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Mitochondrial DNA Replication in Health and Disease

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1. Introduction

Mitochondria are dynamic, semi-autonomous organelles that play a diverse role in cellular physiopathology, being involved in bioenergetics, ROS generation/signaling and redox balance, β-oxidation of free fatty acids, Ca^{2+} homeostasis, thermogenesis, and essential anabolic pathways (fatty acids, cholesterol, urea, haem and bile acids). They contain their own, mitochondrial DNA (mtDNA) which is one of the main points in favor of the hypothesis of the endosymbiotic origin of these organelles (Lang et al., 1999). The human mitochondrial genome, a 16.5 kb circular DNA consisting of a heavy and a light chain, contains 37 genes, 13 of which encode proteins involved in the mitochondrial electron transport chain (ETC), 22 of which encode transfer RNA and the remaining 2 genes encode ribosomal RNA. A mammalian somatic cell contains between 1000 and 10000 copies of mtDNA arranged in covalently closed circular molecules. There are considerable physiological variations in the mtDNA content in any given human tissue, however the mechanism of these modulations and their clinical relevance are still not clear. Like bacterial chromosomal DNA, mtDNA is organized in DNA-protein structures called nucleoids. Several proteins seem to be involved in the maintenance of these structures. The most widely studied is Transcription Factor A (TFAM) which has a clear structural role and is necessary for nucleoid stabilization.

2. Replication of mtDNA

The replication of mtDNA is wholly dependent on the nucleus. The minimal mtDNA replication apparatus consists of DNA polymerase γ (Pol γ) and two replication factors: mitochondrial single-stranded DNA binding protein (SSB) and the Twinkle helicase. Pol γ is the only known DNA polymerase present in mammalian mitochondria (there are 16 DNA polymerases in the eukaryotic cell) and carries out both DNA replication and DNA repairing function (Bebenek & Kunkel, 2004; Sweasy et al., 2006). The presence of a specific mitochondrial DNA polymerase was suggested in the late 1960s with the discovery of a polymerase in mitochondrial fractions that exhibited distinct characteristics from known mammalian DNA polymerases (Kalf et al., 1968). Several years later, a novel human polymerase was identified in HeLa cells that could utilize DNA/RNA primer templates

(Fridlender et al., 1972) which was eventually identified as mitochondrial DNA polymerase (Bolden et al., 1977). The holoenzyme of Pol γ consists of a catalytic subunit encoded by *POLG* (located at the chromosomal locus 15q25) and a dimeric form of an accessory subunit p55 encoded by *POLG2* (located at the chromosomal locus 17q24.1), which all together form the Pol γ holoenzyme (Yakubovskaya et al., 2006). Pol γ is a 140kDa enzyme that possesses DNA polymerase but also additional intrinsic activities such as 3´-5´ proofreading exonuclease activity and 5´ deoxyribonucleic phosphate lyase activity, which are responsible for base excision repair (Graziewicz et al., 2006) (Fig.1). Initial pre-steady state kinetic analyses of Pol γ demonstrated that the catalytic subunit of this enzyme alone was somewhat inefficient, with relatively weak binding to DNA (39nM) and a slow maximum rate of polymerization ($3.5s^{-1}$). Processivity of the enzyme was estimated to be about 50-100 nucleotides (Graves et al., 1998; Longley et al., 1998). Thus, it became clear that the catalytic subunit was insufficient for successful DNA replication. An accessory subunit was purified and described as a 55kDa protein required for tight DNA binding and processing DNA synthesis (Lim et al., 1999). Kinetic analysis showed that the accessory and the catalytic subunit bind with a K_d of 35nM and that this association enhances enzyme processivity from several hundreds to thousands of nucleotides. This enhancement was not linked to a significant decrease in the dissociation rate of the holoenzyme from the primer/template (Johnson et al., 2000). However, the accessory subunit provides a 3.5-fold increase in DNA binding affinity and a 6-fold decrease in K_d for dATP incorporation. The accessory subunit has also been suggested to play a role in primer recognition (Fan et al., 1999) and its ability to bind nucleic acids, particularly dsDNA, has also been demonstrated, which is very uncommon for processing factors. This feature points to a function of the accessory unit not directly related with mtDNA synthesis; namely it has been suggested to have a role in maintenance of the mitochondrial genome, specifically by organization of mtDNA in nucleoids (Di Re et al., 2009). Pol γ has high base substitution fidelity due to high nucleotide selectivity and 3´-5´ exonucleolytic proofreading. It is particularly efficient in base incorporation in short repetitive sequences in which a missinsertion has been estimated to occur only once in every 500000 nucleotides (Longley et al., 2001). However, for copying homopolymeric sequences longer than 4 nucleotides, Pol γ has lower frameshift fidelity, which can lead to replication errors and frameshift mutations in mtDNA. Importantly, Pol γ contains an intrinsic 3´-5´ exonuclease activity that contributes to its replication fidelity.

The exonuclease activity is also efficient in repairing buried mismatches. Several additional factors have also been reported to contribute to mtDNA replication and/or repair, such as mitochondrial DNA-directed RNA polymerase (POLRMT), RNA-DNA hybrid-specific RNase, Topoisomerase I and IIIα, 5´-3´ Flap endonuclease, 5´-3´ exonuclease, uracil DNA glycosylase and 8-oxo-dG glycosylase, among others (Table 1)(Copeland, 2010).

