Mitochondrial toxicity of antiviral drugs

Long-term treatment with antiviral nucleoside analogue drugs, such as AZT, can give rise to delayed and at times severe mitochondrial toxicity. Although these toxic effects are manifest in many tissues, a common disease mechanism can explain the diverse clinical events. A better understanding of these disorders will shed light on genetic mitochondrial diseases and lead to the design of safer and more effective antiviral drugs.

WILLIAM LEWIS & MARINOS C. DALAKAS

Mitochondrial diseases are multisystem disorders with unique genetics. Changes in mitochondrial structure, function and molecular biology occur in a very diverse group of disorders including viral infections, diabetes mellitus, heart disease, Parkinson's disease and even the biologic process of ageing[1,2]. Recently, clinical and experimental events in mitochondrial toxicity caused by antiviral nucleoside analogues (ANAs) were found to resemble those of genetic mitochondrial diseases.

The ANA acyclovir was the archetypal nucleoside analogue for the treatment of herpes virus infections with minimal toxicity[3]. Other ANAs, some of which are used to treat human immunodeficiency virus type 1 (HIV-1) and hepatitis B virus (HBV) infection, include zidovudine (AZT), zalcitabine (ddC), didanosine (ddI), stavudine (D4T), and 2'-deoxy-3'-thiacytidine (3TC)[4-6].

In structure, ANAs resemble natural nucleotide bases that serve as the building blocks in DNA. The pharmacologic effectiveness of ANAs depends upon their relatively selective interference with viral DNA replication in the absence of significant toxicity to cellular DNA replication in the patient. Toxicity occurs when cellular DNA polymerases are inhibited and the subcellular toxic target is frequently the mitochondrial DNA polymerase (DNA pol-γ) in selected tissues.

Although short-term ANA therapy appears to be relatively safe, long-term therapy has revealed unique toxic effects on oxidative phosphorylation in various tissues. Features of this toxicity suggest that defective mitochondrial DNA (mtDNA) replication may be the common event. These features resemble those that occur in genetic mitochondrial diseases and include mitochondrial myopathy, cardiomyopathy, neuropathy, lactic acidosis, exocrine pancreas failure, liver failure and bone marrow failure[2,4-6]. The clearest example is AZT mitochondrial myopathy[7] with a prevalence of up to 20% in long-term AZT-treated AIDS patients. Manifestations of the disorder relate to defective mitochondrial gene expression and to ANA triphosphates' (ANATP's) inhibition of mitochondrial DNA pol-γ in the target tissues[7,8]. Recent experience with the fluorinated ANA fialuridine [FIAU, 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouridine] have further dampened expectations for the long-term safety of ANA treatments. FIAU yielded serious toxicity and was linked to several deaths in an aborted clinical trial for chronic hepatitis B virus (HBV). Toxic manifestations of the FIAU treatment included profound lactic acidosis, hepatic failure, fat accumulation in the liver, coma, skeletal and cardiac myopathy, pancreatitis and peripheral neuropathy[9-12].

In spite of the established effectiveness of ANAs in AIDS, enthusiasm has diminished because long-term therapy causes multi-organ side-effects to striated muscle[7], peripheral nerves[13], pancreas[14], liver[15] and heart[16]. In some cases, reversal of symptoms corresponds to cessation of therapy; in others, toxicity persists. In this review we present evidence that ANA-induced inhibition of mitochondrial DNA pol-γ partially explains the pathophysiologic changes to mtDNA replication.

The potential impact of ANA mitochondrial toxicity in viral infections other than HIV may be formidable given their widespread use. For example, chronic hepatitis due to HBV (which afflicts approximately 250 million people worldwide) is currently treated with the ANA 3TC in clinical studies (ref. 17). ANA-induced mitochondrial toxicity offers insights into the mechanisms of mitochondrial and genetic diseases. Studies using ANAs in animal models explain normal and abnormal mitochondrial function.

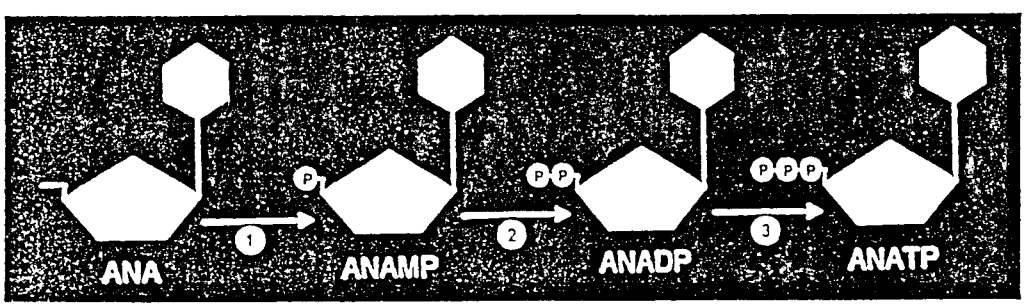

Fig. 1 Phosphorylation of thymidine analogue ANAs. ANA phosphorylation occurs intracellularly and thymidine kinase (1) is the enzyme responsible for the first anabolic phosphorylation to ANAMPs. ANAMPs are phosphorylated by thymidylate kinase (2) to ANADPs and these in turn are phosphorylated to ANATPs by nucleoside diphosphate kinase (3) or other enzymes.

Cellular metabolism of ANAs

Nucleoside kinases are enzymes responsible for the intracellular activation of ANAs. Activation is accomplished using the same enzymes that phosphorylate the natural ribonucleosides and deoxyribonucleosides of RNA and DNA[18]. AZT, ddC and FIAU are phosphorylated by cellular

thymidine kinase (TK); (Fig. 1, Step (1)). In mammals, two tissue-specific forms of TK exist: TK1 is cytosolic, with low activity in skeletal muscle, and TK2 is mitochondrial, with higher activity in muscle[17]. The tissue distribution of these two forms of TK may relate to the selective toxicity of some ANAs. After monophosphorylation, ANAs are phosphorylated by other cellular enzymes, to ANATPs (Fig. 1, Steps (2) and (3)) where they function as inhibitors of viral DNA polymerases by competing with the natural substrates or by leading to chain termination of viral DNA.

Effect Of ANAs on mitochondria

Interactions with DNA polymerases. On a structural basis, ANATPs could inhibit mammalian nuclear DNA polymerases (DNA pol-α, DNA pol-β, DNA pol-δ, and DNA pol-ε) and DNA pol-γ used for mtDNA replication. Both the mechanisms for DNA polymerization and the choice of nucleotide substrates are similar among eukaryotic DNA polymerases, and some ANATPs have been shown to inhibit nuclear DNA pols *in vitro*[20]. However, neither clinical nor laboratory evidence support inhibition of nuclear DNA polymerases by ANATPs, whereas it strongly suggests that ANATPs do inhibit the mitochondrial DNA pol-γ. This relatively selective polymerase inhibition may in part explain ANA toxicity to mtDNA replication.

Role of DNA pol-γ. One important clinical and experimental observation in some ANA toxicities is a decreased steady-state mtDNA abundance. This may relate to the function of DNA pol-γ, a nuclear-encoded DNA polymerase for mtDNA replication[21]. The *polymerase* function of DNA pol-γ is fundamental, but an associated 3′→5′ *exonuclease* is also present. DNA pol-γ is *processive*. Processivity allows each DNA pol-γ molecule to replicate each mtDNA molecule to completion after one initiation event.

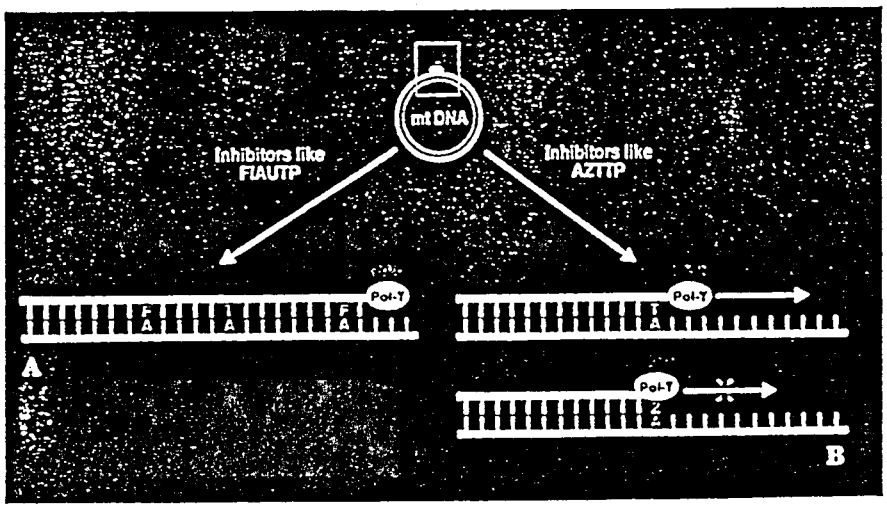

Fig. 2 Proposed mechanism of ANA toxicity to mtDNA (small double circle), with attached DNA pol-γ (in block) in which ANATPs serve as inhibitors of mtDNA replication. *a*, Enlarged view of interaction of mtDNA with DNA pol-γ. Incorporation of ANAs into mtDNA as alternate substrates for DNA pol-γ. FIAUTP serves as an alternate substrate for thymidine triphosphate with DNA pol-γ and is incorporated (inserted into mtDNA as F) into the nascent chain because it possesses a 3′-OH group. The nascent mtDNA can be extended beyond the inserted FIAU. *b*, In contrast, ANATPs (like AZTTP) that lack a 3′-OH also compete with thymidine triphosphate as substrates of DNA pol-γ. The latter ANAs terminate mtDNA synthesis (inserted into DNA as Z) because bases cannot be added to the nascent chain after AZT is inserted.

Processivity of DNA pol-γ may also relate to *heteroplasmy* (an intracellular mix of normal and mutant mtDNA molecules)[22]. Since DNA pol-γ is processive, mtDNA deletion mutants (shortened mtDNA) may be replicated more quickly than native mtDNA. As a result, the proportion of mtDNA mutants may increase and yield functional changes beyond a certain threshold. In some ways, analogies exist between this mechanism of ANA toxicity (as with AZT therapy) and accumulated mtDNA deletions seen in heritable mitochondrial illnesses[23].

ANA toxicity and its relationship to ANA structure. ANA toxicity can be classified based on ANA chemical structure and fundamental chemical properties. In one case, phosphorylated ANAs could be internalized into nascent mtDNA by their substitution for the natural base. In another, mtDNA chain termination could be pivotal[24] (Fig. 2).

The first form of inhibition (represented by ANAs like FIAU), involves ANA incorporation at internucleotide linkages, with, for example, fialuridine substituting for the natural base, thymidine (Fig. 2*a*). ANATPs (like FIAUTP) that contain 3′-hydroxyl groups (3′-OHs) serve as competitive, alternate substrates for DNA pol-γ in mtDNA synthesis (by substituting for thymidine triphosphate). The 3′-OH on members of this class of ANAs enables DNA pol-γ to extend the mtDNA chain after the ANA is incorporated into mtDNA. No mitochondrial postreplicational repair mechanism exists to remove the internally incorporated ANA, and as a result, mtDNA damage is essentially permanent. Moreover, changes in mtDNA caused by fialuridine incorporation could lead to alterations in mitochondrial transcription and ultimately to defective mitochondrial polypeptide synthesis.

In the second case, ANATPs (like AZTTP) also compete with natural thymidine triphosphate at the nucleotide binding site of DNA pol-γ (as above). However, their incorporation into mtDNA terminates nascent mtDNA non-competitively, because they lack the 3′-OH group necessary for nascent mtDNA chain extension with a new base (Fig. 2*b*). This correlates mechanistically with mixed inhibition (competitive and non-competitive inhibition[25]) of DNA pol-γ by AZTTP *in vitro*. It follows that reversibility of ANA toxicity may be possible if the toxic ANA can be removed from the mtDNA terminus (possibly by the exonuclease associated with DNA pol-γ).

Clinical manifestations of ANA toxicity

It is self-evident that ANAs, like all drugs, have side-effects. However, the prevalent and at times serious ANA mitochondrial toxic side-effects are particularly broad ranging with respect to their tissue target and mechanisms of toxicity.

Haematologic toxicity. The most common side effect of AZT is haematologic toxicity, occurring in up to 21% of patients[26,27]. The clinical importance of this effect relates to the fact that it limits AZT ther-

apy. Clinical manifestations of AZT haematologic toxicity include anaemia, leukopenia, thrombocytopenia and bone marrow suppression. AZT produces significant loss of haematopoietic precursors in the peripheral blood before affecting bone marrow itself. This toxicity is attributed to inhibition of cellular DNA polymerases[7], (possibly DNA pol-γ) or to depletion of thymidine. Effects of AZT on bone marrow cultures include time- and dose-dependent inhibition of marrow precursors[29].

Clinical trials used recombinant human erythropoietin (r-HuEPO) to treat the anaemia associated with HIV infection and AZT therapy[30]. In immunosuppressed mice, AZT-induced erythroid toxicity was ameliorated by hemin and stem factor[31] and 2',3'-dideoxythymidine reversed AZT-induced bone marrow toxicity[32]. AZT-induced perturbation of deoxyribonucleotide pools suggests the use of benzylacyclouridine to reverse AZT haematotoxicity[33].

Analogies exist between AZT myelotoxicity and one heritable mtDNA illness. Mitochondrial deletions and bone marrow failure are main components of Pearson's marrow-pancreas syndrome[34]. It should be noted also that an important manifestation of didanosine (ddI) toxicity is acute pancreatitis; exocrine pancreas dysfunction is part of Pearson's syndrome.

Myopathy. Clinically, AZT is the aetiologic agent in a skeletal myopathy in AIDS. The myopathy presents with fatigue, myalgia, muscle weakness, wasting and elevated serum creatine kinase[7,35]. We reported that zidovudine induces a mitochondrial myopathy with "ragged red fibres"[7,35] seen microscopically (Fig. 3). Ragged red fibres are characteristic histopathologic changes in the skeletal muscle of patients with mitochondrial myopathies and result from subsarcolemmal accumulation of mitochondria. By transmission electron microscopy the mitochondria are enlarged and swollen, contain disrupted cristae, and paracrystalline inclusions (Fig. 4)[36–39].

Biochemical assays performed on muscle homogenates reveal abnormal mitochondrial respiratory function. Enzyme histochemical analysis of patients' muscle biopsies shows partial deficiency of cytochrome *c* oxidase activity[40,41] (complex IV) and a high lactate/pyruvate ratio (consistent with abnormal mitochondrial function) is seen in the blood of patients with AZT-myopathy[42]. Assessment of muscle metabolism *in vivo* using magnetic resonance spectroscopy shows marked phosphocreatine depletion with slow recovery only in AZT-treated, HIV-positive patients[43]. This suggests impaired oxidative metabolism as a result of AZT-induced abnormalities in mitochondria.

