

## Pharmacology of Nucleoside and Nucleotide Reverse Transcriptase Inhibitor–Induced Mitochondrial Toxicity

**Thomas N. Kakuda, PharmD**

*Antiviral Pharmacology Laboratory, Department of Experimental and Clinical Pharmacology, College of Pharmacy, University of Minnesota, Minneapolis, Minnesota*

### ABSTRACT

**Objective:** This paper reviews the function of the mitochondria and the mechanisms by which nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs) cause mitochondrial toxicity.

**Background:** Highly active antiretroviral therapy (HAART) reduces rates of morbidity and mortality due to HIV disease. However, long-term treatment with these drugs may be associated with adverse effects. Nucleoside and nucleotide analogues are potent inhibitors of HIV reverse transcriptase and have become the cornerstone of HAART. Unfortunately, these drugs have also been shown to inhibit cellular polymerases, most notably mitochondrial DNA polymerase  $\gamma$ .

**Results:** Studies of the NRTIs in enzyme assays and cell cultures demonstrate the following hierarchy of mitochondrial DNA polymerase  $\gamma$  inhibition: zalcitabine  $\geq$  didanosine  $\geq$  stavudine  $>$  lamivudine  $>$  zidovudine  $>$  abacavir. In vitro investigations have also documented impairment of the mitochondrial enzymes adenylate kinase and the adenosine diphosphate/adenosine triphosphate translocator. Inhibition of DNA polymerase  $\gamma$  and other mitochondrial enzymes can gradually lead to mitochondrial dysfunction and cellular toxicity. The clinical manifestations of NRTI-induced mitochondrial toxicity resemble those of inherited mitochondrial diseases (ie, hepatic steatosis, lactic acidosis, myopathy, nephrotoxicity, peripheral neuropathy, and pancreatitis). Fat redistribution syndrome, or HIV-associated lipodystrophy, is another side effect attributed in part to NRTI therapy. The morphologic and metabolic complications of this syndrome are similar to those of the mitochondrial disorder known as multiple symmetric lipomatosis, suggesting that this too may be related to mitochondrial toxicity. The pathophysiology of less common adverse effects of nucleoside analogue therapy, such as diabetes, ototoxicity, and retinal lesions, may be related to mitochondrial dysfunction but have not been adequately studied.

**Conclusion:** NRTIs can block both HIV reverse transcriptase and mitochondrial DNA polymerase  $\gamma$ . Inhibition of the latter enzyme is the most likely cause of the adverse effects associated with these drugs.

**Key words:** adverse effects, anti-HIV drugs, DNA polymerase, HIV, mitochondria, nucleoside and nucleotide reverse transcriptase inhibitors. (*Clin Ther.* 2000;22:685–708)

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## INTRODUCTION

Current guidelines for antiretroviral therapy recommend the use of 2 nucleoside reverse transcriptase inhibitors with a protease inhibitor or a nonnucleoside reverse transcriptase inhibitor. Alternative regimens include 3 nucleoside reverse transcriptase inhibitors.<sup>1,2</sup> The pivotal role played by nucleoside reverse transcriptase inhibitors in therapy is based on the results of randomized clinical trials demonstrating virologic and immunologic improvement in HIV-infected persons receiving 2 compared with 1 of these agents.<sup>3-9</sup> Studies have also shown efficacy with the use of 3 nucleoside reverse transcriptase inhibitors,<sup>10,11</sup> although these regimens may not be potent in persons with a viral load of >100,000 copies/mL.<sup>1,2</sup> Use of these agents as part of a regimen of highly active antiretroviral therapy (HAART) has reduced morbidity and mortality due to HIV disease as well as the incidence of opportunistic infections.<sup>12</sup> Despite these advances in HIV pharmacotherapy, it has become evident that antiretroviral therapy alone is insufficient to eradicate the virus; continuous HIV suppression is likely to require long-term treatment.<sup>13</sup>

Adverse effects associated with HAART are likely to pose a significant obstacle to long-term pharmacotherapy, particularly with nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs), since they are the foundation on which current therapy is based.<sup>14</sup> Recognized adverse effects of NRTIs include anemia, cardiomyopathy, gastrointestinal distress, drug-induced hypersensitivity, myopathy, nephrotoxicity, pancreatitis, ototoxicity, peripheral neuropathy, retinal lesions, and hepatic steatosis with or without lactic acidosis. Lactic acidosis and encephalopathy have also been

reported in children exposed to NRTIs for the prevention of vertical transmission of HIV.<sup>15,16</sup> Furthermore, NRTIs have recently been implicated in the etiology of fat redistribution syndrome, commonly referred to as HIV-associated lipodystrophy.<sup>17-19</sup>

Despite these seemingly different toxicities, a common pathway may be involved—NRTI-induced, tissue-specific mitochondrial toxicity (Table 1).<sup>14,20,21</sup> This hypothesis is based on *in vitro* and *in vivo* studies reviewed herein demonstrating impaired mitochondrial function with exposure to NRTIs. Moreover, the clinical manifestations of NRTI toxicity resemble the phenotypic expression of known mitochondrial diseases.<sup>14,20,21</sup> For example, the fat redistribution syndrome is attributed in part to NRTI toxicity. Although the etiology of this syndrome is unknown, its morphologic and metabolic complications are similar to the mitochondrial disease multiple symmetric lipomatosis (Madelung's disease). It is speculated that NRTI-induced mitochondrial toxicity may be a factor in the development of fat redistribution syndrome.<sup>22,23</sup> If NRTIs are to be used in the treatment of HIV, a better understanding of their toxicology is needed.

The purpose of this paper is to review the function of the mitochondria and the mechanisms by which NRTIs cause mitochondrial toxicity.

## FUNCTION OF THE MITOCHONDRIA

Mitochondria are organelles existing in all cells except red blood cells. Structurally, they have a double membrane, circular DNA molecules, and mitochondrion-specific transcription, translation, and protein-assembly systems. These characteristics suggest that mitochondria

Table I. Adverse effects of nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs).

Drug	Adverse Effect	Evidence*	Comments
All NRTIs	Hepatic steatosis	+++	Reported in children receiving NRTIs for the prevention of vertical transmission <sup>16</sup> Possibly related to mitochondrial toxicity <sup>22,23</sup>
	Lactic acidosis	+++	
	Encephalopathy	+	
	Lipodystrophy	±	
Abacavir sulfate	Hypersensitivity	–	
Adefovir dipivoxil <sup>†</sup>	Nephrotoxicity	±	Dipivoxil ester depletes carnitine <sup>24</sup>
Didanosine	Diabetes	±	
	Pancreatitis	+	
	Peripheral neuropathy	++	
Lamivudine	Pancreatitis	±	Reported in children
Lodenosine <sup>†</sup>	Hepatotoxicity	±	Clinical trials halted <sup>25</sup>
Stavudine	Pancreatitis	±	
	Peripheral neuropathy	++	
Zalcitabine	Ototoxicity	±	
	Peripheral neuropathy	++	
Zidovudine	Anemia	+	
	Myopathy	+++	

Adapted from Brinkman and Kakuda.<sup>14</sup>

\*Strength of evidence is indicated as follows: – = not likely to be related to mitochondrial toxicity; ± = possibly related to mitochondrial toxicity but not well studied; + = supported by limited data; ++ = supported by in vitro data and some in vivo data; +++ = supported by in vitro and in vivo data.

