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Mitochondrial DNA Impairment in NRTIs-Associated Cardiomyopathy¹

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Summary

NRTIs are cornerstones of antiretroviral therapy which have significantly improved HIV/AIDS treatment. Side effects from NRTIs exist. A principal toxicity of NRTIs relates to chronic and cumulative NRTI mitochondrial toxicity in various tissues, including heart. Over the last decade, in vitro and in vivo studies have sought to define mechanisms of NRTI-related CM using sophisticated, cardiac-targeted transgenic models. The HIV/AIDS epidemic is global. NRTIs in combination therapies have afforded long-term survival for infected individuals, but with increased risk for toxic side effects and clinical symptoms including CM.

The acquired immune deficiency syndrome (AIDS) is a global epidemic that continues to escalate. Recent World Health Organization estimates include over 33 million people currently diagnosed with HIV/AIDS. Another 20 million HIV-infected individuals died over the past quarter century. Antiretrovirals are effective treatments that changed the outcome of HIV infection from a fatal disease to a chronic illness. Cardiomyopathy (CM) is a bona fide component of HIV/AIDS with occurrence that is higher in HIV+ individuals. CM may result from individual or combined effects of HIV, immune reactions or toxicities of prolonged antiretrovirals. Nucleoside reverse transcriptase inhibitors (NRTIs) are the cornerstone of antiretroviral therapy. Despite pharmacological benefits of NRTIs, NRTI side effects include increased risk for CM. Clinical observations, in vitro and in vivo studies support various mechanisms of CM. This review highlights some of the hypotheses and focuses on mitochondrial-associated pathways of NRTI- related CM.

Introduction and Overview

For over a quarter century, the global epidemic of HIV/AIDS has escalated. An estimated 33.2 million people worldwide are infected (1) with over 20 million AIDS-related deaths. Rates of new infections grow and approximately 4.3 million people were infected in 2006. Deaths from AIDS-related illnesses in 2006 totaled 2.9 million. HIV/AIDS is the leading cause of death worldwide for all people ages 15-59. Of the 33.2 million people infected, 2.3 million are children, and low and middle income patients constitute 95% of all HIV-infected people. Sub-Saharan Africa is the region with the highest infectious prevalence with about 24.7 million people living with HIV infection (62.5% of all people who are infected reside there). Eastern Europe and Central Asia have the fastest growing number HIV-infected people where an estimated 1.7 million people infected with HIV live (a 20-fold increase in less than a decade) (2). India represents the largest population in a single nation with ~2.5 million people infected

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(1). In Latin America and the Caribbean, over 2 million people are infected and 75% live in the Dominican Republic and Haiti (3).

Untreated AIDS was rapidly fatal early in the epidemic. The most notable success story of antiretroviral therapy was the unambiguous effective prevention of vertical transmission (mother to child, 99%) with early zidovudine (AZT) treatment of HIV⁺ mothers (4). An obvious benefit of effective treatment for patients with HIV/AIDS is prolonged life. Unfortunately, a consequence of prolonged life with HIV/AIDS is increased prevalence of therapeutic side effects that may relate in part to HIV infection, prolonged antiretroviral treatment, or both. Nucleoside reverse transcriptase inhibitors (NRTI) have reported side effects that include toxicity to mitochondria. Current World Health Organization (WHO) guidelines recommend a non-nucleoside reverse transcriptase inhibitor (NNRTI) along with two NRTIs as the treatment of choice for first-line antiretroviral therapy (5). In impoverished, developing countries this often includes use of stavudine (d4T) and zidovudine (AZT) which unfortunately may lead to lipoatrophy, lactic acidosis, polyneuritis and serious anemia. Thus, the epidemic of HIV/AIDS, at least in the developed world, has evolved into a chronic illness that requires regular monitoring and therapy(6).

Mitochondria are cellular organelles that receive scientific and clinical attention because of their importance to cell energy production (7) through the complexes of the electron transport chain (ETC), diseases (8,9), aging (7), and cell death (10). Mitochondria possess their own unique DNA (11) (mtDNA). Inherited diseases of mtDNA replication include nuclear gene mutations that produce mtDNA alterations and cause mitochondrial depletion syndrome (MDS), progressive external ophthalmoplegia (PEO), ataxia-neuropathy, or mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) (12).

