Available online at www.sciencedirect.com
SCIENCE DIRECT®
Antiviral Research 58 (2003) 217–225
Antiviral Research
www.elsevier.com/locate/antiviral

Evaluation of mitochondrial DNA content and enzyme levels in tenofovir DF-treated rats, rhesus monkeys and woodchucks

Greg Biesecker*, Susan Karimi, John Desjardins, Dennis Meyer, Beth Abbott, Ray Bendele, Frank Richardson

OSI Phamaceuticals, 2860 Wilderness Place, Boulder, CO 80301, USA

Received 6 September 2002; accepted 10 December 2002

Abstract

The antiviral compound tenofovir DF (Gilead Sciences) was evaluated for possible mitochondrial toxicity in rats, rhesus monkeys and woodchucks. Animals were treated by oral gavage with tenofovir DF, and the levels of mitochondrial enzymes cytochrome *c* oxidase and citrate synthase were assayed. In rats (6/group) treated daily for 28 days with 300 mg/kg tenofovir DF the enzyme levels were unchanged versus control in liver, kidney, and skeletal muscle. In a parallel study, rats (6/group) were treated with 40 mg/kg of the antiviral adefovir dipivoxil (Gilead Sciences) and enzyme levels were also unchanged versus control. In rhesus monkeys (6/group) treated daily with 30 mg/kg or 250 mg/kg tenofovir DF for 56 days, and in woodchucks (6/group) treated daily with 15 mg/kg or 50 mg/kg tenofovir DF for 90 days, the enzyme levels were unchanged in liver, kidney, skeletal muscle and cardiac muscle. Mitochondrial DNA (mtDNA) content was determined in tissue from treated versus control animals by utilizing a quantitative real-time PCR (QPCR) technique, where the relative ratios of mitochondrial cytochrome b gene to the genomic actin gene were measured. The relative mtDNA content from rats, rhesus monkeys and woodchucks were unchanged in the various treatment groups. Variations in mtDNA content between animals in the same treatment group were noted. The actual species-dependent mitochondria/genomic ratios were estimated from the QPCR assay. In summary, treatment with tenofovir DF, or with adefovir dipivoxil, did not affect mtDNA content or level of mitochondrial enzymes, and no liver, muscle or renal microscopic abnormalities were observed in tenofovir-treated animals.

Keywords: Cytochrome b; Mitochondrial DNA; Tenofovir DF

1. Introduction

A variety of nucleoside analogs have been developed for treatment of viral infections, including HIV and hepatitis B. Although highly efficacious, these compounds have been associated with adverse effects including neuropathy, myopathy, pancreatitis, hepatic steatosis, and possibly lipodystrophy (Brinkman et al., 1998, 1999; Carr and Cooper, 2000; John et al., 2001; Moyle, 2000). Implicated in the production of the adverse effects are interference with mitochondrial function based on abnormal morphology of muscle mitochondria, depletion of mitochondrial-encoded enzyme subunits and decreased mitochondrial gene number (Brinkman et al., 1998; Lewis et al., 2001; White, 2001).

FIAU mediated liver failure in humans following chronic treatment (Lewis et al., 1997) and in woodchucks after eight weeks of treatment (Tennant et al., 1998) is one example of nucleoside analog induced mitochondrial injury. FIAU, an inhibitor of viral DNA polymerase, is incorporated internally into DNA (Richardson et al., 1994) and causes mitochondrial DNA (mtDNA) depletion in several species (Morton, 1998). Other nucleoside analogs associated with mitochondrial injury include the HIV nucleotide reverse transcriptase inhibitor (NRTI) zidovudine (AZT), which has been associated with mitochondrial myopathy with histological features of ragged-red fibers, and with mtDNA depletion in HIV patients (Arnaudo et al., 1991; Masanes et al., 1998; Pezeshkpour et al., 1991) and in rat skeletal muscle (Lewis et al., 1992), and the NRTI zalcitabine (ddC), didanosine (ddI) and stavudine (d4T) (Bissuel et al., 1994; Dubinsky et al., 1989; Miller et al., 2000). AZT through perinatal AZT exposure also has been implicated in pediatric mitochondrial dysfunction including mitochondrial respiratory-chain defects that were associated with neurological symptoms and death (Blanche et al., 1999), although a larger study did not find this association (The Perinatal Safety Review Working Group, 2000). A study of maternal-fetal exposure

* Corresponding author. Tel.: +1-303-530-5205; fax: +1-303-444-0672.
E-mail address: gbiesecker@osip.com (G. Biesecker).

0166-3542/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0166-3542(03)00005-6

Table 1
Pre-clinical summary

Animal (number)	Treatment (duration)	Major treatment-related clinical observations
Rat (Sprague-Dawley, 6/group)	300 mg/kg tenofovir DF (28 days) 6× human dose (AUC)	Slight ↑BUN, ALT, AST; normal creatinine, lactate. ↓Urinary phosphorus and ↑calcium. Minimal renal proximal tubular epithelial karyomegaly. No liver or muscle microscopic abnormalities
	40 mg/kg adefovir dipivoxil (28 days)	Normal weight gain. ↓Reticulocyte and erythrocyte count. ↑Serum urea, creatinine, ALT, AST. ↓Urinary creatinine and ↑urinary phosphorus, calcium, protein and glucose. Normal lactate. Renal cortical tubular nephrosis. Moderate duodenal epithelial hyperplasia
Monkey (rhesus, 6/group)	30 mg/kg tenofovir DF (56 days), 250 mg/kg tenofovir DF (56 days) 5× human dose (AUC)	Normal weight gain. Dose-related ↓urinary and serum phosphorus Day 7, ↑phosphorus ∼7 days after initiating phosphate supplementation, to end at approximate prestudy values. ALT and AST ↑ ∼3-fold and 2-fold, respectively, Day 7, and remained ↑ to end of study. Normal lactate. Minimal liver centrilobular hypertrophy (250 mg/kg), no muscle or renal abnormalities
Woodchuck (6/group)	15 mg/kg tenofovir DF (90 days), 50 mg/kg tenofovir DF (90 days) maximum achievable dose	Normal weight. No dose-dependent or biologically or toxicologically important changes in hematological and clinical chemistry analytes. Slight lactate elevation in high dose group at one time point. No treatment-related histopathologic changes

to AZT in *patas* monkeys also showed evidence for mitochondrial dysfunction including respiratory-chain defects and mtDNA reduction (Gerschenson et al., 2000).

