ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1992, p. 2061–2065
0066-4804/92/092061-05$02.00/0

Vol. 36, No. 9

Ultrastructural Changes Associated with Reduced Mitochondrial DNA and Impaired Mitochondrial Function in the Presence of 2′3′-Dideoxycytidine

L. D. LEWIS,[1] F. M. HAMZEH,[1] AND P. S. LIETMAN[1,2]*

Division of Clinical Pharmacology, Departments of Medicine[1] and Pharmacology and Molecular Sciences,[2] The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received 19 July 1991/Accepted 9 June 1992

Incubation of Molt-4 cells in 4 μM 2′3′-dideoxycytidine did not produce a significant change in the mitochondrial ultrastructure after 4 days; however, by 12 days, the mitochondrial ultrastructure was distorted, with condensed cristae or vacuolization, or both. Concentration-dependent decreases in both cell growth (mean 50% inhibitory concentration, 4.70 ± 0.5 μM) and mitochondrial DNA content (mean 50% inhibitory concentration, 0.46 ± 0.06 μM) occurred after incubation with 2′3′-dideoxycytidine for 4 days.

2′3′-Dideoxycytidine (DDC), a deoxynucleoside analog, has activity against human immunodeficiency virus type 1 (HIV-1) in vitro (15) and in vivo (14, 25). The selective effect of DDC triphosphate (DDC-TP) on the HIV-1 reverse transcriptase is thought to be due to the lower K_i for the viral reverse transcriptase (K_i = 0.26 μM) as opposed to higher K_is for the host nuclear alpha and beta DNA polymerases (K_is = 110 and 2.6 μM, respectively) (7, 24). DDC-TP also inhibits the mitochondrial gamma DNA polymerase (K_i = 0.016 μM) and does so at concentrations that are comparable to or less than the concentrations necessary to inhibit the viral reverse transcriptase (3, 24). Cells cultured with DDC at concentrations that are comparable to those that can be obtained therapeutically in human plasma (12) produce intracellular concentrations of DDC-TP (4, 24) that are capable of inhibiting the mitochondrial gamma DNA polymerase.

The major toxic effect of DDC that limits the dose that can be used is a painful, reversible, primarily sensory peripheral neuropathy, but DDC can also cause granulocytopenia and thrombocytopenia (13). The toxicity of DDC in humans suggests that it must have another site of activity in vivo, apart from inhibiting the HIV-1 reverse transcriptase. Studies of the effect of DDC (and zidovudine [AZT]) on isolated rat liver mitochondria revealed a reduction in the incorporation of radioactive deoxynucleoside triphosphates into mitochondrial DNA (22). DDC-TP caused a reduction of tritiated thymidine uptake by mitochondria isolated from HeLa cells (27). Chen and Cheng (2) reported a time-dependent, reversible reduction in the Molt-4 cell growth rate, with a concomitant reversible reduction in the mitochondrial DNA content and an increase in the lactate content in the culture medium. The in vitro observations of Simpson et al. (22) and Chen and Cheng (2) led to their proposal that the mitochondrion, particularly the replication of mitochondrial DNA, is a potential site for the toxic action of DDC and, possibly, for other anti-HIV-1 nucleoside analogs. Interestingly, peripheral neuropathy (16, 17) and hematopoietic suppression with granulocytopenia and thrombocytopenia (19) have been reported in patients with genetic defects in their mitochondrial DNAs.

We investigated the effects of DDC on the mitochondrial structure, biogenesis, and function in Molt-4 cells. We also investigated the effects of DDC on the rate of growth and mitochondrial morphology in HEL cells (a human erythroleukemic cell line).