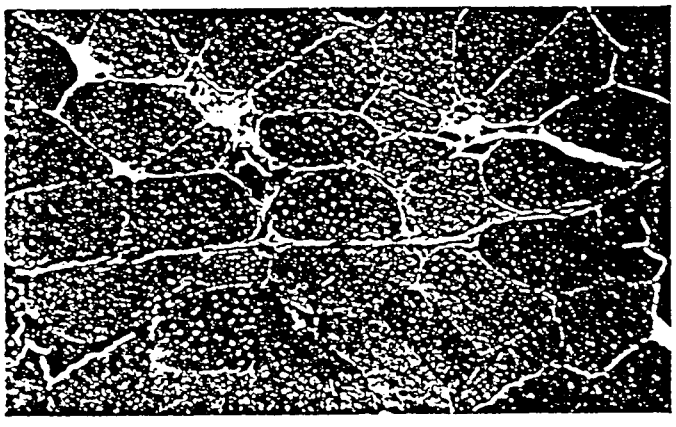

Fig. 3. Cross section of a snap-frozen muscle biopsy from a patient with muscle weakness, myalgia and fatigue after 8 months of AZT-therapy. Note several "ragged-red" fibres with subsarcolemmal cracks shown to be due to mitochondrial myopathy and mtDNA depletion. A few degenerated fibres are noted. The tiny vacuoles in a few fibres represent accumulated fat. Trichrome stain (x 320).

Because of mitochondrial dysfunction in AZT-induced myopathy, long-chain fatty acids may be mobilized ineffectively for β-oxidation. Consequently, fat accumulates intracellularly and can be seen histopathologically[44] (Fig. 5). Muscle biopsies from AZT-myopathy patients contain decreased mtDNA[45], and this correlates with decreased mtDNA, mtRNA, polypeptide synthesis and altered histochemistry and ultrastructure in animal systems[36–39]. These morphological, functional and molecular changes are, however, absent in AZT-naive, myopathic AIDS patients' muscle samples[7,35,36,44,45]. The pathological changes in AZT-induced myopathy are reversed when AZT treatment is discontinued and clinical improvement accompanies these histological improvements and reversal[7,35]. Pathologic and clinical reversibility of AZT myopathy is consistent with AZT terminating nascent mtDNA (Fig. 2*b*)[6].

AZT myopathy develops slowly after at least 6 months of therapy and occurs in up to 17% of AZT-treated patients[46]. It occurs not only with the high-dose therapy but with current low-dose regimens. In paediatric populations with AIDS, AZT myopathy is less frequently recognized and may be masked by coexistent encephalopathy[47].

Experimental evidence in non-HIV-infected tissues supports the myotoxicity of AZT. In various human cell lines, AZT exposure causes a reduction in mtDNA abundance and gives rise to abnormal mitochondria with extensive lipid accumulation in human myotubes[39,48,49]. Rats treated with AZT develop ultrastructural abnormalities in their skeletal and cardiac muscle mitochondria associated with depression of muscle mtDNA and mitochondrial polypeptide synthesis, impaired cytochrome *c* reductase and an uncoupling effect[37–39,48,49].

Cardiotoxicity. Dilated cardiomyopathy related to AZT or other antiretroviral therapy was reported in AIDS patients. Cessation of the presumed toxic ANA therapy resulted in improved left ventricular function on echocardiographic examinations[50]. AZT cardiomyopathy occurs after prolonged treatment and includes congestive heart failure, left ventricle dilation and reduced ejection fractions. Endomyocardial biopsies show intramyocytic vacuoles, myofibrillar loss, dilated sarcoplasmic reticulum and mitochondrial cristae disruption[51]. In AZT-treated paediatric AIDS patients, it has been reported that impaired cardiac function was not attributed to AZT therapy[52]. AZT-skeletal myopathy is also uncommon in children with AIDS[47].

Hepatic toxicity. Hepatic toxicity of AZT, ddI and ddC was reported recently[10,52,53] and may relate to ANA toxic effects on liver mitochondria. Fatal hepatomegaly with intracellular fat accumulation[54], lactic acidosis[52] and adult Reye's syndrome[53] in AZT-treated HIV-seropositive patients is linked to AZT-induced hepatotoxicity. Clinical features resemble those seen in FIAU toxicity (see below), and some uncommon metabolic illnesses in which neutral fat accumulates in liver cells.

Peripheral neuropathy. An axonal peripheral neuropathy is caused by zalcitabine (ddC) and stavudine (D4T) (2',3'-didehy-

dro-2',3'-dideoxythymidine[7]. It is characterized by painful tingling sensations (dysesthesias) in the feet and toes, loss of tendon reflexes (areflexia), distal sensory loss and mild muscle weakness. Electrophysiologic studies confirm axonal involvement. Histological findings in nerve biopsies include axonal degeneration. We observed mitochondria with disrupted cristae in some nerve axons. These findings resemble the mitochondrial "Schwannopathy" induced in the peripheral nerves of rabbits fed ddC[8]. The dideoxynucleoside-related neuropathy reverses when ddC or ddI is discontinued. However, clinical evidence suggests that these agents can aggravate pre-existing neuropathy related to HIV.

The role of tissue selectivity in ANA toxicity is highlighted with the contrast between AZT myopathy and ddI or ddC neuropathy. Interestingly, AZT does not cause neuropathy and neither ddI nor ddC exacerbate AZT myopathy. When AZT is discontinued, myopathy improves even in the face of continued ddI or ddC therapy[9]. ANA tissue selectivity may be related to differential phosphorylation of ANAs or specificity of cellular kinases for ANA phosphorylation in different tissues.

ANA neurotoxicity of ddC (and ddI) has been confirmed in tissue cultures. AZT decreases mtDNA in myoblasts and lymphoblasts[9,37]. ddC and ddI decrease mtDNA abundance, cause

Fig. 4. Transmission electron micrograph of a muscle biopsy from a patient with AZT-myopathy. Note mitochondrial enlargement and destruction of cristae with vacuolization. Intramitochondrial paracrystalline inclusions (arrowheads) are prominent (original magnification x 14,000).

destruction of mitochondria and increase lactate production in a neuronal cell line (PC 12) (ref. 58). Rabbits treated with ddC develop an axonopathy with abnormal Schwann cell mitochondria and decreased myelin mRNA[38] *in vivo*. Rats treated with ddI develop an axonal neuropathy with characteristic electrophysiological abnormalities, and ultrastructural changes in the axonal mitochondria.

Neuropathy also occurs with FIAU treatment. FIAU-induced neuropathy has a later onset and persists longer than that of ddC or ddI. Toxic mechanisms may relate to the triphosphate of FIAU inhibiting DNA pol-γ and fialuridine incorporating into mtDNA (Fig. 1*a*), which may explain the persistence of FIAU neurotoxicity after ANA therapy is stopped.

Toxicity of FIAU. The documented anti-HBV activity of FIAU[39] was the basis for a clinical trial in patients with chronic HBV. However, serious FIAU toxicity occurred in clinical trials (including liver failure requiring liver transplantation and the death of some patients)[10–12]. Toxic manifestations included profound lactic acidosis, hepatic failure, skeletal and cardiac myopathy, pancreatitis and neuropathy. Pathologic findings from autopsies and liver explants showed marked micro- and macrovesicular steatosis (D. Kleiner, pers. commun.).

In vitro, FIAU increases lactate abundance in myotube and hepatoblast culture medium, alters mitochondrial ultrastructure and causes accumulation of intracellular neutral fat. In contrast to AZT treatment, FIAU-induced changes appear to be irreversible and are consistent with FIAU's incorporation into mtDNA. FIAUTP competitively inhibits hepatic DNA pol-γ[40]. Woodchucks infected with hepatitis and treated with FIAU develop hepatic steatosis (B. Tennant, pers. commun.) and we found neutral lipid droplets in their myocardium. Rodents, canines and primates incorporate FIAU into DNA[41].

A hypothesis common to ANA toxicities

At first glance, ANA toxicities to diverse target tissues (including cardiac and skeletal muscle, peripheral nerve, liver and pancreas) do not show an obvious pattern linking features of the illnesses. A unifying hypothesis (that we will call the 'DNA pol-γ hypothesis') addresses this. It was suggested in part by work of Cheng[57], Wallace[22], and Wright and Brown[24]. The hypothesis states that manifestations of ANA toxicity in selected tissues reflect the combined effects of four principal factors: the subcellular availability and abundance of the ANA in the target tissue; the ability of cellular thymidine kinase to use the ANA as a competitive alternate substrate and for the ANA to become monophosphorylated intracellularly; the ability of the ANA triphosphate to inhibit DNA pol-γ either by serving as a competitive alternate substrate and incorporating into mtDNA or by terminating the nascent mtDNA chain non-competitively; and the metabolic requirements in the target tissues for oxidative phosphorylation.

In essence, the DNA pol-γ hypothesis suggests that DNA pol-γ function is pivotal to mitochondrial DNA homeostasis. Alterations in the abundance of substrates for DNA pol-γ may result in altered mtDNA genomes and lead to altered mitochondrial polypeptide expression and ultrastructural changes like those in AZT myopathy. In some ways, parts of the hypothesis serve as corollaries to the oxidative phosphorylation (OXPHOS) paradigm of Wallace[22]. As such, ANA toxicity may serve as a model to study genetic illnesses of mitochondria in which disturbed mtDNA replication is crucial. This was exemplified

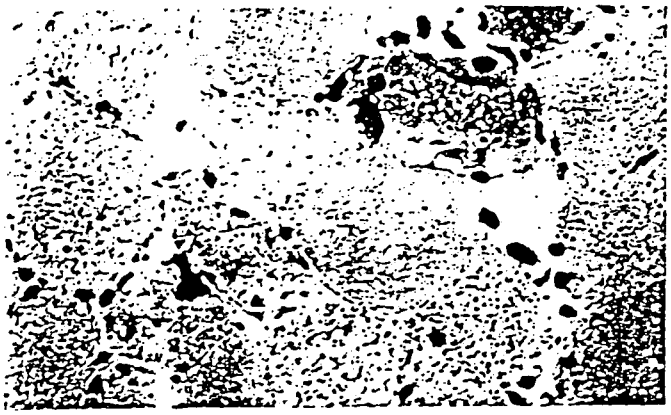

Fig. 5 Cross-section of snap-frozen muscle biopsy from a patient with AZT-myopathy shows abundant orange-staining lipid droplets. (Oil red O stain, original magnification ×560).

with AZT-induced mitochondrial myopathy's resemblance to the genetic mitochondrial DNA-depleting syndromes[62,63].

Analogies to mitochondrial diseases and clinical implications

Genetic mitochondrial illnesses include those with point mutations, mtDNA deletions or duplications, and mtDNA depletion[62–64]. These syndromes include Kearns-Sayre syndrome; myoclonus epilepsy and ragged red fibres syndrome; Leber hereditary optic neuropathy; mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; mitochondrial DNA deletion syndromes and cytochrome *c* oxidase deficiency; Leigh disease; chronic progressive external ophthalmoplegia; Alper syndrome; and mtDNA depletion syndrome)[62–66].

OXPHOS is the main source of mitochondrial energy in many target tissues of ANA toxicity[63]. OXPHOS subunits are encoded by mtDNA. Experience with ANAs suggests that defective OXPHOS may relate to defective mitochondrial gene expression in selected target tissues. Consequently, defining molecular events in acquired OXPHOS defects, such as AZT myopathy, can help to elucidate mechanisms of some hereditary OXPHOS defects.

Pathologically defining ANA-induced changes is necessary to pinpoint subcellular targets of ANA mitochondrial toxicity. Such an approach was employed successfully in elucidating molecular mechanisms of some mitochondrial illnesses[66]. By understanding processes involved in mtDNA replication, more effective ANAs may be designed for antiviral therapy. Conversely, mitochondrial toxic ANAs serve as experimental tools to clarify mtDNA replication mechanisms, and thus based on clinical and experimental studies of ANA toxicity, mitochondrial pharmacology has become an integral part of mitochondrial medicine.

Department of Pathology and Laboratory Medicine
University of Cincinnati College of Medicine
Cincinnati, Ohio 45267-0529, USA (W.L.)

Medical Neurology Branch
National Institutes of Neurological Disorders and Stroke
NIH, Bethesda, Maryland 20892, USA (M.C.D.)

