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MINIREVIEW

Mitochondrial DNA Depletion, Oxidative Stress, and Mutation: Mechanisms Of Dysfunction from Nucleoside Reverse Transcriptase Inhibitors

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Mitochondrial Dysfunction Hypothesis

Clinical, pharmacological, cell, and molecular biological evidence links altered mitochondrial (mt) DNA replication to the toxicity of nucleoside reverse transcriptase inhibitors (NRTI) (Brinkman et al, 1999; Carr et al, 1999; Kakuda, 2000; Moyle, 2000; Swartz, 1995). Damage in various tissue targets results in organ-specific pathological changes or systemic effects that include elevated plasma lactate. Elevated plasma lactate now is seen commonly with combined antiretroviral therapy (HAART) (Carr et al, 1999; Moyle, 2000). Mitochondrial toxicity of NRTIs was established by previous investigations (Brinkman et al, 1998; Kakuda et al, 1999; Lewis, 1998; Lewis et al, 1991, 1992; Lewis and Dalakas, 1995; Swartz, 1995), but the clinical impact of NRTI toxicity in patients with AIDS remains controversial. Long-term side effects of NRTIs may be more common because of increased AIDS survival. Heightened clinical awareness raises the index of suspicion. In turn, this may impact on increased prevalence. Table 1 summarizes evidence for the toxicity of various NRTIs. Mechanistic analysis is included below.

Three interrelated mechanisms may be operative in NRTI toxicity (Fig. 1). The first was observed clinically and experimentally. Its principal feature was *energy deprivation* secondary to mtDNA depletion. Concomitant with or resulting from mitochondrial energy deprivation is the second key event: *mitochondrial oxidative stress*. Evidence for this stems from in vivo studies with NRTIs and from correlative data in other systems. Lastly, *mtDNA mutations* may result from oxidative mtDNA damage, aberrant mtDNA replication, and altered mtRNA transcription. Together, these form the "mitochondrial dysfunction hypothesis." This hypothesis takes into account all of the pathophysiological events that are important in NRTI toxicity. In some ways, it is analogous to approaches that examine defects in genetic mitochondrial illnesses in which the defective mitochondrial gene product, oxidative stress, and the environment contribute to disease pathogenesis (Schapira and Cooper, 1992).

mtDNA, DNA Polymerase-γ, and NRTI Toxicity

Nuclear DNA encodes 80% of the oxidative phosphorylation genes (OXPHOS, the principal source of myocardial energy), but 13 OXPHOS gene products are encoded by mtDNA (reviewed in Wallace, 1992a). Although mitochondrial *genetic* diseases result from point mutations or deletions of mtDNA, *acquired* defects in mtDNA replication resulting from NRTI's inhibition of mtDNA replication may yield phenotypic OXPHOS defects that mimic the genetic illnesses.

DNA polymerase-γ (DNA pol-γ) is the mtDNA replication enzyme in eukaryotic cells. Substantial homology exists among DNA pol-γ extracted from fly, frog, and human. DNA pol-γ is an enzyme encoded by the nuclear genome that contains two subunits, a large 125 to 140 kDa subunit containing catalytic activity for polymerase and exonuclease and a smaller accessory subunit of 41 to 55 kDa required for processive synthesis (Carrodeguas et al, 1999; Gray and Wong, 1992; Insdorf and Bogenhagen, 1989; Lim et al, 1999; Wang et al, 1997; Wernette and Kaguni, 1986). The accessory subunit provides tighter DNA binding of the complex, thus allowing highly processive DNA synthesis (Lim et al, 1999).

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Table 1. Mitochondrial Toxicity of NRTIs

NRTI	Anatomic or Tissue Target	Clinical Evidence for Mitochondrial Toxicity	Experimental Evidence for Mitochondrial Toxicity
AZT	Skeletal muscle and heart muscle. Lactic acidosis with anaerobic metabolism in many tissues including liver.	Mitochondrial myopathy including ragged red fibers; decreased muscle mtDNA, paracrystals; phosphocreatine depletion in exercised patients. Cardiomyopathy with cardiac dilatation and failure; mitochondrial cristae dissolution. Markedly elevated serum lactate; Reye's syndrome-like findings. Hepatomegaly with fatty change (steatosis). Pediatric AIDS patients are relatively resistant. Dalakas et al, 1990 Arnaudo et al, 1991 Chattha et al, 1993 d'Amati et al, 1992 Freiman et al, 1993 Herskowitz et al, 1992 Jolliet and Widmann, 1990 Kohler and Lewis, 1995 Lewis, 1998 Lewis and Dalakas, 1995 Lewis et al, 2000 Lipshultz et al, 2000 Sinnwell et al, 1995	Decreased mtDNA in vitro. Decreased mtDNA, mtRNA, mitochondrial polypeptides, and mitochondrial ultrastructural damage in vivo. Low K_i for AZTTP with mammalian DNA pol-γ. Failure of exonulcleolytic excision of terminally incorporated AZT. Mixed (competitive and noncompetitive) K_i with cardiac DNA pol-γ against various templates. Benbrik et al, 1997 Cherrington et al, 1995 Corcuera et al, 1996 Corcuera Pindado et al, 1994 Eriksson et al, 1995 Hobbs et al, 1995 Martin et al, 1994 Nusbaum and Joseph, 1996 Semino-Mora et al, 1994 Schroder et al, 1996 Wang et al, 1996 Oxidative stress de la Asuncion et al, 1998 Gerschenson et al, 2000 Szabados et al, 1999 AZT inhibits adenylate kinase: Barile et al, 1994 AZT inhibits Adenine nucleoside translocator: Barile et al, 1997 AZT inhibits NADH-cytochrome c reductase: Modica-Napolitano, 1993 AZT inhibits mitochondrial permeability transition: Elimadi et al, 1997 AZT inhibits NADH oxidase Pereira et al, 1998 AZT treatment has no effect on mitochondria Herzberg et al, 1992
DDC	Peripheral nerve	Painful peripheral neuropathy in 50—100% of patients Berger et al, 1993 Dubinsky et al, 1989 Merigan et al, 1989 Yarchoan et al, 1990	Inhibition of mtDNA replication in MOLT cells. Mitochondrial structural changes and lipid accumulation in nerves of DDC-treated rabbits. Anderson et al, 1992, 1994 Chen and Cheng, 1992 Chen and Cheng, 1989 Chen et al, 1991 Cherrington et al, 1995 Eriksson et al, 1995 Feldman et al, 1992 Feldman and Anderson, 1994 Keilbaugh et al, 1993 Kukhanova et al, 1995 Martin et al, 1994 Starnes and Cheng, 1987 Tsai et al, 1994
3TC			Kinetics with HeLa DNA pol-γ Hart et al, 1992 Martin et al, 1994
Carbovir			K_m with gapped duplex DNA Parker et al, 1991 White et al, 1989