Pol γ has three main roles related to disease.

- Synthesis and repair, the origin of most spontaneous mtDNA mutations are believed to be due to errors produced by Pol γ. Comparison of the mutation spectrum from *in vivo* sources with DNA copied *in vitro* by purified human Pol γ has revealed that over 85% of the *in vivo* mutations can be recapitulated *in vitro* (Zheng et al., 2006).
- Inherited mutations in *POLG*- more than 150 disease-associated mutations have been described in this gene (Copeland, 2010).
- Mitochondrial toxicity induced by NRTI drugs- Pol γ is the only DNA polymerase that is sensitive to the nucleoside analogues used for HIV treatment (Lim et al., 2003; Lewis et al., 2006).

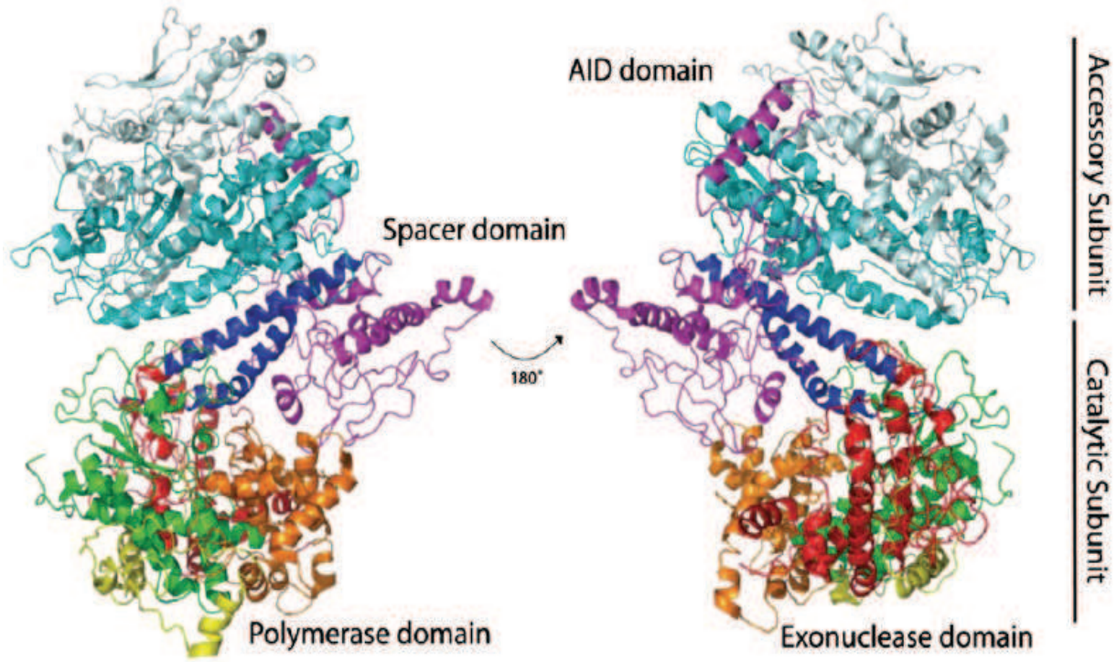


Fig. 1. Crystal structure of the Pol γ holoenzyme. Canonical right hand organization of the polymerase domain: fingers (orange), palm (green) and thumb (blue). Additional domains: mitochondrial localization sequence (yellow), exonuclease (red) and spacer (purple). Dimeric accessory subunit: proximal (cyan) and distal (light cyan) monomers (From Bailey & Anderson, 2010).

2.1 Mechanism of mtDNA replication and repair

Although mtDNA replication was identified as far back as 1972, it was only in the last decade that researchers began to understand its complex mechanism. Basically, two models have been proposed for replication of the mitochondrial genome: the strand-displacement theory and the strand-coupled theory. The strand-displacement theory suggests that replication is performed in one direction in a continuous manner without requiring the processing of Okazaki fragments on the displaced strand (Clayton, 1982). Copying of the mitochondrial genome begins at the origin of replication of the heavy strand DNA in the non-coding D-loop region of the mitochondrial genome, displacing the light chain until progressing two thirds of the way around the circular DNA. Synthesis of the light chain then begins after the formation of a stem-loop structure of the displaced heavy chain which forms the replication origin of the light strand DNA (Shadel et al., 1997). The strand-coupled model suggests that the synthesis occurs bidirectionally from multiple sites of initiation in a zone near the origin of the heavy chain replication (Holt et al., 2000; Bowmaker et al., 2003). Of note, there is a high prevalence of ribonucleotides in the lagging strand during mtDNA replication, which has more recently led to an alternative view of the strand-displacement theory termed RITOLS (RNA incorporated throughout the lagging strand) replication (Yasukawa et al., 2006; Holt, 2009). In this process, large patches of RNA protect the displaced strand during one-directional DNA synthesis. Short RNA templates are used as primers to complete replication of the lagging strand. This phenomenon may explain the lag between synthesis of the heavy and light chains of mtDNA.