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1. Luft, R. The development of mitochondrial medicine. *Proc. natn. Acad. Sci. U.S.A.* 91, 8731–8738 (1994).
2. Wallace, D.C. Mitochondrial genetics: a paradigm for aging and degenerative diseases? *Science* 256, 628–632 (1994).
3. Elion, G.B. Acyclovir: Discovery, mechanism of action, and selectivity. *J med. Virol. (supp 1)* 1, 2–6 (1993).
4. McGowan, J.J. *et al.* Overview of the preclinical development of an antiretroviral drug, 2',3'-dideoxyinosine. *Rev. infect. Dis.* 12, S513–S521 (1990).
5. Prusoff, W., Lin, T-S, Pivazyan, A., Sun, A.S. & Birks, E. Empirical and rational approaches for development of inhibitors of the human immunodeficiency virus - HIV-1. *Pharmac. Ther.* 60, 315–329 (1993).
6. Mitsuya, H., Yarchoan, R. & Broder, S. Molecular targets for AIDS therapy. *Science* 249, 1533–1544 (1990).
7. Dalakas, M.C., Illa, I., Pezeshkpour, G., Laukaitis, J., Cohen, B. & Griffin, J. Mitochondrial myopathy caused by long term zidovudine therapy. *New Engl. J. Med.* 322, 1098–1105 (1990).
8. Lewis, W., Simpson, J.F. & Meyer, R.R. Cardiac mitochondrial DNA polymerase-γ is inhibited competitively and noncompetitively by phosphorylated zidovudine. *Circulation Res* 74, 344–348 (1994).
9. Cui, L., Toon, S., Schinazi, R.F. & Sommadossi, J-P. Cellular and molecular events leading to mitochondrial toxicity of 1-[2'-deoxy-2'-fluoro-β-D-arabinofuranosyl]-5-iodouracil in human liver cells. *J. clin. Invest.* 95, 555–563 (1995).
10. Brahams, D. Deaths in US fialuridine trial. *Lancet* 343, 1494–1495 (1994).
11. Marshall, E.. Drug trial deaths deemed unavoidable. *Science* 264, 1530 (1994).
12. Macilwain, C. US health agencies at odds over trial deaths. *Nature* 369, 433 (1994).
13. Rozencweig, M. *et al.* Overview of phase 1 trials of 2',3'-dideoxyinosine (ddI) conducted on adult patients. *Rev. infect. Dis.* 12, S570–S575 (1990).
14. Butler, K.M. *et al.* Dideoxyinosine in children with symptomatic human immunodeficiency virus infection. *New Engl. J. Med.* 324, 137–144 (1993).
15. Freiman, J.P., Helfert, K.E., Hamrell, M.R. & Stein, D.S. Hepatomegaly with severe steatosis in HIV-seropositive patients. *AIDS* 7, 379–385 (1993).
16. d'Amati, G., Kwan, W. & Lewis, W. Dilated cardiomyopathy in a zidovudine treated AIDS patient. *Cardiovasc. Pathol.* 1, 317–320 (1992).
17. Chang, C-N, Skalski, V., Zhou, J.H. & Cheng, Y-C. Biochemical pharmacology of (+)-and (−)-2',3'-dideoxy-3'-thiacytidine as anti-hepatitis B virus agents. *J. biol. Chem.* 267, 22414–22420 (1992).
18. Reichard, P. Interactions between deoxyribonucleotide and DNA. *Annu. Rev. Biochem.* 57, 349–374 (1988).
19. Munch-Petersen, B., Cloos, L., Tyrsted, G. & Eriksson, S. Diverging substrate specificity of pure human thymidine kinase 1 and 2 against antiviral dideoxynucleosides. *J. biol. Chem.* 266, 9032–9038 (1991).
20. Fry, M. & Loeb, L.A. *Animal Cell DNA Polymerases* 221(CRC Press, Boca Raton, Florida, 1986).
21. Wang, T.S.F. Eukaryotic DNA polymerases. *Annu. Rev. Biochem.* 60, 513–552 (1991).
22. Wallace, D.C. Diseases of the mitochondrial DNA. *Annu. Rev. Biochem.* 61, 1175–1212 (1992).
23. Wallace, D.C. Mitochondrial DNA sequence variation in human evolution and disease. *Proc. natn. Acad. Sci. U.S.A.* 91, 8739–8746 (1994).
24. Wright, G.E. & Brown, N.C. Deoxyribonucleotide analogs as inhibitors and substrates of DNA polymerases. *Pharmac. Ther.* 47, 447–497 (1990).
25. Dixon, M. & Webb, E.C. *Enzymes* 3rd edn. (Academic, New York, 1979).
26. Richmann, D.D. *et al.* The toxicity of AZT in the treatment of patients with AIDS and AIDS-related complex. *New Engl. J. Med.* 317, 192–197 (1987).
27. Yarchoan, R., Mitsuya, H., Myers, C.E. & Broder, S. Clinical pharmacology of 3'-azido-2',3'-dideoxythymidine (zidovudine) and related dideoxynucleosides. *New Engl. J. Med.* 321, 726–738 (1989).
28. Nickel, W., Austermann, S., Bialek, G. & Grosse, F. Interactions of azidothymidine triphosphate with the cellular DNA polymerases α, δ, and ε and with DNA Primase. *J. biol. Chem.* 267, 848–854 (1992).
29. Dainiak, N., Worthington, M., Riordan, M.A., Kreczko, S. & Goldman, L. 3'-Azido-3'-deoxythymidine (AZT) inhibits proliferation in vitro of human haematopoietic progenitor cells. *Br. J. Haematol.* 69, 299–304 (1988).
30. Scadden, D.T., Zon, L.I. & Groopman, J.E. Pathophysiology and management of HIV-associated hematologic disorders. *Blood* 74, 1455–1463 (1989).
31. Hamburger, A.W. & Chen, R-B. Amelioration of azidothymidine-induced erythroid toxicity by hemin and stem cell factor in immune-suppressed mice. *Exp. Hematol.* 22, 348–352 (1994).
32. Yoshida, Y. & Yoshida, C. Reversal of azidothymidine-induced bone marrow suppression by 2',3'-dideoxythymidine as studied by hemopoietic clonal culture. *AIDS Res. hum. Retrovir.* 6, 929–932 (1990).
33. Falcone, A., Darnowski, J.W., Ruprecht, R.M., Chu, S.H., Brunetti, I. & Calabresi, P. Different effect of benzylacyclouridine on the toxic and therapeu-

tic effects of azidothymidine in mice. *Blood* 76, 2216–2221 (1990).
34. Rotig, A. *et al.* Pearson's marrow-pancreas syndrome. *J. clin. Invest.* 86, 1601–1608 (1990).
35. Arnaudo, E., Dalakas, M.C., Shanske, S., Moraes, C.T., DiMauro, S. & Schoen, E.A. Depletion of muscle mitochondrial DNA in AIDS patients with zidovudine-induced myopathy. *Lancet* 337, 508–510 (1991).
36. Pezeshkpour, G., Illa, I. & Dalakas, M.C. Ultrastructural characteristics and DNA immunocytochemistry in human immunodeficiency virus and zidovudine-associated myopathies. *Hum. Pathol.* 22, 1281–1288 (1991).
37. Lewis, W., Chomyn, A., Gonzalez, B. & Papoian, T. Zidovudine induces molecular, biochemical and ultrastructural changes in rat skeletal muscle mitochondria. *J. clin. Invest.* 89, 1354–1360 (1992).
38. Lewis, W. *et al.* Mitochondrial ultrastructural and molecular changes induced by zidovudine in rat hearts. *Lab Invest.* 65, 228–236 (1991).
39. Lamperth, L., Dalakas, M.C., Dagani, F., Anderson, J. & Ferrari, R. Abnormal skeletal and cardiac mitochondria induced by zidovudine (AZT) in human muscle in vitro and in an animal model. *Lab Invest.* 65, 742–751 (1991).
40. Dalakas, M.C. Carnitine loss and AZT-myopathy. *Ann. Neurol.* 36, 680–681 (1994).
41. Chariot, P. & Gherardi, R. Partial cytochrome *c* oxidase deficiency and cytoplasmic bodies in patients with zidovudine myopathy. *Neuromusc. Dis.* 5, 357–363 (1991).
42. Chariot, P. *et al.* Blood lactate/pyruvate ratio is a non invasive test for the diagnosis of zidovudine myopathy. *Arthritis Rheumat. 37*, 583–586 (1994).
43. Weissman, J.D., Constantinides, I., Hudgins, P. & Wallace, D.C. ^{31}P magnetic resonance spectroscopy suggests impaired mitochondrial functions in AZT-treated HIV-infected patients. *Neurology* 42, 619–623 (1992).
44. Dalakas, M.C., Monzon, M.E., Bernardini, I., Gahl, W.A. & Jay, C.A. Zidovudine-induced mitochondrial myopathy is associated with muscle carnitine deficiency and lipid storage. *Ann. Neurol.* 35, 483–487 (1994).
45. Mhiri, C. *et al.* Zidovudine myopathy: a distinctive disorder associated with mitochondrial dysfunction. *Ann. Neurol.* 29, 606–614 (1991).
46. Peters, R.S., Winer, J., Landon, D.N., Stotter, A. & Pinching, A.J. Mitochondrial myopathy associated with chronic zidovudine therapy in AIDS. *Q. J. Med.* 86, 5–15 (1993).
47. Jay, C. & Dalakas, M.C. Myopathies and neuropathies in HIV-infected adults and children. in *Pediatric Aids* (Williams & Wilkins, Baltimore, 1994).
48. Semino-Mora, M.C., Leon-Monzon, M.E. & Dalakas, M.C. The effect of L-carnitine on the AZT-induced destruction of human myotubes. Part 1: L-Carnitine prevents the myotoxicity of AZT in vitro. *Lab. Invest.* 70, 102–112 (1994).
49. d'Amati, G. & Lewis, W. Zidovudine azidothymidine causes early increases in mitochondrial ribonucleic acid abundance and induces ultrastructural changes in cultured mouse muscle cells. *Lab. Invest.* 71, 879–884 (1994).
50. Herskowitz, A., Willoughby, S.B., Baughman, K.L., Schulman, S.P. & Bartlett, J.D. Cardiomyopathy associated with antiretroviral therapy in patients with HIV infection: a report of 6 cases. *Ann. intern. Med.* 116, 311–313 (1992).
51. Lipschultz, S.E., Gray, L., Sanders, S.P., Hale, A.R., McIntosh, K. & Colan, S.D. Cardiac structure and function in children with human immunodeficiency virus infection treated with zidovudine. *New Engl. J. Med.* 327, 1260–1265 (1992).
52. Chatta, G., Arieff, A.I., Cummings, C. & Tierney, L.M. Lactic acidosis complicating the acquired immunodeficiency syndrome. *Ann. intern. Med.* 118, 37–39 (1993).
53. Jolliet, P. & Widman, J.J. Reye's syndrome in adult with AIDS. *Lancet* 335, 1457 (1990).
54. Dubinsky, R.M., Yarchoan, R., Dalakas, M.C. & Broder, S. Reversible axonal neuropathy from the treatment of AIDS and related disorders with 2',3'-dideoxycytidine (ddC). *Muscle Nerve* 12, 856–860 (1989).
55. Anderson, T.D. *et al.* Mitochondrial schwannopathy and peripheral myelinopathy in a rabbit model of dideoxycytidine neurotoxicity. *Lab Invest.* 70, 724–739 (1994).
56. Jay, C. Ropka, M. & Dalakas, M.C. The drugs 2',3'-dideoxyinosine (ddI) and 2',3'-dideoxycytidine (ddC) are safe alternatives in people with AIDS with zidovudine-induced myopathy. *J. AIDS* 7, 630–631 (1994).
57. Chen, C-H. & Cheng, Y-C. Delayed cytotoxicity and selective loss of mitochondrial DNA in cells treated with the anti-human immunodeficiency virus compound 2',3'-deoxythymidine. *J. biol. Chem.* 264, 11934–11937 (1989).
58. Keilbaugh, S.A., Prusoff, W.H. & Simpson, M.V. The PC 12 cell as a model for studies of mechanism of induction of peripheral neuropathy by anti-HIV-1 dideoxynucleoside analogs. *Biochem. Pharmacol.* 42, RS–R8 (1991).
59. Fourel, I., Li, J., Hantz, O., Jacquet, C., Fox, J.J. & Trepo, C. Effects of 2'-fluorinated arabinosyl-pyrimidine nucleosides on duck hepatitis B virus DNA level in serum and liver of chronically infected ducks. *J. med. Virol.* 37, 122–126 (1992).
60. Lewis, W., Simpson, J.F., Meyer, R.R., Colacino, J.M. & Perrino, F.W. Mammalian DNA polymerases α, β, γ, δ, and ε incorporate fialuridine (FIAU) monophosphate into DNA and are inhibited competitively by FIAU triphosphate. *Biochemistry* 33, 14620–14624 (1994).
61. Richardson, F.C., Engelhardt, J.A. & Bowsher, R.R. Fialuridine accumulates in DNA of dogs, monkeys, and rats following long-term oral administration. *Proc. natn. Acad. Sci. U.S.A.* 91, 12003–12007(1994).
62. Moraes, C.T. *et al.* mtDNA depletion with variable tissue expression: an novel genetic abnormality in mitochondrial diseases. *Am. J. hum. Genet.* 48, 492–501 (1991).
63. *Progress in Neuropathy* Vol 7 (eds. Sato, T. & DiMauro, S.) (Raven Press, New York, 1991).
64. Morgan-Hughes, J.A. Mitochondrial diseases. in *Myology* (eds. Engel, A.G. & Franzini-Armstrong, C.) 1610–1660 (McGraw Hill, New York, 1994).
65. Shoffner, J.M.V.I. & Wallace, D.C. Oxidative phosphorylation diseases. Disorder of two genomes. *Adv. hum. Genet.* 19, 267–330 (1990).
66. Moraes, C.T. *et al. Mitochondrial DNA in human pathology.* (eds. DiMauro, S. & Wallace, D.C.) 97–108 (Raven, New York, 1993).

Depletion of muscle mitochondrial DNA in AIDS patients with zidovudine-induced myopathy

ENRICA ARNAUDO MARINOS DALAKAS SARA SHANSKE
CARLOS T. MORAES SALVATORE DIMAURO ERIC A. SCHON

Long-term zidovudine therapy in patients with human immunodeficiency virus (HIV) infection can cause a destructive mitochondrial myopathy with histological features of ragged-red fibres (RRF) and proliferation of abnormal mitochondria. In 9 zidovudine-treated patients with this myopathy we found severely reduced amounts (up to 78% reduction *vs* normal adult controls) of mitochondrial DNA (mtDNA) in muscle biopsy specimens by means of Southern blotting. In 2 HIV-positive patients who had not received zidovudine, muscle mtDNA content did not differ from that in the 4 controls. Depletion of mtDNA seems to be reversible, since 1 patient showed a substantial reduction in RRF and a concomitant pronounced increase in muscle mtDNA content after zidovudine therapy was discontinued. Depletion of muscle mtDNA is probably due to zidovudine-induced inhibition of mtDNA replication by DNA polymerase gamma and is not a secondary effect of HIV infection.

Lancet 1991; **337**: 508–10.

Introduction

Neuromuscular complications in patients with human immunodeficiency virus (HIV) infection have been ascribed to the effects of the virus per se[1] and have also been associated with zidovudine therapy. Zidovudine-related changes include an inflammatory myopathy with abundant ragged-red fibres (RRF) and proliferation of enlarged mitochondria.[2,3] Because dideoxy nucleoside triphosphates, including zidovudine,[4] can serve as substrates for DNA polymerase gamma,[5] which is responsible for the replication of mitochondrial DNA (mtDNA), zidovudine therapy might inhibit mtDNA replication. We[3] previously suggested that such inhibition could have contributed to the mitochondrial abnormalities seen in our patients; to study the effect of zidovudine therapy on mtDNA in vivo, we carried out a molecular genetic analysis on muscle mtDNA from the zidovudine-treated patients with mitochondrial myopathy.

Patients and methods

Patients

We analysed freshly frozen muscle biopsy specimens from 9 HIV-positive patients who had been treated with zidovudine for 9 to 18 months, and from 2 HIV-positive patients who had not received the drug. For non-HIV- patient controls, we used muscle biopsy specimens from 4 normal adults. All specimens were processed for muscle enzyme histochemistry and electron microscopy, as previously reported.[3]

DNA analyses

Total DNA was prepared from about 50 mg of muscle tissue by a modification[6] of the method of Davis et al.[7] 5µg of DNA was digested with *Pvu*II; after electrophoresis through an 0·8% agarose gel the sample was transferred to nitrocellulose (Southern blotting).[8] The filters were hybridised with two probes. One probe was the entire mitochondrial genome, isolated as described previously,[9] and labelled with ^{32}P by random priming[10] (2 ng labelled to a specific activity of 4×10^9 cpm/µg; 5×10^4 cpm/ml used). The other probe was clone pB,[11] containing nuclear-encoded 18S rDNA sequences on a 5·8 kb *Eco*RI fragment (20 ng labelled to a specific activity of $2{\cdot}4 \times 10^8$ cpm/µg; 1×10^5 cpm/ml used). Prehybridisation (30 ml), overnight hybridisation, and washing were carried out at 65°C.[7] The filters were exposed to Kodak 'X-Omat R' films at −70°C with an intensifying screen (Cronex, DuPont).