In one study with HIV patients, mtDNA depletion in peripheral blood cells versus non-infected controls was greatest in anti-retroviral treated HIV-infected patients, although mtDNA was reduced also in non-treated HIV-infected individuals (Cote et al., 2002). For patients in this study the reduction of mtDNA preceded symptomatic hyperlactatemia, and discontinuation of the anti-retroviral therapy led to increased mtDNA content.

Nucleoside analogs may cause mitochondrial damage through interfering with mtDNA replication or through inhibiting synthesis of essential mitochondrial proteins (Kakuda, 2000). Nucleoside analogs can inhibit the mitochondrial-specific DNA polymerase γ (Martin et al., 1994) and can incorporate into replicating DNA to cause chain termination. In cell culture assays some NRTI, especially AZT (Pan-Zhou et al., 2000), cause delayed toxicity (Birkus et al., 2002) depletion of mtDNA, inhibit mitochondrial enzymes, and cause a rise in lactate (Birkus et al., 2002). DNA incorporation also reflects the efficiency of nucleotide analogue excision (Feng et al., 2001; Lim and Copeland, 2001) and depends on the efficiency and intracellular site of NRTI phosphorylation (Zhu et al., 2000). While analogs incorporated in mtDNA can be removed by excision, repair mechanisms for RNA have not been established (Sawyer and Van Houten, 1999). Nucleoside analogs could also interact with other nucleotide binding proteins and interfere with mitochondrial function (Dolce et al., 2001).

In this study, we investigated mitochondrial function and content in rats, rhesus monkeys and woodchucks that were orally administered the newly approved nucleotide RT inhibitor, tenofovir DF (Viread—Gilead Sciences) (Barditch-Crovo et al., 2001), with the aim of evaluating potential for in vivo mitochondrial injury of this new class of HIV RT inhibitors. We evaluated the effects of tenofovir DF treatment on levels of mitochondrial enzymes cytochrome *c* oxidase and citrate synthase in liver, kidney and muscle. These mitochondrial enzymes also were assayed in tissue from animals treated by chronic administration with a closely related nucleotide analog adefovir dipivoxil (Gilead Sciences), a compound in development for treatment of hepatitis B (Cullen et al., 2001; Delmas et al., 2002; Staschke and Colacino, 2001). Tissues from treated animals were also evaluated for mtDNA content by real time quantitative PCR (QPCR) assay. We developed this assay to accurately measure the tissue mtDNA content and thus mitochondria number by measuring the amount of a mitochondrial gene, cytochrome b, relative to a nuclear gene, actin.

In three animal studies reported here the levels of mitochondrial enzymes and mtDNA content showed no evidence of mitochondrial damage or depletion at any dose level following daily oral administration of tenofovir DF. Rats and monkeys were treated at 5–6 times the human dose, based on blood levels (AUC), in order to detect possible drug related pathology (Table 1). Woodchucks were treated at the highest dose that could be humanely administered (based on volume and low solubility) for 90 days, which was sufficient to elicit mitochondrial toxicity and hepatic failure in FIAU-treated woodchucks (Tennant et al., 1998). The lack of adverse mitochondrial effects were further supported by the lack of light or electron (monkeys only) microscopic findings, or clinical chemistry changes (including lack of lactic acidosis) that would be associated with mitochondrial injury. These results are consistent with a recent report, where tenofovir was evaluated on human cultured cells and was shown less toxic than other NRTI in common use, based on

lactate synthesis, mitochondrial enzyme levels and mtDNA content (Birkus et al., 2002; Cihlar et al., 2002).

2. Materials and methods

2.1. Animal treatment groups

Rats, rhesus monkeys and woodchucks were treated by oral dosage of tenofovir DF or with adefovir dipivoxil, which were prepared by Gilead Sciences (Foster City, CA), or with control vehicle (Table 1). Tissues examined in this report were obtained from toxicology studies and represent the highest drug treatment group and control group for each study. Control animals (6/group) were treated with vehicle on the same schedule as the drug treatment and necropsies were performed at the same time point for drug treated and control animals. Each treatment and control group comprised equal numbers of male and female animals.

Rats were treated by oral gavage once a day for 28 days with 300 mg/kg tenofovir DF (male Sprague-Dawley rats—6/group, Charles River Labs, Portage, MI), or 40 mg/kg adefovir dipivoxil. Rhesus monkeys (housed at Sierra Biomedical, Inc., Sparks, NV) were treated by nasogastric gavage with 30 or 250 mg/kg/day tenofovir DF for 56 days (3 male and 3 female rhesus monkeys/group). Woodchucks (housed at Marmotech, Inc., Ithaca, NY) were dosed orally with a syringe and attached tube at 15 or 50/mg/kg/day for 90 days (3 male and 3 female per group); ninety-five percent or more of the dose was consumed. The control vehicle for rats and monkeys was 50 mM citrate, pH 2–2.4. The control vehicle for woodchucks was a 1:2 mixture of water and molasses (Table 1).

2.2. Sample collection

Samples of liver, kidney, skeletal muscle and cardiac muscle were taken at necropsy. The samples were cut into ≈100 mg cubes, flash frozen in liquid nitrogen, stored individually at −80 °C, and later processed for enzyme analysis or mtDNA quantitation.