Function	Gene	Protein	Size (kDa)	Chromosome locus
Core replication	POLG	DNA polymerase γ	140	15q25
	POLG2	DNA polymerase γ accessory subunit	55	17q23-24
	SSB	Single stranded DNA binding protein	15	7q34
	PEO1 (Twinkle)	Helicase	77	10q24
Replication and repair	DNA ligase III	Ligase	96	17q11.2-12
	RNase H1	RNA-DNA hybrid specific RNase	32	19p13.2
	Topo I	Topoisomerase I	67	8q24.3
	Topo IIIα	Topoisomerase IIIα	112	17p12-11.2
	Fen-1	5´-3´Flap endonuclease	43	11q12
	DNA2	5´-3´DNA/RNA endonuclease/exonuclease	130	10q21.3-q22.1
	ExoG	5´-3´exonuclease	41	3p21.3
DNA repair	UDG	Uracil DNA glycosylase	27.5	12q23-q24.1
	OGG1	8-oxo-dG glycosylase	38	3p26.2
	NTH1	Thymine glycol glycosylase	34	16p13.3
	MUTYH	glycosylase	60	1p34.3-p32.1
	NEIL1	Fapy glycosylase	44	15q4.2
	APE1	Ap endonuclease	35	14q11.2-q12
	APE2	Ap endonuclease	57	Xp11.22

Table 1. Gene products required for mtDNA replication and repair. Ap (apurinic, apyrimidinic); Fapy (2,6-diamino-4-hydroxy-formamido-pyrimidine) (Modified from Copeland, 2010).

mtDNA repair is limited to base excision repair (BER), for which the mitochondrion is equipped with several glycosylases that recognize base damage. Mitochondrial excision base repair can be performed via two pathways: single-nucleotide-BER (SN-BER) and long-patch BER (LP-BER) (Copeland & Longley, 2008). In both repair pathways, a damaged base is recognized and cleaved by a specific glycosylase, leaving an abasic site that is further cleaved on the 5´ end by AP nuclease to generate a nick with a 5´ deoxyribose phosphate (dRP) flap. During SN-BER, Pol γ fills the gap and cleaves the 5´dRP moiety prior to ligation. LP-BER seems to need the activity of additional proteins such as 5´-3´Flap endonuclease (FEN-1) (Liu et al., 2008) and 5´-3´DNA/RNA endonuclease/exonuclease (DNA2) (Zheng et al., 2008).

Current efforts are focused not only on elucidating the process of replication but particularly on identifying the factors involved in mtDNA repair and maintenance. This special interest is due to the observation that mtDNA depletion and/or mutation underlies a constantly growing list of human pathologies (Wanrooij & Falkenberg, 2010).

3. Inherited mitochondrial diseases which involve impaired DNA replication

Mitochondrial depletion syndrome (MDS) is a heterogeneous group of inherited disorders, characterized by a decreased amount of mtDNA in a specific tissue. The most severely affected organs include the brain, muscle and liver. This syndrome includes a wide spectrum of clinical disorders ranging from well-known diseases such as progressive external ophthalmoplegia (PEO) to rare tricarboxylic acid (TCA) cycle abnormalities. Typically, MDS are devastating and usually lethal diseases of infancy or early childhood and show autosomal recessive inheritance (Suomalainen & Isohanni, 2010). Since 1999, a dozen genes linked to MDS and related disorders have been described including mutations in the essential genes of mtDNA replication machinery: *POLG*, *POLG2* and *TWINKLE*. *POLG* is the most common of the genes that cause MDS and is believed to be the cause of 25% of described mitochondriopathies. Nearly 150 pathogenic mutations have been found in *POLG (*Copeland, 2010) which result in highly heterogenous disorders, such as PEO, Parkinson´s disease, Alpers syndrome, sensory ataxic neuropathy, mitochondrial neurogastrointestinal encephalomyopathy, dysarthria, Charcot-Marie-Tooth syndrome and ophthalmoparesis. In addition, mutations in several nuclear genes encoding enzymes involved in the mitochondrial nucleotide metabolism can cause depletions of mtDNA, resulting in mitochondrial syndromes. These include mitochondrial thymidine kinase (TK2), a pyrimidine nucleoside kinase essential to post-mitotic cells for phosphorylation of pyrimidine nucleosides, deoxyguanosine kinase (DGUOK), an enzyme necessary for mitochondrial purine nucleoside salvage pathways, adenine nucleotide translocator (ANT1), and mitochondrial deoxynucleotide carrier (DNC) (Suomalainen & Isohanni, 2010). Indeed, the fact that many genes involved in nucleotide salvage pathways and nucleotide transport are responsible for mitochondrial diseases suggests that imbalanced nucleotide pools are detrimental to mtDNA replication. The inherited mitochondrial diseases involving mtDNA replication are characterized by a long range of overlapping and progressing clinical symptoms, most commonly lactic acidosis, muscle weakness and myopathy which can lead to ataxia, polyneuropathy with epilepsy, cognitive delay and sensory impairment (ophthalmoplegia, deafness) as well as liver and gastrointestinal alterations (dysmotility) (Copleand, 2008; Copeland, 2010). According to the manifestations of the disease, MDS can be divided into three categories: myopathic, encephalomyopathic and hepatocerebral. To illustrate these effects, two inherited *POLG*-originated diseases are described. PEO, a mitochondrial disorder characterized by mtDNA depletions and/or accumulation of mutated mtDNA, has a late onset (between 18 and 40 years of age) and results in progressive weakening of the external eye muscles, leading to blepharoptosis and ophthalmoparesis (Copeland, 2008). PEO patients also manifest skeletal muscle weakness and wasting accompanied by exercise intolerance. This disease is also associated with specific neurologic syndromes such as familial forms of spastic paraplegia, spinocerebellar disorders, and sensorimotor peripheral neuropathy. The variants of this disorder involve both autosomal dominant (adPEO) and recessive (arPEO) forms, as both the nuclear and the mitochondrial genome are implicated in this pathogenesis. Importantly, several mutations in *POLG*, the first of which was described in 2001, are involved in the development of PEO. Alpers syndrome is another disease caused by mutations in *POLG* (Copeland, 2008). This is a rare but very severe and usually lethal autosomal recessive MDS disease that appears within the first few years of life. Patients exhibit progressive spastic quadri-paresis, progressive cerebral degeneration leading to mental deterioration, cortical blindness, deafness and liver failure.

