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Effects of apricitabine and other nucleoside reverse transcriptase inhibitors on replication of mitochondrial DNA in HepG2 cells

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Abstract

Several nucleoside reverse transcriptase inhibitors are associated with mitochondrial toxicity resulting from inhibition of DNA polymerase-γ. This study compared the effects on mitochondrial DNA of apricitabine (previously referred to as AVX754 or SPD754), a novel cytidine analogue under development for the treatment of human immunodeficiency virus (HIV)-1 infection, and other reverse transcriptase inhibitors. Human HepG2 hepatoblastoma were cultured for up to 16 days with test compounds at concentrations of 0.3–300 μM. Mitochondrial DNA replication was assessed by means of a duplex nucleic acid sequence-based amplification technique, which measures the ratio of the number of mitochondrial DNA copies to the number of genomic DNA copies. Apricitabine and tenofovir had no effect on the mitochondrial DNA content. In contrast, alovudine, zalcitabine, didanosine and stavudine markedly reduced mitochondrial DNA content, whereas abacavir, emtricitabine, lamivudine and zidovudine produced slight increases in mitochondrial DNA, which may reflect an adaptive cellular response to mitochondrial dysfunction. These results suggest that apricitabine shows a favorable mitochondrial toxicity profile, which is important for long-term clinical use. Further studies are warranted to define the clinical implications of these findings.

Keywords: Apricitabine; Mitochondrial DNA; NRTI

1. Introduction

Nucleoside reverse transcriptase inhibitors are potent inhibitors of human immunodeficiency virus (HIV) replication, and represent a central component of highly active antiretroviral therapy regimens. However, these agents are also able to inhibit human DNA polymerases, including DNA polymerase-γ, a cellular DNA polymerase that is responsible for the replication of mitochondrial DNA (Chen and Cheng, 1989; Chen et al., 1991). Inhibition of mitochondrial DNA replication results in delayed toxicity which is manifested clinically by a variety of adverse effects, including peripheral neuropathy, cardiomyopathy, hepatic steatosis, lactic acidosis, hyperlactemia, anaemia, neutropenia, diabetes and lipodystrophy (Brinkman et al., 1998, 1999; Brinkman and Kakuda, 2000; Walker, 2001; Walker and Brinkman, 2001a,b). Therefore, new nucleoside reverse transcriptase inhibitors should undergo preclinical testing to assess their potential for mitochondrial toxicity.

Apricitabine is a novel deoxycytidine analogue (previously referred to as AVX754 or SPD754) that is currently under clinical development for the treatment of HIV-1 infection. This compound has shown excellent clinical anti-HIV activity when used as short-term (10 days) monotherapy in treatment-naïve patients with predominantly wild-type virus (Cahn et al., 2006), and in vitro studies have shown that this activity is retained against viruses with the M184V mutation and multiple thymidine-associated mutations (TAMs) (Bethell et al., 2003). Further in vitro studies showed that apricitabine had no effect on mitochondrial DNA in human HepG2 and MT-4 cells, when incubated for 28 days at concentrations of up to 200 μM, whereas dideoxycytidine gave rise to large reductions at concentrations below 10 μM (Gu et al., 2001). In these studies, however, mitochondrial DNA was measured by Southern blotting, which may be subject to cumulative errors resulting from

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separate amplification and hybridization of genomic and mitochondrial DNA. Since a duplex nucleic acid sequence-based amplification assay is now available (Timmermans et al., 2006), allowing faster and more reliable measurement of mitochondrial DNA, this technique was used to assess the mitochondrial toxicity of apricitabine and other nucleoside reverse transcriptase inhibitors. HepG2 cells were used in this study since this cell line has been widely used in previous studies of the effects of antiretroviral drugs on mitochondrial function (Pan-Zhou et al., 2000; Birkus et al., 2002; Walker et al., 2002; Nerurkar et al., 2003; Velsor et al., 2004).

2. Materials and methods

The following cytidine analogue and non-cytidine analogue reverse transcriptase inhibitors were used in this study: apricitabine (ATC), zalcitabine (ddC), lamivudine (3TC), emtricitabine (FTC), zidovudine (AZT), alovudine (FLT), stavudine (d4T), tenofovir (TFV), didanosine (ddI) and abacavir (ABC). ATC and TFV were obtained from Shire Biochem Inc. (Laval, Canada), ABC and 3TC from the National Institutes of Health Reagent Program, and FTC from Moravek Biochemicals (Brea, CA, USA). Human HepG2 hepatoblastoma cells were obtained from ATCC (Manassas, VA, USA). All other chemicals and biological agents were obtained from Sigma (Zwijndrecht, The Netherlands) unless otherwise stated.