2.3. Enzyme assays

Tissues for enzyme assays were homogenized in 0.25 M sucrose, pH 7.0, on ice, for 60–120 s. Samples were centrifuged at 4 °C for 10 min at 700 × *g* (1700 rpm—Sorvall), and then supernatants were poured off and aliquots taken for analysis. All assays were run as described (Richardson et al., 1999) on freshly homogenized tissue. The nuclear-encoded enzyme citrate synthase was assayed as originally reported (Robinson et al., 1987), and the mitochondrial—and nuclear-encoded enzyme cytochrome *c* oxidase (Wharton and Tzagoloff, 1967) was assayed with modification to allow for quantitation in a 96-well plate format. Total protein was assayed by the method of Lowery (DC assay kit, BioRad Laboratories, Hercules, CA).

2.4. mtDNA quantitation

mtDNA was quantitated by measuring the ratio of a mitochondrial gene (cytochrome b) to a nuclear gene (actin) by QPCR (Gibson et al., 1996; Heid et al., 1996). Sequences for each gene were obtained from GenBank and homologous primers were made that would produce a 100–200 bp amplicon. Forward and reverse cytochrome b and actin primer sequences were designed using the primer design software Primer Express™ (Applied Biosystems, Foster City, CA).

Forward primers were appended with a molecular beacon probe (Nazarenko et al., 1997; Tyagi and Kramer, 1996) comprising a hairpin loop sequence with a 5′-fluorescent molecule, FAM, and a dabcyl quencher, TAMARA. All primers were obtained from Operon, Inc. (Alameda, CA). FastStart Taq DNA polymerase was obtained from Roche Diagnostics (Indianapolis, IN), and dNTPs from Behringer Mannheim (Mannheim, Germany). Total DNA was isolated from tissue (Wizard prep—Promega, Madison, WI) and suspended in Tris–EDTA (TE) buffer. Liver DNA from one normal (untreated) animal was isolated in sufficient quantity to use as a standard.

Primer sets were (forward and reverse): rat actin = (5′-CATCATGTTTGAGACCTTCAACACCC-3′ and 5′-CATCTCTTGCTCGAAGTCTAGG-3′), rat cytochrome b = (5′-CATGACAAATATCCGAAAATC-3′ and 5′-CCTCCTCAGATTCATTCGAC-3′); primate actin = (rat forward and 5′-CGTAGCTCTTCTCCAGGGAGG-3′), and primate cytochrome b = (5′-CATGATACCAATACGCAAATC-3′ and 5′-CGTGTGAGAGTGGGGCTGC-3′); woodchuck actin = (rat forward and 5′-CCTAGACTTCGAGCAAGAGATG-3′), and woodchuck cytochrome b = (5′-GCTACAGCTTTCATAGGCTATG-3′ and 5′-CTATGACTAGAGCTGCGATG-3′). The molecular beacon was 5′-OGAGCGAAGCZ-3′, where O = FAM and Z = dabcyl.

The DNA gene content in a given sample was measured by QPCR using an ABI Prism Sequence Detection System (Applied Biosystems) with the molecular beacon probe. The PCR reaction mix contained the sample, dNTPs, Taq, reverse primer, and the single-stranded, quenched, molecular beacon-conjugated forward primer. The fluorescence was monitored in real-time and plotted as Δ*R*n (change in fluorescence of FAM relative to the ROX internal standard) versus the PCR cycle number. For each reaction the cycle was determined for which the signal exceeded a threshold value (=C_t which was determined as 10 standard deviations over background). Standard curves were constructed with the C_t plotted versus two-fold dilutions of a liver extract, starting with a 1:20 dilution of liver extract for actin and with a 1:100 dilution of liver extract for cytochrome b, and with actin or cytochrome b primers, respectively. This plot of C_t versus input was linear over 3–4 logs of template. Each PCR reaction plate contained a cytochrome b standard and an actin standard; and samples were run in quadruplicate (rat and rhesus monkey) or triplicate (woodchuck) with either cytochrome b or actin primers.

Sample values were calculated relative to a batch prep of liver DNA made from a control normal animal for each study. The same standard was used for both the actin and cytochrome b assay, where each was assayed with appropriate primers. With this method the relative cytochrome b/actin ratios were determined for each sample. The relative ratios for the different studies were normalized to a rhesus monkey sample for which the actual gene ratio was estimated as ten.

2.5. Estimated species-dependent cytochrome b/actin ratios

The actual gene ratios in the control liver standards were estimated from the sample dilutions required to produce equal C_t values. The liver standards were total extracts containing both genomic and mtDNA. For a QPCR reaction the cycle number at which the product signal exceeds a ten-fold standard deviation above background (i.e. the C_t) is proportional to the input template quantity (Heid et al., 1996). Since each cycle doubles the amount of template, with more input template there are fewer cycles required to reach C_t. Therefore, the ratio of the two dilutions of liver standard required to yield equal C_t values for the actin and cytochrome b reactions estimates the ratio of these two genes in the tissue.

2.6. Assay variation

Assay results were evaluated by calculating the coefficient of variance (CV) for replicate sample assays run on the same plate (intra-assay variation). For rhesus monkeys the CV also was calculated for repeat assays that were run on different plates, and usually on different days (inter-assay variation).

2.7. Statistical analysis

Treatment groups pre-study and at each study time point were compared by Shapiro-Wilks and Brown-Forsythe tests to characterize the normality and homogeneity of variance among the groups. If either of these tests was significant, $P \leq 0.05$, the ranks of the data rather than the data values were used in further analysis. Analysis of variance with Dunnett's tests was performed to test for differences between the control group and each of the treatment groups. Dunnett's test was also performed to compare each treatment group to its own baseline. All comparisons were considered to be significant if $P \leq 0.05$.

3. Results

3.1. Treatment groups

Tenofovir DF was administered orally to rats, rhesus monkeys and woodchucks for 28, 56, and 90 days, respectively. Adefovir dipivoxil was administered by oral gavage to rats only for 28 days. For drug treated animals there were no adverse effects on body weights or body weight gains as compared to control animals. Serum lactate concentrations were similar to control animals for all species (Table 1). A notable clinical chemistry alteration in rhesus monkeys was a reduction in the serum phosphate concentration, without concurrent hyperphosphaturia, which normalized following phosphate supplementation despite continued tenofovir DF treatment. There were no histopathologic lesions of the type that have been associated with delayed mitochondrial toxicity in humans including pancreatitis, hepatic microvesicular steatosis, ragged-red fibers in muscle and peripheral neuropathy (Table 1). Clinical pathology and microscopic alterations indicative of renal tubular nephrosis were noted in rats treated with adefovir dipivoxil and which are believed to result from inhibition by adefovir of organic ion transporters (Cihlar et al., 2001; Ho et al., 2000).