4. mtDNA replication and NRTI

The most widely studied class of drugs that inhibit mtDNA replication, thus generating drug-related toxicities, are the nucleoside analog reverse transcriptase inhibitors (NRTI) (Fig.2). This was the first family of drugs approved by FDA for treatment of HIV infection (zidovudine, 1987). The combined antiretroviral approach currently employed in HIV therapeutics was introduced in 1996 and is known as Highly Active Antiretroviral Therapy (HAART) or Combination Antiretroviral Therapy (cART). It involves the use of one or two NRTI and one Non-Nucleoside Analogue Reverse Inhibitor (NNRTI) or one protease inhibitor (Zolopa, 2010). In this way, NRTI constitute the cornerstone of current HIV therapy. They are administered as prodrugs that must be transported into the cell and phopshorylated to the metabolically active triphosphate in order to exert their therapeutic effect. These drugs are pharmacological analogues of native nucleosides that can be

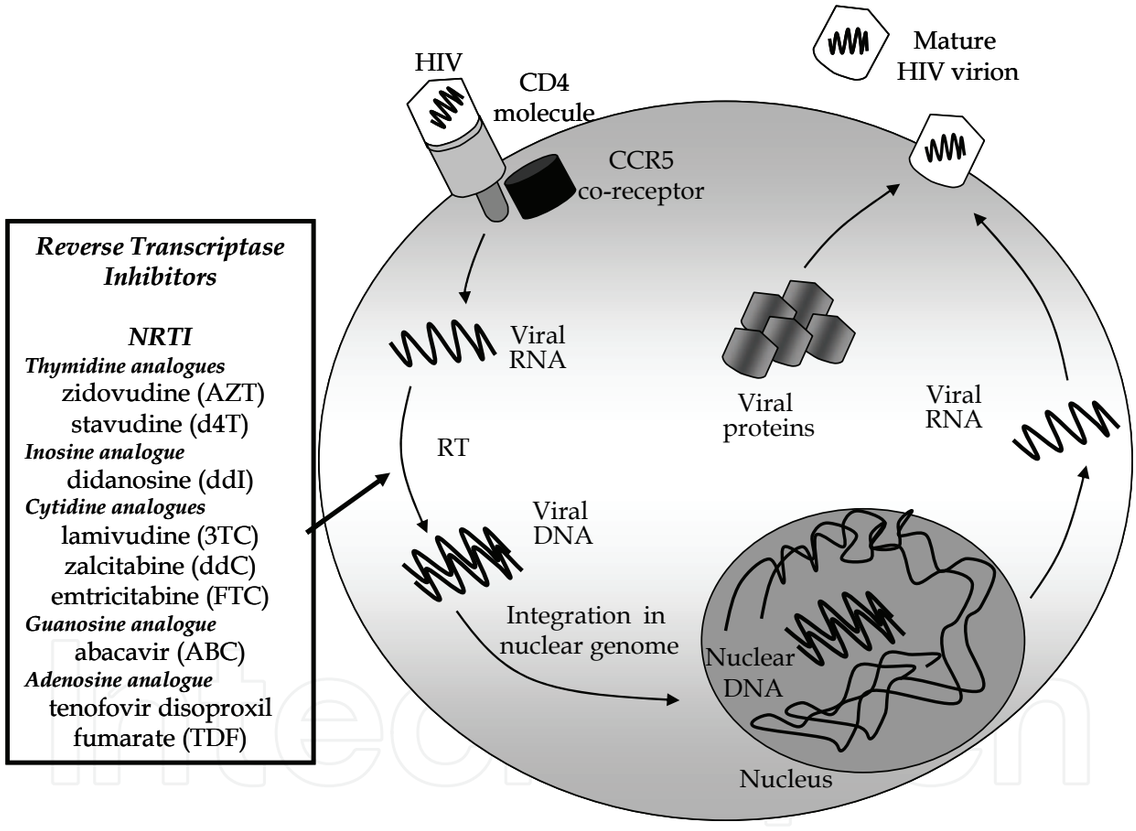


Fig. 2. The interference of NRTI drugs with the life cycle of HIV.
Reverse Transcriptase Inhibitors are a group of antiretroviral drugs which inhibit the viral reverse transcriptase, a crucial enzyme of the HIV life cycle. This enzyme reverse-transcribes the viral RNA genome into DNA, which is then integrated into the host genome and replicated along with it. This drug group comprises nucleoside analogues (NRTI) and non-nucleoside analogues (NNRTI). NRTI are administered as prodrugs and act as competitive inhibitors whereas NNRTI which do not require intracellular activation exert a non-competitive inhibitory action by acting at an allosteric, non-substrate binding site and thereby inducing a conformational change which impairs the enzyme's catalytic activity.

incorporated into proviral DNA during DNA replication by reverse transcriptase. Because they lack the 3´-OH group, their incorporation results in the termination of viral DNA replication. However, the triphosphate forms of the analogues have also been shown to be substrates for Pol γ and can also provoke termination of the DNA chain during mtDNA replication, an effect which can alter mitochondrial function. It has been postulated that NRTI inhibits Pol γ through several mechanisms or a combination of them: 1) termination of the mtDNA chain due to incorporation of NRTI in the growing strand without the 3´-OH group; 2) direct competitive inhibition of Pol γ without incorporation in the nascent DNA chain, as they compete with natural nucleotides for such incorporation; 3) alteration of Pol γ synthesis fidelity -induction of errors during mtDNA replication by inhibition of the exonucleolytic proof-reading function of Pol γ; 4) decrease in mtDNA reparatory exonuclease activity as NRTI resists exonucleolytic removal. Additional effects on mtDNA synthesis have also been suggested. Regardless of the mechanism by which mtDNA replication is compromised, it ultimately interferes with the synthesis of essential proteins of the mitochondrial ETC (Chiao et al., 2009).