All nucleoside reverse transcriptase inhibitors were dissolved in 100% dimethyl sulphoxide (DMSO), to a concentration of 300 mM; the final DMSO concentration in all assays was 0.1%.

2.1. Cell culture

HepG2 cells were cultured in six-well plates at an initial concentration of 2×10^6 cells per well, and exposed to the test compounds at concentrations of 0.3, 1, 3, 10, 30, 100 and 300 μM in duplicate; 0.1% DMSO and culture were used as controls. The cells were incubated at 37 °C under 5% CO_2. The culture medium consisted of Minimal Essential Medium (MEM: Invitrogen-Gibco, Paisley, United Kingdom) supplemented with 10% fetal bovine serum albumin, sodium pyruvate (1 mM), 100,000 μg/ml streptomycin sulphate (Invitrogen-Gibco) and 100,000 U/ml penicillin G. The adherent cells were washed with phosphate-buffered saline (PBS) before being split by trypsinization with 1× trypsin solution (Invitrogen-Gibco) every third day. After counting the number of cells in the samples, 3×10^5 cells were taken for analysis of mitochondrial DNA. These cells were dispensed into lysis buffer (bioMerieux, Boxtel, The Netherlands) and stored at −80 °C prior to analysis. During counting the relative number of dead cells that remained in the culture after washing was determined by Trypan blue exclusion. The absolute number of dead cells in the adherent culture could not be determined, as the cells would have had to be trypsinized before counting, in turn necessitating thorough washing of the cells with PBS to eliminate residual proteins, which would have removed the dead cells. From each sample, 2×10^6 live cells were incubated as a fresh culture with the respective compound in the culture medium.

Cells were monitored almost every day for growth characteristics such as cell density, cell shape and the relative number of dead cells by light microscopy. Monitoring of cytotoxicity by light microscopy was performed as a non-invasive, qualitative check of culture growth, rather than an accurate quantitative estimation of the cytotoxicity of the antiretroviral agents under study.

2.2. Mitochondrial DNA assay

Mitochondrial DNA was measured by means of the Retina^{TM} Mitox^{TM} assay (Timmermans et al., 2006). This assay measures the ratio of mitochondrial DNA copies to nuclear genomic DNA copies: since human cells contain two genomic DNA copies per cell, the number of mitochondrial copies can be calculated from this ratio. The assay is based on a duplex nucleic acid sequence-based amplification (NASBA), which amplifies both genomic and mitochondrial DNA in a single reaction. This duplex amplification improves the accuracy of the assay, since genomic and mitochondrial DNA are equally affected by the conditions within the amplification tube.

Details of the assay have been published previously (Timmermans et al., 2006). In brief, nucleic acids from an equivalent of 3000 cells were added to an amplification system containing primers and molecular beacons specific for human mitochondrial DNA and nuclear DNA: the use of molecular beacons for both nuclear and mitochondrial DNA allowed reactions to be followed in real time (Tyagi and Kramer, 1996). The primer set for mitochondrial DNA spanned an RNA splice-site, with one primer at the 3′-end of 16S RNA and the other in the tRNA-lys downstream of the mitochondrial transcription terminator. The P1 primer included a naturally occurring MspI restriction enzyme site. The sequences of this primer pair were 5′-AATTCTAATACGACTCACTA-TAGGGAAGAAC*CGGGCTCTGCCATCTTAA-3', where *indicates the MspI restriction enzyme site, for P1 and 5′-GTAATCCAGGTCGGTTTCTA-3′ for P2. For the amplification of nuclear snRP U1A DNA, the P1 primer was located in an intron, and the P2 primer in the adjacent exon sequence; again, the P1 primer included a naturally occurring MspI restriction enzyme site. The sequences of these primers were 5′-AATTCTAATACGACTCACTATAGGGAGAGGCC*CGG-CATGTGGTGCATAA-3′ for P1, where * indicates the restriction site, and 5′-TGCGCCTCTTTCGGGTGTT-3′ for P2. The molecular beacon sequences and fluorescence labels for the U1A amplicon and the mitochondrial DNA amplicon were 5-carboxyfluorescein (FAM)-5′-CGCA-TGCTGTAACCACGCACTCTCCTCGCATCCG-3′-dabcyl and 5-(and-6)-carboxy-X-rhodamine (ROX)-5′-CGTACGTG-ATATCATCTCAACTTAGTATCGTACG-3′-dabcyl, respectively. The primers were synthesized in large batches and purified to purities exceeding 90% by bioMerieux.