To quantify the mtDNA and nuclear rDNA signals, we scanned the nitrocellulose filters with a 'Betascope' 603 blot analyser (Betagen), and counted the mtDNA (16·6 kb) and rDNA (12·0 kb) signals after subtracting the background counts on each filter (taken from regions adjacent to the hybridising fragments in each lane).

An unpaired two-tailed Student's *t*-test was used for comparisons between groups.

Results

All the specimens from zidovudine-treated patients showed RRF with the modified Gomori trichrome stain;[12] this finding was confirmed by electron microscopy[2] (see table). No abnormal mitochondria were noted histologically in samples from the HIV-positive patients who had not received zidovudine.

In our initial investigations, with the mitochondrial genome probe, we sought to determine whether long-term zidovudine treatment caused qualitative abnormalities of muscle mtDNA. There was no abnormality in mtDNA size, at the level of discrimination of this analysis (data not shown). Nevertheless, although the Southern blots were consistently normal there seemed to be a quantitative decrease in the mtDNA signal of the zidovudine-treated patients by comparison with the controls. Consequently we used the second probe as an internal control for each sample to correct for differences in the amount of DNA loaded in each lane; rDNA genes are useful for this purpose because there are about 450 genes encoding the 18S/28S rRNAs in each diploid cell (J. Silvester, personal communication), so the time-scales for detection of the rDNA hybridisation signal and for the signal arising from the large numbers of mitochondrial genomes present in each cell are similar. Moreover, two *Pvu*II sites flank the genes, generating rDNA-containing 12·0 kb fragments (J. Silvester, personal communication) that are easily observable on Southern blots of *Pvu*II-digested total DNA. Since the rDNA content/cell varies only slightly among individuals, the

ADDRESSES: **Department of Neurology** (E. Arnaudo, MD, S. Shanske, PhD, S. DiMauro, MD, E. A. Schon, PhD), **and Department of Genetics and Development** (C. T. Moraes, MSc, E. A. Schon), **Columbia University College of Physicians and Surgeons, New York; and Neuromuscular Diseases Unit, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland, USA** (M. Dalakas MD). Correspondence to: Dr E. A. Schon, Department of Neurology, Columbia University, 630 West 168th Street, New York, NY 10032, USA.

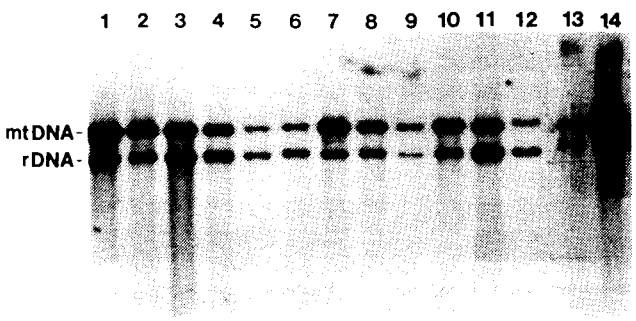
QUANTIFICATION OF HYBRIDISING BANDS SHOWN IN THE FIGURE

—	Months on zidovudine	% RRF	mtDNA/ rDNA	% change *vs* control average
*Controls**				
C1	0	0	2·6	..
C2	0	0	2·6	..
C3	0	0	2·2	..
C4	0	0	2·1	..
AIDS patients				
P1†	0	0	3·4	+48
P2†	0	0	2·0	−13
P3	12	49·1	1·2	−48
P4	12	1·9	0·9	−61
P5	9	28·3	0·7	−69
P6	12	4·4	0·7	−69
P7	13	8·5	1·8	−22
P8	11	14·2	1·4	−39
P9	18	8·3	0·6	−74
P10	10	7.6	0·5	−78
P11	14	16·0	1·7	−26
P11‡	..	6·6	4·1	+78

*C3 and C4 not shown in the figure.
†AIDS patients not on zidovudine.
‡Second biopsy 4 months after discontinuation of zidovudine.

mtDNA:rDNA ratio can be used to normalise the mtDNA signal in each lane so that the relative mtDNA content of various samples can be compared.

Using this method, we confirmed that mtDNA was decreased in all the zidovudine-treated patients with myopathy and RRF by comparison with the controls. In the experiment shown in the figure and the table, the mtDNA:rDNA ratio in the 4 normal adults was 2·3 (SD 0·2, range 2·1–2·6), whereas the ratio in the zidovudine-treated patients with RRF was 1·1 (SD 0·5, range 0·5–1·8). The decrease in mtDNA content in the zidovudine-treated patients ranged from 22% in patient 7 to 78% in patient 10, with an average depletion of 54%; the depletion relative to the controls was statistically significant ($t = 6·4$; $p < 0·001$). This result was confirmed in 5 other zidovudine-treated patients who had RRF and myopathy (data not shown). As shown in the table, there was no apparent difference between the mtDNA:rDNA ratios of the 2 AIDS patients who were not on zidovudine (figure, lanes 7 and 10; average 2·7, SD 1·0) and those of the controls (lanes 1 and 2). The sample size was too small for statistical comparison.



Autoradiograph of Southern blot hybridisation of muscle mtDNA.

The hybridisation bands are denoted at left. Normal controls C1 and C2 (see table) are in lanes 1 and 2, respectively. AIDS patients P1 (lane 7) and P2 (lane 10) were not treated with zidovudine; patients P3-11 (lanes 3, 4, 5, 6, 8, 9, 11, 12, 13,) were treated with zidovudine. Note analysis of DNA from specimen taken from patient P11 before (lane 13) and after (lane 14) zidovudine therapy was discontinued; these results are from a second Southern blot treated identically, and simultaneously, with the blot shown in lanes 1–12. (See table for quantification of hybridising signals.)

The effect of zidovudine on the amount of mtDNA was also shown in the HIV-positive patient P11 who had a first muscle biopsy while taking zidovudine and a second after the drug had been discontinued for 4 months. The mtDNA:rDNA ratio was 1·7 in the first specimen (figure, lane 13), when the patient had myopathy, RRF, and raised blood creatine kinase; in the second specimen (lane 14), when he had improved clinically and the RRF percentage had decreased substantially, the ratio increased by a factor of 2·4, to 4·1 (see table).

There was no apparent correlation between RRF numbers in the specimens from long-term zidovudine-treated patients (expressed as a percentage of total muscle fibres examined) and the mtDNA:rDNA ratio in the same samples. There was likewise no obvious correlation between mtDNA depletion and duration of zidovudine therapy.

Discussion

Nucleoside analogues such as zidovudine that lack a 3′-OH group inhibit the infectivity and cytopathic effect of retroviruses, including HIV-1,[1] because their incorporation into the viral DNA leads to premature termination of the elongating DNA chain.[13] Thus, it is not the enzyme itself that is inhibited but elongation of the daughter-strand template; the better a DNA polymerase uses a substrate such as zidovudine, the greater will be the inhibition of DNA replication.[4] We have found that HIV-positive patients with zidovudine-induced myopathy and RRF have a pronounced reduction in mtDNA content in affected muscle. This observation accords with the finding that zidovudine is readily incorporated into mtDNA by DNA polymerase gamma in vitro.[4] The depletion of up to 78% of muscle mtDNA in these patients is most probably due to the chain-termination property of this agent.[1]

We believe that the depletion of mtDNA is a direct result of zidovudine and not a consequence of HIV-1 infection. Thus: (a) Southern blotting showed a significantly reduced amount of mtDNA in muscle from zidovudine-treated patients compared with controls; (b) in the HIV-positive patient in whom biopsies were done before and after zidovudine therapy there was a clear relation between clinical and histological improvement and recovery in mtDNA content; and (c) the specimens from 2 HIV-positive patients with myopathy who had not been treated with zidovudine had normal or increased amounts of mtDNA.

Why should muscle be specifically affected? Muscle is a long-lived tissue, and muscle fibres are multinucleated syncytia of fused myoblasts. Consequently, an initial reduction in mtDNA in a few mitochondria will persist and will have an effect at sites in muscle far removed from the initial zidovudine-induced event. In muscle, defective mitochondria remain within myofibres, where they coexist with normal mitochondria. In other, non-syncytial tissues—eg, liver or kidney—cells containing defective mitochondria may be rapidly and easily eliminated from the cell population without compromising the respiratory requirements of the tissue as a whole.

Since the half-life of mitochondria is measured in weeks,[14] and since the timing of mtDNA replication varies among mitochondria, the effects of zidovudine are probably confined to the few mtDNAs that are replicating at any one time. Conversely, other mtDNAs may replicate successfully at times when the circulating concentration of zidovudine is low; in this respect the half-life of ingested zidovudine in vivo is only about an hour.[15] These observations may explain

why the mtDNA content in our patients was not eliminated completely. Moreover, individual mitochondria contain multiple mtDNAs, with an average of 2–10 mtDNAs per organelle;[16] perhaps only those mitochondria in which all mtDNAs fail to replicate successfully become abnormal.

That the main side-effects of zidovudine therapy are apparently localised to muscle and blood may not be coincidental. Abnormalities associated with large deletions and duplications of human mtDNA are manifested either in muscle (as ocular myopathy and Kearns-Sayre syndrome, two progressive external ophthalmoplegias characterised by RRF in muscle,[6,17]) or in blood (as Pearson's pancreas/marrow syndrome[18]), even though such deletions are present in all body tissues.[19]

The 2 HIV-positive patients who did not have RRF had mtDNA values that were not appreciably lower than those of the controls. These results suggest that HIV itself does not cause either the abnormal mitochondria or the decline in mtDNA, and that depletion of muscle mtDNA is qualitatively related to the morphological appearance of RRF (as seen with trichrome stain) and of abnormal mitochondria (by electron microscopy). Although the lack of correlation between the RRF numbers and reduction in muscle mtDNA content indicates that zidovudine does not directly affect the number of muscle mitochondria, this finding accords with the ubiquitous mitochondrial abnormalities noted by electron microscopy. We suggest that once zidovudine has caused a reduction in muscle mtDNA content, mitochondria proliferate, resulting in RRF, but continuing mitochondrial proliferation may be a secondary event. Reversibility of the destructive effects of zidovudine in patient 11 indicates that affected mitochondria can recover once they are able to replicate their mtDNA normally.

There are two ways in which RRF numbers can increase in muscle sections: (a) via an increase in the number of initial "foci" where mitochondria begin to proliferate; or (b) because more fibres contain RRF as a result of proliferation of mitochondria through a fibre longitudinally (ie, over some time). Since the development of RRF and the decrease in mtDNA seem to take some months in HIV-positive patients, the decrease in mtDNA may be generalised to mitochondria throughout the entire muscle (irrespective of RRF content) and RRF may contain very few mtDNAs per mitochondrion. This suggestion accords with the widespread and readily detectable abnormal mitochondria with paracrystalline inclusions found by electron microscopy.

These observations should be borne in mind when symptom-free HIV-positive individuals are treated with zidovudine, and also if zidovudine is to be used prophylactically in individuals who may be at risk of AIDS but who are not HIV-positive. Finally, we note that dideoxycytidine (ddC) and dideoxyinosine, two other inhibitors of HIV replication, are being used to treat AIDS patients, and that ddC causes a selective loss of mtDNA in vitro.[5,20]

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REFERENCES

1. Dalakas MC, Pezeshkpour GH. Neuromuscular diseases associated with human immunodeficiency virus infection. *Ann Neurol* 1988; **235**: 38–48.
2. Dalakas M, Pezeshkpour GH. AZT-induced destructive inflammatory myopathy with abnormal mitochondria (DIM-Mi): study of seven patients. *Neurology* 1989; **39** (suppl 1): 152.
3. Dalakas M, Illa I, Pezeshkpour GH, et al. Mitochondrial myopathy caused by long-term AZT (zidovudine) therapy: management and differences from HIV-associated myopathy. *N Engl J Med* 1990; **322**: 1098–105.
4. Simpson MV, Chin CD, Keilbaugh SA, Lin T-s, Prusoff WH. Studies on the inhibition of mitochondrial DNA replication by 3′-azido-3′-deoxythmidine and other dideoxynucleoside analogs which inhibit HIV-1 replication. *Biochem Pharmacol* 1989; **38**: 1033–36.
5. Zimmerman W, Chen SM, Bolden A, Weissbach A. Mitochondrial DNA replication does not involve DNA polymerase alpha. *J Biol Chem* 1980; **255**: 11847–52.
6. Zeviani M, Moraes CT, DiMauro S, et al. Deletions of mitochondrial DNA in Kearns-Sayre syndrome. *Neurology* 1988; **38**: 1339–46.
7. Davis LG, Dibner MD, Battey JF, eds. Basic methods in molecular biology. New York: Elsevier, 1986: 47–50.
8. Southern E. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 1975; **98**: 503–17.
9. Palva TK, Palva ET. Rapid isolation of animal mitochondrial DNA by alkaline extraction. *FEBS Lett* 1985; **192**: 267–70.
10. Feinberg AP, Vogelstein B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 1983; **132**: 6–13.
11. Wilson G, Hollar B, Waterson J, Schmickel R. Molecular analysis of cloned human 18S ribosomal DNA segments. *Proc Natl Acad Sci USA* 1978; **75**: 5367–71.
12. Engel WK, Cunningham GG. Rapid examination of muscle tissue. *Neurology* 1963; **13**: 919–23.
13. Byars N, Kidson C. DNA chain termination by 2′, 3′-dideoxythymidine in replicating mammalian cells. *Biochemistry* 1975; **14**: 3159–64.
14. Kadenbach B. Half-lives of cytochrome c from various organs of the rat. *Biochim Biophys Acta* 1969; **186**: 399–401.
15. Physicians Desk Reference 44. Oradell, NJ: Medical Economics, 1990: 799.
16. Bogenhagen D, Clayton DA. The number of mitochondrial deoxyribonucleic acid genomes in mouse L and human HeLa cells. *J Biol Chem* 1974; **249**: 7991–95.
17. Holt IJ, Harding AE, Morgan-Hughes JA. Deletions of mitochondrial DNA in patients with mitochondrial myopathies. *Nature* 1988; **331**: 717–19.
18. Rotig A, Colonna M, Bonnefont JP, et al. Mitochondrial DNA deletion in Pearson's marrow/pancreas syndrome. *Lancet* 1989; i: 902–03.
19. Shanske S, Moraes CT, Lombes A, et al. Wide-spread tissue distribution of mitochondrial DNA deletions in Kearns-Sayre syndrome. *Neurology* 1990; **40**: 24–28.
20. Chen C-H, Cheng Y-C. Delayed cytotoxicity and selective loss of mitochondrial DNA in cells treated with the anti-human immunodeficiency virus compound 2′, 3′-dideoxycytidine. *J Biol Chem* 1989; **264**: 11934–37.