The "Pol γ hypothesis" holds that NRTI treatment disrupts the OxPhos process thereby generating an energy defect and triggering subsequent alterations in the mitochondrial function such as increase in ROS production, reduced ATP synthesis, electron leakage, changes in the mitochondrial membrane potential and ROS generation, alterations which lead to further cellular damage (Fig.3),(Kohler & Lewis, 2007). Clinical experience with NRTI-including therapy has revealed the appearance of several side effects ranging from hyperlactatemia and lactic acidosis to lipodystrophy, myopathy, peripheral neuropathy, bone marrow suppression, insulin resistance and diabetes, as well as hepatosteatosis and pancreatitis, some of which develop into life-threatening condition (Kakuda, 2000). The first report of NRTI-induced mitochondrial effects, described in 1990, was myopathy in patients treated with zidovudine, who exhibited ragged red muscle fibers and reduced mtDNA content (Dalakas et al., 1990). Cardiomyopathy and bone marrow suppression were also described.

Kinetic *in vitro* studies have reported that dideoxynucleotides can be substrates for Pol γ nearly as efficiently as natural deoxynucleotides and thus the proposed hierarchy of mitochondrial toxicity for the approved NRTI is: zalcitabine >didanosine >stavudine >> lamivudine >tenofovir >zidovudine >abacavir (Lim & Copeland, 2001). Once incorporated into DNA, terminal NRTI can be removed by the intrinsic exonuclease activity of Pol γ, however this action is quite inefficient particularly in the case of dideoxynucleotides. For, example, zidovudine is unlikely to be incorporated into DNA by Pol γ, but once incorporated its removal is very inefficient which may explain the strong zidovudine-induced mtDNA depletion observed *in vitro* (Lim et al., 2003).

In contrast, removal of 3´- terminal lamivudine residues is 50% as efficient as natural 3´-termini. This, together with the lower degree of lamivudine incorporation in the mtDNA chain, predicts reduced toxicity for this analogue, a finding which is supported by *in vivo* observations. Pol γ discrimination against specific NRTI drugs, as illustrated in the examples, is considered the basis of the mitochondrial toxicity induced by these compounds and is a major rationale in the design of new antiretroviral nucleoside analogs.

Three aminoacid residues in human Pol γ (Tyr951, Tyr955 and Glu895) are thought to account for the selection of dNTPs and, therefore, NRTI (Lim et al., 2003). For example, a