3.2. Assay for cytochrome c oxidase and citrate synthase

Tissue from treated and control animals were analyzed for citrate synthase, which is encoded in the nucleus and transported to the mitochondria, and for cytochrome *c* oxidase, which comprises 10 subunits encoded by nuclear DNA and three subunits encoded by the mtDNA (Cooper et al., 1991). A decrease in enzyme levels or in the ratio of the two enzymes would suggest the occurrence of mitochondrial injury. Enzyme activities were measured in tissue homogenates and normalized to protein concentration. Tissue from rats, rhesus monkeys and woodchucks were assayed by the same procedure.

Rats were treated with 300 mg/kg/day tenofovir DF, or 40 mg/kg/day adefovir dipivoxil, for 28 days. Cytochrome *c* oxidase levels were very similar in liver extracts from control, tenofovir DF- or adefovir-treated rats (Table 2). In extracts from kidney and muscle the cytochrome *c* oxidase levels had greater inter-animal variability, but the enzyme levels in treated animals were statistically not different from control values. Citrate synthase enzyme levels (Table 2) in liver, kidney and muscle were very similar and not statistically different in control and treated animals.

Tissue extracts from rhesus monkeys treated with tenofovir DF at 30 mg/kg or at 250 mg/kg/day for 56 days were assayed for cytochrome *c* oxidase and citrate synthase as above (Table 2). Enzyme levels in extracts from liver, kidney, skeletal and cardiac muscle were equivalent. The greatest variations in cytochrome *c* oxidase levels were measured in cardiac muscle, but the enzyme level variations between control and treatment groups were not dose-related and were statistically not different. The citrate synthase levels were equivalent between control and treated animals. There was less inter-animal variation in citrate synthase levels within the same tissue as compared to the cytochrome *c* oxidase levels.

Woodchucks were treated with tenofovir DF at 15 mg/kg or at 50 mg/kg/day for 90 days, and cytochrome *c* oxidase and citrate synthase levels were measured for liver, kidney,

Table 2
Assay for cytochrome *c* oxidase and citrate synthase in tissue from rats, rhesus monkeys and woodchucks treated with tenofovir DF, and rats treated with adefovir dipivoxil

	Liver (S.D.)	Kidney	Skeletal muscle	Cardiac muscle
Rat				
Cytochrome *c* oxidase (U/mg protein)				
Control (citrate) (6[a])	35.7 (15.7)	28.8 (19.9)	23.7 (31.0)	
TDF[b] (300 mg/kg) (6)	43.7 (25.5)	25.5 (20.0)	9.2 (6.6)	
ADV[c] (40 mg/kg) (5)	41.2 (24.0)	34.0 (20.8)	8.5 (3.9)	
Citrate synthase (μmol CoA/min/mg protein)				
Control (citrate) (6)	0.071 (0.017)	0.20 (0.02)	0.23 (0.22)	
TDF (300 mg/kg) (6)	0.080 (0.021)	0.23 (0.04)	0.17 (0.08)	
ADV (40 mg/kg) (5)	0.064 (0.016)	0.21 (0.09)	0.28 (0.21)	
Rhesus monkey				
Cytochrome *c* oxidase (U/mg protein)				
Control (citrate) (6[a])	8.9 (3.1)	24.4 (16.6)	12.0 (11.3)	66.1 (37.3)
TDF[b] (30 mg/kg) (6)	9.9 (7.0)	28.8 (7.3)	6.4 (3.8)	75.6 (16.8)
TDF (250 mg/kg) (6)	8.1 (2.9)	33.2 (9.6)	12.9 (7.0)	46.4 (16.1)
Citrate synthase (μmol CoA/min/mg protein)				
Control (citrate) (6)	0.048 (0.008)	0.23 (0.046)	0.14 (0.056)	0.79 (0.26)
TDF (30 mg/kg) (6)	0.042 (0.020)	0.31 (0.062)	0.12 (0.017)	0.96 (0.35)
TDF (250 mg/kg) (6)	0.057 (0.007)	0.27 (0.053)	0.10 (0.020)	0.91 (0.17)
Woodchuck				
Cytochrome *c* oxidase (U/mg protein)				
Control (citrate) (6[a])	2.69 (1.49)	10.8 (2.4)	23.9 (10.0)	26.6 (7.8)
TDF[b] (15 mg/kg) (6)	2.67 (1.28)	9.35 (2.68)	27.9 (6.8)	21.6 (7.4)
TDF (50 mg/kg) (6)	2.02 (1.40)	7.47 (3.61)	20.2 (10.0)	19.1 (6.8)
Citrate synthase (μmol CoA/min/mg protein)				
Control (citrate) (6)	0.051 (0.021)	0.124 (0.023)	0.133 (0.019)	0.677 (0.082)
TDF (15 mg/kg) (6)	0.056 (0.017)	0.120 (0.022)	0.166 (0.048)	0.720 (0.251)
TDF (50 mg/kg) (6)	0.052 (0.014)	0.115 (0.029)	0.127 (0.035)	0.563 (0.074)

The values for treated animals were not statistically different from values for control animals.
[a] Number of animals.
[b] TDF: tenofovir DF.
[c] ADV: adefovir dipivoxil.

skeletal muscle and cardiac muscle (Table 2). The cytochrome *c* oxidase and citrate synthase levels were equivalent in control versus treatment groups. There appeared to be a small decrease in enzyme levels for cardiac muscle in the highest treatment group, but this difference was not statistically significant.