<sup>†</sup>Not approved by the US Food and Drug Administration.

may have been independent organisms at one time. Their existence in cells is likely to be the result of a symbiotic relationship between a primitive oxidative bacterium (eventually giving rise to the mitochondrion) and a prototypic eukaryotic cell originating >1 billion years ago.

The primary role of the mitochondrion is to provide energy to the cell in the form of adenosine triphosphate (ATP). The number of mitochondria residing in a cell depends on the cellular need for

energy; for example, a single hepatocyte, myocyte, or adipocyte can contain hundreds to thousands of mitochondria, whereas a platelet may have only a few. Some cells, such as adipocytes, are differentiated histologically by the number of mitochondria they contain (eg, brown fat cells have more mitochondria than white fat cells).

The observation that mitochondria regulate programmed cell death (apoptosis) further supports the existence of a symbi-

otic relationship between the mitochondrion and the cell.<sup>26</sup> Other important biochemical functions of the mitochondrion include synthesis of heme and bile acid, ammonia detoxification, cholesterol metabolism, ethanol metabolism, estrogen and testosterone synthesis, and cellular calcium homeostasis.

Mitochondria efficiently generate energy using intracellular fatty acids and glucose as fuel sources (Figure 1). Long-chain fatty acids enter the mitochondrion via the carnitine-palmitoyl transferase shuttle; medium- and short-chain fatty acids do not depend on this transport system and freely penetrate the mitochondrion. Once

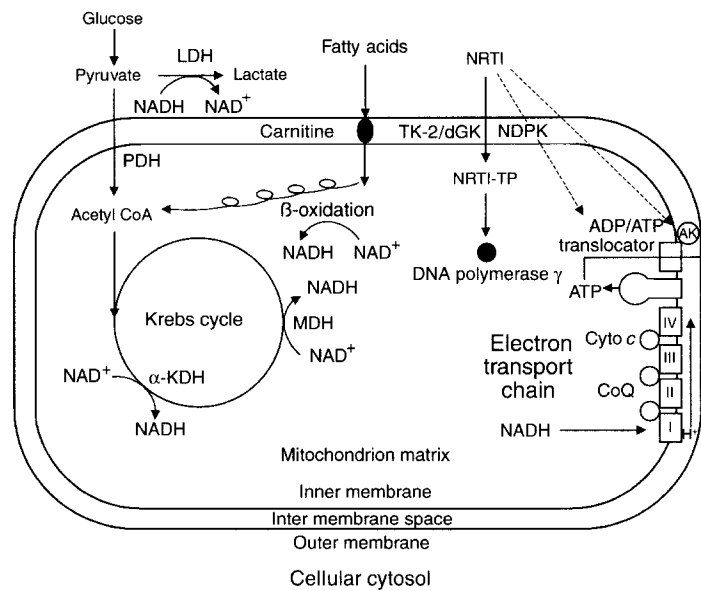


Figure 1. Mitochondrial structure. Glucose and fatty acids are the raw material for energy production. Glucose is converted by glycolysis to pyruvate, which then enters the mitochondrion. Long-chain fatty acids enter the mitochondrion with the assistance of carnitine. Reduced nicotinamide adenine dinucleotide (NADH) is used by the electron transport chain (consisting of complexes I–IV) to produce adenosine triphosphate (ATP). Activated nucleoside and nucleotide reverse transcriptase inhibitors (NRTI-TP) can inhibit DNA polymerase  $\gamma$ , the enzyme responsible for mitochondrial DNA replication. Studies also suggest that NRTIs may impair adenylate kinase (AK) and the adenosine diphosphate (ADP)/ATP translocator. Loss of mitochondrial function leads to energy loss, intracellular lipid accumulation, and production of lactate from anaerobic respiration. Acetyl CoA = acetyl coenzyme A;  $\alpha$ -KDH = alpha-ketoglutarate dehydrogenase; CoQ = coenzyme Q; cyto *c* = cytochrome *c*; dGK = deoxyguanosine kinase; LDH = lactate dehydrogenase; MDH = malate dehydrogenase; NAD<sup>+</sup> = nicotinamide adenine dinucleotide; NDPK = nucleoside diphosphate kinase; PDH = pyruvate dehydrogenase; TK-2 = thymidine kinase–2.

inside the mitochondrion, fatty acids undergo beta-oxidation, a repeating cycle of 4 reactions: flavin adenine dinucleotide (FAD)-linked dehydrogenation and hydration, nicotinamide adenine dinucleotide (NAD<sup>+</sup>)-linked dehydrogenation, and thiolysis. Each repetition of the cycle shortens the fatty acid chain by 2 carbon atoms and generates acetyl coenzyme A (acetyl CoA) along with reduced NAD<sup>+</sup> (NADH) and reduced FAD.

Glucose enters the mitochondrion in the form of pyruvate, which subsequently is converted to acetyl CoA by pyruvate dehydrogenase. Acetyl CoA from glucose and fatty acid metabolism then feeds into the Krebs oxaloacetate (tricarboxylic acid) cycle—the major product of the Krebs cycle is NADH. NADH generated from beta-oxidation and the Krebs cycle provides the necessary substrate for oxidative phosphorylation (OXPHOS). The synthesis of ATP in the mitochondria by OXPHOS involves 4 energy-transducing enzyme complexes, all representing inner-mitochondrial membrane-bound proton pumps. The electron transport chain (ETC) consists of complex I (NADH-ubiquinone-oxidoreductase, or NADH dehydrogenase), complex II (succinate-ubiquinone reductase, or succinate dehydrogenase), complex III (cytochrome bc<sub>1</sub>), and complex IV (cytochrome oxidase). Ubiquinone (coenzyme Q), which accepts electrons and protons as it is reduced to ubiquinol, shuttles from complexes I and II to complex III. Similarly, cytochrome *c* shuttles electrons from complex III to complex IV. Together, these coordinated series of proteins pass along electrons derived from NADH, forming a proton gradient ( $\Delta\mu_{\text{H}^+}$ ) within the inner mitochondrial membrane. A fifth enzyme, complex V (F<sub>1</sub>F<sub>0</sub> ATPase, or ATP synthase) uses the proton gradient to generate ATP

from adenosine diphosphate (ADP) and inorganic phosphate. ATP is then released into the cytosol by ADP/ATP translocase in exchange for ADP in the cytoplasm.<sup>27</sup>

Mitochondria are almost exclusively inherited from the maternal oocytes. Each mitochondrion contains 2 to 10 copies of mitochondrial DNA (mtDNA) plus the necessary proteins for nucleic acid synthesis, phosphorylation, transcription, and translation. Human wild-type mtDNA is a double-stranded, covalently closed, circular molecule consisting of 16,569 base pairs; it is located within the mitochondrial matrix. Mitochondrial DNA accounts for <2% of total cellular DNA and is the only extrachromosomal DNA found in a cell. Unlike nuclear DNA (nDNA), mtDNA contains few noncoding sequences such as introns, is not protected by histones, and does not recombine. Replication of mtDNA is semi-independent from nDNA replication—the enzyme responsible for mtDNA replication is DNA polymerase  $\gamma$ , an nDNA-encoded enzyme. DNA polymerase  $\gamma$  is highly error prone compared with nuclear polymerases and can introduce mutations in mtDNA at a rate 10 times greater than that with nuclear polymerases. Because of the multigenomic nature of mitochondria, a unique situation may arise in which wild-type and mutant mtDNA coexist, a condition known as heteroplasmy.<sup>28</sup>

The mtDNA genome encodes 13 proteins, 22 transfer RNAs and 2 ribosomal RNAs (12S and 16S ribosomes). Interestingly, the genetic code used by the mitochondria is slightly different from that used by nDNA. For example, the codon UGA encodes tryptophan in the mitochondria but is a termination codon in the nucleus. The 13 proteins encoded by mtDNA are essential subunits of the ETC

and ATP synthase. The remaining 65 proteins necessary to create the full complement of the ETC are encoded by nDNA and transported into the mitochondria. Thus, a functional mitochondrion requires both intact nDNA and mtDNA. Defects in either component can lead to impaired mitochondria and mitochondrial disease.