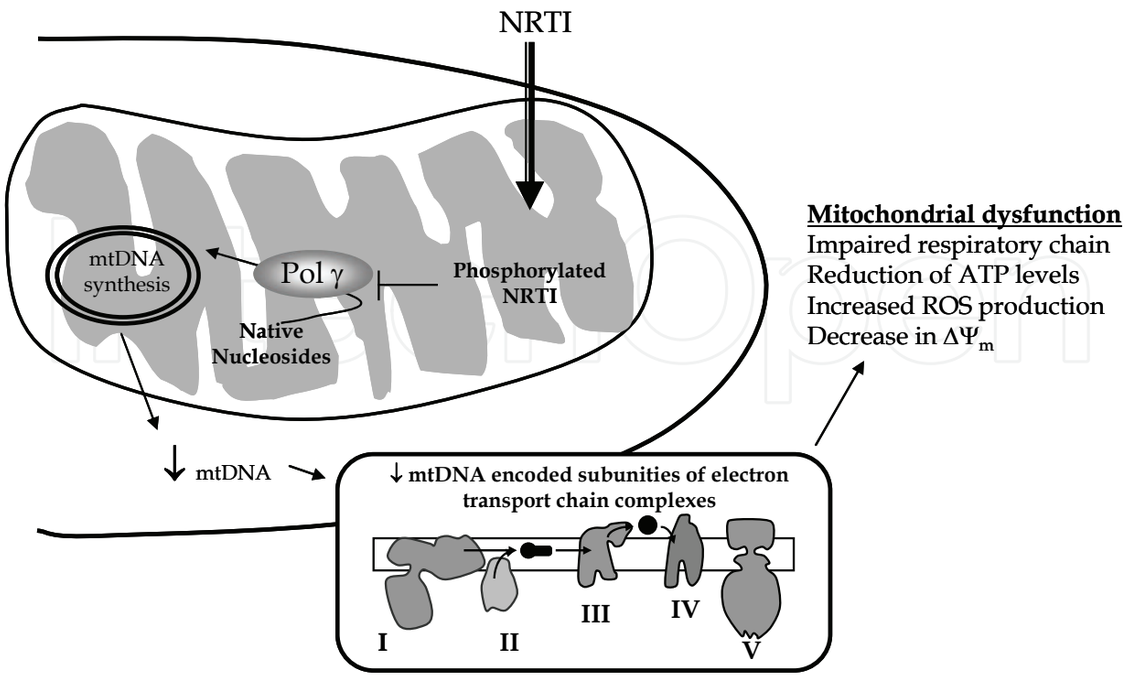


Fig. 3. The effect of NRTI drugs on Pol γ and its consequences for mitochondrial function. NRTI drugs are prodrugs which are phosphorylated intracellularly and the generated triphosphate form inhibits Pol γ in a competitive fashion. This undermines mtDNA synthesis with a consequent depletion of the mtDNA-encoded subunits of the mitochondrial electron transport chain. Such an effect leads to impairment of the mitochondrial function manifested as compromised oxidative phosphorylation, a reduction in mitochondrial membrane potential and induction of oxidative stress.

single tyrosine in motif B of human Pol γ, Tyr951, has been shown to cause dideoxynucleoside and stavudine sensitivity. Substitution of this Tyr residue with phenylalanine reduces the inhibition by dideoxynucleotides or stavudine by several thousand-fold with only minor effects on the overall function of Pol γ (Lim et al., 2003). It was hypothesized that the phenolic hydroxyl group of the tyrosine residue could substitute the missing 3´-OH of the bound ddNTP, thus allowing its efficient incorporation. Tyr955 and Glu895 seem to interact with the rigid sugar rings of stavudine and abacavir. Interestingly, discrimination against zidovudine does not seem to be related to any of these aminoacid residues at the active site of Pol γ. Moreover HIV-1 reverse transcriptase mutants derived from zidovudine-resitant viruses harbor changes in aminoacid residues outside the active site and the drug resistance conferred by these mutations could be due to subtle structural changes in Pol γ (Lim et al., 2003).

Additional effects of NRTI on mtDNA synthesis have also been suggested. Therefore, given that conversion of the monophosphate to the triphosphate form of NRTI inside the mitochondria is rather inefficient, it is possible that the monophosphorylated forms can accumulate within the mitochondrial matrix reaching extremely high (mM) levels which could have unspecific inhibitory effects on mtDNA synthesis, such as decreased mtDNA replication fidelity induced by the inhibition of the exonuclease function of Pol-γ, and inhibition of mtDNA replication, mediated by the reduction of thymidine phosphorylation,

a necessary substrate for DNA synthesis (Walker et al., 2003). *In vivo* millimolar accumulation has been shown for the phosphorylated form of zidovudine (Frick et al., 1988). Moreover, interactions with host proteins during the process of activation of NRTI inside the cell, allow additional mechanisms for mitochondrial toxicity of these drugs.