3.3. QPCR technique

A quantitative real-time PCR technique was developed to evaluate the cellular content of mtDNA. Advantages of this technique are: high sensitivity; a signal directly proportional to input DNA template; linear over 3–7 logs; a closed tube, one enzyme reaction; and potential for multiplex and robotic setup. Primers were chosen to amplify a 100–200 bp product and were evaluated with a total DNA extract of rat liver. The sample was amplified for 40 cycles and the product evaluated by gel electrophoresis. A single template-dependent product was detected for both actin and cytochrome b (data not shown). Similar analyses were run for rhesus monkey and woodchuck primers, and all primer sets yielded a single amplification product. Control, template-minus reactions gave no detectable product. The ratios of cytochrome b/actin genes were estimated from the gel analysis and were consistent with the QPCR results.

For the QPCR reaction a molecular beacon probe was appended to the 5′-end of the forward primer. The beacon comprises a stem-loop with a FAM fluorescence probe at the 5′-end and a dabcyl quencher at the 3′-end of the stem (the quencher is at the junction of the molecular beacon with the 5′-end of the forward primer). We chose this molecular beacon reporter for consistency of primer design across the different species studied and for lower background (Nazarenko et al., 1997).

3.4. mtDNA quantitation

The ratio of cytochrome b/actin, as criteria of mtDNA content and mtDNA damage, was measured in DNA extracts from liver, kidney and muscle from treated and control rats, rhesus monkeys and woodchucks (Table 3). In samples from rats the cytochrome b/actin ratio was highest for muscle; the variation between animals also was greatest in muscle so the apparent increase with treatment was not significant.

Table 3
Assay for mtDNA in tissue from rats, rhesus monkeys and woodchucks treated with tenofovir DF, and rats treated with adefovir dipivoxil

	mtDNA (relative cytochrome b/actin ratio[a])			
	Liver (S.D.)	Kidney	Skeletal muscle	Cardiacmuscle
Rat				
Control (citrate) (6[b])	9.1 (4.3)	5.1 (2.6)	8.3 (6.8)	N/D
TDF[c] (300 mg/kg) (6)	8.4 (4.5)	4.7 (1.7)	14.0 (13.0)	N/D
ADV[d] (40 mg/kg) (5)	9.8 (6.4)	5.7 (2.4)	16.0 (8.3)	N/D
Rhesus monkey				
Control (citrate) (5)	2.6 (2.6)	2.1 (2.2)	2.7 (2.9)	6.5 (5.5)
TDF (30 mg/kg) (6)	2.6 (2.0)	1.7 (1.1)	1.4 (1.1)	6.6 (3.6)
TDF (250 mg/kg) (6)	1.6 (1.8)	1.1 (1.1)	2.0 (2.8)	5.4 (5.1)
Woodchuck				
Control (citrate) (6)	12.2 (2.9)	8.4 (2.6)	5.1 (2.4)	11.9 (8.8)
TDF (15 mg/kg) (6)	14.0 (6.6)	7.1 (5.0)	6.1 (2.2)	14.8 (6.3)
TDF (50 mg/kg) (6)	12.6 (6.4)	7.5 (1.6)	5.9 (2.9)	11.0 (6.8)

The values for treated animals were not statistically different from values for control animals.
[a] Compared to control liver DNA.
[b] Number of animals.
[c] TDF: tenofovir DF.
[d] ADV: adefovir dipivoxil.

mtDNA content in treated versus control animals was very similar in rat liver and kidney. In rhesus monkeys the mtDNA content was very similar in all tissues from treated versus control animals. The cytochrome b/actin ratio was similar in liver, kidney and skeletal muscle, and highest in cardiac muscle. In samples from woodchucks the mtDNA content was greater in liver and cardiac muscle, and was less in kidney and skeletal muscle. No significant differences were measured between control and treated animals for any of the tissues within the three studies.

For tissues from each species and within each group there was a broad range of values measured. This broad range reflects both individual metabolic or energetic variations, and also properties of the technique; QPCR has a wide dynamic range as compared to semi-quantitative techniques, including blot analysis and quantitation of PCR plateau value. In tissue from one rhesus monkey there was a total absence of cytochrome b signal in all tissues, which could result from a mutation in the cytochrome b gene for that individual. The actin signal from this animal was similar to other animals within the group.

To evaluate intra- and inter-assay variability the coefficient of variation (CV) was calculated for assay groups (Table 4). The intra-assay CV for this assay averaged 26%. This CV is higher than reported for QPCR assay performed with plasmid DNA (Heid et al., 1996) as tissue samples have higher levels of interfering contaminants than plasmid preparations, and also the amount of template DNA is lower in tissue extracts. Since variation between individuals was 10- to 100-fold, a CV of 26% does not greatly affect the results.

The inter-assay CV for one group of rhesus monkey samples repeated on different days was somewhat higher at 44%, in part because these were the first samples run with this technique. The lowest CV values were obtained with woodchuck samples that were run last, and the average intra-assay CV for this group of 18% should be representative of this kind of assay.

Table 4
Intra- and inter-assay CV

Species	Gene	Liver	Kidney	Skeletal muscle	Cardiac muscle	Average
Intra-assay CV (%)						
Rat	Actin	27	32	27	N/D	29
	Cytochrome b	30	28	27	N/D	28
Monkey	Actin	20	35	33	27	29
	Cytochrome b	23	33	45	39	35
Woodchuck	Actin	25	14	17	13	17
	Cytochrome b	24	12	24	19	20
Inter-assay CV (%)						
Monkey	Actin	24	23	60	48	39
	Cytochrome b	45	61	53	61	55

Table 5
Estimated species-dependent cytochrome b/actin ratio

Species	Average ratio[a]	Standard deviation (number of repeats[b])
Rat	74.8	39.2 (23)
Rhesus monkey	19.6	17.5 (12)
Woodchuck	1577	1610 (9)

[a] Based on equivalent C_t values.
[b] Repeat samples of liver DNA standard.

A large inter-animal variation in mtDNA content was somewhat unexpected based on literature reports, however, recent studies have reported a wide range of values for mtDNA from human PBMC samples when assayed either using a RNA amplification technique (van Gemen et al., 2001) or with a QPCR technique (Cote et al., 2002).