From The Lancet

Street man-traps

The ordinary traffic of the streets is surely associated with dangers enough without the addition of such as result from careless mismanagement of the very roadway itself. Yet these are not wanting. We do not refer merely to any risk of the season such as that occasioned by non-clearance of snow-laden thoroughfares, but to a cause of mischief ever latent and even more hazardous—namely, the trap-door which ought to cover the entrance to the cellarage of many warehouses, but which is too often left agape, with no sufficient barrier or voice of warning to turn the steps of any unwary foot passenger. Such neglect is highly culpable, even in broad daylight, but the degree of blame as well as the possibility of most serious injury are immensely increased by such perilous conditions as are added by the twilight of evening or the too common incident of a London fog. . . . Almost identical in character with this form of street danger is that arising from the half-open coal-cellar plate, which may be occasionally noticed in front of private dwellings.

(Jan 10, 1891)

Hypothesis

Mitochondrial toxicity induced by nucleoside-analogue reverse-transcriptase inhibitors is a key factor in the pathogenesis of antiretroviral-therapy-related lipodystrophy

Kees Brinkman, Jan A Smeitink, Johannes A Romijn, Peter Reiss

Highly active antiretroviral therapy (HAART) can induce a characteristic lipodystrophy syndrome of peripheral fat wasting and central adiposity. HIV-1 protease inhibitors are generally believed to be the causal agents, although the syndrome has also been observed with protease-inhibitor-sparing regimens. Here, we postulate that the mitochondrial toxicity of the nucleoside-analogue reverse-transcriptase inhibitors plays an essential part in the development of this lipodystrophy, similar to the role of mitochondrial defects in the development of multiple symmetrical lipomatosis.

Since 1996, treatment with a combination of at least three antiretroviral drugs has become the standard of care for patients with HIV-1 infection in industrialised countries. In most cases, treatment regimens include two nucleoside-analogue reverse-transcriptase inhibitors (NRTIs), and one or more protease inhibitors. The use of these new highly active antiretroviral therapies (HAART) has led to unsurpassed reductions in HIV-1-related morbidity and mortality, which in substantial proportions of patients is sustained over the long term. Unfortunately, the widespread introduction of protease-inhibitor-containing therapies in clinical practice has also been associated with an increasing recognition of unusual adverse events, of which the lipodystrophy syndrome is one of the most debilitating and worrisome.

Lipodystrophy

Although a consensus case-definition of the lipodystrophy syndrome has not yet been formulated, it seems to be characterised by a (self-reported) wasting of peripheral fat, mostly of the distal extremities and facial region; this is often combined with a more central accumulation of fat, which is especially noticeable in the dorsocervical area ("buffalo hump"),[1,2] the breasts,[3] and inside the abdominal cavity.[4] Hyperlipidaemia and insulin resistance are additional characteristics of the syndrome.[5] The clinical presentation indicates that there are differential effects on adipose tissue at different locations (subcutaneous *vs* intra-abdominal fat). Although the functional characteristics of the adipose tissue in these two compartments are different,[6] the molecular biology of the differentiation of these two types of adipocytes has not been fully characterised in non-HIV-1-infected individuals. This has hampered the elucidation of the HAART-related lipodystrophy syndrome so far.

In most published reports, the suggestion is that protease inhibitors are the main cause of the development of the syndrome. Carr and colleagues have, so far, offered the most comprehensive hypothetical explanation for the pathogenesis of the syndrome.[7] Crucial to this hypothesis is the inhibition by protease inhibitors of several host-cell proteins involved in lipid and carbohydrate metabolism; this explanation is based on the substantial degree of amino-acid sequence homology between the HIV-1 protease and these human proteins. Carr and colleagues suggested that protease inhibitors induce apoptosis of peripheral adipocytes by binding cytoplasmic retinoic-acid binding protein-1, a molecule that mediates cis-9-retinoic acid stimulation of the retinoic X receptor, normally leading to adipocyte differentiation.[7] In addition, protease inhibitors may inhibit the synthesis of cis-9-retinoic acid that is catalysed by cytochrome P450-3A. Within adipocyte nuclei, cis-9-retinoic acid functions as a heterodimer with peroxisome proliferator activated receptor type gamma (PPAR-γ).[7] That PPAR-γ is preferentially expressed in peripheral rather than central fat might explain the wasting of peripheral fat in particular. Abnormal release of fat from peripheral sites would lead to hyperlipidaemia. This release is reinforced by protease-inhibitor-mediated inhibition of yet another host protein—ie, lipoprotein receptor-related protein, which is involved in hepatic and endothelial clearance of chylomicrons and triglycerides. Impaired storage of peripheral fat by default may result in fat accumulation in the more central parts of the body.[7]

In-vitro, protease inhibitors seem to interfere in adipocyte metabolism via altered retinoid signalling,[8] but further proof for Carr's hypothesis is still missing. Furthermore, it does not explain reports of the lipodystrophy syndrome in HIV-1-infected patients who never used protease inhibitors.[1,3,9] This suggests that alternative or at least additional pathogenic mechanisms are involved.

Benign or multiple symmetrical lipomatosis

Recently, a similarity was noted between HAART-related lipodystrophy and benign or multiple symmetrical lipomatosis (MSL),[10,11] also called Madelungs disease or

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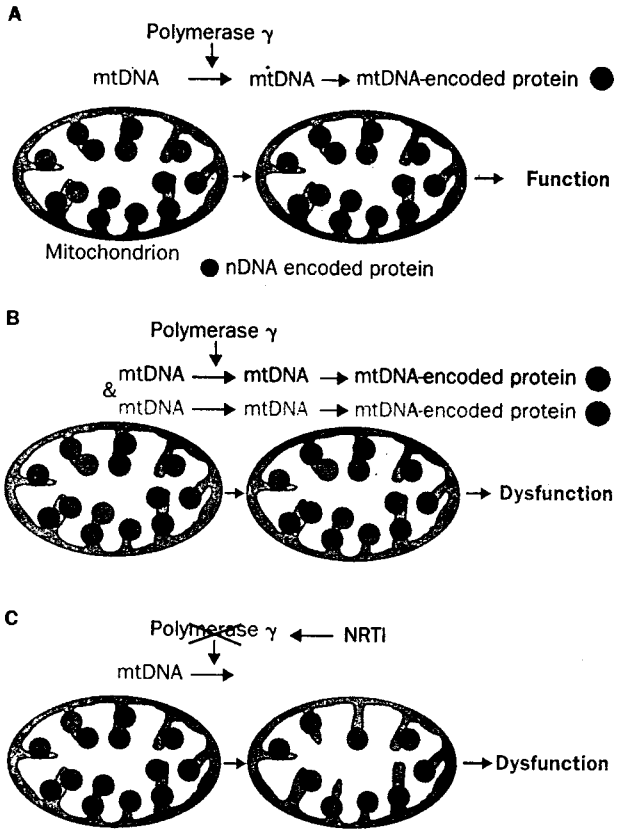
See Commentary Page 1046

Department of Internal Medicine, Onze Lieve Vrouwe Gasthuis, 1e Oosterparkstraat 279, 1091 HA Amsterdam (K Brinkman MD); **Department of Pediatrics, University Hospital Nijmegen** (J A Smeitink MD); **Department of Endocrinology, Leiden University Medical Center** (Prof J A Romijn MD); **and Department of Infectious Diseases, Tropical Medicine and AIDS, Academic Medical Center, Amsterdam** (P Reiss MD), **Netherlands**

Correspondence to: Dr Kees Brinkman
(e-mail: K.Brinkman@OLVG.nl)



Relation between mitochondrial DNA and mitochondrial function

In the normal situation (A) mitochondrial DNA (mtDNA) encodes for proteins (blue circles) in the respiratory chain, situated at the inner mitochondrial membrane. Most respiratory enzymes are encoded by nuclear DNA (nDNA) (red circles). Replication of mtDNA is regulated by the enzyme DNA polymerase γ. In inherited mitochondrial diseases (B), parts of mtDNA have been mutated or even deleted (green), which results in altered mtDNA-encoded proteins (green circles), leading to mitochondrial dysfunction. During NRTI treatment (C), DNA polymerase γ will be inhibited, leading to a depletion of mtDNA and mtDNA-encoded proteins and dysfunctioning mitochondria.

Launois-Bensaude adenolipomatosis. Clinically, this syndrome has been distinguished into two types. In MSL type 1, patients generally have a low body-mass index and show symmetrical accumulation of non-encapsulated masses of fatty tissue, especially in the subcutaneous regions of the neck and shoulders and inside the mediastinum; in addition there is pronounced atrophy of subcutaneous fat in the extremities, thereby resembling the HAART-related lipodystrophy. By contrast, patients with MSL type 2 are usually overweight and show a more diffuse lipomatosis. Since both MSL types 1 and 2 may be accompanied by hypertriglyceridaemia and insulin resistance, MSL has been termed a triglyceride storage disease.[12] This is supported by the observation that lipomatous tissue from patients with MSL shows a defective lipolytic response to adrenergic stimulation.[13] Since patients with MSL do not have abnormal intra-abdominal fat accumulation, MSL type 1 and HAART-related lipodystrophy may not be necessarily identical, but may merely represent different parts of a spectrum.

Aetiology

In addition to alcohol overconsumption, peripheral neuropathy is found in almost all patients with MSL,[12] and this feature points to the possibility that one of the main causes of MSL is mitochondrial dysfunction.[14] Several reports have shown that in MSL there are point mutations at the nucleotide position 8344 in the mitochondrial DNA (mtDNA) or multiple or single mtDNA deletions, leading to an impaired function of the oxidative phosphorylation complex IV (cytochrome c oxidase).[15–19] Since brown adipose tissue has the highest content of mitochondria,[20] a defect in the enzymes of the respiratory chain can easily prompt a decrease in its high fat turnover, leading to the development of lipomas. Brown adipose tissue is particularly present in regions that are affected in MSL type I.[13,21]

Mitochondrial toxicity of NRTIs

Apart from the inherited mtDNA defects, depletion of mtDNA may also be acquired. The only enzyme that is responsible for mtDNA replication, DNA polymerase γ, is inhibited to a varying extent by NRTIs used in HAART.[22,23] Through this mechanism, NRTIs can easily induce depletion of mtDNA, resulting also in depletion of mtDNA-encoded mitochondrial enzymes and this will finally lead to mitochondrial dysfunction (figure). In fact, nearly all side-effects that have been attributed to the use of NRTIs, such as polyneuropathy, myopathy, cardiomyopathy, pancreatitis, bone-marrow suppression, and lactic acidosis, greatly resemble the spectrum of clinical manifestations seen in inherited mitochondrial diseases.[23]

A few studies have shown (with muscle biopsies) the occurrence of mitochondrial dysfunction during zidovudine monotherapy in selected patients with drug-induced myopathy.[24,25] Of the other NRTIs, mitochondrial toxicity has only been shown in vitro, when tested as single agents.[23] So far, there are no studies that have addressed this issue in clinical practice, but it is likely that a combination of NRTIs will synergistically give rise to any form of mitochondrial dysfunction.

Hypothesis

Since HAART almost always includes at least two NRTIs and since HAART-related lipodystrophy has been described in patients not taking protease inhibitors, but only NRTIs, we hypothesise that NRTIs have a key role in the pathogenesis of this syndrome. We propose that the mitochondrial toxicity of these drugs is the responsible mechanism, leading to similar metabolic disturbances as those found in MSL type I.

Protease inhibitors may very well aggravate this metabolic process through additional mechanisms, as suggested by others.[7] The use of NRTIs might even turn out to be the initiating essential factor, since HAART-related lipodystrophy was only observed in patients treated with protease inhibitors when they received NRTIs at the same time. The cause of HAART-related lipodystrophy would then be based on a multifactorial, cascadic process, in which both NRTIs and protease inhibitors play a deleterious part.

Testing the hypothesis

First, a working case-definition of the syndrome needs to be established, enabling objective rating and quantification of the features. Although such a case definition was recently suggested,[26] during the 1st International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV (June 26–28, 1999, San Diego, USA) an attempt to reach a consensus upon the criteria was not successful. A link between the observed metabolic perturbations and the

changes in body composition was felt not to be definitely proven, and, therefore, it was felt better only to define a list with observational items. Future studies need to determine which items of the list might become criteria in the definition.

Second, prospective studies need to be done to show epidemiological evidence for the role of protease inhibitors, NRTIs, or a combination of both in the development of the syndrome. In these studies, either NRTIs or protease inhibitors have to be excluded. At present, there are only a few studies that have not included NRTIs. One such trial is the Prometheus study,[27] which studied a double protease inhibitor combination (saquinavir/ritonavir) with or without the NRTI stavudine. Although it was not evaluated systematically, the investigators had the impression that lipodystrophy was seen more often in patients tested with stavudine than in patients treated with protease inhibitors alone (E H Gisolf, Academic Medical Center, Amsterdam, Netherlands). Since NRTIs will remain the cornerstone of HAART in the near future, it will be difficult to study this issue in a proper, prospective fashion.

There are several studies of protease-inhibitor-spacing regimens, in which double NRTI plus non-NRTI combinations, or even triple NRTI combinations are used. These studies might disclose to what extent NRTIs alone can induce the syndrome. During the Lipodystrophy Workshop in San Diego (see above), data from observational cohorts were presented that show a strong association between the time of exposure to NRTIs and the development of the syndrome.[28-30] Furthermore, more cases were presented of lipodystrophy features in protease-inhibitor-naïve patients;[28,31,32] in one of these studies the physical features of the syndrome seemed to be indistinguishable from the lipodystrophy in patients treated with protease inhibitors, although hyperlipidaemia and insulin-resistance occurred less frequently.[28] Recently, Saint-Marc and colleagues claimed a special role for stavudine over and above other NRTIs in the development of lipodystrophy,[33] and, during the workshop, reversibility of peripheral fat wasting upon interruption of stavudine therapy only was described.[34]

Third, the occurrence of mitochondrial dysfunction during (combination) NRTI therapy has to be further investigated. Most likely, this needs to be done at tissue level, since the different tissue-specific pharmacodynamics of every NRTI and the different metabolic characteristics of the specific cell-types produce different tissue-specific toxicity profiles of every individual NRTI.[22,23] Invasive procedures, such as biopsies of muscle, nerve, or adipose tissue, are not suitable for large-scale, prospective investigations, so animal studies might better serve this purpose. Furthermore, in-vitro studies on different cell-lines, including adipocytes, might further elucidate the extent of mitochondrial dysfunction induced by NRTIs individually or in combination.