Cells with defective mitochondria typically have intracellular lipid droplets due to the unused fatty acids. Lactic acidosis (with or without an elevated anion gap and altered bicarbonate) may also occur as aerobic respiration (OXPHOS) shifts to anaerobic respiration (glycolysis). In general, disease develops when a threshold of >85% loss of mitochondrial function is reached; this threshold effect, however, is likely to be tissue-specific, with energy-dependent cells more likely to be affected first.<sup>29-31</sup> Mitochondrial diseases are typically genetic in origin but can be chemically induced—NRTIs are among the known mitochondrial toxins.

### PHARMACOLOGY OF NUCLEOSIDE AND NUCLEOTIDE REVERSE TRANSCRIPTASE INHIBITORS

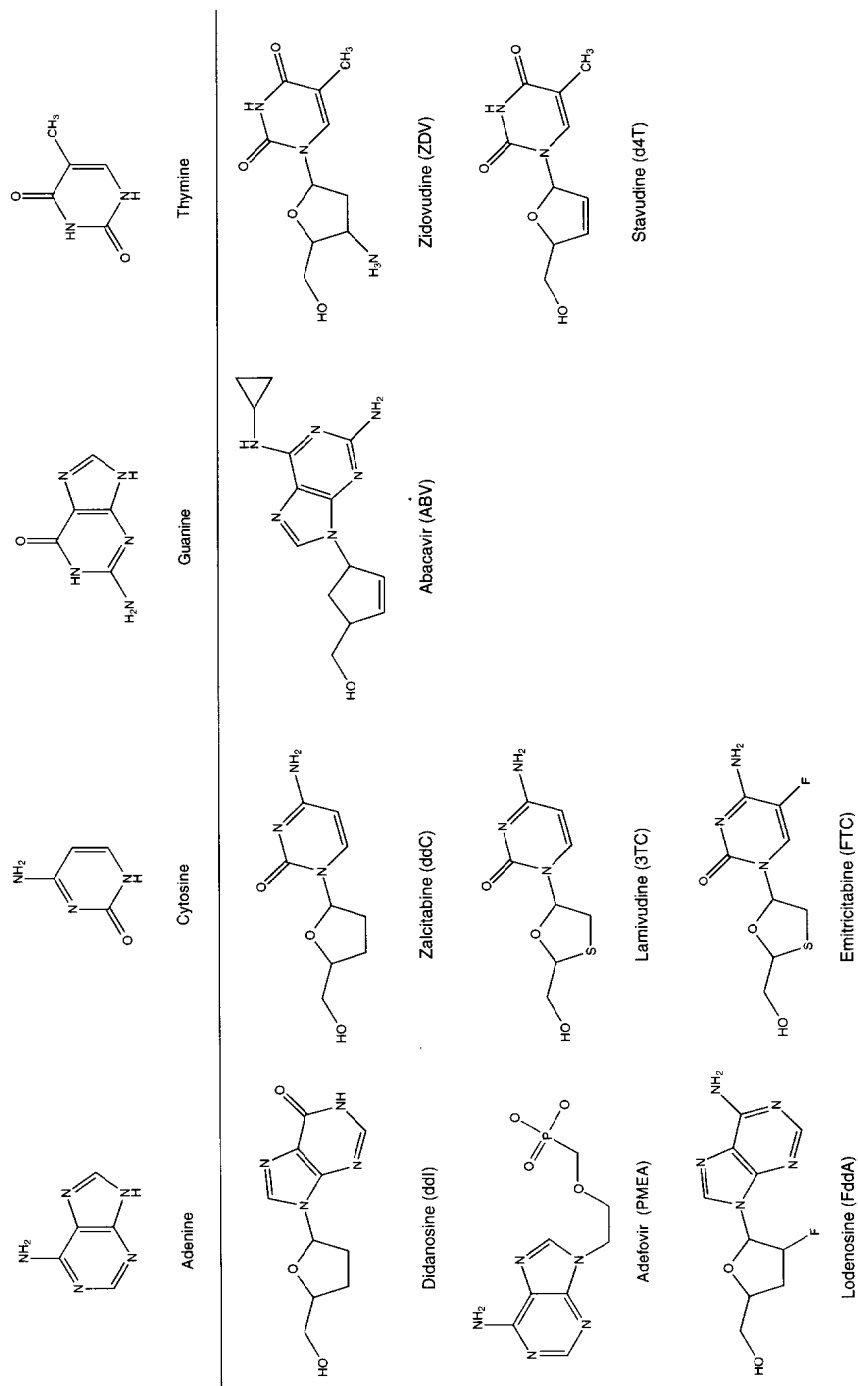
NRTIs are derivatives of adenosine, cytidine, guanosine, and thymidine (Figure 2). As such, they provide an alternative substrate for DNA polymerases. The important chemical modification of these compounds is the 3'-OH of the deoxyribose sugar, which normally forms the 5- to 3-phosphoester bond with the next nucleic acid. The lack of an available 3'-OH, or in the case of zidovudine, modification to an azido group (N<sub>3</sub>), prevents the attachment of subsequent nucleic acids by DNA polymerase. Thus, NRTIs can

impair transcription in 2 ways: (1) by competing with endogenous nucleic acids for incorporation; and (2) by prematurely terminating chain elongation once incorporated. HIV reverse transcriptase (HIV-RT), an RNA-dependent DNA polymerase, is particularly sensitive to the effects of NRTIs.<sup>32</sup>

### Phosphorylation

Like nucleic acids, NRTIs require a series of phosphorylation steps (Figure 3) before they can be used—the final form used by DNA polymerase is the triphosphorylated (TP) moiety. This stepwise activation process occurs inside the cell and is mediated by a coordinated series of enzymes. Zidovudine and stavudine, for example, are initially phosphorylated by thymidine kinase to their monophosphorylated (MP) derivatives zidovudine-MP and stavudine-MP, respectively; the corresponding diphosphate (DP) of zidovudine or stavudine is formed by thymidylate kinase.

The first step in emtricitabine, lamivudine, and zalcitabine phosphorylation is catalyzed by cytosolic deoxycytidine kinase. The second step is catalyzed by cytidine-MP kinase to form the corresponding DP.<sup>32</sup> Cytidylyl transferase can add choline or ethanolamine to zalcitabine-DP, forming the liponucleotide adducts zalcitabine-DP choline and zalcitabine-DP ethanolamine, respectively.<sup>33</sup> These metabolites appear to be unique to zalcitabine. Didanosine is initially phosphorylated by 5'-nucleotidase to form didanosine-MP and then deaminated by adenylosuccinate synthetase and lyase to form dideoxyadenosine-MP. Phosphorylation of dideoxyadenosine-MP to dideoxyadenosine-DP and adefovir (PMEA, a



691 Figure 2. Chemical structures of the nucleic acids and selected nucleoside and nucleotide reverse transcriptase inhibitors.