Continued

Table 1. Continued

NRTI	Anatomic or Tissue Target	Clinical Evidence for Mitochondrial Toxicity	Experimental Evidence for Mitochondrial Toxicity
DDI	Peripheral nerve	Painful peripheral neuropathy in 3—22% of patients Cooley et al, 1990 Lambert et al, 1990	Distorted cristae and decreased mtDNA in CEM cells Cui et al, 1997 Martin et al, 1994 Medina et al, 1994 Youssef and Badr, 1992
D4T	Peripheral nerve	Painful peripheral neuropathy in 55% of patients Browne et al, 1993 Cohen et al, 1994	Distorted cristae and decreased mtDNA in CEM cells Cui et al, 1997 Martin et al, 1994 Medina et al, 1994
FIAU	Liver, skeletal, and cardiac muscle, peripheral nerve	Lactic acidosis; hepatic failure and steatosis; renal failure; skeletal and cardiac myopathy; peripheral neuropathy Stevenson et al, 1995	FIAU incorporation into mtDNA in vivo and in vitro. Mitochondrial structural defects and intracellular fat accumulation in vitro and in vivo. Competitive K_i of FIAUTP, FMAUTP with DNA pol-γ (0.02 μM). Cui et al, 1995 Klecker et al, 1994 Lewis et al, 1994a, 1996, 1997 Lewis and Tankersley, 1995 Richardson et al, 1994 Tennant et al, 1998

NRTIs, nucleoside reverse transcriptase inhibitors; AZT, zidovudine; DDC, zalcitabine; 3TC, lamivudine; DDI, didanosine; D4T, stavudine.

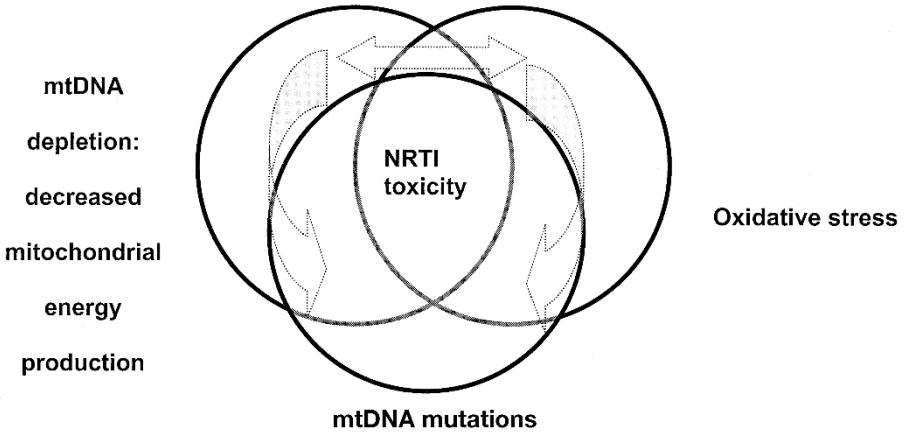

Figure 1.
Mitochondrial dysfunction includes the interaction of decreased energy production, mitochondrial oxidative stress, and mtDNA mutations. The initiating step seems to be decreased energy production, based on clinical and biological manifestations. mtDNA, mitochondrial DNA; NRTI, nucleoside reverse transcriptase inhibitors.

In the mitochondrial dysfunction hypothesis, the *polymerase* function of DNA pol-γ is fundamental to the first proposed mechanism of NRTI toxicity, ie, decreased energy production secondary to decreased mtDNA abundance. When DNA pol-γ activity is inhibited by NRTI triphosphates, mtDNA depletion results. This also suggests that NRTI toxicity may be cumulative and toxic manifestations increase with duration of exposure.

The two-subunit DNA pol-γ is highly *processive* because of its accessory subunit. This high processivity allows the DNA pol-γ complex to replicate the entire mitochondrial genome in one binding event (Lim et al, 1999). Processivity of DNA pol-γ may relate in part to *heteroplasmy*, an intracellular or intramitochondrial mix of normal and mutant mitochondrial DNA molecules (Gray and Wong, 1992; Wang et al, 1997) in NRTI toxicity in which truncated mtDNA fragments may be synthesized.