The final composition of the amplification reaction mixture was: tris–HCl (40 mM, pH 8.3), $MgCl_2$ (12 mM), KCl (90 mM), dithiothreitol (DTT; 5 mM), dNTPs (1 mM each), rNTPs (2 mM each), DMSO (15%, v/v), oligonucleotide U1A nDNA-P1 (0.2 μM), oligonucleotide U1A nDNA-P2 (0.2 μM), oligonu-

cleotide mtDNA-P1 (0.3 μM), oligonucleotide mtDNA-P2 (0.3 μM), U1A-beacon (0.04 μM), mtDNA-beacon (0.04 μM) and MspI restriction enzyme (2 units). All reactions were performed at 37 °C for 12 min, after which the mixture was heated to 95 °C for 3 min. The mixture was then cooled to 41 °C and the following enzyme mixture added for nucleic acid amplification (Kievits et al., 1991): bovine serum albumin [BSA] (2.1 mg), RNase H (0.08 units), T7 RNA polymerase (64 units), AMV-RT (9.6 units) and sorbitol (0.376 M). Amplification was performed for 60 min in a fluorescence reader at 41 °C and fluorescence measured every 30 s: excitation and emission wavelengths were 485 and 518 nm, respectively, for FAM and 578 and 604 nm, respectively, for ROX. Calibration curves containing 6×10^3 nuclear DNA plasmids and 1.2×10^5–9.6×10^6 mitochondrial DNA plasmids (equivalent to 20, 40, 200, 800 and 1600 copies of mitochondrial DNA per cell) were included in each assay. All assays were performed twice, within each assay the duplicates were not permitted to have a coefficient of variation above 25%: if the coefficient of variation was higher, the assay was repeated. In this manner a total of four individual measurements of mitochondrial DNA content were recorded at each time point for a given experiment. A total of six samples whose coefficient of variation remained above 25% after re-analysis were excluded: these exclusions did not affect the overall results.

2.3. Data analysis

For each of the compounds tested, the relative mitochondrial DNA content per cell was calculated and plotted against time. The data were entered into Excel files and subjected to linear regression analysis (Microsoft Corporation, Richmond, WA). Analyses were checked using SPSS 11.5.2 software (SPSS Inc., Chicago, IL).

No formal statistical comparisons were made, but clear increases or decreases were deemed to have occurred when mtDNA fell outside of the 95% confidence intervals (CI) for samples take on day 0 before exposure to test compounds.

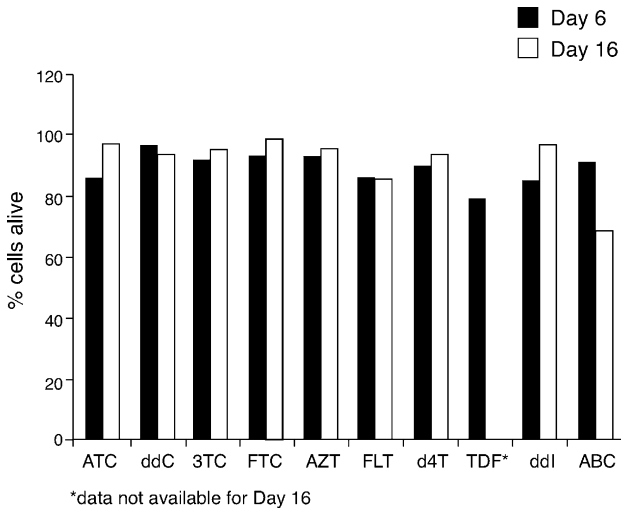

Fig. 1. Relative proportion of live cells on treatment with reverse transcriptase inhibitors at 300 μM, determined by Trypan blue exclusion. A semi-quantitative assessment of cell viability was made, in which the number of dead cells was scored as follows 5%: hardly any dead cells; 20%: <25% dead cells; 40%: 25–50% dead cells; 75%: >50% dead cells. The proportion of live cells was then determined by subtraction of these figures from 100%.