Molt-4 and HEL cells were obtained from the American Type Culture Collection, Rockville, Md.; they were grown in RPMI 1640 medium (GIBCO) and supplemented with 2 mM L-glutamine and 10% fetal bovine serum (GIBCO). Logarithmically growing cells were placed in either 12-well Corning plates (short-term [4-day] incubations) or 75-cm^2 Corning flasks (long-term incubations) at an initial density of 2×10^5 cells per ml with the designated concentrations of DDC (Sigma Chemical Co., St. Louis, Mo.). Cells were incubated at 37°C in 5% CO_2–95% air. After 2 days, each well was supplemented with 50% (by volume) fresh RPMI 1640 medium with the designated concentration of DDC (for the long-term experiments, 100% [by volume] fresh RPMI 1640 medium with the designated concentration of DDC was added to each flask). Cells were harvested and counted (Coulter counter), and cell viability was measured (trypan blue dye exclusion) after 4 days of incubation. In the long-term experiments, cells were then reseeded at the initial density, fresh medium was added every 2 days, and counting, harvesting, and reseeding were done every 4 days. Aliquots of cells and media were stored at −80°C prior to DNA or biochemical analyses.

Measurement of the lactate concentration of the cell culture medium was performed by using a diagnostic pyruvate-lactate measurement kit (Sigma). Samples were run in duplicate and reassayed if the duplicates differed by more than 10%. Mitochondrial DNA was extracted from platelets by the method of Shuster et al. (21). A mitochondrial DNA probe was prepared by using an oligolabeling kit (Pharmacia, Piscataway, N.J.), and the probe was used for DNA-DNA dot blot hybridization as described by Hamzeh et al. (9).

Cell suspensions of untreated and DDC-treated Molt-4 and HEL cells were centrifuged at 1,000 × *g* for 7 min. The cell pellets were washed twice with 0.1 M phosphate buffer (pH 7.4) and were then fixed in 2% glutaraldehyde–1% paraformaldehyde in 0.1 M phosphate buffer, starting at room temperature and then cooling to 4°C. After 1 h, the cell pellets were washed three times for 15 min each time in phosphate buffer. The cell pellets were then postfixed for 1 h with 1% osmium tetroxide–0.1 M phosphate buffer. The cells were

* Corresponding author.