It could be argued that mtDNA depletion caused by NRTIs resemble (pharmacologically) genetic mtDNA depletion syndromes (MDS). MDS are autosomal recessive disorders in which quantitative mtDNA depletion is a crucial factor, rather than the accumulation of mtDNA mutations. This class of heterogeneous disorders characteristically involves tissue-specific reduction in mtDNA abundance (13,14). For example, hepatocerebral MDS include progressive liver failure, neurological abnormalities, hypoglycemia and increased plasma lactate. In parallel, these are also clinical features found in mitochondrial toxicity caused by FIAU (15,16). These genetic illnesses also show features of decreased activity of electron-transport-chain complexes (I, III, IV, V) and mtDNA depletion that resemble those seen with AZT and FIAU toxicity (17,18). mtDNA depletion in patients suggest that the salvage-pathway enzymes are intimately involved in the maintenance of mitochondrial dNTP pools required for mtDNA replication, and argues for that fundamental similarity between MDS and NRTI mitochondrial toxicity. Muscle weakness, liver failure and multi-system involvement, with lactic acidosis are common manifestations for MDS and resemble those of NRTI mitochondrial toxicity (19,20) in which heart muscle, skeletal muscle, liver, and peripheral nerve are targets. Acquired diseases include the susceptibility of mtDNA replication machinery to NRTI toxicity (19,20). NRTI-induced injuries can result in a broad spectrum of diseases including: lipoatrophy, fatty liver, hyperlactatemia, peripheral muscle involvement, neuropathy, rare vascular neurological disorders such as MELAS-like syndromes, and cardiomyopathy.

Notably, the role of heart disease in HIV/AIDS patients is an evolving clinical manifestation that has only recently been addressed as a secondary effect of HIV/AIDS and antiretroviral therapies (21-23). Among cardiovascular diseases, cardiomyopathy (CM) warrants investigation to define the mechanisms involved and the relationship of CM to HIV/AIDS and antiretroviral therapy. This review highlights some of the recent studies and developed hypotheses based on *in vitro* and *in vivo* animal models as well as clinical studies.

1. Cardiomyopathy (CM) in HIV/AIDS

CM is a clear complication of HIV/AIDS. It was first described in 1986 and examined frequently thereafter (24-28) (reviewed in (29,30)). The pathogenesis of CM is not fully understood but its clinical features in HIV/AIDS are similar to those of idiopathic CM in HIV negative individuals. Additionally, the difference between LV dysfunction and CM *per se* in AIDS may be somewhat arbitrary since similar diagnostic criteria were not used to identify illness. For the purpose of this review, "LV dysfunction" and CM in HIV/AIDS are used interchangeably. Currie et al. reported an association between cardiac dysfunction and lymphocytic myocarditis in AIDS early in the epidemic (31). Both HIV negative blood donors and patients with hematological malignancy served as controls to high risk lifestyles or matched HIV positive patients. Even though their data did not indicate a direct relationship between CM in HIV/AIDS and high risk activities, or nonspecific manifestation of a chronic illness, it is rational to consider any co-morbid conditions or risk behavior before attributing HIV/AIDS as the sole etiological factor for CM. The differential diagnosis of CM in HIV/AIDS includes LV dysfunction secondary to ischemic heart disease, diabetes or hypertension, hypersensitivity reactions to drugs or foreign material and coronary spasm from cocaine use (32). Importantly, CM in HIV/AIDS has a poor prognosis (33). An algorithm to define HIV/AIDS CM includes positive serology, LV dysfunction and pericardial effusion (34).

As mentioned, the terms LV dysfunction and CM in AIDS are not clearly distinguished clinically. Epidemiological data suggest prevalence of heart muscle disease of about 15%, ~4% for a CM and 6.4% for isolated LV dysfunction in HIV/AIDS, with high percentage of symptomatic patients. CM in HIV/AIDS patients is associated with poor survival compared to patients with structurally normal hearts, even after correcting for CD4 cell count (35). In a one-year study of patients admitted to the intensive care unit of an urban hospital 6% of HIV/AIDS patients had echocardiographically documented CM with a mortality rate of 25% (36).

It is believed that heart failure is partially due to altered energetics, therefore it is reasonable to compare CM to an energy starved engine (37), and LV hypertrophy is part of the continuum to CM (38). As mentioned earlier, the pathogenesis of CM in HIV/AIDS patient is not fully understood, however several factors, individually or in combination may play a role in this process. These factors include HIV-1 virus and its corresponding immunological events, therapeutics, and co-morbidity resulting from life style. Autoimmune processes associated with HIV/AIDS may play a role in development of CM HIV/AIDS (39). Cardiac specific auto-antibodies against β -myosin were significantly higher in HIV positive patients compared to HIV negative individuals. The level was also significantly higher in HIV⁺ patients with CM compared to HIV⁺ patients with normal heart (31). Presence of these cardiac specific auto-antibodies may compromise normal contractile function of cardiac tissues, and could be a contributing factor in CM.