3. Results

3.1. Cell culture experiments

The relative proportion of live cells on days 6 and 16, determined by Trypan blue exclusion, is shown in Fig. 1. For all compounds tested except for ABC on day 16, at least 75% of cultured cells were alive when counted.

Although the cells remained viable and continued to grow in the presence of each compound at each concentration tested, cellular proliferation was reduced at concentrations of ≥100 μM with all of the compounds tested except for ABC and d4T, where increased proliferation was apparent (Table 1). At concentrations below 100 μM, proliferation was slightly increased in the presence of FTC, d4T and ABC, and no consistent effect was seen with ATC, ddC, AZT, FLT and TFV. All concentrations of

Table 1
Total counted cell numbers[a] by light microscopy on days 6 and 16 for cells treated with reverse transcriptase inhibitors at 300 μM

Agent (μM)	Apricitabine (ATC)		Zalcitabine (ddC)		Lamivudine (3TC)		Emtricitabine (FTC)		Zidovudine (AZT)	
	Day 6	Day 16	Day 6	Day 16	Day 6	Day 16	Day 6	Day 16	Day 6	Day 16
1	84	107	33	68	65	63	87	146	55	48
10	144	77	62	29	99	99	81	127	89	93
100	145	62	48	24	106	73	99	80	45	29
300	107	98	114	11	110	56	71	54	70	44

	Alovudine (FLT)		Stavudine (d4T)		Tenofovir (TFV)		Didanosine (ddI)		Abacavir (ABC)	
	Day 6	Day 16	Day 6	Day 16	Day 6	Day 16	Day 6	Day 16	Day 6	Day 16
1	49	66	75	103	112	61	65	89	57	121
10	68	27	66	128	34	63	62	111	57	72
100	89	28	99	62	76	51	137	65	57	109
300	39	37	88	100	122	74	50	23	49	52

[a] Data are counted cell numbers in the visual field of the counting chamber.

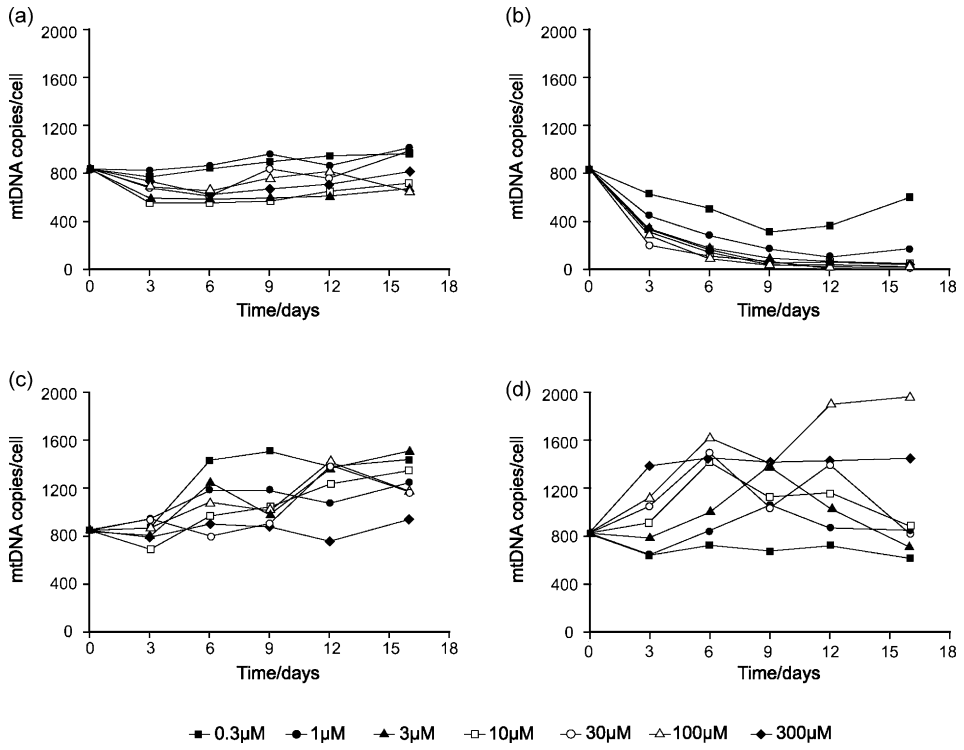

Fig. 2. Changes in mitochondrial DNA content over time in HepG2 cells cultured with deoxycytidine analogue reverse transcriptase inhibitors. (a) Apricitabine; (b) zalcitabine; (c) lamivudine; (d) emtricitabine.