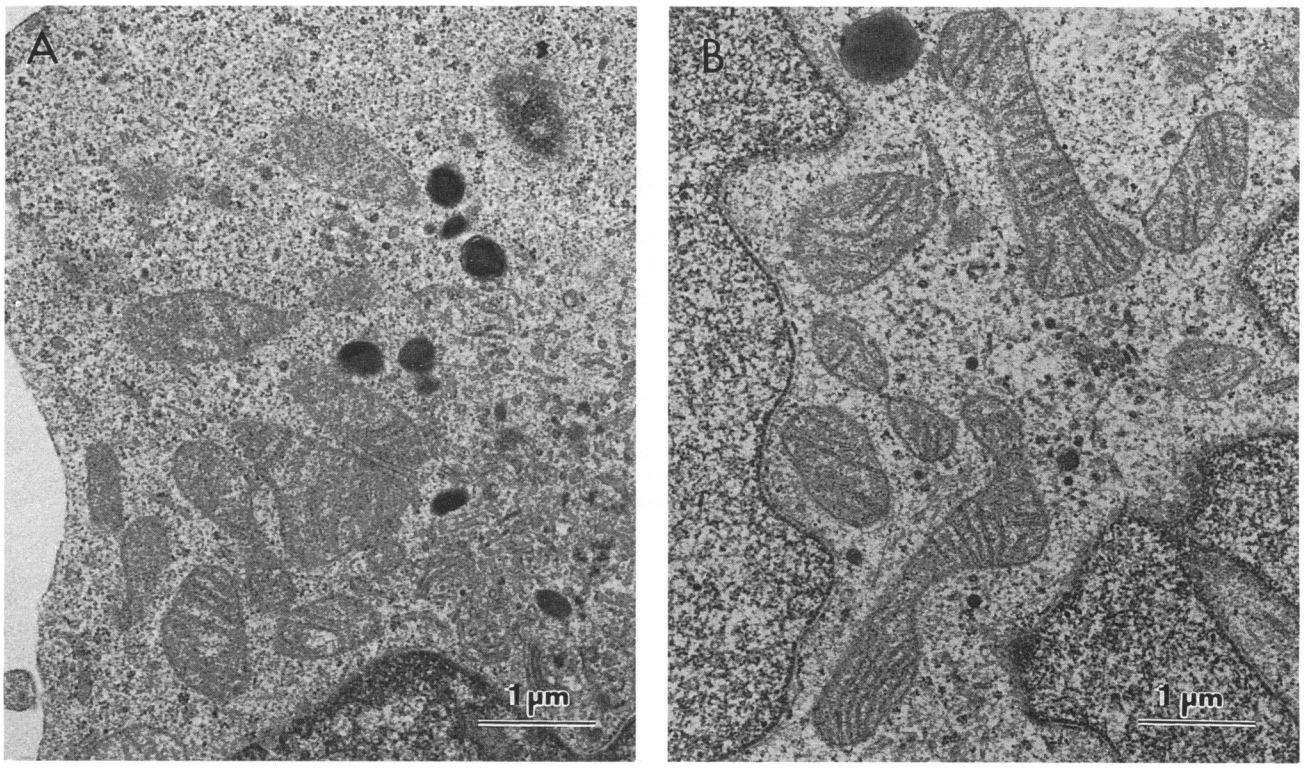


FIG. 1. Electron micrographs showing the mitochondrial ultrastructures of untreated control Molt-4 cells (A) and Molt-4 cells treated with 4 μM DDC (B) after 4 days in vitro incubation. Magnifications, ×17,700.

then washed in three changes of phosphate buffer at room temperature, dehydrated through graded alcohol concentrations, and embedded in Poly/812. Sections were cut with an MJO Diatome diamond knife on an LKB-V ultramicrotome, stained with uranyl acetate and lead citrate (Fahmy's), and viewed at 100 kV on a Philips 410 transmission electron microscope (all reagents required for the preparation of electron microscopy sections were obtained from Polysciences Inc.).

Cell growth, mitochondrial DNA content, lactate content of the medium, and mitochondrial morphology after short-term incubation with DDC. After 4 days of incubation, we found that DDC produced a concentration-dependent inhibition of cell growth, with a mean 50% inhibitory concentration of 4.7 ± 0.5 μM (n = 3). There was only a minimal (<15%) reduction in Molt-4 cell viability during incubation with DDC, and the minimal reduction occurred only at the highest DDC concentration used (8 μM). Simultaneously, DDC produced a concentration-dependent increase in the lactate content of the culture medium of the Molt-4 cells, with 4 μM DDC producing a mean 55% ± 8% (n = 3) increase above control values. DDC also produced a concomitant decrease in the mitochondrial DNA content of Molt-4 cells, with a mean 50% inhibitory concentration of 0.46 ± 0.06 μM (n = 3).

Electron micrographs of untreated control and DDC-treated (4 μM) Molt-4 cells incubated for 4 days are shown in Fig. 1A and B, respectively. There were no significant differences in the mitochondrial ultrastructural morphologies or numbers of Molt-4 cells incubated in the absence or presence of DDC (4 μM).

Effect of long-term incubation of Molt-4 cells with 4 μM DDC on mitochondrial ultrastructure, growth rate, and lactate content of the tissue culture medium. Figure 2A demonstrates untreated control Molt-4 cells, and Fig. 2B and C demonstrate Molt-4 cells treated with 4 μM DDC for 12 days. There were considerable changes in the mitochondrial ultrastructures of DDC-treated cells. The general organization of the mitochondrial inner membrane with cristae in transverse alignment was lost, and the matrix density was increased. Some cells had mitochondria with abnormally "whorled," lamellarlike, concentrically arranged cristae; the cristae were condensed and severely distorted, and there was a marked increase in the density of the matrix. In other DDC-treated cells, the mitochondria had a condensed matrix, with central vacuoles and very distorted cristae arranged concentrically. The doubling times of Molt-4 cells treated with 4 μM DDC increased to 261 h (control, 28.3 h) over the course of 12 days, with a simultaneous increase in the lactate content of the tissue culture medium to a mean of 210% above control values.