Finally, even if mitochondrial dysfunction can be shown in adipose tissue, it remains to be proven that this event leads to the clinically observed HAART-related lipodystrophy. To clarify in vivo the molecular mechanisms involved, animal experiments are inevitable: the lipodystrophy syndrome takes months to develop and short-term in-vitro experiments may therefore not be appropriate. Studies in mice with targeted gene disruptions might prove valuable in this respect.

Assays have to be developed so that the mitochondrial dysfunction can be assessed at tissue-specific levels—not only to prove the proposed hypothesis, but also, more importantly, to detect mitochondrial dysfunction during NRTI therapy early enough to prevent deleterious side-effects. Only then will HAART have the potential to become a truly long-term successful treatment.

References

1 Lo JC, Mulligan K, Tai VW, Algren H, Schambelan M. "Buffalo hump" in men with HIV-1 infection. *Lancet* 1998; **351**: 867–70.

2 Roth VR, Kravcik S, Angel JB. Development of cervical fat pads following therapy with human immunodeficiency virus type 1 protease inhibitors. *Clin Infect Dis* 1998; **27**: 65–67.

3 Gervasoni C, Ridolfo AL, Trifirò G, et al. Redistribution of body fat in HIV-infected women undergoing combined antiretroviral therapy. *AIDS* 1999; **13**: 465–71.

4 Miller KD, Jones E, Yanovski JA, Shankar R, Feuerstein I, Falloon J. Visceral abdominal-fat accumulation associated with use of indinavir *Lancet* 1998: **351**: 871–75.

5 Carr A, Samaras K, Burton S, et al. A syndrome of peripheral lipodystrophy, hyperlipidaemia and insulin resistance in patients receiving HIV protease inhibitors. *AIDS* 1998; **12**: F51–58.

6 Arner P. Not all fat is alike. *Lancet* 1998; **351**: 1301–02.

7 Carr A, Samaras K, Chisholm DJ, Cooper DA. Pathogenesis of HIV-1-protease inhibitor-associated peripheral lipodystrophy, hyperlipidaemia, and insulin resistance. *Lancet* 1998; **351**: 1881–83.

8 Lenhard JM, Weiel JE, Paulik MA, Lehmann JM, Koszalka G, Furfine ES. Indinavir enhances retinoic acid signaling: nelfinafir, saquinavir, and ritonavir inhibit retinoid effects in vitro. 6th Conference on Retroviruses and Opportunistic Infections, Chicago 1999; abstr 665.

9 Madge S, Kinloch de Loes S, Mercey D, Johnson MA, Weller IVD. Lipodystrophy in patients naive to protease inhibitors. *AIDS* 1999; **13**: 735–37.

10 Hengel RL, Watts NB, Lennox JL. Benign symmetric lipomatosis associated with protease inhibitors. *Lancet* 1997; **350**: 1596.

11 Teplitsky V, Halabe A. Fat distribution in AIDS. *N Engl J Med* 1999; **340**: 969–70.

12 Enzi G. Multiple symmetric lipomatosis: an updated clinical report. *Medicine* 1984; **63**: 56–64.

13 Enzi G, Inelmen E, Baritussio A, Dorigo P, Prosdocimi M, Mazzoleni F. Multiple symmetric lipomatosis. A defect in adrenergic-stimulated lipolysis. *J Clin Invest* 1977; **60**: 1221–29.

14 Berkovic SF, Andermann F, Shoubridge EA, et al. Mitochondrial dysfunction in multiple symmetrical lipomatosis. *Ann Neurol* 1991; **29**: 566–69.

15 Larsson NG, Tulinius MH, Holme E, et al. Segregation and manifestations of the mtDNA tRNA(Lys)A→G(8344) mutation of myoclonus epilepsy and ragged-red fibers (MERRF) syndrome. *Am J Hum Genet* 1992; **51**: 1201–12.

16 Silvestri G, Ciafaloni E, Santorelli FM, et al. Clinical features associated with the A→G transition at nucleotide 8344 of mtDNA ("MERRF mutation"). *Neurology* 1993; **43**: 1200–06.

17 Klopstock T, Naumann M, Scchalke B, et al. Multiple symmetric lipomatosis: abnormalities in complex IV and multiple deletions in mitochondrial DNA. *Neurology* 1994; **44**: 862–66.

18 Campos Y, Mártin MA, Navarro C, Gordo P, Arenas J. Single large-scale mitochondrial DNA deletion in a patient with mitochondrial myopathy associated with multiple symmetric lipomatosis. *Neurology* 1996; **47**: 1012–14.

19 Becker-Wegerich P, Steuber M, Olbrisch R, Ruzicka T, Auburger G, Hofhaus G. Defects of mitochondrial respiratory chain in multiple symmetric lipomatosis. *Arch Dermatol Res* 1998; **290**: 652–55.

20 Nedergaard J, Lindberg O. The brown fat cell. *Int Rev Cytol* 1982; **74**: 187–286.

21 Zancanaro C, Sbarbati A, Morroni M, et al. Multiple symmetric lipomatosis. Ultrastructural investigation of the tissue and preadipocytes in primary culture. *Lab Invest* 1990; **63**: 253–58.

22 Lewis W, Dalakas MC. Mitochondrial toxicity of antiviral drugs. *Nat Med* 1995; **1**: 417–22.

23 Brinkman K, ter Hofstede HJM, Burger DM, Smeitink JA, Koopmans PP. Adverse effects of reverse transcriptase inhibitors: mitochondrial toxicity as common pathway. *AIDS* 1998; **12**: 1735–44.

24 Dalakas MC, Illa I, Pezeshkpour GH, et al. Mitochondrial myopathy caused by long-term zidovudine toxicity. *N Engl J Med* 1990; **322**: 1098–105.

25 Arnaudo E, Dalakas M, Shanske S, Moraes CT, DiMauro S, Schon EA. Depletion of muscle mitochondrial DNA in AIDS patients with zidovudine-induced myopathy. *Lancet* 1991; **337**: 508–10.

26 Carr A, Samaras K, Thorisdottir A, Kaufmann GR, Chisholm DJ, Cooper DA. Diagnosis, prediction, and natural course of HIV-1

protease-inhibitor-associated lipodystrophy, hyperlipidaemia, and diabetes mellitus: a cohort study. *Lancet* 1999; **353**: 2093–99.

27 Gisolf EH, de Wolf F, Pelgrom J, et al. Treatment of ritonavir (RTV)/saquinavir(SQV) versus RTV/SQV/stavudine(d4T) (the Prometheus study): preliminary results. 12th World AIDS Conference, Geneva, 1998; abstr 291.

28 Carr A, Miller J, Law M, Cooper DA. A syndrome of lipodystrophy (LD), lactic acidaemia and liver dysfunction associated with HIV nucleoside analogue reverse transcriptase inhibitor therapy: a contribution to PI-treated LD syndrome. *Antiviral Ther* 1999; **4** (suppl 2): 19–20.

29 Mallal S, John M, Moore C, James I, Mckinnon E. Protease inhibitors and nucleoside analogue reverse transcriptase inhibitors interact to cause subcutaneous fat wasting in patients with HIV infection. *Antiviral Ther* 1999; **4** (suppl 2): 28–29.

30 Viard JP, Rakotoambinina B. Lipodystrophic syndromes in a cohort of HIV-1-infected patients receiving HAART with a protease inhibitor. *Antiviral Ther* 1999; **4** (suppl 2): 32–33.

31 Mercié P, Malvy D, Daucourt V, et al. Case report of lipodystrophy observations in patients naive of protease inhibitor treatment, Acquitaine cohort, 1999. *Antiviral Ther* 1999; **4** (suppl 2): 27–28.

32 Galli M, Ridolfo AL, Gervasoni C, Ravasio L, Adorni F, Moroni M. Incidence of fat tissue abnormalities in protease inhibitor-naive patients treated with NRTI combinations. *Antiviral Ther* 1999; **4** (suppl 2): 29.

33 Saint-Marc T, Partisani M, Poizot-Martin I, et al. A syndrome of peripheral fat wasting (lipodystrophy) in patients receiving long-term nucleoside analogue therapy. *AIDS* 1999; **13**: 1659–67.

34 Saint-Marc T, Touraine JL. Reversibility of peripheral fat wasting (lipoatrophy) on stopping stavudine therapy. *Antiviral Ther* 1999; **4** (suppl 2): 31.

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Cellular and mitochondrial toxicity of zidovudine (AZT), didanosine (ddI) and zalcitabine (ddC) on cultured human muscle cells

Eric Benbrik[a], Patrick Chariot[a,b,c,*], Sylvie Bonavaud[a], Mustapha Ammi-Saïd[b,d], Eric Frisdal[e], Christiane Rey[a], Romain Gherardi[a,c], Georgia Barlovatz-Meimon[a]

[a]*Groupe d'Etudes et de Recherches sur le Muscle et le Nerf (GERMEN: ER 269 et 315, Université Paris XII), Faculté de Médecine, 94000 Créteil, France*

[b]*Service de Toxicologie, Hôpital Henri Mondor, 94000 Créteil, France*

[c]*Département de Pathologie (Neuropathologie), Hôpital Henri Mondor, 94000 Créteil, France*

[d]*Service d'Explorations Fonctionnelles, Hôpital Henri Mondor, 94000 Créteil, France*

[e]*INSERM U 296, Créteil, France*

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Abstract

Zidovudine (AZT), didanosine (ddI) and zalcitabine (ddC) are the reference antiretroviral therapy in patients with AIDS. A toxic mitochondrial myopathy can be observed in patients treated with AZT, but not with ddI and ddC. All 3 compounds can inhibit mitochondrial (mt)DNA polymerase and cause termination of synthesis of growing mtDNA strands and mtDNA depletion. The propensity to injure particular target tissues is unexplained. In our work, cultured muscle cells prepared from human muscle biopsies, were exposed to various concentrations of AZT (4–5000 μmol/l), ddI (5–1000 μmol/l) and ddC (1–1000 μmol/l) for 10 days. We evaluated cell proliferation and differentiation and measured lipid droplet accumulation, lactate production and respiratory chain enzyme activities. All 3 compounds induced a dose-related decrease of cell proliferation and differentiation. AZT seemed to be the most potent inhibitor of cell proliferation. AZT, ddI and ddC induced cytoplasmic lipid droplet accumulations, increased lactate production and decreased activities of COX (complex IV) and SDH (part of complex II). NADHR (complex I) and citrate sinthase activities were unchanged. Zalcitabine (ddC) and, to a lesser extent, ddI, were the most potent inhibitors of mitochondrial function. In conclusion, AZT, ddI and ddC all exert cytotoxic effects on human muscle cells and induce functional alterations of mitochondria possibly due to mechanisms other than the sole mtDNA depletion. Our results provide only a partial explanation of the fact that AZT, but not ddI and ddC, can induce a myopathy in HIV-infected patients. AZT myopathy might not simply result from a direct mitochondrial toxic effect of crude AZT. © 1997 Elsevier Science B.V.

Keywords: Mitochondria; Lipid accumulation; Zidovudine; Didanosine; Zalcitabine; Muscle cells; Cell culture

1. Introduction

Nucleoside analogs, such as zidovudine (AZT), didanosine (ddI) and zalcitabine (ddC), are the reference antiretroviral drugs in patients with AIDS. These analogs cannot form 3′ phosphodiester linkages, and thus terminate DNA elongation when incorporated into a growing DNA strand (Lewis et al., 1992). They inhibit the reverse transcriptase and the mitochondrial DNA polymerase (gamma polymerase) responsible for mitochondrial (mt)DNA replication (Yarchoan et al., 1989).

Long-term administration of these drugs can induce reversible neuromuscular disorders. AZT can induce a mitochondrial myopathy with ragged-red fibers (Dalakas et al., 1990; Mhiri et al., 1991; Grau et al., 1993), partial cytochrome *c* oxidase (COX) deficiency (Chariot and Gherardi, 1991; Chariot et al., 1993) and depletion of mtDNA (Arnaudo et al., 1991) in muscle and increased

*Corresponding author. Tel.: +33 (01) 49812734; fax: +33 (01) 49812733. Address reprint requests to the Department of Pathology, Hôpital Henri Mondor, 94000 Créteil, France.

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blood lactate/pyruvate ratios (Chariot et al., 1994a). Both ddI and ddC induce painful distal axonal polyneuropathies (Yarchoan et al., 1990; Berger et al., 1993), also possibly related to mitochondrial dysfunction (Chen et al., 1991; Feldman and Anderson, 1994; Martin et al., 1994).

Several points remain unclear about mitochondrial toxicity of nucleoside analogs. (1) Animals receiving AZT showed mild structural and functional mitochondrial abnormalities (Lamperth et al., 1991; Lewis et al., 1991) but ragged-red fibers were not documented, raising questions about the ability of AZT to induce mitochondrial myopathy in the absence of concurrent HIV infection (Reyes et al., 1992; Engbretson, 1996). (2) Studies of mitochondrial function of human muscle cells exposed to AZT have yielded mixed results: mitochondrial dysfunction was reported to be detectable when cells were exposed to concentrations similar to those observed in serum of AZT receivers (Lamperth et al., 1991). Another group failed to demonstrate any dysfunction until exposure to a concentration 1000 times higher (Herzberg et al., 1992). (3) The propensity to injure particular target tissues, namely muscle in the case of AZT and peripheral nerve in the case of ddI and ddC, is unexplained.

In the present study, we intended to compare the in vitro myotoxicity of the three main nucleoside analogs. Would in vitro myotoxicity parallel clinical myotoxicity? Our general objective was to determine if assessment of toxicity on cultured human muscle cells might be used as a predictive test for clinical myotoxicity in early phases of research and development in antiretroviral therapy. We compared the effects of AZT, ddI and ddC on proliferation, differentiation, lipid accumulation, lactate production and mitochondrial enzyme activities in cultured human muscle cells.

2. Methods

2.1. Muscle cultures

Human muscle biopsies were obtained from healthy patients undergoing orthopedic surgery. Primary cultures were prepared as described (Barlovatz-Meimon et al., 1994). Culture medium was changed every fifth culture day.

Zidovudine (AZT, Burroughs Wellcome, Research Triangle, North Carolina: 4–5000 μmol/l), ddI (Bristol-Myers, Wallingford, CT: 5–1000 μmol/l) and ddC (Hoffman La Roche, Basel, Switzerland: 1–1000 μmol/l) were added to culture medium every fifth day from the fourth day after seeding. Cultures were evaluated after 2, 4, 6 and 10 days of exposure to nucleoside analogs.