While CM associated with HIV-1 infection is well-recognized, it is unclear what role is played by HIV infection of the cardiomyocyte. An early study on human tissues from autopsy suggested cardiac myocytes were directly infected, although precise cell identification was obscured by the hybridization signal (40). Findings from a prospective longitudinal study from two hundred fifteen patients also suggested, but failed to demonstrate direct role of the virus in CM (41). Subsequent *in vitro* and *in situ* studies of human tissues demonstrated invasion of cardiomyocyte tissue but only direct infection of inflammatory cells, such as dendritic cells (42,43).

NRTIs contribute to cardiac dysfunction in TG models of HIV/AIDS (NL4-3Δ *gag/pol* TG) with mono-NRTI and combined NRTI therapy (44-46). Our group and others suggest that NRTIs (*e.g.* AZT) caused or contributed to CM through mitochondrial toxicity (19) by

inhibition of the mtDNA replicase, DNA pol- γ . Since thirteen of mitochondrial electron transport chain polypeptides are encoded by mtDNA, resultant decreases in mtDNA replication ultimately lead to energy defects and oxidative stress. NRTIs were implicated in the development of HIV-related CM (47-51). Investigators evaluated the frequency and clinical course of CM in HIV-infected populations. In one study, underlying cardiac pathologies occurred in 82% of HIV positive patients (52). A newer ongoing study evaluates the frequency and clinical course of myocardial dysfunction and heart failure in a HIV-infected population with and without therapy (53).

The role of AZT on mtDNA toxicity has been examined *in vitro* and *in vivo*. One study showed AZT decreased mtDNA replication, caused mitochondrial skeletal myopathy in a dose dependent fashion (54), and interfered with mitochondrial oxidative metabolism that eventually resulted in decreasing the energy for muscle contraction (55). Specific ultrastructural and histological changes are documented in human and animal studies and include mitochondrial abnormalities and ragged, red fibers. Similar changes also have been documented in the myocardium of an HIV-1 transgenic mouse treated with AZT (45). The exact role of AZT in the pathogenesis of human CM with HIV/AIDS remains incompletely understood. Nonetheless, clinical NRTI mitochondrial toxicity and CM is documented from fialuridine (FIAU (1-[2-deoxy-2-fluoro- β -D-arabinofuranosyl]-5-iodouracil)) (15,56,57), and a purine (FDDA; (2'-fluoro-2',3'-dideoxyadenosine)) which was to be a salvage NRTI (58, 59), but clinical trials terminated because of mitochondrial side effects.

Empirical evidence supports CM from AZT. Small numbers of HIV positive patients have developed LV dysfunction that improved following NRTI discontinuation (48). Didanosine appears toxic, and mitochondrial toxicity from NRTIs is becoming a problem (60-62) (47, 63-65). Side effects of NRTIs to fetal cardiac development/performance are documented. Mitochondrial toxicity in hearts of mice followed perinatal exposure to NRTIs (66,67).

2. Role of NRTIs on HIV and Host Cell

Recognition of the causative agent of AIDS as the retrovirus, human immunodeficiency virus - type 1 (HIV-1), led to therapeutic strategies to arrest the replicative cycle of the HIV-1 virus (reviewed in (68)). AZT was the first drug that inhibited HIV reverse transcriptase (RT) (69). Currently, NRTIs are cornerstones of AIDS therapy in the developed world, and were introduced into the developing world recently.

NRTIs are pharmacological analogs of native nucleosides for DNA replication. As such, they can be subdivided into pyrimidine and purine analogs. Like native nucleosides, NRTIs require activation by intracellular phosphorylation to active triphosphates (Figure 1). NRTI triphosphates are incorporated by RT into proviral DNA. Pharmacological activity of this class of drugs relates to premature termination of proviral DNA based on NRTI triphosphate's lack of 3' OH group.

Despite the pharmacological effects of NRTI inhibition of HIV replication, NRTI activity also was found to cause side effects. High-dose AZT caused toxicity to bone marrow (70) and disabling skeletal muscle myopathy early in the epidemic (71), however, lower dosed AZT decreased prevalence of those side effects (72). The addition of ddI and ddC to the pharmacopoeia offered agents with decreased toxicity to bone marrow compared to AZT, but those latter agents exhibited pancreatitis and peripheral neuropathy as prominent, sometimes lethal, side effects.