Because of the high processivity of DNA pol-γ, deletion mutants (truncated mtDNA templates) may be replicated more quickly and efficiently than native mtDNA counterparts (Lim et al, 1999; Wang et al, 1997). The abundance of defective mtDNA may increase to a point at which a threshold of energy depletion may be reached and symptoms become manifested. This *threshold effect* on energetics in NRTI toxicity is analogous to that seen with heritable mitochondrial illnesses, including those that include mtDNA depletion (Moraes et al, 1991; Wallace, 1992a,

1992b). One potential defense against NRTI toxicity exists in the 3'→5' exonuclease within the enzyme. This exonucleolytic function (Kunkel and Mosbaugh, 1989; Kunkel and Soni, 1988) is inhibited by nucleoside 5'-monophosphates (Kaguni et al, 1988).

Mitochondrial Energy Depletion: The First Step in Mitochondrial Dysfunction from NRTI Therapy

Zidovudine (AZT 3'-azido-2',3'-deoxythymidine), zalcitabine (ddC 2',3'-dideoxycytidine), didanosine (ddI 2',3'-dideoxyinosine), stavudine (d4T 2',3'-didehydro-3'-dideoxythymidine), and lamivudine (3TC; 3 thiacytidine; cis-1-[2'-hydroxymethyl-5'-(1, 3oxathiolanyl)] cytosine) are formidable NRTIs that also serve as tools in vitro and in vivo in biomedical and cell biological models of inhibition of DNA pol-γ.

Agents that showed promise in AIDS salvage therapy (including fluoro-dideoxyadenosine [FDDA], 2'-fluoro-2',3'-dideoxyadenosine) or in treatment of chronic hepatitis B infection, such as fialuridine (1-[2-deoxy-2-fluoro-β-D-arabinofuranosyl]-5-iodouracil), were extremely toxic to liver, skeletal, and cardiac muscle, and peripheral nerve in clinical trials. The extent of mitochondrial toxicity was so profound that premature death and hepatic failure in some patients required early termination of clinical trials and abandonment of these pharmacological agents because of mitochondrial toxicity.

The mitochondrial dysfunction hypothesis is an expansion of earlier work. In essence, it incorporates the "DNA pol-γ hypothesis" (Lewis and Dalakas, 1995), oxidative stress, and "acquired" mtDNA mutations as events in a pathophysiological continuum related to energy depletion. *Acquired* mitochondrial diseases may affect mtDNA replication at the level of competition with native nucleotide pools and at the nucleotide binding site of the polymerase. They ultimately result in depletion of mtDNA in affected tissues. The phenotypic results include deleterious effects on mitochondrial structure and function in selected targets, much like what may be expected in some genetic mitochondrial illnesses. Depletion of mtDNA is important to the toxic process mechanistically and seems to be a diagnostic hallmark (Arnaudo et al, 1991; Lewis et al, 1992). The NRTI monophosphate serves as either a mtDNA chain terminator or a nonfunctional base in newly replicating mtDNA (Lewis and Dalakas, 1995).

The DNA pol-γ hypothesis (Lewis and Dalakas, 1995) relates clinical observations, and biochemical, pharmacological, and pathological data. For example, one aspect of the hypothesis focuses on energy deprivation. An analogous hypothesis was effectively used by Katz (1998) to explain the role of mitochondrial alterations in the development of low output congestive heart failure. The inability of mitochondria to function normally in that setting relates to decreased cardiac performance. Energy deprivation, possibly the initiating step of NRTI toxicity based on mtDNA depletion, relates decreased energy abundance in tissues (eg, heart) to decreased abundance of normal, functional mitochondria. The *OXPHOS paradigm*, articulated by Wallace (1992a, 1992b), states that tissue requirements for oxidative phosphorylation and threshold effects of dysfunction appear to be integral to the development of symptoms in genetic illnesses of mtDNA. It follows that NRTI toxicity is cumulative in its effect on mtDNA. Clinical thresholds may be crucial in the acquired forms of mitochondrial illnesses that result from NRTI toxicity.

Corollaries of the DNA pol-γ hypothesis suggest that other pharmacological and cell biological processes are disturbed to yield mtDNA depletion. The subcellular availability and abundance of the NRTI in the target tissue, the ability of the NRTI to become monophosphorylated, and its subsequent ability to serve as a substrate for phosphorylation to the triphosphate are all crucial to the clinical events. The ability of the NRTI triphosphate to inhibit DNA pol-γ and the metabolic requirements of the tissues for oxidative phosphorylation also play key roles.

Oxidative Stress: The Second Step

Although energy depletion from altered mtDNA replication in NRTI toxicity is a logical consequence (Lewis et al, 1991, 1992, 1994a, 1994b, 1996, 1997, 2000; Lewis and Dalakas, 1995), related events of oxidative stress also impact on energetics and mtDNA replication. Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ie, superoxide, hydrogen peroxide, lipid peroxides, hydroxyl radical, and peroxynitrite) and the cellular antioxidant defenses that prevent damage from those moieties (Betteridge, 2000). Mitochondria are logical targets for oxidative stress based on their ability to generate reactive oxygen species and may be primarily involved in oxidative stress associated with AIDS treatment. Chronic AZT treatment induces oxidative damage of skeletal muscle in mice (de la Asuncion et al, 1998) and in rats treated acutely with AZT (Szabados et al, 1999).