Effect of long-term incubation with 2 μM DDC on HEL cell mitochondrial ultrastructure. To prove that the mitochondrial ultrastructural changes caused by DDC were not limited to one cell line, we studied the effect of DDC on HEL cell mitochondrial ultrastructure. After 16 days, untreated control HEL cells had a cell doubling time of 30.4 h, while that of HEL cells treated with 2 μM DDC was 183.7 h. Electron micrographs of the mitochondria from untreated control cells and HEL cells treated with 2 μM DDC are shown in Fig. 3A and in Fig. 3B and C, respectively. DDC-treated HEL cells showed very abnormal mitochondria, with condensed distorted cristae or vacuolization very

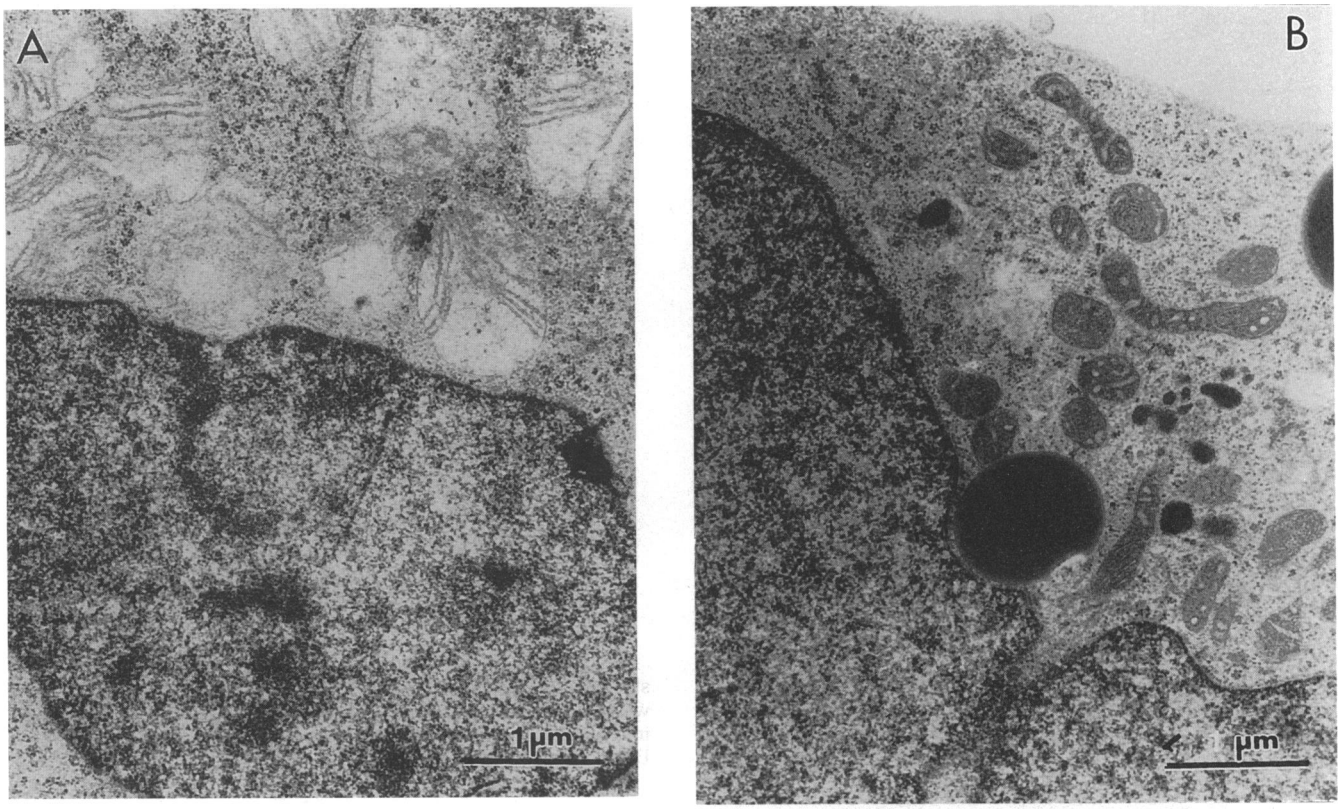
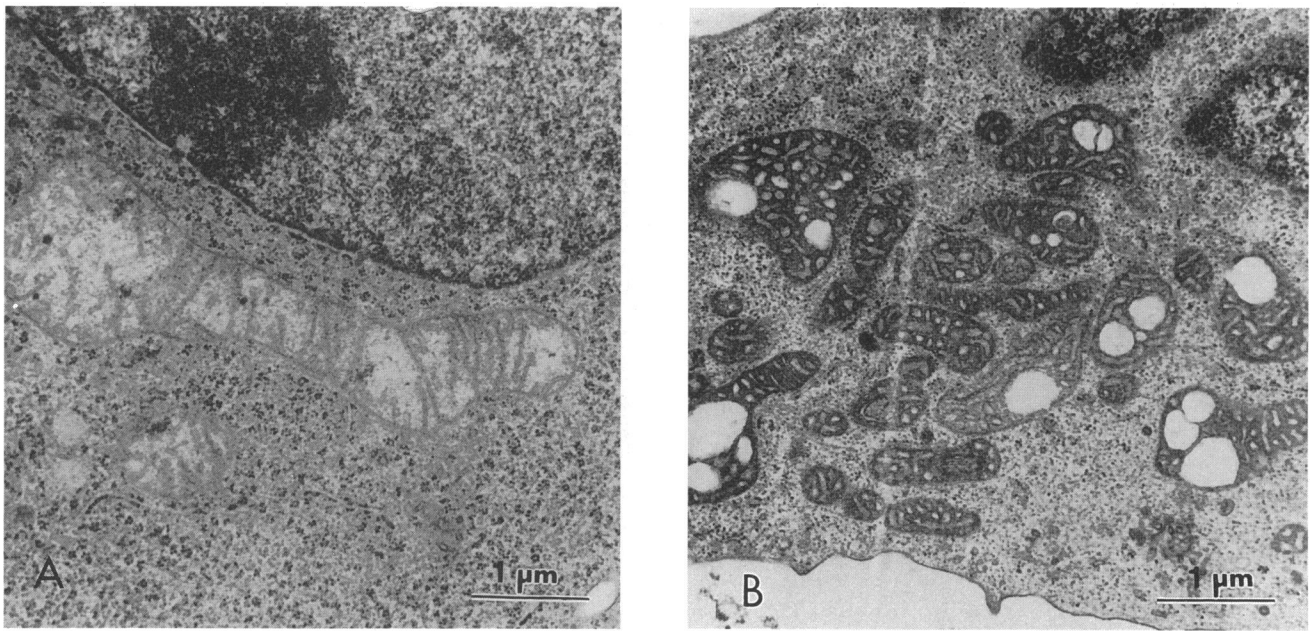


FIG. 2. Electron micrographs showing the mitochondrial ultrastructures of untreated control Molt-4 cells (A) and Molt-4 cells treated with 4 μM DDC (B and C) after 12 days in vitro. The changes in the mitochondrial ultrastructures of the cells were found in approximately 85% of DDC-treated cells. Magnifications, ×17,700.

similar to the abnormalities found in the Molt-4 cells treated with 4 μM DDC for 12 days.