Clinical experience, pharmacological, cell and molecular biological evidence all link defective mitochondrial (mt-) DNA replication to mitochondrial toxicity of NRTIs (19,30,73-76). Early studies aimed at determining the mechanism(s) underlying NRTI-induced toxicity focused on

the chemical structure, and the related biological activity of these antiretrovirals. In addition to the intended inhibitory action on viral RT, NRTI triphosphates were found to inhibit the polymerase function of DNA polymerase gamma (pol- γ) (56,77). DNA pol- γ is part of an assembly of proteins and enzymes responsible for the replication of mtDNA. DNA pol- γ is composed of a large catalytic subunit that has DNA polymerase and exonuclease activity, the latter of which allow for proofreading of the growing mtDNA strand and increases the fidelity of replication (78,79). In contrast to the cellular replicative DNA polymerases, DNA pol- γ is uniquely sensitive to inhibition by NRTI triphosphates (80-85). This inhibition occurs with mixed or competitive kinetics depending on NRTI structure (86). The relative inhibitory effects of NRTI triphosphates on eukaryotic DNA polymerases have been determined (76) (Figure 2a). Second only to HIV-RT, DNA pol- γ is a primary target for NRTI-inhibition, which can lead to mitochondrial toxicity. Kinetic analysis of pol- γ inhibition *in vitro* revealed a hierarchy of mitochondrial toxicity for the various NRTIs (87,88) (Figure 2b). K_m values, for example, are in the micromolar range for AZT and 3TC suggesting only moderate incorporation by eukaryotic polymerases as compared with native nucleotides and dTTP, which demonstrate incorporation at the nanomolar range. In addition to a high K_m for 3TC, it also has the lowest k_{cat} . This suggests that not only is it not incorporated well by pol- γ , but it is also very quickly excised.

The “DNA pol- γ hypothesis” (19) postulated that inhibition of pol- γ leads to the depletion of mtDNA and thereby causes mitochondrial dysfunction through defective electron transport. It underscores the pathophysiological importance of intracellular and intramitochondrial phosphorylation of NRTIs by cellular kinases to active moieties (Figure 1), inhibition of pol- γ by NRTI triphosphates and mtDNA depletion in tissue targets. The initial hypothesis was expanded to include additional pathophysiological contributions from mtDNA mutations and mitochondrial oxidative stress into the “mitochondrial dysfunction hypothesis” (20).

Various mechanisms have been proposed to explain acquired mitochondrial toxicity from NRTIs. These include direct inhibition of DNA pol- γ without incorporation; nascent chain termination after NRTI-TP incorporation into mtDNA; alteration of fidelity of DNA synthesis; persistence of incorporated analogs in mtDNA due to inefficient excision; or combinations thereof. Many of these proposed mechanisms are documented *in vitro* ((87-89)), but clinical proof is lacking or incomplete.

3. Models to Define Mechanisms of NRTI-Related CM

As mentioned, several mechanisms have been proposed to explain the toxicity related to NRTIs, particularly related to mitochondrial toxicity. The incidence of clinical CM associated with NRTI treatment was identified early (48,51). This NRTI-induced CM appears to be potentially reversible, after discontinuation of the toxic drug or substitution with a less toxic moiety. Genetic backgrounds, stage of HIV/AIDS progression, pre-existing heart conditions, co-infections, and treatment combinations offer complexities to human patient assessments rendering clinical determination of causative mechanisms of CM virtually impossible.

An AIDS transgenic (TG) murine model was created using a replication defective HIV-1 construct, hemizygous NL4-3 Δ gag/pol (90,91). Following 35 days of treatment with AZT, TG and WT mice both revealed dilation of the LV chamber, with increased dilation present in the AZT-treated TG than in the AZT-treated WT (45). Ultrastructural changes in cardiac myocytes were also observed.

Mitochondrial biogenesis is necessary to sustain cell and organ function, particularly in tissues that rely heavily on ATP-dependent energy, such as skeletal muscle or heart. It was hypothesized that disruption of cardiac mitochondrial biogenesis may directly impact cardiac function, leading to CM. Figure 3 outlines three key targets in the intramitochondrial

phosphorylation or active transport of NRTIs. These targets have been recently studied using *in vivo* TG mice to determine the specific role of each in NRTI activation or transport. Ultimately, these may play a critical role in disrupting nucleoside pool homeostasis and mtDNA replication. These genes were transgenically cardiac-targeted to the heart using the α -myosin heavy chain (α -MyHC) promoter. This results primarily in transcriptional activation in cardiomyocytes in the adult mouse.