Under physiological conditions, reactive oxygen species are produced principally by the mitochondria during OXPHOS. During OXPHOS, approximately 2% to 4% of electron flux results in the reduction of oxygen to superoxide instead of water. Mitochondria generate superoxide and hydrogen peroxide (Boveris and Chance, 1973; Freeman and Crapo, 1981; Turrens et al, 1982). Importantly, disruption of electron flow through the electron transport complexes can greatly increase reactive oxygen production. Superoxide is generated at both the NADH dehydrogenase and the ubiquinone Q-cytochrome b complexes (Fig. 2). Mitochondrial hydrogen peroxide is rapidly formed from superoxide by spontaneous dismutation ($10^5\ M^{-1}\ s^{-1}$) or by mitochondrial manganese superoxide dismutase ($10^9\ M^{-1}\ s^{-1}$). Hydroxyl radicals have been detected at sites where superoxide and hydrogen peroxide are formed in the mitochondria (Nohl et al, 1982) and are produced in liver mitochondria from septic rats (Taylor et al, 1995).

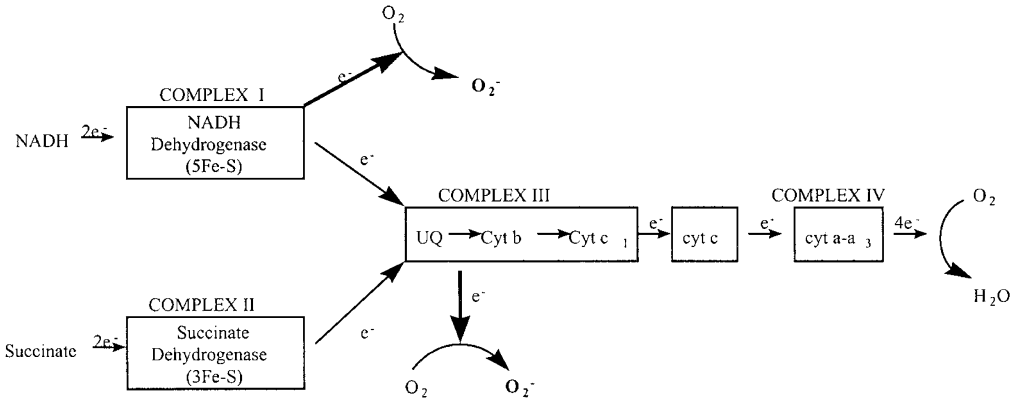

Figure 2.
Simplified scheme of the mitochondrial respiratory chain showing potential sites of superoxide anion (O_2^-) formation. Cyt, cytochrome; UQ, ubiquinone; Fe-S, iron sulfur center.

Mitochondria concentrate iron for incorporation into cytochromes and non–heme-iron proteins (Tangeras et al, 1980). Mitochondrial iron is found in iron (Fe-S)-containing enzymes such as aconitase. Reactive oxygen species can release Fe (II) from aconitase (Flint et al, 1993) and this Fe (II) can bind to mtDNA and provide a site for the production of powerful oxidants immediately adjacent to this critical target. Thus, oxidative stress affects mtDNA replication by altering mtDNA templates through oxidation. It should be noted that DNA pol-γ is very susceptible to oxidative degeneration in vitro and may reflect the biological phenomenon.

Nitric oxide is another reactive oxygen species that is generated by both calcium-calmodulin–dependent (constitutive) and calcium-independent (inducible) forms of nitric oxide synthase. Nitric oxide can affect energy production because it has been shown to tightly bind and inhibit cytochrome oxidase (Brown, 2000). Nitric oxide's direct toxicity is modest but is greatly enhanced by reaction with superoxide to form peroxynitrite (Beckman and Koppenol, 1996). The protonation and decomposition of peroxynitrite to more reactive species is an important mechanism by which nitric oxide can damage DNA (Radi et al, 1991). Fortunately, mitochondria contain antioxidants to protect against damage from reactive oxygen species. A manganese superoxide dismutase (MnSOD) catalyzes the dismutation of superoxide into hydrogen peroxide and oxygen at a rate that approaches the diffusion limit. MnSOD eliminates superoxide but generates hydrogen peroxide that can also produce injury (Buckley et al, 1987). Mitochondria eliminate hydrogen peroxide principally by endogenous glutathione peroxidase. This enzyme converts hydrogen peroxide to water and oxidizes glutathione (Asayama et al, 1996; Marinho et al, 1997). Glutathione reductase and NADPH (from the pentose shunt) recycle oxidized glutathione. Under normal conditions these enzyme systems prevent the build-up of hydrogen peroxide and limit the formation of potentially more reactive species (eg, hydroxyl radical) (Halliwell and Gutteridge, 1985).