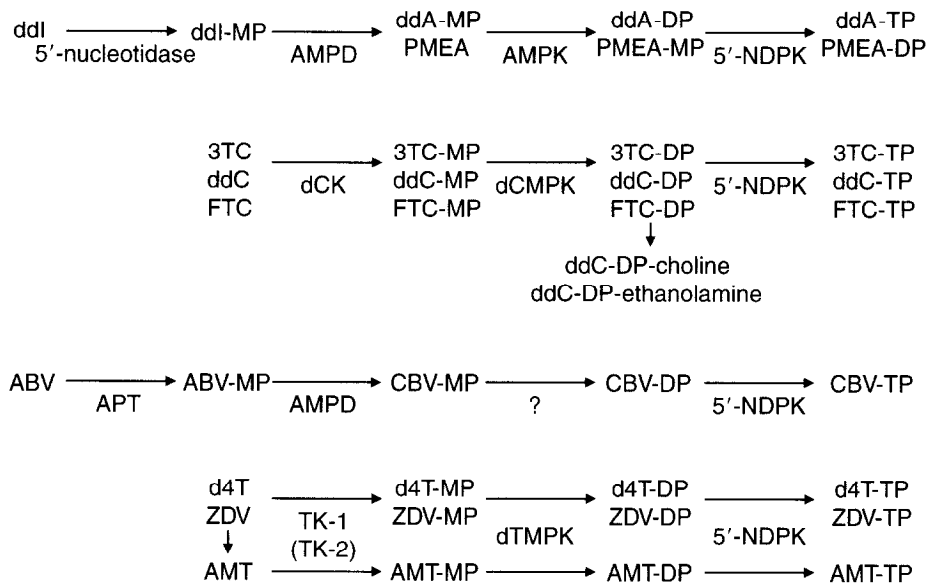


Figure 3. Phosphorylation pathways for selected nucleoside and nucleotide reverse transcriptase inhibitors. ddI = didanosine; MP = monophosphate; AMPD = adenosine monophosphate deaminase; ddA = dideoxyadenosine; PMEAs = adefovir; AMPK = adenosine monophosphate kinase; DP = diphosphate; NDPK = nucleoside diphosphate kinase; TP = triphosphate; 3TC = lamivudine; ddC = zalcitabine; FTC = emtricitabine; dCK = deoxycytosine kinase; dCMPK = deoxycytosine monophosphate kinase; ABV = abacavir; APT = adenosine phosphotransferase; CBV = carbovir; d4T = stavudine; ZDV = zidovudine; AMT = 3'-amino-3'-deoxythymidine; TK = thymidine kinase; dTMPK = deoxythymine monophosphate kinase (thymidylate kinase).

nucleotide RTI) to adefovir-MP is catalyzed by adenosine-MP kinase. The final step in phosphorylation, which is probably common to all NRTIs, is catalyzed by nucleoside diphosphate kinase.<sup>32</sup> Abacavir has a unique intracellular phosphorylation pathway that first involves addition of a phosphate by adenosine phosphotransferase to form abacavir-MP; cytosolic deamination of abacavir-MP forms carbovir-MP, which is subsequently phosphorylated twice to form carbovir-TP, the active moiety.<sup>34</sup>

The intracellular phosphorylation of NRTIs is subject to both enzymatic and cellular regulation. Thymidylate kinase, for example, is the rate-limiting enzyme for zidovudine activation but not for stavudine activation; the rate-limiting enzyme for stavudine phosphorylation is thymidine kinase.<sup>35</sup> Furthermore, nucleoside DP kinase may represent a second rate-limiting enzyme in the final production of zidovudine-TP.<sup>36</sup> Intracellular concentrations of zidovudine-MP and zidovudine-DP that are greater than those

of zidovudine-TP have been observed *in vitro*, reflecting the enzymatic bottlenecks that occur with this drug. On the other hand, intracellular concentrations of stavudine-MP, stavudine-DP, and stavudine-TP *in vitro* are similar because the rate-limiting enzyme occurs at the initial phosphorylation step. The difference in rate-limiting enzymes has an important clinical implication, in that further increases in plasma concentrations of zidovudine are unlikely to cause further elevation of intracellular zidovudine-TP concentrations, whereas increases in plasma concentrations of stavudine will probably cause an increase in intracellular stavudine-TP formation.<sup>32</sup> Rate-limiting enzymes for the other NRTIs, if they exist, have not been adequately described.

Cellular regulation of NRTIs depends on whether the cell is postmitotic (resting) or active. Resting cells preferentially phosphorylate lamivudine, zalcitabine, and didanosine, whereas activated cells preferentially phosphorylate thymidine analogues (eg, stavudine and zidovudine).<sup>37,38</sup> Cytosolic thymidine kinase-1 (TK-1) is expressed only during the S-phase and probably accounts for the latter effect. In cells that are resting, such as cardiac and skeletal myocytes and neurons, expression of thymidine kinase occurs in the mitochondrion. Mitochondrial thymidine kinase-2 (TK-2) is constitutively expressed in the mitochondria of all cells and is an important enzyme for cellular pyrimidine biosynthesis. Although stavudine and zidovudine have a much greater affinity for TK-1 than for TK-2, TK-2 may play a role in activating either of these drugs. Moreover, some cells, such as monocytes/macrophages and peripheral blood lymphocytes, intrinsically lack TK-1; in these cells, phosphorylation of

thymidine analogues is likely to occur through TK-2.

The other mitochondrial enzyme involved in phosphorylation of nucleic acids and their derivatives is deoxypyrimidine kinase. Unlike the differences in affinity for TK-1 and TK-2 between stavudine and zidovudine, zalcitabine has a similar affinity ( $K_m$ ) for cytoplasmic deoxycytidine kinase and mitochondrial deoxypyrimidine kinase (mean  $K_m \pm SD = 180 \pm 30$  and  $120 \pm 20 \mu M$ , respectively).<sup>39</sup> Mitochondrial NRTI activation may partly explain the toxicity profile of this class of drugs.

#### **MECHANISMS OF NUCLEOSIDE AND NUCLEOTIDE REVERSE TRANSCRIPTASE INHIBITOR-INDUCED MITOCHONDRIAL TOXICITY**

##### *DNA Polymerases*

The selectivity and specificity of NRTIs for HIV-RT are important, because cellular replication depends on DNA polymerases. There are 5 major human cellular DNA polymerases:  $\alpha$ ,  $\beta$ ,  $\sigma$ ,  $\epsilon$ , and  $\gamma$ . DNA polymerase  $\gamma$  is located in the mitochondrion; the remaining polymerases are located in the nucleus. The primary function of DNA polymerases  $\beta$  and  $\sigma$  is DNA repair, whereas DNA polymerases  $\alpha$  and  $\epsilon$  are responsible for nDNA transcription. Each of these polymerases can inadvertently incorporate an NRTI instead of the endogenous nucleotide. Consequently, the toxicity of NRTIs is most likely related to the indirect effects of these drugs on nDNA or mtDNA. Zidovudine, for example, has been shown to be incorporated into cellular DNA both *in vitro* and *in vivo*.<sup>40,41</sup> Reported inhibitory constants ( $K_i$ ) for the zidovudine-TP inhibition of deoxythymidine-MP incorpora-