Cell proliferation was assessed by counting viable cells using trypan blue exclusion on trypsinized cells (Freshney, 1994). Approximately 100 viable cells from each well were counted, using an hemocytometer. We also evaluated cultures for cell differentiation and fusion during the first 10 days. Cell differentiation, expressed as the number of nuclei included in myotubes per cm^2, was assessed in 24 well culture plates by May-Grünwald-Giemsa staining. Myotubes were defined as cells containing at least 3 nuclei (Florini et al., 1988; Lagord et al., 1993). Cell fusion was evaluated by determining the fusion index, defined as the percentage of nuclei included within myotubes (Delaporte et al., 1986; Lagord et al., 1993). All nuclei were counted in each myotube in 2 randomly chosen areas, each area corresponding to a quarter of a well. After 10 days of culture, approximately 2500 myotubes and 5000 nuclei from each well were counted. Lipid accumulation was evaluated by Soudan red, a staining equivalent to oil-red-O (Gabe, 1968).

2.2. Biochemical studies

Enzyme activities were measured by spectrophotometry in trypsinized cells. Citrate synthase activity was determined after subtraction of acetyl-CoA hydrolase activity (Srere, 1969; Robinson et al., 1987). Complex I was estimated by NADH reductase activity assessed by the decrease of ferricyanide reduction (Hatefi, 1978). Complex II was assessed by succinate dehydrogenase (SDH) activity, expressed as formazan absorbance per mg of protein (Chrzanowska-Lightowlers et al., 1993). Complex IV (cytochrome *c* oxidase, COX) activity was measured as described (Cooperstein and Lazarow, 1951), with minor modifications (Herzberg et al., 1992). Proteins were measured by the method of Lowry (Lowry et al., 1951).

To evaluate the reproducibility of the biochemical data, we measured the activities of COX, NADH reductase, SDH and citrate synthase in 3 different cultures for each nucleoside analog at a concentration of 1000 μmol/l.

We determined lactate concentration in the culture medium in all experiments using an analyzer that was specific for L-lactate (Model 640 lactate analyzer, Roche, Basel, Switzerland), as described in Chariot et al. (1994b). The amount of lactate in the medium was expressed as mmoles of lactate production/10^6 cells/day.

2.3. Statistical analysis

Analysis of variance (ANOVA), Student's unpaired *t*-test and the coefficient of correlation *R* were used for statistical analysis. A $p<0.05$ was considered significant.

3. Results

3.1. Effect of AZT, ddI and ddC on cell proliferation and differentiation

Cultures exposed to AZT, ddI and ddC showed a remarkable decrease in cell proliferation assessed by

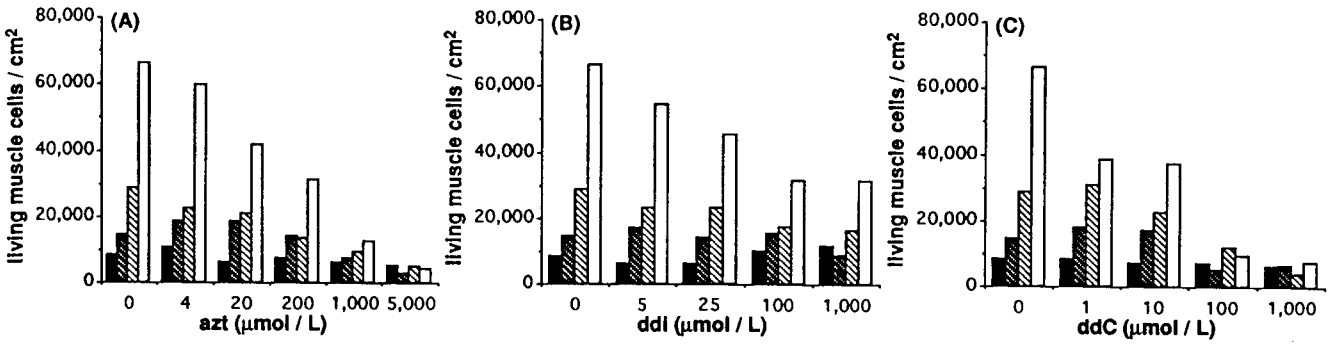

Fig. 1. Effects of AZT, ddI and ddC on muscle cell proliferation. The toxicity of (A) AZT, (B) ddI, and (C) ddC after exposures of 2 days (■), 4 days (▨), 6 days (▧) and 10 days (□) is shown. Nucleoside analogs were added to the culture medium from the fourth day after seeding. Proliferation was measured using trypan blue exclusion on trypsinized cells.

counting viable cells using trypan blue (Fig. 1). The decrease of proliferation was observed after 10 day exposures to AZT, ddI or ddC, at concentrations ≥20, 100 and 100 µmol/l, respectively. The concentrations of AZT, ddI and ddC required to inhibit cell proliferation by 50% (IC_{50}) were approximately 200, 1000 and 100 µmol/l after 6 days of incubation and 100, 500 and 20 µmol/l after 10 days.

Cell differentiation decreased after a 6 day exposure to AZT (≥200 µmol/l), ddI (≥100 µmol/l) and ddC (≥100 µmol/l). The concentrations of AZT, ddI and ddC required to inhibit cell differentiation by 50% (IC_{50}) were approximately 80, 100 and 90 µmol/l after 6 days of incubation. Cell fusion decreased after a 6 day exposure to AZT (≥200 µmol/l) or ddI (≥100 µmol/l). However, cell fusion was not modified by exposure to ddC (Fig. 2).

3.2. Effect of AZT, ddI and ddC on lipid droplet accumulation

All three compounds induced progressive accumulation of lipid droplets in myoblasts or myotubes, the most extensive accumulation being observed with ddC (Fig. 3).

3.3. Effect of AZT, ddI and ddC on mitochondrial function

Biochemical analysis showed decreased respiratory chain activities after 10 day exposures to 1000 µmol/l of AZT, ddI and ddC. Both COX and SDH activities decreased significantly after exposure to AZT, ddI and ddC (COX: ANOVA, $F=6.3$; $p<0.01$ for AZT, ddI and ddC; SDH: ANOVA, $F=21.4$; $p<0.01$ for AZT, ddI and ddC). Complex I and citrate synthase activities were unchanged. No significant changes of any enzyme activity studied were observed after 6 day exposures (Table 1).

A 50% decrease of COX activity was observed with concentrations of AZT, ddI and ddC≥5000 µmol/l, 25 µmol/l and 10 µmol/l, respectively (Fig. 4). The decrease of COX activity correlated with the concentration of AZT after 6 day ($R=0.89$, $p=0.02$) and 10 day ($R=0.90$, $p=0.02$) exposures. The correlation was not significant after exposure to ddI and ddC. A 50% decrease of SDH activity was observed with concentrations of AZT, ddI and ddC≥200 µmol/l, 100 µmol/l and 100 µmol/l, respectively (not shown). The decrease of SDH activity did not correlate significantly with the concentration of AZT, ddI

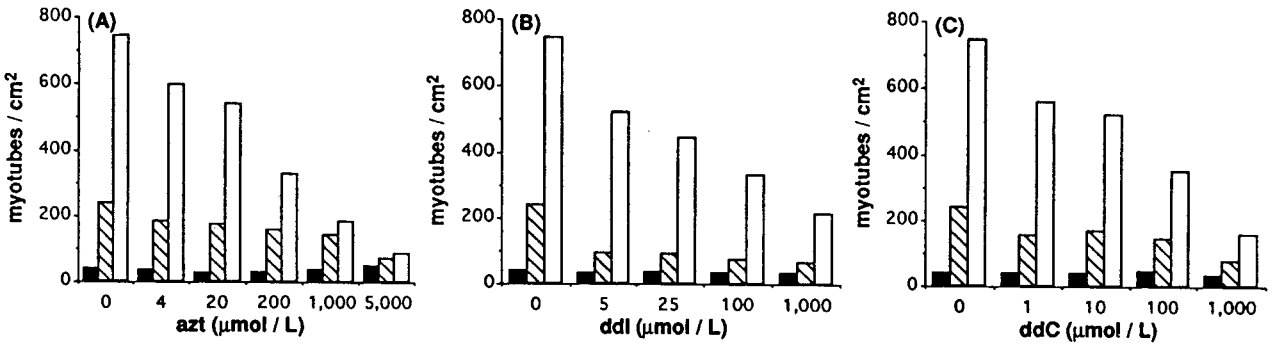

Fig. 2. Effects of AZT, ddI and ddC on muscle cell differentiation. The toxicity of (A) AZT, (B) ddI, and (C) ddC after exposures of 2 days (■), 4 days (▨) and 6 days (□) is shown. Nucleoside analogs were added to the culture medium from the fourth day after seeding. Cells were stained using May-Grünwald-Giemsa.

Fig. 3. Lipid droplet accumulation. Exposure of cultured human muscle cells to nucleoside analogs for 10 days induced lipid accumulation, which was particularly marked after exposure to ddC. (A) AZT, 5000 μmol/l, (B) ddC, 1 μmol/l (Soudan red, original magnification: ×200).

or ddC. No changes of complex I and citrate synthase activities were observed at any concentration tested.

An increased lactate production was observed after exposure to AZT, ddI or ddC, as compared to control cells (Fig. 5). The approximate concentrations of AZT, ddI and ddC corresponding to a 50% increase of lactate production after 10 day exposures were 20, 25 and 10 μmol/l, respectively. The lactate production correlated positively with the concentration of AZT and ddC after 6 day exposures (R=0.98, p<0.001 and R=0.97, p=0.007, respectively) and with the concentration of AZT after 10 day exposures (R=0.99, p<0.001). Lactate production after exposure to 1000 μM of ddC was approximately 50% higher than that observed after exposure to the same concentration of AZT or ddI (Fig. 5).

Table 1
Effects of AZT, ddI and ddC on mitochondrial enzyme activities in cultured muscle cells

Day 6[a]	Control	AZT	ddI	ddC
NADHR[b]	0.56±0.23	0.57±0.34	0.38±0.13	0.45±0.24
SDH[c]	1.07±0.48	0.55±0.09	0.67±0.14	0.54±0.04
COX[d]	0.39±0.12	0.27±0.12	0.25±0.05	0.23±0.05
CS[e]	59.3±27.2	49.4±29.6	54.3±27.8	35.5±21.1
Day 10[a]	Control	AZT	ddI	ddC
NADHR[b]	0.43±0.13	0.51±0.24	0.40±0.12	0.46±0.23
SDH[c]	1.75±0.19	1.09±0.03[f]	0.68±0.07[f]	0.48±0.12[f]
COX[d]	0.63±0.12	0.34±0.11[f]	0.17±0.03[f]	0.19±0.04[f]
CS[e]	91.1±25.8	54.5±18.2	48.3±13.7	34.5±19.8

Cells were exposed to 1000 μmol/l of AZT, ddI or ddC. Enzyme activities are expressed as specific activities. Values are means of 3 experiments±S.E.M.

[a] Days of exposure to AZT, ddI, ddC or medium (control culture).

[b] NADH reductase: μmol ferricyanide reduced min^{-1} mg $protein^{-1}$.

[c] Succinate dehydrogenase (SDH): formazan absorbance at 550 nm mg $protein^{-1}$.

[d] Cytochrome c oxidase (COX): k×mg $protein^{-1}$; $k=2.3 \log A_{(time\ 0)}/A_{(time\ 0+1\ min)}$ min^{-1}.

[e] Citrate synthase (CS): nmol of DTNB oxidized min^{-1} mg $protein^{-1}$. For each nucleoside analog, differences were determined by analysis of variance (ANOVA), followed by Dunnett's procedure for post hoc comparisons to control values if the ANOVA showed a significant difference among groups. p<0.05 was considered significant.

[f] (p<0.01).

4. Discussion

In the present study, human muscle cells exposed to AZT, ddI and ddC showed decreased proliferation and differentiation, marked lipid droplet accumulation and increased lactate production. COX (complex IV) and SDH (part of complex II) activities decreased while NADH reductase (complex I) was unchanged. Concentrations of nucleoside analogs necessary to induce a decrease of COX activity after a 10 day exposure were lower for ddI and ddC than for AZT.

Human plasma peak concentrations in patients treated with AZT, ddI and ddC are approximately the concentrations at which these compounds inhibit the replication of HIV in T cells in vitro, namely 5, 10 and 0.5 μmol/l, respectively (Yarchoan et al., 1989). With short exposures to similar or slightly higher concentrations of these drugs, we and others observed decreased proliferation rates of myogenic cells or other cell types (Lamperth et al., 1991; Chen et al., 1991; Herzberg et al., 1992; Faraj et al., 1994). In our study, the potencies in inhibiting proliferation of myogenic cells were in the order ddC>AZT>ddI, and the

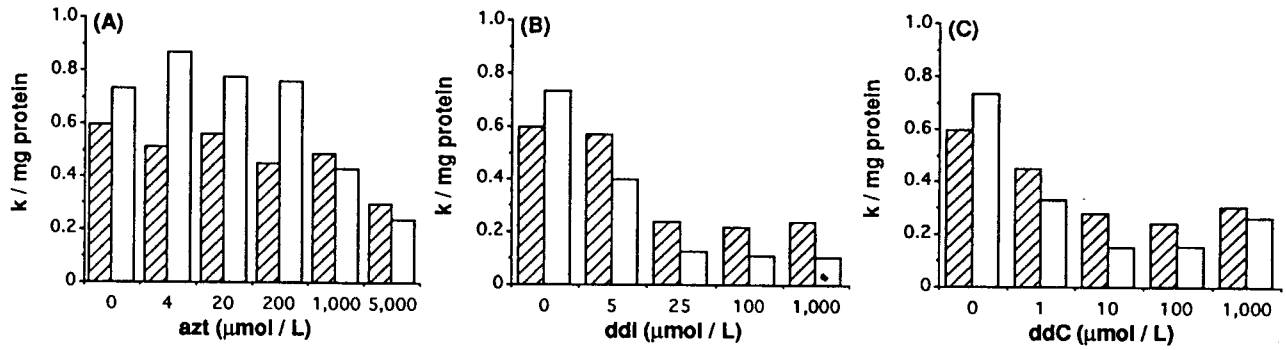

Fig. 4. Effects of AZT, ddI and ddC on cytochrome c oxidase activity. Cultured human muscle cells were exposed to nucleoside analogs for 6 days (▨) and 10 days (□).

three analogs had similar potencies in inhibiting differentiation. However, the existence of a 20 fold difference between plasma concentrations of AZT and ddC in patients with AIDS suggest that AZT could act as the most potent inhibitor of myogenic cell proliferation.

All three nucleoside analogs induced cytoplasmic accumulation of lipid droplets, the most extensive accumulation being observed with ddC. Such an accumulation has been previously observed in the muscle of patients with AZT myopathy (Chariot and Gherardi, 1991; Dalakas et al., 1994) and in muscle cells exposed to AZT in vitro (Semino-Mora et al., 1994a,b). An increased lactate production was observed after exposure to all three analogs, reflecting functional alterations of mitochondria, as previously reported in other cell models (Chen et al., 1991).