Mutations of mtDNA: The Third Step

In addition to NRTI-induced energy deprivation, oxidative damage to mtDNA by respiration-linked reactive oxygen species may relate to damage of cardiac myocytes and development of cardiomyopathy (CM) (reviewed in Linnane et al, 1989; Miquel, 1992). Reactive oxygen species such as hydrogen peroxide, hydroxyl radical, and others are generated close to the inner membrane of the mitochondria and can react with and oxidize mtDNA (Freese et al, 1967). This makes the mtDNA a likely target for oxidative stress.

mtDNA from rat liver has more than 100 times the level of oxidative DNA damage than does nuclear DNA. Differences in oxidative damage between nDNA and mtDNA may relate to (a) lack of known repair enzymes for mtDNA error excision, (b) a lack of histones protecting mtDNA, and (c) a subcellular proximity of mtDNA to these oxidants. Exposure of DNA to superoxide-generating systems causes extensive strand breakage and degradation of deoxyribose (Brawn and Fridovich, 1981). Additionally, peroxynitrite is a potent initiator of DNA strand breaks (Szabo et al, 1996) and causes DNA base modifications (Spencer et al, 1996). On a mass-action basis, random mtDNA mutations would likely inactivate complex I, because of the significant contribution from mtDNA-encoded elements. Moreover, deficiency of complex I proteins could amplify superoxide formation and increase oxidative stress (Cortopassi et al, 1996).

Oxidation of mtDNA by hydroxyl radicals results in the formation of the oxidized base 8-hydroxydeoxyguanosine (8-OHdG). 8-OHdG is present in hepatic mtDNA at 16-fold higher levels than in corresponding nuclear DNA (Richter, 1988; Richter et al, 1988). In human hearts, similar observations were made (Hayakawa et al, 1992). Base modification can lead to mispairing and point mutation (Pavlov et al, 1994). It follows stochastically that, during any given oxidative event, mtDNA will sustain more damage than nuclear DNA (Ames et al, 1993; Yakes and Van Houten, 1997). The number of oxidative hits in rat DNA is estimated at about 100,000 per cell per day. Enzymes for nuclear

DNA repair efficiently remove most, but not all, of the adducts in nDNA (Ames et al, 1993). Although most of the components of a mitochondrial base excision repair system have been identified (Pinz and Bogenhagen, 1998), it is unclear how efficiently this repair removes the wide spectrum of adducts that may result from oxidative damage. Mitochondrial oxidative damage was supported indirectly by the coexistence of malondialdehyde on (or near) the inner mitochondrial membrane (Fleming et al, 1982). Its interaction with mtDNA could lead to cross-linking, deletion errors in transcription, or mtDNA polymerization. In oxidative stress, abundance of 8-OHdG is higher in mtDNA than in nuclear DNA (Kuchino et al, 1987). This may relate to the abundance of mtDNA deletions (Hattori et al, 1991; Hayakawa et al, 1992). A random accumulation of mtDNA defects may result in myocytes with an array of oxidative capacity ranging from normal to severely impaired. This would effectively produce a myocardial "bioenergy mosaic" in NRTI-treated cells in the aging heart (Linnane et al, 1992). Such a mosaic may be absent during histochemical analysis in which a spectrum of activity may be seen in a given tissue (eg, myocardial cytochrome c oxidase activity as a function of aging) (Muller-Hocker, 1989). Pathophysiological events would not occur until the threshold of damage were severe enough to affect organ function (Wallace, 1992a).

NRTI Pharmacological Classification

Pharmacologically, NRTIs have been divided into classes of mtDNA replication inhibitors according to the relative importance of DNA chain termination, or the internalization of the analog into nascent mtDNA and substitution for the natural base (Kakuda, 2000; Parker and Cheng, 1994; Wright and Brown, 1990). One class inhibits mtDNA replication in ways that resemble the action of fialuridine (FIAU). This suggests that the NRTI monophosphate is incorporated into mtDNA. FIAU, FIAC (1-[2-deoxy-2-fluoro-β-D-arabinofuranosyl]-5-iodocytosine), FMAU (1-[2-deoxy-2-fluoro-β-D-arabinofuranosyl]-5-methyluracil), and FEAU (1-[2-deoxy-2-fluoro-β-D-arabinofuranosyl]-5-ethyluracil) each demonstrated efficacy in viral disease models (Fourel et al, 1990), and many of their triphosphates inhibit mammalian DNA pol-γ in vitro (Lewis et al, 1994a). With these agents, *competition* with the native nucleotide and NRTI at the nucleotide binding site of DNA pol-γ appears to be a critical event.

The second type of NRTI is represented by some dideoxynucleosides, such as AZT, ddC, and D4T. With such agents, 5'-triphosphates are substrates for mtDNA synthesis by DNA pol-γ. They *compete* with the natural nucleotides (as above) and also *terminate* nascent mtDNA chains because they lack 3'-hydroxyl groups (3'-OH) for continued mtDNA polymerization. mtDNA replication defects from NRTI toxicity may be reversible in some cases. The 3'$\rightarrow$5' exonuclease of DNA pol-γ may excise the inserted NRTI monophosphate at the time of its insertion into nascent mtDNA if the NRTI is recognized as an erroneous substitute. If the NRTI is not capable of being recognized, it remains in the chain and mtDNA synthesis ceases because the 3'-OH necessary for DNA replication is absent in dideoxy NRTIs.

Terminally incorporated AZT monophosphate is not removed by the 3'$\rightarrow$5' exonuclease of *Saccharomyces cerevisiae* DNA pol-γ (Eriksson et al, 1995). Similar results were found using porcine DNA pol-γ against dideoxynucleotide termini (Longley and Mosbaugh, 1991). Human DNA pol-γ removes chain terminators poorly compared with normal nucleotides. AZT-monophosphate is the most persistent chain terminator to DNA pol-γ exonuclease activity (Lim and Copeland, 2001). It is possible that this could be significant mechanistically in the observed mitochondrial toxicity of DNA by AZT and related NRTIs.