4.1 Pol-γ independent mitochondrial action of NRTI

HAART has dramatically reduced AIDS-related morbidity and mortality and has converted HIV-infection into chronic rather than a mortal disease (in the pre-HAART era a HIV-infected individual was expected to survive only 7 years). However, the adverse reactions associated with the long-term use of this therapy (rash and hypersensibility reactions, hepatotoxicity, metabolic disturbances including lipodystrophy, hyperlactatemia, and CNS toxic effects) have become a major concern. As a result, research efforts are now focused on understanding the cellular mechanisms underlying these effects. Most of NRTI-induced side effects have been attributed to their mitotoxic potential which has mainly been believed to originate from the inhibitory action of these drugs on Pol γ. However, other mitochondrial mechanisms and targets responsible for NRTI-induced mitotoxicity have also been suggested. There is evidence of NRTI-induced mitochondrial dysfunction unrelated to mtDNA depletion. Zidovudine has been shown to inhibit thymidine phopshorylation, ADP/ATP translocase and adenylate kinase, to provoke a decrease in cytochrome *c* oxidase (Complex IV) expression, and to enhance oxidative stress (Maagaard & Kvale, 2009). *In vivo* studies have demonstrated that treatment with this drug leads to a disrupted cardiac mitochondrial ultrastructure and a diminished expression of mitochondrial cytochrome b mRNA, as well as induction of oxidative stress in heart mtDNA (Sardao et al., 2008). In addition, in cultured rat hepatocytes, stavudine, but not zidovudine or zalcitabine, impairs fatty acid oxidation in the absence of mtDNA depletion (Igoudjil et al., 2008). Moreover, mitochondrial import of nucleoside drugs may also be related to their toxicity in this organelle. Some of the nucleoside channels have been shown to transport stavudine, zalcitabine, zidovudine and didanosine (Yamamoto et al., 2007; Baldwin et al., 2005) and nucleoside drugs are also the subject of several other transporters, including organic cation and anion transporters and multi-drug-resistant proteins with potential implication in toxicity (Leung & Tse, 2007). Recently, mitochondrial bioenergetics has been directly linked to NRTI-induced mitotoxicity, independently of mtDNA replication. *In vitro* exposure to zidovudine has revealed a direct interaction with cellular bioenergetics by impairing mitochondrial respiration through inhibition of Complex I of the ETC (Lund & Wallace, 2008).

Hepatotoxicity has emerged as one of the most common adverse events associated with HAART and constitutes a major problem in the management of HIV-patients. In certain clinical trials, up to 10% of patients receiving cART exhibited a severely increased liver enzymes level which is a major cause of therapy discontinuation (Jain, 2007). The implication of mitochondria in these events and particularly the drug-induced mitochondrial effects that occur independently of Pol γ is still not clear. We employed a human hepatoma cell line, Hep3B (ATCC HB-8064) to assess the potential direct and Pol γ–independent involvement of mitochondria in hepatic side effects. Preliminary studies were performed in which cells were treated with NRTI (Sequoia Research Products) at therapeutic concentrations during a short period of time in order to avoid any effects due to a decrease in mtDNA content. Subsequently, several parameters of mitochondrial function

including mitochondrial respiration, generation of ATP and mitochondrial ROS production were determined. Electrochemical measurement of oxygen (O_2) consumption was performed using a Clark-type O_2 electrode (Rank Brothers, Bottisham, UK). Cells (3-5x10^6) were placed in a gas-tight chamber containing 1mL respiration buffer (Hank's balanced salt solution, HBSS) and agitated at 37ºC. Measurements were recorded using the Duo.18 data acquisition device (WPI, Stevenage, UK), immediately after addition of the drugs. The adenosine triphosphate (ATP) concentration (nmol/mg protein) was determined using Bioluminescence Assay Kit HSII (Roche, Mannheim, Germany) and a Fluoroskan microplate reader (Thermo Labsystems, Thermo Scientific, Rockford, IL). For these measurements, cells were incubated for 1h with the NRTI under study. Protein concentrations were determined with the BCA protein assay kit. ROS production was analyzed in cells seeded in a black 96-well plate. The fluorescent probe DCFH-DA (2´,7´-dichlorodihydrofluorescein diacetate, 2.5 μM) was added for 30 minutes, cells were washed with HBSS before addition of the NRTI drug and fluorescence was detected at 5-minute intervals over a 1h period using a Fluoroskan. Rotenone (100 μM) or exogenous hydrogen peroxide (H_2O_2, 100 μM) were used as a positive control. We observed that abacavir but not lamivudine significantly reduced mitochondrial respiration and ATP production (Fig.4). However no significant changes were detected regarding ROS production with either of the drugs (results not shown) (Blas-Garcia, 2010). Other preliminary studies conducted in our laboratory have revealed that clinically relevant concentrations of another NRTI, didanosine, also lead to alterations in the mitochondrial function of Hep3B cells, detected as decreased O_2 consumption and ATP generation, but in the absence of an increase in ROS production (unpublished data).