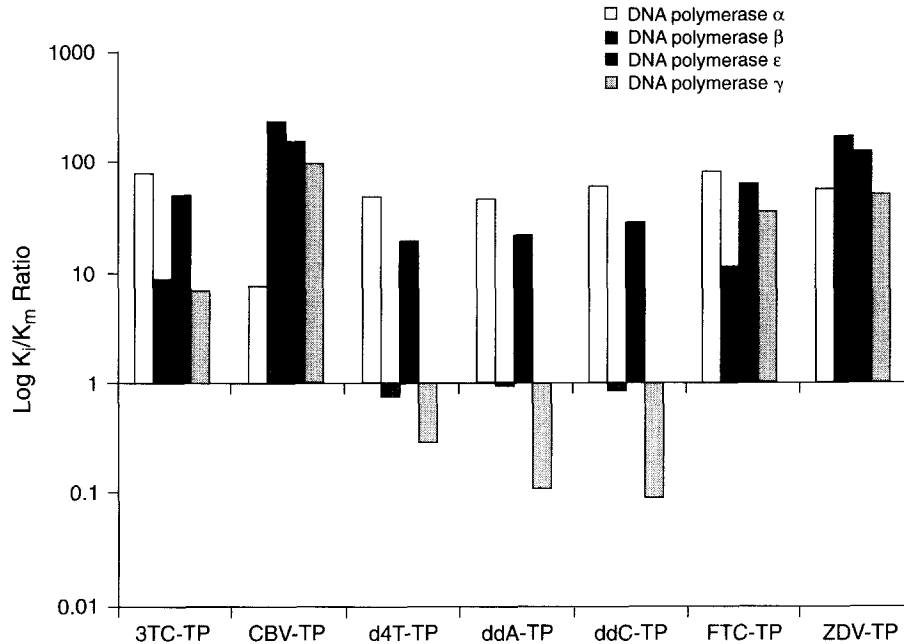


Figure 4. Effect of nucleoside reverse transcriptase inhibitors on polymerase  $\alpha$ ,  $\beta$ ,  $\epsilon$ , and  $\gamma$ . Adapted from Martin et al.<sup>44</sup> TP = triphosphate; 3TC = lamivudine; CBV = carbovir; d4T = stavudine; ddA = dideoxyadenosine; ddC = zalcitabine; FTC = emtricitabine; ZDV = zidovudine.

tion into endonuclease-activated DNA range from 45 to 650  $\mu\text{M}$ , and from 0.67 to 810  $\mu\text{M}$  for polymerase  $\alpha$  and  $\beta$ , respectively.<sup>40,42-44</sup> Although DNA incorporation has been studied only with zidovudine, it is likely to occur with all NRTIs. The most comprehensive in vitro study of the effect of NRTIs on polymerase activity was performed by Martin et al,<sup>44</sup> who compared 16 NRTIs (only selected NRTIs are discussed here) and their effects on DNA polymerase  $\alpha$ ,  $\beta$ ,  $\epsilon$ , and  $\gamma$  (Figure 4).

The ratio of  $K_i$  to  $K_m$  ( $K_i/K_m$ ) provides a means of comparing the potency of enzyme inhibition, with smaller ratios suggesting greater enzyme inhibition. Based

on the  $K_i/K_m$ , DNA polymerase  $\gamma$  and  $\beta$  were generally more sensitive to inhibition than were DNA polymerase  $\alpha$  and  $\epsilon$ . The calculated  $K_i/K_m$  for DNA polymerase  $\beta$  was  $<1$  for the TPs of didanosine, stavudine, and zalcitabine (dideoxyadenosine-TP, stavudine-TP, and zalcitabine-TP, respectively); in comparison, these ratios ranged from 9.3 to 240 for lamivudine, carbovir, emtricitabine, and zidovudine (lamivudine-TP, carbovir-TP, emtricitabine-TP, and zidovudine-TP, respectively). Results for DNA polymerase  $\gamma$  are discussed in the following paragraph. The implications of these findings are unclear. In persons receiving long-term zidovudine

monotherapy, only a slight amount of zidovudine (<0.005%) was found incorporated into the DNA of peripheral blood mononuclear cells.<sup>41</sup> The investigators concluded that the long-term toxicity of NRTIs may include genotoxicity and/or cytotoxicity.

Mitochondrial DNA polymerase  $\gamma$  has both DNA- and RNA-dependent DNA polymerase activity.<sup>45</sup> The latter effect resembles HIV-RT activity and may increase DNA polymerase  $\gamma$  susceptibility to NRTIs. Mechanisms of NRTI-induced mitochondrial toxicity include competitive inhibition; incorporation into mtDNA, causing premature chain termination (ie, mtDNA deletions); impairment of mitochondrial enzymes (ie, deficiency in ETC subunits encoded by mtDNA, or a direct effect of NRTIs on adenylate kinase and ADP/ATP translocase); uncoupling of OXPHOS; and triggering of mitochondrial-induced apoptosis. Table II summarizes the available *in vitro* and *in vivo* data.<sup>33,39,43-81</sup>

### **Zalcitabine**

Inhibition of mitochondrial DNA polymerase  $\gamma$  by an NRTI was first described by Starnes and Cheng<sup>39</sup> in an *in vitro* study of zalcitabine. DNA polymerase  $\gamma$  was exposed to zalcitabine-TP, and the enzymatic reaction was observed. The  $K_i$  of zalcitabine-TP was determined to be 0.016 to 0.008  $\mu\text{M}$ , whereas the  $K_m$  for endogenous deoxycytidine-TP was 0.31 to 0.1  $\mu\text{M}$  ( $K_i/K_m = 0.05$ ). Zalcitabine appeared to be a potent inhibitor of DNA polymerase  $\gamma$  activity. Similar results were found in other studies ( $K_i/K_m = 0.09$ <sup>44</sup> and 0.07<sup>50</sup>). Toxicity of zalcitabine in cell cultures is primarily attributed to the TP moiety and its ability to inhibit polymerase

activity compared with other zalcitabine metabolites.<sup>59</sup>

Further studies have demonstrated that zalcitabine significantly reduces mtDNA content in cell cultures. For example, mtDNA in peripheral neurons (PC-12) cultured with 10  $\mu\text{M}$  zalcitabine was reduced by >75%.<sup>73</sup> This same study detected zalcitabine-MP but not zalcitabine-TP in isolated mitochondria, suggesting that zalcitabine-TP is most likely formed in the cytoplasm and transferred into the mitochondria.<sup>73</sup> This suggestion was later confirmed by a study in rabbit liver mitochondria—uptake of zalcitabine-TP into mitochondria was concentration dependent.<sup>33</sup> These investigators also discovered that zalcitabine liponucleotides gain preferential entry into mitochondria and may serve as an intramitochondrial reservoir for phosphorylated zalcitabine.

Kukhanova et al<sup>52</sup> determined that zalcitabine-TP impairs mtDNA polymerase  $\gamma$  more efficiently than it impairs its intended target, HIV-RT. This may explain the poor tolerability of this drug among HIV-infected patients. Clinical studies of zalcitabine have identified its major dose-limiting effect as peripheral neuropathy, which is also a side effect of didanosine and stavudine.<sup>82</sup> The mechanism of NRTI-induced peripheral neuropathy has not been adequately studied in humans. There have been anecdotal reports of biopsy-proven mitochondrial damage in patients with NRTI-induced peripheral neuropathy,<sup>83</sup> but more extensive investigation is needed. The only available animal data come from nerve biopsies obtained from rabbits exposed to high doses of zalcitabine (35 mg/kg) for 16 weeks. Pathologic examination showed mitochondrial alterations in Schwann cells of sciatic and tibial nerves and dorsal root ganglia.<sup>78</sup> The investigators

Table II. In vitro and in vivo studies of nucleoside and nucleotide reverse transcriptase inhibitor (NRTI)-induced mitochondrial toxicity.