In the case of non–dideoxy-NRTIs, the situation has molecular similarities to the biological condition. Accordingly, use of these compounds has potentially more hazardous consequences, some of which have been observed in humans. Postreplicational repair mechanisms that remove the internally incorporated NRTI monophosphate are incompletely understood, and damage to mtDNA is likely to be more difficult to reverse.

This biochemical point was substantiated in a tragic National Institutes of Health (NIH) clinical trial using FIAU in patients with chronic hepatitis b virus infection. mtDNA replication was profoundly disturbed, principally in the liver, and this resulted in many patient deaths (with or without liver transplant). Incorporated FIAUMP (or FIAU metabolites) could be released from degraded DNA. We demonstrated that "adenosine tracts" were analogous to template-related "hot spots" that were particularly sensitive to mtDNA replication inhibition and resembled those found in some heritable mitochondrial diseases (Wallace, 1992a, 1992b) because they altered mtDNA synthesis. Adenosine tracts in DNA templates inhibit the incorporation of fialuridine monophosphate into DNA in vitro (Lewis et al, 1994b).

Experimental NRTI Toxicity

Table 1 highlights some of the studies in the literature. It should be noted that a few studies have implicated other mechanisms in NRTI mitochondrial toxicity. These include inhibition of adenine nucleotide translocator, NADH-cytochrome-reductase, mitochondrial permeability transition, and NADH oxidase.

In studies that suggested mtDNA replication was affected, kinetics were performed. Incubation of AZT triphosphate with DNA pol-γ in vitro resulted in mixed kinetics with a competitive K_i of 1.8 $\pm$ 0.2 μM and a noncompetitive K_i of 6.8 $\pm$ 1.7 μM. In studies by others, AZT triphosphate inhibited DNA pol-γ activity by approximately 30% at 4 μM, compared with 80% inhibition of reverse transcriptase activity at the same concentration in vitro (Konig et al, 1989). A mixed inhibition pattern also was determined for inhibition of cardiac DNA pol-γ by D4T triphosphate, but with a much lower K_is (Lewis and Tankersley, 1995).

Human DNA pol-γ is inhibited by 50% with 20 μM AZT triphosphate in a reverse transcriptase assay (Lim and Copeland, 2001). Gray et al (1995) showed that Hela cell DNA pol-γ incorporated 3TC triphosphate nearly as well as ddC triphosphate into DNA. Martin et al (1994) compared the inhibition kinetics of human DNA polymerase α, β and found DNA pol-γ to be highly inhibited by dideoxynucleotide triphosphates, D4T triphosphate, and fluoro-substituted analogs. AZT triphosphate, 3TC triphosphate, and carbovir triphosphate were moderate inhibitors of DNA pol-γ in vitro. Martin et al (1994) also demonstrated that ddC, D4T, and FIAU inhibited mtDNA synthesis in vivo below known physiological levels.

AZT Myopathy: Dideoxy NRTI Toxicity

AZT causes a cumulative mitochondrial skeletal myopathy in adult AIDS patients (Dalakas et al, 1990). It is a bona fide complication, (Groopman, 1990; Till and MacDonell, 1990) with characteristic microscopic "ragged red fibers" (Shoubridge, 1994) and ultrastructural paracrystalline inclusions (Dalakas et al, 1990) that result from subsarcolemmal accumulation of mitochondria in the skeletal muscle with long-term, high-dose treatment in adult AIDS patients. Mitochondria are enlarged and swollen ultrastructurally and contain disrupted cristae and occasional paracrystalline inclusions (Lamperth et al, 1991; Lewis et al, 1991; Lewis and Dalakas, 1995; Pezeshkpour et al, 1991).

Extracts of muscle biopsy specimens of AZT-treated patients revealed decreased skeletal muscle mtDNA. Mitochondrial dysfunction in AZT-induced myopathy results in inefficient use of long-chain fatty acids for β-oxidation. Fat droplets accumulate. AZT myopathy develops after at least 6 months of therapy and occurs in up to 17% of treated patients (Dalakas et al, 1994; Peters et al, 1993). Jay and Dalakas (1994) showed that it occurs with the high-dose therapy and with current low-dose regimens. In pediatric populations with AIDS, AZT skeletal myopathy is less frequently observed and may be masked by coexisting encephalopathy.

Clinical features include fatigue, myalgia, muscle weakness, wasting, and elevation of serum creatine kinase (Arnaudo et al, 1991; Dalakas et al, 1994). Clinical improvement accompanies histologic improvement and reversal of zidovudine-induced mtDNA changes (Arnaudo et al, 1991; Dalakas et al, 1994). Pathologic changes in AZT myopathy are reversible following discontinuation of the drug. Serum analytes that are elevated include lactate dehydrogenase, creatine kinase, and serum glutamic-oxaloacetic transaminase. These changes occur after prolonged therapy (Bessen et al, 1988). Exercise decreases muscle phosphocreatine (detected by ^{31}P nuclear magnetic resonance) in AZT-treated patients (Sinnwell et al, 1995). Abnormal mitochondrial respiratory function is found. Enzyme histochemical analysis of muscle biopsies shows partial deficiency of cytochrome c oxidase activity (Chariot and Gherardi, 1991; Dalakas et al, 1994). A high lactate/pyruvate ratio (consistent with abnormal mitochondrial function) is seen in the blood of patients with AZT myopathy (Chariot et al, 1994). Assessment of muscle metabolism in vivo using magnetic resonance spectroscopy shows marked phosphocreatine depletion with slow recovery only in AZT-treated, HIV-positive patients (Sinnwell et al, 1995).