Our findings confirm the observations of Simpson et al. (22) and Chen and Cheng (2) that DDC causes a decrease in mitochondrial DNA content and impairs the mitochondrial function in Molt-4 cells. The reduction in mitochondrial DNA content may be due to the inhibition of gamma DNA polymerase by DDC-TP (4, 24) or chain termination of nascent mitochondrial DNA. Interestingly, Keilbaugh et al. (10) tested mouse erythroleukemic cells and found that the incorporation of radioactive dATP into mitochondria isolated from AZT-treated mouse erythroleukemic cells was reduced, again suggesting impaired mitochondrial DNA replication.

Significant distortion of the mitochondrial ultrastructure of Molt-4 cells occurred after 12 days but not after 4 days of incubation with DDC. These delayed changes in mitochondrial ultrastructure suggest that the initial defect caused by DDC is a reduction in the mitochondrial DNA content. This could produce concomitant functional impairment followed by delayed mitochondrial ultrastructural changes. Chloramphenicol, a known inhibitor of mitochondrial protein synthesis, and ethidium bromide, an inhibitor of mitochondrial DNA synthesis, produce similar mitochondrial changes in HeLa and L cells (11). Incubation with chloramphenicol for 3 to 20 days or ethidium bromide for 2 to 8 days caused inhibition of cell growth. Simultaneously, there was enlargement of the mitochondria, with either condensation or vac-



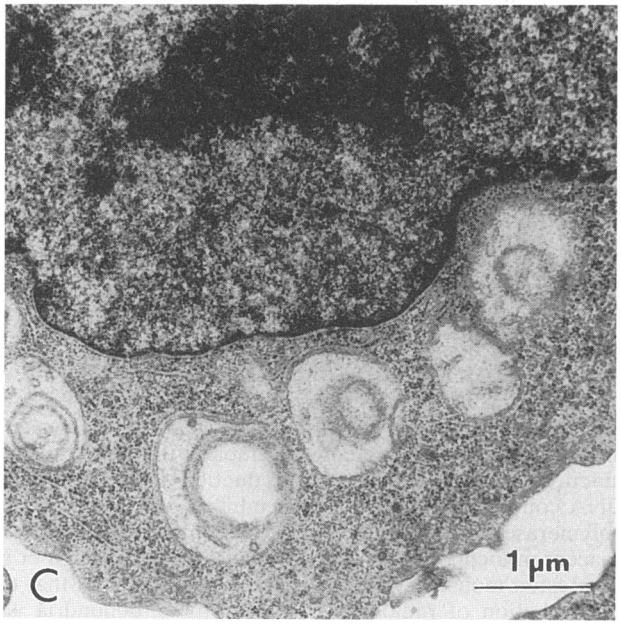


FIG. 3. Electron micrographs showing the mitochondrial ultrastructures of untreated control HEL cells (A) and HEL cells treated with 2 μM DDC (B and C) after 16 days in vitro. The changes in mitochondrial ultrastructures were noted in approximately 85% of DDC-treated cells. Magnifications, ×15,472.

uolization of the cristae and distortion of the inner membrane. In humans, chloramphenicol induces reversible bone marrow toxicity (20) and, rarely, neurotoxicity (peripheral neuropathy [1, 18] and optic neuritis [8]). The bone marrow toxicity of chloramphenicol has been associated with mitochondrial ultrastructural abnormalities, producing a condensed configuration of the matrix and cristae (23) or cristae arranged in concentric rings (26). The mitochondrial ultrastructural changes described for cells incubated with chloramphenicol and ethidium bromide are similar to our findings with Molt-4 and HEL cells incubated in tissue culture with DDC.

AZT has also been implicated as producing mitochondrial dysfunction in humans. Preliminary evidence has suggested that long-term treatment (mean duration, 12.8 months) of AIDS patients with AZT may produce a mitochondrial myopathy (6). Electron microscopy of muscle fibers from patients with AIDS suspected of having AZT myopathy revealed enlarged mitochondria with vacuolization and paracrystalline inclusion bodies. In comparison with controls, the muscle fibers of patients with AZT myopathy exhibited up to a 78% reduction in mitochondrial DNA content (5).