Model	NRTI(s) Studied	Effect of NRTI(s) on Model Studied	Reference
Enzyme assays			
DNA polymerase $\gamma$ on activated calf thymus DNA	ddC-TP	$K_i/K_m = 0.05$	39
DNA polymerase $\gamma$ on gapped duplex DNA template	CBV-TP ZDV-TP	$K_m = 100$ M $K_m = 11$ M	46
DNA polymerase $\gamma$	ZDV-TP	$K_i/K_m = 5$ and 13 for poly(rA)-oligo(dT) template and activated calf thymus DNA, respectively	47
DNA polymerase $\gamma$ on gapped duplex DNA template	CBV-TP	$K_i/K_m = 88.46$	48
DNA polymerase $\gamma$ on activated calf thymus DNA	3TC-TP ddA-TP ddC-TP ZDV-TP	$K_m = 43.8 \pm 16.4$ $\mu$ M $K_m = 0.068 \pm 0.008$ $\mu$ M $K_m = 0.031 \pm 0.006$ $\mu$ M $K_m = 147 \pm 24$ $\mu$ M	43
Bovine cardiac DNA polymerase $\gamma$ on poly(A)-oligo(dT) template	ZDV	$K_i/K_m = 2.57$ and 8.14 for competitive and noncompetitive inhibition, respectively	49
HeLa S3 DNA polymerase $\gamma$ on activated calf thymus DNA	3TC-TP CBV-TP d4T-TP ddA-TP ddC-TP FTC-TP ZDV-TP	$K_i/K_m = 23.53$ $K_i/K_m = 116.67$ $K_i/K_m = 0.28$ $K_i/K_m = 0.11$ $K_i/K_m = 0.09$ $K_i/K_m = 35.29$ $K_i/K_m = 51.18$	44
DNA polymerase $\gamma$ on activated calf thymus DNA	ddC-TP PMEA-DP PMPA-DP ZDV-TP	$K_i/K_m = 0.07$ $K_i/K_m = 1.35$ $K_i/K_m = 82.64$ $K_i/K_m = 33.89$	50
Yeast DNA polymerase $\gamma$ on activated calf thymus DNA	ddC-TP ZDV-TP	$K_m = 0.020 \pm 0.009$ $\mu$ M $K_m > 6$ $\mu$ M	51
DNA polymerase $\gamma$ on M13mp19 phage DNA	ddC-TP	Concentration-dependent inhibition of DNA polymerase $\gamma$ activity; greater inhibition of DNA polymerase $\gamma$ than HIV reverse transcriptase	52
DNA polymerase $\gamma$	ZDV-TP	$K_i = 0.86$ $\mu$ M	45
Adenylate kinase	ZDV	10 $\mu$ M ZDV reduced ATP formation by 30%	53
ADP/ATP translocator	ZDV	ZDV inhibited ADP/ATP translocase	54

(continued)

Table II. (Continued)

Model	NRTI(s) Studied	Effect of NRTI(s) on Model Studied	Reference
Isolated mitochondria Mitochondria from Sprague-Dawley rat brain, liver, and skeletal muscle	ZDV	Concentration-dependent inhibition of NADH-cytochrome <i>c</i> reductase activity and NADH-linked respiration	55
Mitochondria from Wistar rat liver	ZDV-TP	Concentration-dependent inhibition of mitochondrial swelling; ZDV-TP inhibited mitochondrial permeability transition ( $IC_{50} = 3 \mu\text{M}$ )	56
Mitochondria from Wistar rat kidney, liver, and cardiac and skeletal muscle	ZDV	Concentration-dependent inhibition of NADH oxidase activ- ity; no change in succinate oxidase or NADH- $K_3\text{Fe}(\text{CN})_6$ oxidoreductase activity; concentration-dependent inhibi- tion of $F_1F_0$ -ATPase in cardiac and skeletal muscle but not kidney or liver	57
Cell cultures T-lymphoblastic cell (CEM)	d4T ddC ddI ZDV	Concentration-dependent decrease in mtDNA content: ddC > d4T > ZDV > ddI; concentration-dependent increase in lactic acid production	58
T-lymphoblastic cell (CEM)	ddC	Potency of ddC metabolites on mtDNA synthesis: ddC-TP > ddC-DP > ddC-MP > ddC = ddc-DP-choline	59
T-lymphoblastic cell (CEM)	d4T ddC ddI	Concentration-dependent decrease in cell viability, mtDNA content, and mitochondrial morphology; ddC > d4T > ddI	60
T-lymphoblastic cell (CEM)	ddC ddI FddA	$IC_{50} = 0.022 \pm 0.02 \mu\text{M}$ $IC_{50} = 290 \pm 120 \mu\text{M}$ $IC_{50} > 500 \mu\text{M}$ Concentration-dependent increase in lactic acid: ddC > ddI = FddA	61
T-lymphoblastic cell (CEM)	CBV ddC	0.5 M ddC decreased mtDNA >80% after 9 days, whereas 50 M CBV had no effect; increased lactic acid production with ddC but not with CBV	62
T-lymphoblastic cell (Molt-4F)	ddC	5-fold decrease in mtDNA with 0.2 $\mu\text{M}$ after 2 days; increased lactic acid production	63
T-lymphoblastic cell (Molt-4)	3TC d4T ddC FTC ZDV	$IC_{50} > 200 \mu\text{M}$ $IC_{50} = 10 \mu\text{M}$ $IC_{50} = 0.002 \mu\text{M}$ $IC_{50} > 100 \mu\text{M}$ $IC_{50} > 100 \mu\text{M}$ Concentration-dependent decrease in mtDNA content: ddC > d4T > FTC = ZDV > 3TC	44
Human myeloid leukemia cell (HL-60)	ZDV	2% of ZDV detected in mitochondria	64
Friend mouse erythroleukemic cell	ZDV	1 $\mu\text{M}$ inhibited mtDNA replication by 43.8%	65

(continued)

Table II. (Continued)

Model	NRTI(s) Studied	Effect of NRTI(s) on Model Studied	Reference
Friend mouse erythroleukemic cell	ZDV	10 $\mu$ M inhibited OXPHOS by 45.5%; no uncoupling effect; no effect of ZDV on adenylate kinase	66
Human myocytes	ZDV	No effect of ZDV on mtDNA content or number of deletions; no effect on mitochondrial citrate synthase or cytochrome <i>c</i> oxidase	67
Human myocytes	ZDV	Concentration-dependent lipid accumulation; morphologic abnormalities in mitochondria attenuated with carnitine	68
Human myocytes	ZDV	Abnormal mitochondrial morphology; including paracrystalline inclusions	69
Human myocytes	ddI ddC ZDV	Increased lipid accumulation, lactate production; decreased succinate dehydrogenase and cytochrome <i>c</i> oxidase activity; no change in NADH reductase. Inhibition of myocyte proliferation: ddC > ZDV > ddI	70
Rat pheochromocytoma cell (PC12)	ddC ddI	Concentration-dependent decrease in mtDNA content: ddC > ddI	58
Rat pheochromocytoma cell (PC12)	ddC	10 $\mu$ M ddC inhibited mtDNA replication by 55%; uridine/pyruvate reduced inhibition	71
Rat pheochromocytoma cell (PC12)	ddC	Concentration-dependent increase in lactate production; no uncoupling effect	72
Rat pheochromocytoma cell (PC12)	3TC d4T ddC ddI ZDV	25 $\mu$ M (but not 1 or 10 $\mu$ M) ddC inhibited cell proliferation; d4T, ddI, and ZDV did not inhibit cell proliferation; d4T, ddC, and ddI exhibited concentration-dependent inhibition of neurite regeneration	73
Wild-type (NF551) and Kearns-Sayre fibroblasts (TC558)	ddC ZDV	Concentration- and duration-dependent depletion of wild-type mtDNA in heteroplasmic mitochondria	74
Spinal cord, spinal ganglia, and myocytes from Sprague-Dawley rats	ZDV	Concentration- and duration-dependent morphologic alterations in mitochondria	75
U937 monoblastoid cells	ddC	Concentration-dependent uptake of ddC-TP into mitochondria; ddC-DP-choline may act as reservoir	33
Animal models Sprague-Dawley rat heart and liver	ZDV	Rates exposed to ZDV 102 mg/kg for 35 days had morphologic changes in mitochondria that did not reverse 14 days after discontinuation of ZDV; ZDV 29 mg/kg had no effect on the myocardium; no mitochondrial alterations in liver were seen at either dose	76