The cytochrome b/actin ratios were calculated relative to one rhesus liver extract. Therefore, the ratios are not presented as actual number of mitochondria per cell for each tissue, but as relative mtDNA content. However, the actual number of cytochrome b genes relative to actin gene was estimated for repeat assays of the liver extract used as the standard for each species (Table 5). This number was estimated from the point at which the C_t value of the cytochrome b standard curve was equal to the C_t value of the actin standard curve. As the C_t value of any sample is related to the amount of template input, the ratio of cytochrome b to actin could be estimated from the dilution of each sample that produced an equal C_t value.

4. Discussion

Toxicity issues for nucleoside analogs have been controversial, in part because the targets for the toxicity have not been clearly defined and in part because the delayed appearance of the toxic effects. Nucleoside analogs including FIAU and AZT are specific for viral DNA polymerase relative to host cytoplasmic enzymes, and thus should have had a wide therapeutic window. However, severe toxicities have been observed with some of these compounds. For example, FIAU in humans produced severe, and fatal, hepatic toxicity. The irreversible microvesicular steatosis produced by FIAU in humans and woodchucks was associated with mitochondrial toxicity (Lewis et al., 1997; Tennant et al., 1998) and was in part explained by the incorporation of FIAU into mtDNA (Richardson et al., 1994), probably via the mitochondria-specific DNA polymerase γ. The accumulation of mitochondrial defects led to a reduced number of mitochondria and defects in mitochondrial respiratory enzymes.

In HIV the widespread use of NRTI has been associated with increased occurrence of toxic side effects suggesting these compounds also produce mitochondrial injury (Carr and Cooper, 2000; Kakuda, 2000; White, 2001) Mitochondrial dysfunction has been most clearly associated with AZT (Gerschenson et al., 2000) in both humans (Blanche et al., 1999) and in primates (Gerschenson et al., 2000). These results are complicated because therapy naive HIV patients also have disease-related mitochondrial abnormalities (Cote et al., 2002), although these are exacerbated by NRTI treatment.

In this report, we have evaluated mitochondrial parameters in tissues from three species following repeat dosing of the tenofovir DF, a newly approved nucleotide RTI. The dosing was of sufficient duration to model chronic human treatment, as based on the time required to produce mitochondrial toxicity in woodchucks treated with FIAU. We also evaluated effects on mitochondria of a closely related compound, adefovir dipivoxil, which is targeted for treatment of hepatitis B (Cullen et al., 2001; Delmas et al., 2002). Tissues analyzed for mitochondrial parameters were taken from rats, rhesus monkeys and woodchucks that were treated daily by oral administration of tenofovir DF or adefovir dipivoxil (rats only). Tissue samples were assayed for the mitochondrial-encoded enzyme cytochrome *c* oxidase and the nuclear- and mitochondrial-encoded enzyme citrate synthase. In all tissues examined, the citrate synthase levels were unchanged as compared to control animals. Cytochrome *c* oxidase levels were more variable between animals, and apparent differences were noted between treated and control animals in muscle of rats and rhesus monkeys, but these differences were not statistically significant. The absence of mitochondrial enzyme effects in tenofovir DF-treated animals is evidence for absence of mitochondrial injury. In addition, enzyme levels were not significantly affected in rats treated with the related nucleotide analog adefovir dipivoxil.

Mitochondrial functional integrity also was evaluated by quantitation of mtDNA content, as the ratio of mitochondrial cytochrome b gene relative to the nuclear actin gene by quantitative real-time PCR. Cytochrome b/actin ratios measured in tissues from the treated and control rats, rhesus monkeys and woodchucks showed that mtDNA content in each of these tissues was unchanged after treatment with tenofovir DF. By this criteria, the tenofovir DF treatment was not associated with mitochondrial injury. There were no differences measured either for tissues from animals treated with adefovir dipivoxil. Mitochondrial injury sufficient to cause injury to cells and clinical toxicity could require mtDNA reduction greater than 70%, based on both in animal studies (Morton, 1998; White, 2001) and HIV patients (Cote et al., 2002. A 70% reduction in mtDNA content would have been detected in our assay. The adefovir results do not support a report that adefovir treatment produced mitochondrial mediated renal toxicity in one patient (Tanji et al., 2001). This report has been questioned (Bendele and Richardson, 2002) based on the fact that only one patient was enrolled, the patient was on multiple HIV therapies, and other clinical studies have not indicated renal toxicity at the dose given. In addition, the PCR method used was a less quantitative method than the one used in these studies.

Rats, rhesus monkeys and woodchucks did not present with elevated serum lactate, pancreatitis, microvesicular hepatic steatosis, myopathy or peripheral neuropathy (Table 1). As mitochondrial injury could produce these effects, and their absence is consistent with absence of mitochondrial toxicity.

There was high inter-animal variability in mtDNA for all species tested in this study, both control and treated. This result is consistent with two studies that report mtDNA content of human blood cells from HIV infected patients and controls (Cote et al., 2002; van Gemen et al., 2001), where both studies also used real-time amplification assays. In the animal study reported here and in the human studies the variation in the mitochondrial gene/nuclear gene ratio was greater than 10-fold. These results suggest that detecting small but significant differences in mtDNA due to disease or treatment would require a large number of samples. For the three species studied here the actual liver mtDNA content of each was estimated from the QPCR amplification results; there was a great variation between species and this might correlate with the relative sensitivity of each species to mitochondrial toxicity (Morton, 1998).

In summary, no adverse effects related to mitochondrial injury were observed in animals treated with tenofovir DF at doses 6× the human dose over a period of 28, 56 or 90 days, or for adefovir dipivoxil in rats treated for 28 days at a nephrotoxic dose. These preclinical models suggest that neither tenofovir DF nor adefovir dipivoxil should pose a high risk for potential mitochondrial-related effects in humans.

Acknowledgements

We thank Julie Wolf from OSI Pharmaceuticals for the statistical analysis, and Mick Hitchcock for critical reading of the manuscript. This work was supported Gilead Sciences, Inc.