The evidence presented in this report suggests that DDC inhibits mitochondrial biogenesis in Molt-4 cells, resulting in mitochondrial dysfunction and ultrastructural changes. Perhaps inhibition of mitochondrial biogenesis could be the pathophysiological mechanism responsible for the neurological and hematological toxicities of DDC.

We acknowledge the assistance of J. E. Farrington and the Electron Microscopy Resource of the Johns Hopkins Oncology Center, supported by NCI grant 5-P30-CA-06973-28. The NIH-NIAID AIDS Clinical Trials Group provided partial support for L.D.L. and for this project (grant 5U01AI2766805). The work was performed in the Alan Berstein Laboratories of Clinical Pharmacology of The Johns Hopkins University School of Medicine.

REFERENCES

1. **Bomb, B. S., and H. K. Bedi.** 1974. Chloramphenicol induced peripheral neuropathy. J. Assoc. Phys. India **22**:623–625.
2. **Chen, C. H., and Y. C. Cheng.** 1989. Delayed cytotoxicity and selective loss of mitochondrial DNA in cells treated with the anti-human immunodeficiency virus compound 2′,3′-dideoxycytidine. J. Biol. Chem. **264**:11934–11937.
3. **Cheng, Y. C., G. E. Dutschman, K. F. Bastow, M. G. Sarngadharan, and R. Y. Ting.** 1987. Human immunodeficiency virus reverse transcriptase: general properties and its interactions with nucleoside triphosphate analogs. J. Biol. Chem. **262**:2187–2189.
4. **Cooney, D. A., M. Dalal, H. Mitsuya, J. B. McMahon, M.**

Nadkarni, J. Balzarini, S. Broder, and D. G. Johns. 1986. Initial studies on the cellular pharmacology of 2',3'-dideoxycytidine, an inhibitor of HTLV-III infectivity. Biochem. Pharmacol. **35**:2065–2068.

