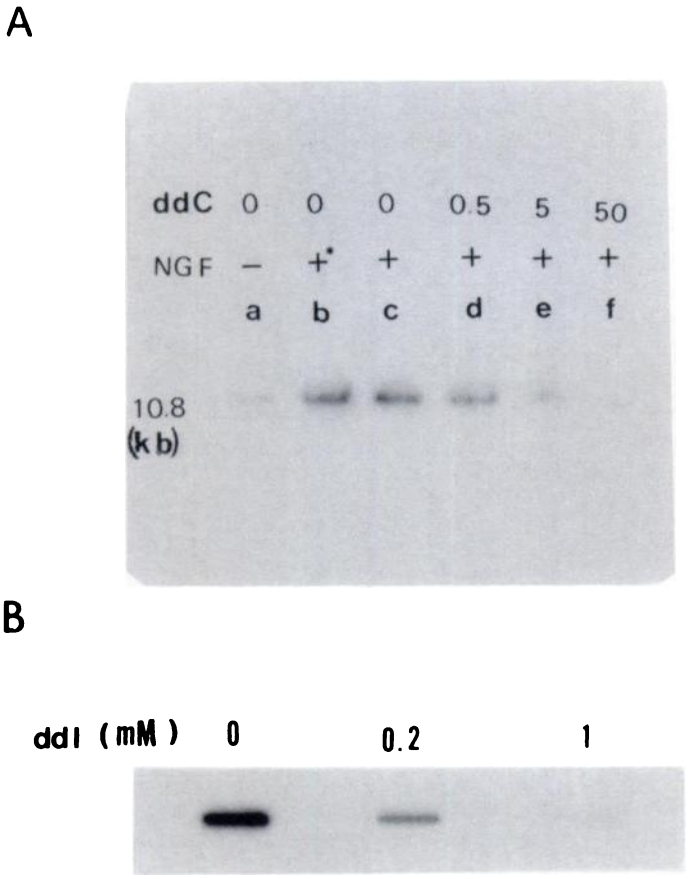

Fig. 4. mtDNA content of PC12 cells. A, PC12 cells were exposed to various concentrations of ddC for 8 days after 2 weeks of treatment with 40 ng/ml NGF. The culture medium, ddC, and NGF were refreshed every 2 days. The mtDNA from PC12 cells was analyzed by Southern blot analysis, as described in Experimental Procedures. B, PC12 cells were treated with ddI for 8 days after 2 weeks of growth in the presence of 40 ng/ml NGF. The mtDNA was detected by slot blot procedure, as described in Experimental Procedures. *, Sample from PC12 cells after 2 weeks of NGF treatment.

for choosing appropriate dosages in the clinic. In this study, we demonstrate that some antiviral nucleoside analogs, such as ddC, ddI, D4T, and D4C, can inhibit mtDNA synthesis at concentrations showing no or less than 50% inhibition of cell growth in a 4-day cell growth assay. This suggests that, at clinical dosages, these compounds may have toxicity toward those tissues that are more susceptible to the mitochondrial toxicity of these drugs. Compounds such as AZT, however, require a much higher dosage to inhibit mtDNA synthesis than to inhibit cell growth or act on other sites (20). Thus, the limiting toxicity for AZT might not be its effect on mtDNA, because the clinical dosage is unlikely to be high enough to deplete the cells of mtDNA, which could lead to delayed cytotoxicity such as peripheral neuropathy or even pancreatitis. Although AZT did not preferentially inhibit the mtDNA of CEM cells, the activity of AZT against the mtDNA of nondividing cells could be very different. Based on the higher turnover rate of mtDNA, compared with that of nuclear DNA (19), AZT could have a preferential effect on mtDNA in quiescent cells. Mitochondrial defects have been related to peripheral neuropathy and many other diseases (21). ddC, ddI, and D4T have been indicated to have toxicity such as peripheral neuropathy. It is possible that the cause of delayed-type toxicity, such as peripheral neuropathy, of antiviral nucleoside analogs at the pharmacological dosages used may be related to the depletion of mtDNA in susceptible tissues.

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