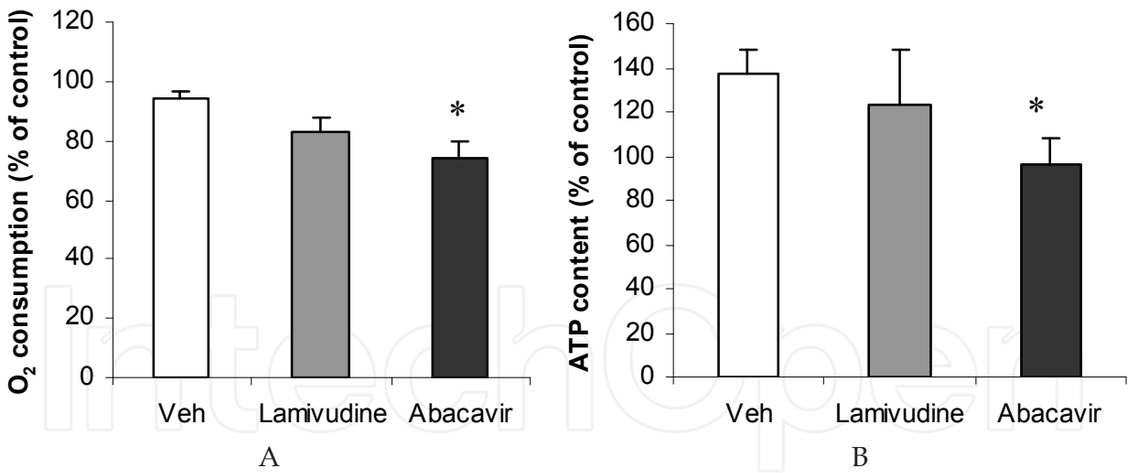


Fig. 4. Acute and Pol γ-independent effect of NRTI drugs (lamivudine and abacavir) on mitochondrial function in Hep3B cells. A) Rate of mitochondrial O_2 consumption determined in a Clark-type O_2 electrode. 10μM of lamivudine or abacavir were added to the chamber immediately after addition of the cell suspension. B) Intracellular ATP concentration studied with a bioluminescence assay in cells treated with 10μM of lamivudine or abacavir for 1h. Data are mean±SEM of 5-6 experiments and are shown as % of the control value (i.e. the value obtained in untreated cells, considered to be 100%). Statistical analysis was performed using the Student´s t-test, *$p<0.05$, vs Vehicle (Veh)-treatment.

These preliminary data lead to several important observations: i) some NRTI, at their clinically employed concentrations, have the potential to directly inhibit the mitochondrial oxidative phosphorylation process, and this occurs in a drug-specific manner; ii) further studies need to be carried out with the aim of analyzing whether these effects are transient or accumulate over time, particularly regarding ROS accumulation which does not seem to be acutely affected. However prolonged and/or more severe impairment of mitochondrial respiration can lead to a progressive increase in ROS generation and the subsequent appearance of oxidative stress; iii) the pathophysiological relevance of the mitochondrial effects elicited by NRTI is unclear and awaits the findings of more detailed studies.

4.2 Factors influencing NRTI-induced mitotoxicity

Several general factors directly influence NRTI-induced mitochondrial toxicities, both related and un-related to mtDNA depletion: i) the subcellular abundance of NRTI, as there is a concentration threshold beyond which these compounds compete with natural moieties; ii) the ability of cellular nucleoside kinases to create nucleoside triphosphate, which is responsible for mtDNA toxicity, and the interaction of nucleoside analogues with the resident proteins during the process of their activation; iii) the existence of a functional threshold as most cells contain a substantial number of mitochondria and therefore, manifestations of cellular injury appear only when a substantial number of malfunctional mitochondria is reached; and iv) the "mtDNA threshold effect" in relation with tissue specificity of OxPhos dependence. The majority of cells have a surplus of mtDNA copies and can withstand significant mtDNA depletion before mitochondrial dysfunction occurs (60-80% of basal levels). In the case of mutations, the threshold varies from 60% for large scale mtDNA deletions to 90% in tRNA point mutations. Nevertheless, the relationship between mtDNA content and NRTI-induced adverse events is unclear. Until recently, quantification of mtDNA in peripheral blood mononuclear cells (PBMC) was employed as a marker of mitochondrial toxicity in HIV patients. However, the accuracy of this measurement regarding toxicity is controversial since several studies have failed to report a decrease in the mtDNA content of PBMC or fat tissue in patients experiencing adverse events such as lipoatrophy (Maagaard & Kvale, 2009). Finally, v) recent advances in pharmacogenomics suggest a link between specific genetic polymorphisms and NRTI toxicity; for instance, R964C (Yamanaka et al., 2007) and E1143G polymorphisms (Chiappini et al., 2009) have been associated with an increased stavudine-induced mitotoxicity whereas mitochondrial haplogroup T has been related to increased peripheral neuropathy in treatment with stavudine and didanosine (Hulgan et al., 2005).

5. Conclusion

Mitochondria contain their own DNA which encodes 13 proteins which are involved in the mitochondrial ETC. The replication of mtDNA is performed by Pol γ, the only mitochondrial DNA polymerase, which consists of a catalytic subunit and a dimeric form of an accessory subunit p55, and operates in conjunction with two replication factors, SSB and Twinkle. A decreased amount of mtDNA, often due to mutations in *POLG*, is a hallmark of mitochondrial depletion syndrome, a heterogeneous group of several severe and usually deadly inherited disorders. Mitochondrial DNA depletion and, consequently, mitochondrial dysfunction are also considered to be the basis of the side effects induced by a class of drugs known as nucleoside analogue reverse transcriptase inhibitors. These drugs are the

cornerstone of the current therapeutic approach employed for treatment of HIV infection. It is believed that the adverse events related to NRTI-containing treatments are mainly due to the mitochondrial toxicity that arises as a result of the inhibitory effect of these drugs on Pol γ. However, Pol γ-independent mitochondrial targets and mechanisms of NRTI-induced toxicity have also been suggested. Using a human hepatic cell line, our group has recently provided *in vitro* evidence of a direct inhibitory effect on mitochondrial respiration and ATP production induced by an acute exposure to certain NRTI such as abacavir and didanosine. No such changes were observed with lamivudine, thus indicating a drug- rather than a class-specific effect. A detailed analysis of these effects is paramount to a better understanding of NRTI-related adverse events. This is of particular clinical relevance given the existence of NRTI that do not exhibit a strong Pol γ -inhibitory action.

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