References

The Perinatal Safety Review Working Group, 2000. Nucleoside exposure in the children of HIV-infected women receiving antiretroviral drugs: absence of clear evidence for mitochondrial disease in children who died before 5 years of age in five United States cohorts. J. Acquir. Immune. Defic. Syndr. 25, 261–268.

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

Barditch-Crovo, P., Deeks, S.G., Collier, A., Safrin, S., Coakley, D.F., Miller, M., Kearney, B.P., Coleman, R.L., Lamy, P.D., Kahn, J.O., McGowan, I., Lietman, P.S., 2001. Phase I/II trial of the pharmacokinetics, safety, and antiretroviral activity of tenofovir disoproxil fumarate in human immunodeficiency virus-infected adults. Antimicrob. Agents Chemother. 45, 2733–2739.

Bendele, R.A., Richardson, F.C., 2002. Adefovir nephrotoxicity and mitochondrial DNA depletion. Hum. Pathol. 33, 574.

Birkus, G., Hitchcock, M.J., Cihlar, T., 2002. Assessment of mitochondrial toxicity in human cells treated with tenofovir: comparison with other nucleoside reverse transcriptase inhibitors. Antimicrob. Agents Chemother. 46, 716–723.

Bissuel, F., Bruneel, F., Habersetzer, F., Chassard, D., Cotte, L., Chevallier, M., Bernuau, J., Lucet, J.C., Trepo, C., 1994. Fulminant hepatitis with severe lactate acidosis in HIV-infected patients on didanosine therapy. J. Intern. Med. 235, 367–371.

Blanche, S., Tardieu, M., Rustin, P., Slama, A., Barret, B., Firtion, G., Ciraru-Vigneron, N., Lacroix, C., Rouzioux, C., Mandelbrot, L., Desguerre, I., Rotig, A., Mayaux, M.J., Delfraissy, J.F., 1999. Persistent mitochondrial dysfunction and perinatal exposure to antiretroviral nucleoside analogues. Lancet 354, 1084–1089.

Brinkman, K., Smeitink, J.A., Romijin, J.A., Reiss, P., 1999. Mitochondrial toxicity induced by nucleoside-analogue reverse-transcriptase inhibitors is a key factor in the pathogenesis of antiretroviral-therapy-related lipodystrophy. Lancet 354, 1112–1115.

Brinkman, K., ter Hofstede, H.J.M., Burger, D.M., Smeitink, J.A.M., Koopmans, P.P., 1998. Adverse effects of reverse transcriptase inhibitors: mitochondrial toxicity as common pathway. AIDS 12, 1735–1744.

Carr, A., Cooper, D.A., 2000. Adverse effects of antiretroviral therapy. Lancet 356, 1423–1430.

Cihlar, T., Birkus, G., Greenwalt, D.E., Hitchcock, M.J., 2002. Tenofovir exhibits low cytotoxicity in various human cell types: comparison with other nucleoside reverse transcriptase inhibitors. Antiviral Res. 54, 37–45.

Cihlar, T., Ho, E.S., Lin, D.C., Mulato, A.S., 2001. Human renal organic anion transporter 1 (hOAT1) and its role in the nephrotoxicity of antiviral nucleotide analogs. Nucleosides Nucleotides Nucleic Acids 20, 641–648.

Cooper, C.E., Nicholls, P., Freedman, J.A., 1991. Cytochrome *c* oxidase: structure, function, and membrane topology of the polypeptide subunits. Biochem. Cell. Biol. 69, 586–607.

Cote, H.C., Brumme, Z.L., Craib, K.J., Alexander, C.S., Wynhoven, B., Ting, L., Wong, H., Harris, M., Harrigan, P.R., O'Shaughnessy, M.V., Montaner, J.S., 2002. Changes in mitochondrial DNA as a marker of nucleoside toxicity in HIV-infected patients. N. Engl. J. Med. 346, 811–820.

Cullen, J.M., Li, D.H., Brown, C., Eisenberg, E.J., Cundy, K.C., Wolfe, J., Toole, J., Gibbs, C., 2001. Antiviral efficacy and pharmacokinetics of oral adefovir dipivoxil in chronically woodchuck hepatitis virus-infected woodchucks. Antimicrob. Agents Chemother. 45, 2740–2745.

Delmas, J., Schorr, O., Jamard, C., Gibbs, C., Trepo, C., Hantz, O., Zoulim, F., 2002. Inhibitory effect of adefovir on viral DNA synthesis and covalently closed circular DNA formation in duck hepatitis B virus-infected hepatocytes in vivo and in vitro. Antimicrob. Agents Chemother. 46, 425–433.

Dolce, V., Fiermonte, G., Runswick, M.J., Palmieri, F., Walker, J.E., 2001. The human mitochondrial deoxynucleotide carrier and its role in the toxicity of nucleoside antivirals. Proc. Natl. Acad. Sci. USA 98, 2284–2288.

Dubinsky, R.M., Yarchoan, R., Dalakas, M., Broder, S., 1989. Reversible axonal neuropathy from the treatment of AIDS and related disorders with 2′,3′-dideoxycytidine (ddC). Muscle Nerve 12, 856–860.

Feng, J.Y., Johnson, A.A., Johnson, K.A., Anderson, K.S., 2001. Insights into the molecular mechanism of mitochondrial toxicity by AIDS drugs. J. Biol. Chem. 246, 23832–23837.

Gerschenson, M., Erhart, S.W., Paik, C.Y., Claire, St.M.C., Nagashima, K., Skopets, B., Harbaugh, S.W., Harbaugh, J.W., Quan, W., Poirier, M.C., 2000. Fetal mitochondrial heart and skeletal muscle damage in *Erythrocebus patas* monkeys exposed *in Utero* to 3′-azido-3′-deoxythymidine. AIDS Res. Hum. Retroviruses 16, 635–644.

Gibson, U.E., Heid, C.A., Williams, P.M., 1996. A novel method for real time quantitative RT-PCR. Genome Res. 6, 995–1001.

Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real time quantitative PCR. Genome Res. 6, 986–994.