FLT initially provoked considerable reductions in cellular proliferation, followed by a recovery at around day 9, although this recovery was not complete at day 16.

The proportion of dead cells was similar to that with 0.1% DMSO and MEM at low concentrations (<10 μM) for all agents with the exception of FLT and ddI, where a small increase in cell death was observed. At 3–30 μM concentrations, increased cell death was observed for FLT, d4T, TFV and AZT. At the highest concentrations (300 μM on days 6 and 16 shown in Fig. 1), increased cell death occurred with ABC in addition to these compounds. However, it should be noted that the wash stage prior to trypsinization and subsequent Trypan blue exclusion assay may have removed some dead cells from the samples, reducing the quantitative accuracy of this assay. As such, trends in these data are best considered qualitatively.

3.2. Mitochondrial DNA analysis

The relative changes in mitochondrial DNA content over time are shown in Figs. 2 and 3. Day 0 samples, taken before seeding the cells and any exposure to compounds, were tested seven times for mtDNA content, resulting in a mean value of 817 ± 188 mtDNA copies per cell, giving 95% CI of 629 and 1005 copies/cell. On day 3, only cultures containing ≥3 μM ddC, ≥30 μM ddI and ≤100 μM FLT had an mtDNA count below the lower 95% CI of day 0 samples. The culture containing 30 μM 3TC had an mtDNA count above the upper 95% CI of the day 0 samples. All other day 3 samples fell within the 95% CI for day 0.

ATC had no obvious effect on mitochondrial DNA content of HepG2 cells during the 16 days of incubation. Some increase in mitochondrial DNA was observed (Fig. 2a), but this was comparable with the normal variation in cells that were not exposed to reverse transcriptase inhibitors. The only other compound for which there was also no effect on mitochondrial DNA observed, was in the cultures exposed to TFV, the only nucleotide analogue in the experiments (Fig. 3d).

Concentration-dependent reductions in the number of copies of mitochondrial DNA per cell to below the level of 600 copies per cell were observed with FLT (Fig. 3b), ddC (Fig. 2b) and to a lesser extent, d4T (Fig. 3c). ddI appeared to have a concentration-dependent effect on mtDNA. No consistent effect was seen for concentrations ≤3 μM, and an initial fall in mtDNA followed by recovery to day 0 levels was seen for concentrations of 10–30 μM. A more substantial initial drop, followed by recovery was seen for the 100 μM concentration, and mtDNA decreased to <100 copies from day 9 onwards, with no recovery, for 300 μM ddI (Fig. 3e). For FLT and ddC, some decrease in mitochondrial DNA was already apparent at day 3, and this may have resulted in an underestimation of the effects of concentrations of 3 μM and above.

Increases in mitochondrial DNA to levels above 1000 copies per cell that were independent of concentration were observed with 3TC (Fig. 2c), FTC (Fig. 2d) and AZT (Fig. 3a). ABC increased mitochondrial DNA content at concentrations up to 30 μM, but no further increase was observed with higher concentrations (Fig. 3f). It should be noted that mitochondrial DNA levels at concentrations of 100 and 300 μM on day 3 were lower