The first experiments employed a cardiac targeted TG expressing the deoxynucleotide carrier (DNC). DNC, an inner mitochondrial membrane protein (92), has a predilection for import of dideoxynucleotides (93), like AZT. The testable hypothesis stated that intramitochondrial transport (via DNC) of phosphorylated NRTIs establishes critical NRTI mass leading to inhibition of mtDNA replication (94). DNC TG mice treated with AZT-HAART (35 days) demonstrated cardiac mitochondrial damage, altered cardiac function, and increased plasma lactate (95). A subsequent study tested the impact of a panel of NRTI monotherapy. AZT and d4T treatment each induced mitochondrial toxicity and CM whereas 3TC treatment had no impact (96).

A recent study challenges this hypothesis. It suggests that Slc25a19 (also called DNC) has a role in transport of thiamine pyrophosphate (97). In this study, knockout of Slc25a19 caused mitochondrial thiamine pyrophosphate depletion, embryonic lethality, CNS malformations, and anemia in mice without a reduction in mitochondrial dNTP or rNTP levels. Thus, the role of DNC in nucleotide or NRTI transport remains unclear, and remains worthy of additional exploration.

The relative toxicities of AZT, d4T, and 3TC correlate with the biological relationship between enzyme inhibition kinetics of DNA pol- γ *in vitro* (Table 1), and the observed changes of mitochondrial damage and mtDNA depletion *in vivo*. Results supported the hypothesis that intramitochondrial transport of NRTIs disrupts the native nucleotide homeostasis, and suggests how NRTIs can lead to disruption of mtDNA replication.

Another potential focus of NRTI mitochondrial toxicity is the cellular nucleoside kinases (both cytoplasmic and intra-mitochondrial) responsible for NRTI phosphorylation to active drugs. The critical first intramitochondrial phosphorylation of pyrimidines is performed by the mitochondrial isoform thymidine kinase 2 (TK2, a nuclear encoded, mitochondrially localized TK in mitotically quiescent cells) (98,99). Since mitochondrial toxicity was demonstrated with the pyrimidine AZT or d4T, TK2 was a logical target for further study. Cardiac targeted TGs overexpressing TK2 exhibited robust, targeted expression in the heart that resulted in increased mtDNA replication in the heart and contributed to functional defects following AZT-HAART treatment (35 days) (100). Results suggest that phosphorylation and dephosphorylation can have a direct role in NRTI toxicity, impacting mtDNA replication. It could be hypothesized that developmental or age-related changes (such as mutations) in these kinases responsible for regulating the phosphorylation and dephosphorylation events may disrupt the mitochondrial homeostasis. Studies using clinically identified mutations in these key kinases are currently under investigation. AZT directly inhibits TK2 activity in the isolated perfused rat heart model (101), suggesting inhibition of TK2 may have a larger roll in reduction of mtDNA by the direct depletion of TTP pools.

TG models of defective mtDNA replication that expressed exonucleolytic proofreading-deficient pol- γ yielded increased mtDNA mutations (102) and CM (103,104). Y955C, a point mutation in pol- γ , causes an authentic human mitochondrial illness called Chronic Progressive External Ophthalmoplegia (CPEO). Cardiac targeted TG overexpressing Y955C developed mtDNA depletion and mitochondrial damage as evidenced by decreased mtDNA abundance

and ultrastructural changes in addition to increased oxidative stress, LV dilation and CM (105).

While these are the main mechanisms of NRTI inhibition of mtDNA replication, other mitochondrial targets worth mentioning are beyond the scope of this review. In particular, the (cAMP)-dependent phosphoregulation of mitochondrial complex I has been shown to be inhibited by NRTIs such as AZT (106,107). NRTIs may have other effects unrelated to mtDNA as suggested by studies that showed stavudine overdosing triggers fat wasting, leptin insufficiency and liver damage by increasing liver triglycerides and plasma aminotransferases (108) as well as 80% depletion in adipocyte mtDNA (109) without impairment of respiratory chain complex.

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Activation/Phosphorylation

Reverse Transcriptase Inhibition

Figure 1. NRTI intracellular activation and mechanisms of HIV RT inhibition

a) HIV-RT >> DNA pol- γ > DNA pol- β > DNA pol- α = DNA pol- ϵ

b) ddC = ddi = FIAU = d4T >> 3TC > AZT > ABC

Figure 2. Relative Inhibitory effects of NRTIs on key viral and cellular polymerases

Figure 3. Focused targeted studies for potential mechanisms of NRTI-induced mitochondrial toxicity leading to CM

AZT is representative NRTI in model. Three targets are highlighted for potential roles in phosphorylation and/or transport of NRTIs in mitochondria.

Table 1

NRTI- TP	K_i (reported)
AZT	$K_i = 1.8 \mu M$; $K_i' = 6.8 \mu M$
d4T	$K_i = 1 \text{ nM}$; $K_i' = 8 \text{ nM}$
3TC	$K_i = 15.8 \mu M$