Taken together, the analysis of mitochondrial enzyme activities, lactate production and lipid droplet accumulation suggests that ddC and, to a lesser extent, ddI, were the most potent inhibitors of mitochondrial function. In our study, COX activity decreased only after exposure to the highest concentrations of AZT (≥1000 μmol/l), a finding already stressed by Herzberg et al. (1992). These results suggest that the effect of AZT on COX activity was much weaker than that on SDH activity or lactate production. Indeed, SDH, a part of complex II, is encoded by nuclear DNA and its impairment may not be related to a direct toxicity of nucleoside analogs on mtDNA. In fact, at least in the case of AZT, the increase of lactate production and the loss of SDH activity support the view that mitochondrial toxicity of antiretroviral nucleoside analogs could be due to mechanisms other than solely the loss of mtDNA, as previously suggested by several studies. Chen et al. (1991) did not find any correlation between the ability of nucleoside analogs to increase lactate production and their potency in mtDNA depletion. In a second study, analysis of oxidative lesions of nucleic acids in an experimental model of AZT toxicity in rodents showed oxidative damage of mtDNA, which might be a prominent mechanism of mitochondrial toxicity of nucleoside analogs (Hayakawa et al., 1991). Some investigators also demonstrated an early effect of AZT on oxidative phosphorylation, not related to the mtDNA loss (Hobbs et al., 1995).

The differences observed between the rather weak effect of AZT on COX activity in our model and the well-demonstrated COX deficiency in patients treated with AZT (Chariot and Gherardi, 1991; Chariot et al., 1993) relate both to the methods used for evaluation of COX activity and to the different models being considered: (i) biochemistry using spectrophotometry on cell extracts, as used in the present in vitro study, is much less sensitive than histochemistry, where a single deficient myocyte can be detected; (ii) it is obviously difficult to compare in vitro

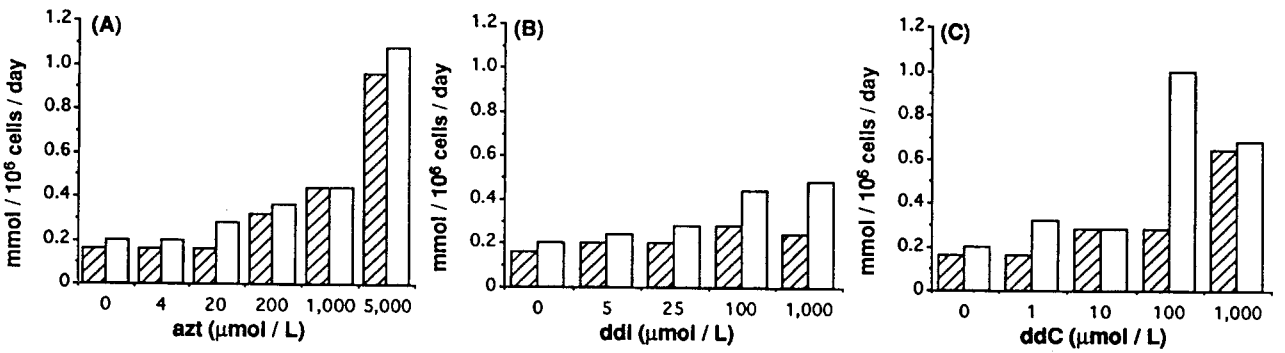

Fig. 5. Effects of AZT, ddI and ddC on lactate production. Cultured human muscle cells were exposed to nucleoside analogs for 6 days (▨) and 10 days (□).

experiments characterized by relatively short-term exposures to the drugs and clinical situations, as observed in patients treated by nucleoside analogs for several years.

Finally, our results provide only a partial explanation of the fact that AZT, but not ddI and ddC, can induce a myopathy in HIV-infected patients. The differences found in vitro between myotoxic and nonmyotoxic compounds are not marked enough to allow us to consider this cell model as a reliable tool to predict clinical myotoxicity of drugs in early phase of evaluation. Some explanations can be proposed. The duration of exposure to drugs in an in vitro model is much shorter than the duration of therapy in patients with AZT myopathy. We speculate that cofactors, such as HIV infection itself or selenium deficiency, could be implicated in the genesis of AZT myopathy (Reyes et al., 1992; Chariot et al., 1994b, 1996, 1997; Chariot and Gherardi, 1995), but this needs to be substantiated. To understand the target organ specificity of the different nucleoside analogs, that are all toxic to mtDNA, it might be useful to compare the subcellular availability and abundance of the nucleoside analogs as competitive alternate substrates for the DNA polymerase gamma and the kinetics of intracellular drug phosphorylation in the different tissues or in in vitro systems (Gosselin et al., 1994; Lewis and Dalakas, 1995).

We conclude that AZT, ddI and ddC all exert cytotoxic effects on human myogenic cells; ddC, and to a lesser extent, ddI, seem to be the most potent inhibitors of mitochondrial function in our model. We speculate that AZT myopathy might not simply result from a direct mitochondrial toxic effect of crude AZT.

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References

Arnaudo, E., Dalakas, M.C., Shanske, S., Moraes, C.T., DiMauro, S. and Schon, E.A. (1991) Depletion of muscle mitochondrial DNA in AIDS patients with zidovudine-induced myopathy. Lancet, 337: 508–510.

Barlovatz-Meimon, G., Bonavaud, S. and Thibert, P. (1994) Culture of specific cell types: Muscle. In: Freshney, R.I. (ed.), Animal Cell Culture, 3rd edn., Wiley-Liss, New York, pp. 329–331.

Berger, A.R., Arezzo, J.C., Schaumburg H.H., et al. (1993) 2′,3′-dideoxycytidine toxic neuropathy: A study of 52 patients. Neurology, 43; 358–362.

Chariot, P. and Gherardi, R. (1991) Partial cytochrome *c* oxidase deficiency and cytoplasmic bodies in patients with zidovudine myopathy. Neuromusc. Disord., 1: 357–363.

Chariot, P., Monnet, I. and Gherardi, R. (1993) Cytochrome *c* oxidase reaction improves histopathological assessment of zidovudine myopathy. Ann. Neurol., 34: 561–565.

Chariot, P., Monnet, I., Mouchet, M., et al. (1994a) Blood lactate/pyruvate ratio as a noninvasive test for the diagnosis of zidovudine myopathy. Arthritis Rheum., 37: 583–586.

Chariot, P., Ratiney, R., Ammi-Said, M., Hérigault, R., Adnot, S. and Gherardi, R. (1994b) Optimal handling of blood samples for routine measurement of lactate and pyruvate. Arch. Pathol. Lab. Med., 118: 695–697.

Chariot, P. and Gherardi, R. (1995) Myopathy and HIV infection. Curr. Opin. Rheumatol., 7: 497–502.

Chariot, P., Lamia, B., Lévy, Y., Dubreuil-Lemaire, M.L., Authier, F.J., Astier, A. and Gherardi, R. (1996) Muscle involvement in HIV-infected patients is associated with marked selenium deficiency. J. Neurovirol., 2: 24 (Abstract).

Chariot, P., Dubreuil-Lemaire, M.L., Zhou, J.Y., Lamia, B., Dumé, L., Larcher, B., Lévy, Y., Astier, A. and Gherardi, R. (1997) Muscle involvement in HIV-infected patients is associated with marked selenium deficiency. Muscle Nerve, in press.

Chen, C.H., Vazquez-Padua, M. and Cheng, Y.C. (1991) Effect of anti-human immunodeficiency virus nucleoside analogs on mitochondrial DNA and its implication for delayed toxicity. Mol. Pharmacol., 39: 625–628.

Chrzanowska-Lightowlers, Z.M.A., Turnbull, D.M. and Lightowlers, R.N. (1993) A microtiter plate assay for cytochrome *c* oxidase in permeabilized whole cells. Anal. Biochem., 214: 45–49.

Cooperstein, S.J. and Lazarow, A. (1951) A microspectrophotometric method for the determination of cytochrome oxidase. J. Biol. Chem., 189: 665–670.

Dalakas, M.C., Illa, I., Pezeshkpour, G.H., Laukaitis, J.P., Cohen, B. and Griffin, J.L. (1990) Mitochondrial myopathy caused by long-term zidovudine therapy. N. Engl. J. Med. 322: 1098–1105.

Dalakas, M.C, Leon-Monzon, M., Bernardini, I., Gahl, W.A. and Jay, C.A. (1994) Zidovudine-induced mitochondrial myopathy is associated with muscle carnitine deficiency and lipid storage. Ann. Neurol., 35: 482–487.

Delaporte, C., Dautreaux, B. and Fardeau, M. (1986) Human myotube differentiation in vitro in different culture conditions. Biol. Cell; 57: 17–22.

Engbretson, B.G. (1996) Effect of zidovudine (AZT) on the structure and function of rat skeletal muscle. Can. J. Physiol. Pharmacol., 74: 679–686.

Faraj, A., Fowler, D.A., Bridges, E.G. and Sommadossi, J.P. (1994) Effects of 2′,3′-dideoxynucleosides on proliferation and differentiation of human pluripotent progenitors in liquid culture and their effects on mitochondrial DNA synthesis. Antimicrob. Ag. Chemother., 38: 924–930.

Feldman, D. and Anderson, T.D. (1994) Schwann cells mitochondrial alterations in peripheral nerves of rabbits treated with 2′,3′-dideoxycytidine. Acta Neuropathol., 87: 71–80.

Florini, J.R., Ewton, D.Z., Ferris, E. and Nadal-Ginard, B. (1988) Muscle cell cultures: Conditions for proliferation and differentiation of muscle cell cultures. In: Baserga, R. (ed.), Cell Growth and Division, A Practical Approach, IRL Press, Oxford, pp. 106–117.

Freshney, R.I. (1994) Measurement of viability and cytotoxicity. In: Freshney R.I. (ed.), Animal Cell Culture, 3rd edn., Wiley-Liss, New York, pp. 287–307.

Gabe, M. (1968) Détection histochimique des lipides et des composés apparentés. In: Techniques Histologiques, Masson, Paris, pp. 447–492.

Gosselin, G., Imbach, J.L. and Sommadossi, J.P. (1994) Antiviral nucleoside analogues and their bioactive phosphorylated metabolites: State of the art review of their detection and tissue monitoring applied to the anti-HIV drug AZT. Bull. Inst. Pasteur, 92: 181–196.

Grau, J.M., Masanes, F., Pedrol, E., Casademont, J., Fernandez-Sola, J. and Urbano-Marquez, A. (1993) Human immunodeficiency virus type 1 infection and myopathy: Clinical relevance of zidovudine therapy. Ann. Neurol., 34: 206–211.

Hatefi, Y. (1978) Preparation and properties of NADH-ubiquinone oxidoreductase (Complex I): NADH-ferricyanide reductase assay. Methods Enzymol., 53: 11–14.

Hayakawa, M., Ogawa, T., Sugiyama, S., Tanaka, M. and Ozawa, R. (1991) Massive conversion of guanosine to 8-hydroxyguanosine in mouse liver mitochondrial DNA by administration of azidothymidine. Biochem. Biophys. Res. Commun., 176: 87–93.

Herzberg, N.H., Zorn, I., Zwart, R., Portegies, P. and Bolhuis, P.A. (1992) Major growth reduction and minor decrease in mitochondrial enzyme activity in cultured human muscle cells after exposure to zidovudine. Muscle Nerve, 15: 706–710.

Hobbs, G.A., Keilbaugh, S.A., Rief, P.M. and Simpson, M.V. (1995) Cellular targets of 3′azido-3′deoxythymidine: An early (non-delayed) effect on oxidative phosphorylation. Biochem Pharmacol, 50: 381–390.

Lagord, C., Carpentier, G., Moraczewski, J., Pons, G., Climent, F. and Martelly, I. (1993) Satellite cell myogenesis is highly stimulated by the kinase inhibitor ISO-H7: Comparison with HA1004 and staurosporine effects. Biochem. Biophys. Res. Commun., 191: 928–936.

Lamperth, L., Dalakas, M.C., Dagani, F., Anderson, J. and Ferrari, R. (1991) Abnormal skeletal and cardiac muscle mitochondria induced by zidovudine (AZT) in human muscle in vitro and in an animal model. Lab. Invest., 65: 742–751.

Lewis, W., Papoian, T., Gonzales, B. et al. (1991) Mitochondrial ultrastructural and molecular changes induced by zidovudine in rat hearts. Lab. Invest., 65: 228–236.

Lewis, W., Gonzalez, B., Chomyn, A. and Papoian, T. (1992) Zidovudine induces molecular, biochemical and ultrastructural changes in rat skeletal muscle mitochondria. J. Clin. Invest., 89: 1354–1360.

Lewis, W. and Dalakas, M.C. (1995) Mitochondrial toxicity of antiviral drugs. Nature Medicine, 1: 417–422.

Lowry, O.H, Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol. J. Biol. Chem., 193: 265–275.

Martin, J.L., Brown, C.E., Matthews-Davis, N. and Reardon, J.E. (1994) Effects of antiviral nucleoside analogs on human DNA polymerases and mitochondrial DNA synthesis. Antimicrob. Ag. Chemother., 38: 2743–2749.

Mhiri, C., Baudrimont, M., Bonne, G. et al. (1991) Zidovudine myopathy: A distinctive disorder associated with mitochondrial dysfunction. Ann. Neurol. 29: 606–614.

Reyes, M.G., Çasanova, J., Varriccio, F., Sequeira, W. and Fresco, K. (1992) Zidovudine myopathy. Neurology, 42: 1252.

Robinson, J.B., Brent, L.G., Sumegi, B. and Srere, P.A. (1987) An enzymatic approach to the study of the Krebs tricarboxylic acid cycle. In: Darley-Usmar, V.M., Rickwood, D. and Wilson, M.T. (eds.), Mitochondria, a Practical Approach, IRL Press, Oxford, pp. 153–170.

Semino-Mora, M.C., Leon-Monzon, M.E. and Dalakas, M.C. (1994a) Effect of L-carnitine on the zidovudine-induced destruction of human myotubes. Part I: L-carnitine prevents the myotoxicity of AZT in vitro. Lab. Invest., 71: 102–112.

Semino-Mora, M.C., Leon-Monzon, M.E. and Dalakas, M.C. (1994b) The effect of L-carnitine on the AZT-induced destruction of human myotubes. Part II: Treatment with L-carnitine improves the AZT-induced changes and prevents further destruction. Lab. Invest., 71: 773–781.

Srere, A. (1969) Citrate synthase. Methods Enzymol., 13: 3–11.

Yarchoan, R., Mitsuya, H., Myers C.E. and Broder, S. (1989) Clinical pharmacology of 3′-azido-2′3′-dideoxythymidine (zidovudine) and related dideoxynucleosides. N. Engl. J. Med., 321: 726–738.

Yarchoan, R., Pluda, J.M., Thomas, R.V. et al. (1990) Long-term toxicity/activity profile of 2′,3′-dideoxyinosine in AIDS or AIDS-related complex. Lancet, 336: 526–529.