(continued)

Table II. (Continued)

Model	NRTI(s) Studied	Effect of NRTI(s) on Model Studied	Reference
Sprague-Dawley rat liver	ddI	20% reduction in respiration state 3; 300% increase in respiration state 4; inhibition of mtDNA synthesis	77
Rabbit peripheral nerve	ddC	Morphologic alterations in Schwann cell mitochondria	78
Rat cardiac and skeletal muscle	ZDV	Reduced muscle activity	79
Sprague-Dawley rat liver	ZDV	Histologic changes associated with altered mitochondrial morphology	80
OF1 mice	ZDV	Mice exposed to ZDV 10 mg/kg for 35 days had 40% higher levels of 8-oxo-dG in liver; mice treated concomitantly with ZDV and vitamins C and E had no change compared with controls	81

ddC = zalcitabine; TP = triphosphate;  $K_i/K_m$  = ratio of the inhibitory constant ( $K_i$ ) to affinity ( $K_m$ ); CBV = carbociclovir; ZDV = zidovudine; 3TC = lamivudine; ddA = dideoxyadenosine; d4T = stavudine; FTC = emtricitabine; PMEA = adefovir; DP = diphosphate; PMPA = tenofovir; ATP = adenosine triphosphate; ADP = adenosine diphosphate; NADH = reduced nicotinamide adenine dinucleotide;  $IC_{50}$  = concentration required for 50% inhibition; ATPase = adenosine triphosphatase; mtDNA = mitochondrial DNA; ddi = didanosine; FddA = lodenosine; OXPHOS = oxidative phosphorylation; 8-oxo-dG = oxidized deoxyguanosine.

concluded that zalcitabine selectively inhibits mtDNA polymerase  $\gamma$  in neurons, causing mitochondrial dysfunction. Others have speculated that the zalcitabine liponucleotides can interfere with the production of sphingomyelin.<sup>84</sup> Liponucleotides, however, have not been described with either stavudine or didanosine. Hearing loss induced by zalcitabine and other NRTIs may also be related to mitochondrial toxicity.<sup>85</sup>

### **Didanosine**

In an enzyme assay,<sup>44</sup> dideoxyadenosine-TP and zalcitabine-TP equally inhibited polymerase  $\gamma$  ( $K_i/K_m = 0.11$ ). Cells exposed to either zalcitabine or didanosine, however, consistently showed greater mitochondrial toxicity with zalcitabine

than didanosine.<sup>44,58,60,70,73</sup> Approximately 10% of zalcitabine is converted to zalcitabine-TP, whereas ~3% of didanosine is metabolized to dideoxyadenosine-TP.<sup>32</sup> This difference in phosphorylation efficiency may account for the discrepancy between the results of enzyme assays that directly use the TP compound and cell culture that is exposed to the prodrug. Adverse effects of didanosine include retinal lesions and dose-related pancreatitis and peripheral neuropathy; didanosine can also cause diabetes and hypertriglyceridemia.<sup>86,87</sup> The etiology of didanosine-induced pancreatitis is unknown, but mitochondrial toxicity is a possible explanation. Two case reports have identified concomitant lactic acidosis and acute pancreatitis.<sup>88,89</sup>

**Zidovudine**

The effect of zidovudine on the mitochondria has been studied extensively. In DNA polymerase  $\gamma$  assays, the  $K_i/K_m$  for zidovudine-TP inhibition ranged from 5 to  $>50$ , depending on the method used.<sup>44,47,50</sup> Various effects of zidovudine have been observed in cell cultures, including intracellular lipid accumulation,<sup>66,68,80</sup> a concentration-dependent increase in lactic acid production,<sup>58,66</sup> and a decrease in mtDNA.<sup>44,58</sup>

**Stavudine**

Stavudine induces similar concentration-dependent decreases in mtDNA, but at much lower doses than zidovudine.<sup>44,60,73</sup> The concentration necessary to inhibit the mtDNA content of CEM cells by 50% ( $IC_{50}$ ) after 4 days of drug exposure was 3  $\mu\text{M}$  for stavudine and 19  $\mu\text{M}$  for zidovudine.<sup>58</sup> In Molt-4 cells,  $IC_{50}$  values for stavudine and zidovudine with respect to mtDNA synthesis were 10 and  $>100$   $\mu\text{M}$ , respectively.<sup>44</sup> The greater likelihood of mitochondrial toxicity with stavudine reflects the more potent  $K_i$  of stavudine-TP compared with zidovudine-TP (0.048–0.005  $\mu\text{M}$  and 8.7–0.7  $\mu\text{M}$ , respectively) on polymerase  $\gamma$  ( $K_m$  for deoxythymidine-TP = 0.17–0.01  $\mu\text{M}$ ).<sup>44</sup> Intramitochondrial stavudine-TP can be detected in isolated PC-12 mitochondria, suggesting that mitochondria can independently phosphorylate stavudine.<sup>73</sup> More importantly, maximum concentrations ( $C_{\text{max}}$ ) of a single 40-mg dose of stavudine in humans have been shown to range from 2.7 to 4.0  $\mu\text{M}$  (0.6–0.9 mg/L),<sup>90</sup> within the range of concentrations producing mitochondrial toxicity in vitro. Major adverse effects of stavudine

include peripheral neuropathy and possibly fat redistribution syndrome.<sup>90</sup>

**Lamivudine and Abacavir**

Lamivudine exerts little or no mitochondrial toxicity in vitro.<sup>43,44</sup> Lamivudine concentrations of up to 10  $\mu\text{M}$  in vitro had no effect on PC-12. Lamivudine-TP had no effect on and was undetectable in isolated mitochondria.<sup>73</sup> Available data for abacavir and its active metabolite, carbovir, indicate that abacavir is the NRTI least likely to inhibit DNA polymerase  $\gamma$ .<sup>44</sup>

**Adefovir Dipivoxil**

Adefovir dipivoxil is unique with regard to its effects on mitochondria. Two mechanisms have been proposed for mitochondrial toxicity with this agent. First, the  $K_i$  for adefovir-DP on DNA polymerase  $\gamma$  has been calculated as 0.97  $\mu\text{M}$ ; in comparison, the  $K_m$  of endogenous deoxyadenosine-TP was 0.72  $\mu\text{M}$  ( $K_i/K_m = 1.35$ ).<sup>50</sup> In the same study, the  $K_i/K_m$  was 0.07 for zalcitabine-TP and 33.89 for zidovudine-TP. Thus, it appears that adefovir's potential for mitochondrial toxicity is likely to fall between that of zalcitabine and zidovudine. Second, the dipivoxil ester covalently binds to carnitine, forming pivoylcarnitine, a renally eliminated metabolite. Carnitine plays an important role in shuttling fatty acids into the mitochondria. The effect of adefovir-induced carnitine depletion on mitochondria requires further evaluation. In clinical trials, adefovir 120 mg/d produced significant nephrotoxicity resembling Fanconi syndrome in 50% of patients after 72 weeks of therapy.<sup>91</sup> It is plausible that the dual effects of adefovir dipivoxil on mitochondria may be involved in this toxicity.