Cardiomyopathy (CM) related to AZT and/or other antiretroviral therapy has been reported in AIDS. Conversely, discontinuation of NRTIs results in improved left ventricular function (Herskowitz et al, 1992). Clinical features of AZT CM resemble some of those previously described. AZT CM occurs after prolonged treatment. Clinical features include congestive heart failure, left ventricle dilatation, and reduced ejection fraction. In general, biopsy data in AZT CM is incomplete. One small study shows ultrastructural changes of intramyocytic vacuoles, myofibrillar loss, dilated sarcoplasmic reticulum, and disruption of mitochondrial cristae (d'Amati et al, 1992).

AZT CM may not be a key finding in neonates treated with AZT but remains a controversial issue. In large-scale studies of pediatric patients with AIDS and of neonates treated with AZT both in utero and perinatally, Lipshultz and colleagues (1992, 2000) reported that impaired cardiac function was not attributed to AZT. Myocardial biopsy findings were absent in any of those reported studies. In parallel, it should be emphasized that AZT-skeletal myopathy is uncommon in children with AIDS (Jay and Dalakas, 1994). Contrasting evidence in other reports suggests that AZT CM in pediatric patients may be more prevalent than previously reported (Domanski et al, 1995). In vivo data from Erythrocebus patas treated with AZT in utero suggest some evidence of a mitochondrial toxicity of AZT to heart and skeletal muscle that resembles those features described in experimental systems with rodents (Gerschenson et al, 2000).

Cell biologically, AZT decreases the abundance of mtDNA in human lymphoblastoid cells (Chen et al, 1991). In vitro, AZT (25μM) inhibits the incorporation of [^{3}H] thymidine by 90% (Chen et al, 1991), and 1 μM AZT inhibits it by 25% to 38% (Simpson et al, 1989). Selective loss of mtDNA occurs in MOLT-4F lymphoblasts exposed to ddC, AZT, and other dideoxynucleosides in vitro (Chen and Cheng, 1989; Chen et al, 1991; d'Amati and Lewis, 1994). In various human and rodent muscle cell lines, exposure to AZT causes abnormal mitochondria with extensive lipid accumulation.

Oral AZT decreases rat cardiac mtRNA and alters mitochondrial ultrastructure (Corcuera Pindado et al, 1994; Lamperth et al, 1991; Lewis et al, 1991, 1992; Semino-Mora et al, 1994). In similarly treated rats, AZT administration decreases mtDNA, mtRNA, and mitochondrial polypeptide expression, and alters mitochondrial ultrastructure in skeletal muscle (Lewis et al, 1992). Mitochondrial changes we observed in the AZT-treated rat heart and muscle are found in striated muscle of hamsters treated with AZT intraperitoneally (Reyes et al, 1991). Rats treated with AZT develop ultrastructural abnormalities in skeletal and cardiac muscle mitochondria associated with depression of

muscle mtDNA and mitochondrial polypeptide synthesis, impaired cytochrome c reductase, and an uncoupling effect (Lamperth et al, 1991; Semino-Mora et al, 1994).

FIAU: Toxicity of a Nondideoxy NRTI

FIAU was the basis for a clinical trial in patients with chronic active hepatitis B virus. Although FIAU exhibits thorough virucidal activity, serious toxicity includes liver failure, (with liver transplantation: Brahams, 1994; Macilwain, 1994; Marshall, 1994; Swartz, 1995). Clinical manifestations include profound lactic acidosis, hepatic failure, skeletal and cardiac myopathy, pancreatitis, and neuropathy. Microvesicular hepatic steatosis is prominent. We show that FIAU triphosphate inhibits DNA pol-γ (competitively with a nanomolar K_i and that FIAU monophosphate is incorporated into DNA (Lewis et al, 1994a, 1996). FIAU and FMAU triphosphate demonstrate nanomolar, competitive K_i values.

HepG2 cells treated with FIAU and FMAU result in each NRTI to be found in nuclear and mtDNA. Ultrastructural defects are found in mitochondria (Cui et al, 1995). mtDNA decrease in abundance in HepG2 cells after 14 days' exposure to FIAU and FMAU (Lewis et al, 1996); FIAU and FMAU, but not FAU, cause mitochondrial structural defects in vitro after at least 2 weeks' treatment. Changes are visible on Oil-red-O–stained HepG2 monolayers. Morphologic changes correlate with lactate abundance in the medium (Levine and Lewis, 1995). In U937 or MOLT-4 cells treated with FIAU, a higher IC_{50} is found, with 1% to 2% replacement of cellular thymidine by fialuridine (Klecker et al, 1994).

Oil-red-O–stained heart samples from FIAU-treated woodchucks reveal neutral lipid droplets in cardiac myocyte cytoplasm. Ultrastructural evidence of mitochondrial destruction is seen. The steady-state abundance of mtDNA in the liver, myocardium, skeletal muscle, and kidney is significantly decreased in tissue samples from fialuridine-treated woodchucks. The magnitude of the decreases varies among the tissues examined (Lewis et al, 1997; Tennant et al, 1998).