5. **Dalakas, M., and I. Illa.** 1990. Mitochondrial myopathy caused by long term zidovudine therapy. N. Engl. J. Med. **323**:994.
6. **Dalakas, M. C., I. Illa, G. H. Pezeshkpour, J. P. Laukaitis, B. Cohen, and J. L. Griffin.** 1990. Mitochondrial myopathy caused by long-term zidovudine therapy. N. Engl. J. Med. **322**:1098–1105.
7. **Furman, P. S., J. A. Fyfe, M. H. St. Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. Nusinoff-Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, and D. W. Barry.** 1986. Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. USA **83**:8333–8337.
8. **Godel, V., P. Nemet, and M. Lazar.** 1980. Chloramphenicol optic neuropathy. Arch. Ophthalmol. **98**:1417–1421.
9. **Hamzeh, F. M., P. S. Lietman, W. Gibson, and G. S. Hayward.** 1990. Identification of the lytic origin of DNA replication in human cytomegalovirus by a novel approach utilizing gancicyclovir induced chain termination. J. Virol. **64**:6184–6195.
10. **Keilbaugh, S. A., J. A. Moschella, C. D. Chin, J. Mladenovic, T.-S. Lin, W. H. Prusoff, R. A. Rowehl, and M. V. Simpson.** 1990. Role of mtDNA replication in the toxicity of 3'-azido-3'-deoxythymidine (AZT) in AIDS therapy and studies on other anti-HIV-1 dideoxynucleosides, p. 159–162. *In* E. Quagliariello, S. Papa, F. Palmieri, and C. Saccone (ed.), Structure, function and biogenesis of energy transfer systems. Elsevier Science Publishers B.V., Amsterdam.
11. **King, M. E., G. C. Godman, and D. W. King.** 1972. Respiratory enzymes and mitochondrial morphology of HeLa and L cells treated with chloramphenicol and ethidium bromide. J. Cell Biol. **53**:127–142.
12. **Klecker, R. W., Jr., J. M. Collins, R. C. Yarchoan, R. Thomas, N. McAtee, S. Broder, and C. E. Myers.** 1988. Pharmacokinetics of 2',3'-dideoxycytidine in patients with AIDS and related disorders. J. Clin. Pharmacol. **28**:837–842.
13. **Merigan, T. C., and G. Skowron.** 1990. Safety and tolerance of dideoxycytidine as a single agent. Results of early-phase studies in patients with acquired immunodeficiency syndrome (AIDS) or advanced AIDS-related complex. Study Group of the AIDS Clinical Trials Group of the National Institute of Allergy and Infectious Diseases. Am. J. Med. **88**:11S–15S.
14. **Merigan, T. C., G. Skowron, S. A. Bozzette, D. Richman, R. Uttamchandani, M. Fischl, R. Schooley, M. Hirsch, W. Soo, C. Pettinelli, et al.** 1989. Circulating p24 antigen levels and responses to dideoxycytidine in human immunodeficiency virus (HIV) infections. A phase I and II study. Ann. Intern. Med. **110**:189–194.
15. **Mitsuya, H., and S. Broder.** 1986. Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2',3'-dideoxynucleosides. Proc. Natl. Acad. Sci. USA **83**:1911–1915.
16. **Peyronnard, J.-M., L. Charron, A. Bellavance, and L. Marchand.** 1980. Neuropathy and mitochondrial myopathy. Ann. Neurol. **7**:262–268.
17. **Pezeshkpour, G., C. Krarup, F. Buchthal, S. DiMauro, N. Bresolin, and J. McBurney.** 1987. Peripheral neuropathy in mitochondrial disease. J. Neurol. Sci. **77**:285–304.
18. **Ramilo, O., B. T. Kinane, and B. H. J. McCracken.** 1988. Chloramphenicol neurotoxicity. Paediatr. Infect. Dis. J. **7**:358–359.
19. **Rotig, A., M. Colonna, J. P. Bonnefont, S. Blanche, A. Fischer, J. M. Saudubray, and A. Munnich.** 1989. Mitochondrial DNA deletion in Pearson's marrow/pancreas syndrome. Lancet **i**:902–903.
20. **Scott, J. L., S. M. Finegold, and G. S. Belthin.** 1965. A controlled double blind study of the haematologic toxicity of chloramphenicol. N. Engl. J. Med. **272**:1137–1142.
21. **Shuster, R. C., A. J. Rubenstein, and D. C. Wallace.** 1988. Mitochondrial DNA in anucleate human blood cells. Biochem. Biophys. Res. Commun. **155**:1360–1365.
22. **Simpson, M. V., C. D. Chin, S. A. Keilbaugh, T. S. Lin, and W. H. Prussoff.** 1989. Studies on the inhibition of mitochondrial replication by 3'-azido-3'-deoxythymidine and other dideoxynucleoside analogs inhibit HIV-1 replication. Biochem. Pharmacol. **38**:1033–1036.
23. **Skinnider, L. F., and F. N. Ghadially.** 1976. Chloramphenicol-induced mitochondrial and ultrastructural changes in hemopoietic cells. Arch. Pathol. Lab. Med. **100**:601–605.
24. **Starnes, M. C., and Y. C. Cheng.** 1987. Cellular metabolism of 2',3'-dideoxycytidine, a compound active against human immunodeficiency virus in vitro. J. Biol. Chem. **262**:988–991.
25. **Yarchoan, R., and S. Broder.** 1987. Development of antiretroviral therapy for the acquired immunodeficiency syndrome and related disorders. A progress report. N. Engl. J. Med. **316**:557–564.
26. **Yunis, A. A., U. S. Smith, and A. Restrapo.** 1970. Reversible bone marrow suppression from chloramphenicol. A consequence of mitochondrial injury. Arch. Intern. Med. **126**:272–275.
27. **Zimmerman, W., S. Min Chen, A. Bolden, and A. Weissbach.** 1980. Mitochondrial DNA replication does not involve DNA polymerase alpha. J. Biol. Chem. **255**:11847–11852.