### **ALTERNATIVE MECHANISMS OF NUCLEOSIDE AND NUCLEOTIDE REVERSE TRANSCRIPTASE INHIBITOR-INDUCED MITOCHONDRIAL TOXICITY**

Although the majority of evidence suggests that NRTIs inhibit mtDNA replication in a manner similar to their inhibition of HIV-RT, there is also evidence for alternative mechanisms. Hayakawa et al<sup>92</sup> proposed that mtDNA mutations may arise from the formation of oxygen-free radicals that attack deoxyguanosine. Two studies using mouse liver confirmed that greater levels of oxidized guanosine are found in mice treated with zidovudine compared with control mice.<sup>81,92</sup> Treatment with antioxidants (vitamins C and E) abrogated the response.<sup>81</sup>

Other explanations for NRTI-induced mitochondrial toxicity include alterations in mitochondrial enzymes other than polymerase  $\gamma$ . Barile et al<sup>53</sup> found that zidovudine may bind to adenylate kinase, an enzyme involved in ATP formation. A 30% decrease in ATP formation was observed with 10  $\mu$ M zidovudine—a concentration several times greater than the  $C_{\max}$  achieved in humans. In a separate study, these investigators also demonstrated that zidovudine inhibited the mitochondrial ADP/ATP translocator.<sup>54</sup> Zidovudine does not affect the following enzymes involved in the Krebs cycle: citrate synthase, isocitrate dehydrogenase, malate dehydrogenase, or succinate dehydrogenase.<sup>67,93,94</sup>

Uncoupling of the ETC from ATP synthesis has been suggested as another mechanism for NRTI-induced mitochondrial toxicity. One study of zidovudine demonstrated a dose-dependent inhibitory effect on NADH-linked respiration and NADH-

cytochrome *c* reductase activity.<sup>55</sup> However, uncoupling could not be produced by zidovudine in a bone marrow model (Friend mouse erythroleukemic cell).<sup>66</sup>

Nucleoside analogues may trigger mitochondrial-induced apoptosis. One pathway of apoptosis involves the stimulation of apoptotic protease activating factor-1 (Apaf-1) by cytochrome *c* and deoxyadenosine-TP. Activated Apaf-1 then activates caspase-9, which then cleaves procaspase-3 to form caspase-3. Caspase-3 initiates further activation of caspases in a feed-forward mechanism (caspase cascade).<sup>95</sup> Triphosphorylated cladribine and fludarabine, both antineoplastic adenine derivatives, are able to substitute for deoxyadenosine-TP and cause cellular apoptosis.<sup>96</sup> In the study by Leoni et al,<sup>96</sup> dideoxyadenosine-TP did not activate Apaf-1 in the presence of cytochrome *c*. Apoptosis, however, has been described as a property of stavudine.<sup>97</sup> How stavudine causes apoptosis, or whether stavudine or other NRTIs activate Apaf-1, is currently unknown. Adipocyte apoptosis is the likely mechanism for fat loss in lipodystrophy,<sup>98</sup> but whether this is related to mitochondrial toxicity requires further study.<sup>23</sup>

### **PHARMACOLOGIC ENHANCERS OF NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR ACTIVITY**

Drugs affecting the phosphorylation and disposition of NRTIs can potentially influence mitochondrial toxicity. Ribonucleotide reductase is a cytosolic enzyme that converts ribonucleotides to deoxyribonucleotides. Hydroxyurea is an inhibitor of this enzyme and is used in the context of HIV treatment to deplete endogenous deoxynucleotide-TP pools; aden-

osine is most prominently affected, and, consequently, adenosine derivatives (eg, adefovir and didanosine) have less competition for incorporation by DNA polymerase.<sup>99</sup> The effect of hydroxyurea and NRTIs on mtDNA replication has not been studied.

### THE POLYMERASE $\gamma$ HYPOTHESIS

Taken together, the data clearly demonstrate that NRTIs cause mitochondrial toxicity. The following sentences summarize what Lewis and Dalakas<sup>20</sup> have referred to as the "DNA pol- $\gamma$  hypothesis," as updated with recent data. The effect of NRTIs on polymerase, in general, is:

$$\text{HIV-RT} \gg \gamma > \beta > \alpha = \epsilon$$

With respect to polymerase  $\gamma$ , the apparent in vitro hierarchy of mitochondrial toxicity for the approved NRTIs is:

$$\begin{aligned} \text{zalcitabine} &\geq \text{didanosine} \geq \text{stavudine} > \\ \text{lamivudine} &> \text{zidovudine} > \text{abacavir} \end{aligned}$$

NRTIs exhibit tissue-specific mitochondrial toxicity, which may reflect differences in their cellular permeation, activation/deactivation, and uptake into mitochondria. The expression of disease is determined by the cellular dependence on mitochondrial function and its threshold. For example, Chen et al<sup>58</sup> demonstrated greater mitochondrial toxicity in CEM cells than in PC-12 cells exposed to zalcitabine. Drug and disease interactions may exacerbate NRTI-induced mitochondrial toxicity.<sup>14</sup>

### CONCLUSIONS

The inhibition of mtDNA polymerase  $\gamma$  by NRTIs is an extension of their pharmaco-

logic activity. Kinases involved in the activation of NRTIs can be found in the cytosol and mitochondria. Once activated, NRTIs are substrates for HIV-RT but can also act as substrates for cellular and mtDNA polymerase at concentrations achieved in vivo. The adverse effects associated with NRTIs are likely to be related to the inhibition of host polymerases. This inhibitory effect of the NRTIs is greatest on mtDNA polymerase  $\gamma$ , the enzyme responsible for maintenance of mtDNA. Inhibition of mtDNA polymerase causes altered mitochondrial structure and function, leading to impaired energy production.

The consequences of mitochondrial toxicity at the cellular level are decreased OXPHOS, intracellular lipid accumulation, and lactic acidosis. Tissue- or organ-specific manifestations of NRTI-induced mitochondrial toxicity include hepatic steatosis with or without lactic acidosis, mitochondrial myopathy, and peripheral neuropathy. Similar conditions are described in acquired or inherited mitochondrial disease. Other adverse effects of NRTIs include anemia, nephrotoxicity, pancreatitis, and possibly fat redistribution syndrome. Correlates for these adverse effects can also be found in mitochondrial diseases, but further studies are required to determine their etiology in association with NRTIs.

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**Address correspondence to:** Thomas N. Kakuda, PharmD, Antiviral Pharmacology Laboratory, Department of Experimental and Clinical Pharmacology, College of Pharmacy, University of Minnesota, 7-152

T.N. KAKUDA

WDH, 308 Harvard Street SE, Minneapolis, MN 55455.

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