Toxicity from Other NRTIs: Varying Tissue Targets

Evidence for the toxicity of NRTIs in different organ systems is presented in tabular form (Table 1). Hepatic toxicity from AZT, ddI, and ddC was reported (Chattha et al, 1993; Freiman et al, 1993; Jolliet and Widmann, 1990). It is presumed to relate to toxicity to liver mitochondria. Fatal hepatomegaly with severe steatosis (Freiman et al, 1993), severe lactic acidosis (Chattha et al, 1993), and adult Reye's syndrome (Jolliet and Widmann, 1990) in AZT-treated HIV seropositive patients are all pathogenetically linked to AZT-induced hepatotoxicity. Clinical features resemble some of those seen in FIAU toxicity. The prevalence of metabolic abnormalities is increasing in AIDS patients treated with NRTI analogs, and the relationship to a variety of metabolic and cardiovascular changes in AIDS is being investigated more closely.

Treatment with certain NRTIs (d4T/3TC) results in anion gap acidosis (Moore et al, 2000a). Moreover, the lactic acidosis/hepatic steatosis syndrome may be more common than previously appreciated in adults (Boubaker et al, 2000; Lonergan et al, 2000; Ter Hofstede et al, 2000) and children (Church et al, 2000) treated with NRTIs. d4T treatment causes lipodystrophy (Saint-Marc et al, 1999). Mechanisms may involve altered mitochondrial biogenesis and/or oxidative changes and possibly adipocyte apoptosis (Harrison, 1997; Lewis and Dalakas, 1995). Recently, we demonstrated arterial dysfunction in FVB/n mice treated with AZT (Sutliff et al, 2000), which may be another important target of toxicity. The use of hydroxyurea in HAART regimens has been associated with neuropathy, pancreatitis, and acute liver failure (Havlir et al, 2000; Moore et al, 2000b).

NRTIs have peripheral neuropathies as side effects (Cohen et al, 1994; Lewis and Dalakas, 1995). Dose-related, painful peripheral neuropathies occur in the majority of patients treated with ddC in doses of 0.03 to 0.09 mg/kg/day (Berger et al, 1993; Dubinsky et al, 1989; Merigan et al, 1989; Yarchoan et al, 1990). Peripheral neuropathy with ddI was unexpected, based upon preclinical data (Anderson et al, 1994). It was observed in 3% to 22% (Cooley et al, 1990; Lambert et al, 1990) of patients after 8 weeks. Peripheral neuropathy occurs in 55% of d4T-treated patients after up to 46 weeks' treatment. Clinically, distal dysesthesias, areflexia, distal sensory loss, and mild muscle weakness are common. Axonal involvement is present. Sural nerve biopsies for patients with ddC neuropathy show axonal degeneration and mitochondria with disrupted cristae. These findings resemble those of experimentally induced neuropathy in ddC-fed rabbits (Anderson et al, 1994). Lamivudine has an associated peripheral neuropathy (Cupler and Dalakas, 1995).

ddC decreases the abundance of mtDNA in PC12 mouse neuronal cell lines in vitro (Keilbaugh et al, 1993) and cultured cells treated with ddC, d4T, or ddI (Medina et al, 1994). We found decreased abundance of mtDNA in HTB11 neuroblastoma cells treated with ddC, ddI, or d4T. In vitro, Dalakas' group showed that AZT increases the abundance of lactate in the culture medium and causes mitochondrial abnormalities in myotubes (Semino-Mora et al, 1994). Although ddC and ddI have not yet been associated with cardiac lesions similar to those associated with AZT (Meyer and Lewis, 1993), administration of ddC to rabbits results in dose-related structural damage to the Schwann cells of the peripheral nerves (Anderson et al, 1992, 1994). This so-called "Schwannopathy" involves mitochondrial changes with some similarities to those found with AZT myopathy.

Summary

NRTI toxicity is now an important clinical problem with long-term significance to AIDS patients. Mechanisms

likely relate to energy depletion, oxidative stress, and mtDNA mutations. Analogously to treatment of other serious infectious agents, combinations of multiple anti–HIV-1 drugs are used to target different viral proteins or points in the virus-host life cycle (Lange, 1995; De Clercq, 1997) and may create combined toxicities to mitochondria. As current clinical guidelines recommend combined therapy, usually including NRTI (Centers for Disease Control and Prevention, 1999), such regimens may be important to the development of mitochondrial toxicity in new tissue targets as treatment is prolonged because of the increased longevity of patients with AIDS. [The following references were cited only in Table 1: Barile et al, 1994, 1997; Benbrik et al, 1997; Browne et al, 1993; Chen and Cheng, 1992; Cherrington et al, 1995; Corcuera et al, 1996; Cui et al, 1997; Dalakas et al, 1990; Elimadi et al, 1997. The following references were cited only in Table 1: Feldman and Anderson, 1994; Feldman et al, 1992; Hart et al, 1992; Hertzberg et al, 1992; Hobbs et al, 1992, 1995; Institute of Medicine (US) Committee to Review the Fialuridine (FIAU/FIAC) Clinical Trials, 1995; Izuta et al, 1991. The following references were cited only in Table 1: Modica-Napolitano, 1993; Nusbaum and Joseph, 1996; Parker et al, 1991; Pereira et al, 1998; Richardson et al, 1994; Schroder et al, 1996; Stevenson et al, 1995; Tsai et al, 1994. The following references were cited only in Table 1: Wang et al, 1996; White et al, 1989.]

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