Ho, E.S., Lin, D.C., Mendel, D.B., Cilar, T., 2000. Cytotoxicity of antiviral nucleotides adefovir and cidofovir is induced by the expression of human organic anion transporter 1. J. Am. Soc. Nephrol. 11, 393.

John, M., Nolan, D., Mallal, S.A., 2001. Antiretroviral therapy and the lipodystrophy syndrome. Antiviral Therapy 6, 9–20.

Kakuda, T.N., 2000. Pharmacology of nucleoside and nucleotide reverse transcriptase inhibitor-induced mitochondrial toxicity. Clin. Ther. 22, 685–708.

Lewis, W., Copeland, W.C., Day, B.J., 2001. Mitochondrial DNA depletion, oxidative stress, and mutation: mechanisms of dysfunction from nucleoside reverse transcriptase inhibitors. Lab. Invest. 81, 777–790.

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

Lewis, W., Griniuviene, B., Tankersley, K.O., Levine, E.S., Montione, R., Engelman, L., de Court, M.A., Hornbuckle, W.E., Gerin, J.L., Tennant, B.C., 1997. Depletion of mitochondrial DNA, destruction of mitochondria, and accumulation of lipid droplets result from fialuridine treatment in woodchucks (*Marmota monax*). Lab. Invest. 76, 77–87.

Lim, S.E., Copeland, W.C., 2001. Differential incorporation and removal of antiviral deoxynucleotides by human DNA polymerase. J. Biol. Chem. 276, 23213–23623.

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

Masanes, F., Barrientos, A., Cebrian, M., Pedrol, E., Miro, O., Casademont, J., Grau, J.M., 1998. Clinical, histological and molecular reversible of zidovudine myopthy. J. Neurol. Sci. 159, 226–228.

Miller, K.D., Cameron, M., Wood, L.V., Dalakas, M.C., Kovacs, J.A., 2000. Lactic acidosis and hepatic steatosis associated with use of stavudine: report of four cases. Ann. Intern. Med. 133, 192–196.

Morton, D.M., 1998. Importance of species selection in drug toxicity testing. Toxicol. Lett. 102 (103), 545–550.

Moyle, G., 2000. Clinical manifestations and management of antiretroviral nucleoside analog-related mitochondrial toxicity. Clin. Ther. 22, 911–936.

Nazarenko, I.A., Bhatnagar, S.K., Hohman, R.J., 1997. A closed tube format for amplification and detection of DNA based on energy transfer. Nucleic Acids Res. 25, 2516–2521.

Pan-Zhou, X.R., Cui, L., Zhou, X.J., Sommadossi, J.P., Darley-Usmar, V.M., 2000. Differential effects of antiretroviral nucleoside analogs on mitochondrial function in HepG2 cells. Antimicrob. Agents Chemother. 44, 496–503.

Pezeshkpour, G., Illa, I., Dalakas, M.C., 1991. Ultrastructural characteristics and DNA immunocytochemistry in human immunodeficiency virus and zidovudine-associated myopathies. Hum. Pathol. 22, 1281–1288.

Richardson, F.C., Tennant, B.C., Meyer, D.J., Richardson, K.A., Mann, P.C., McGinty, G.R., Wolf, J.L., Zack, P.M., Bendele, R.A., 1999. An evaluation of the toxicities of 2′-fluorouridine and 2′-fluorocytidine-HCl in F344 rats and woodchucks (*Marmota monax*). Toxicol. Pathol. 27, 607–617.

Richardson, F.C., Engelhardt, J.A., Bowsher, R.R., 1994. Fialuridine accumulates in DNA of dogs, monkeys, and rats following long-term oral administration. Proc. Natl. Acad. Sci. USA 91, 12003–12007.

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

Sawyer, D.E., Van Houten, B., 1999. Repair of DNA damage in mitochondria. Mutat. Res. 434, 161–176.

Staschke, K.A., Colacino, J.M., 2001. Drug discovery and development of antiviral agents for the treatment of chronic hepatitis B virus infection. Prog. Drug Res. Spec. no. 111–83.

Tanji, N., Tanji, K., Kambham, N., Markowitz, G.S., Bell, A., D'Agati, V.D., 2001. Adefovir nephrotoxicity: possible role of mitochondrial DNA depletion. Hum. Pathol. 32, 734–740.

Tennant, B.C., Baldwin, B.H., Graham, L.A., Ascenzi, M.A., Hornbuckle, W.E., Rowland, P.H., Tochkov, I.A., Yeager, A.E., Erb, H.N., Colacino, J.M., Lopez, C., Engelhardt, J.A., Bowsher, R.R., Richardson, F.C., Lewis, W., Cote, P.J., Korba, B.E., Gerin, J.L., 1998. Antiviral activity and toxicity of fialuridine in the woodchuck model of hepatitis B virus infection. Hepatology 28, 179–191.

Tyagi, S., Kramer, F.R., 1996. Molecular beacons: probes that fluoresce upon hybridization. Nat. Biotechnol. 14, 303–308.

van Gemen, B., Ronde de, A., Casular, M., Timmermans, E., van Dooren, M., Westrop, M., Dobbelaar, I., Bakker, M., Weverling, G.J., Lange, J., Goudsmit, J., Reiss, P., 2001. Quantification of mitochondrial DNA relative to nuclear DNA in peripheral blood mononuclear cells: validation of an accurate duplex assay for use in patients undergoing antiretroviral therapy. Antiviral Therapy 6 (Suppl. 4), 14.

Wharton, D.C., Tzagoloff, A., 1967. Cytochrome oxidase from beef heart mitochondria. In: Estabrook, R.W., Pullman, M.E. (Eds.), Methods Enzymolgy, pp. 245–250.

White, A.J., 2001. Mitochondrial toxicity and HIV therapy. Sex Transm. Infect. 77, 158–173.

Zhu, C., Johansson, M., Karlsson, A., 2000. Incorporation of nucleoside analogs into nuclear or mitochondrial DNA is determined by the intracellular phosphorylation site. J. Biol. Chem. 275, 26727–26731.