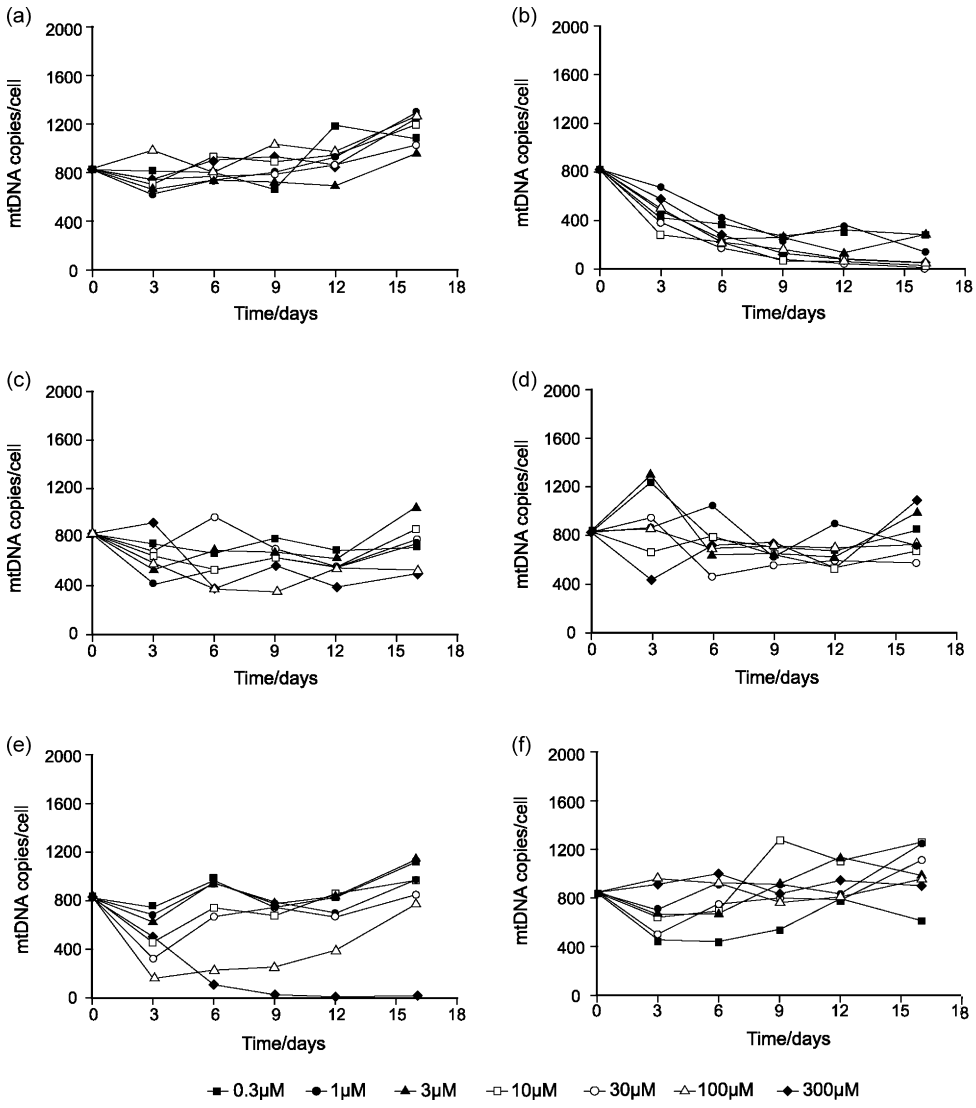

Fig. 3. Changes in mitochondrial DNA content over time in HepG2 cells cultured with non-cytidine analogue reverse transcriptase inhibitors. (a) Zidovudine; (b) alovudine; (c) stavudine; (d) tenofovir; (e) didanosine; (f) abacavir.

than in control cells, and, hence, the effects of these concentrations may have been underestimated.

The variability of the results differed little according to the absolute mitochondrial DNA content of the cells or between different experiments and the standard deviation was generally between 10 and 15% (Fig. 4a). Fig. 4a also emphasised the general trends from Figs. 2 and 3; mtDNA was markedly reduced by ddC, FLT and ddI, and, to a lesser extent, by d4T, and noticeably increased by 3TC, FTC, AZT and ABC. Fig. 4b shows relative changes in mtDNA content for DMSO and MEM control cultures from day 0 to 16. Although more variation was apparent in the mtDNA content of cells cultured in DMSO than in MEM, neither medium significantly affected cellular mtDNA content over the course of the study.

4. Discussion

The results of this study are consistent with and extend those of previous studies of the mitochondrial toxicity of nucleoside reverse transcriptase inhibitors (Birkus et al., 2002; Kakuda, 2000). Based on these results, three groups of compounds can be identified: those that produce reductions in mitochondrial DNA content (ddC, FLT, d4T, ddI), those that increase mitochondrial DNA (3TC, FTC, AZT, ABC), and those with no effect on mitochondrial DNA (ATC, TFV).

Among the compounds that produced reductions in mitochondrial DNA, FLT and ddC had the strongest effects, followed in descending order of potency by ddI and d4T. The effects of ddC in HepG2 cells were comparable with those obtained in previous studies (Birkus et al., 2002; Kakuda, 2000). Inhibition of mitochondrial DNA replication has also been previously reported with FLT in other cell lines; in addition, this agent has been reported to be a stronger inhibitor of granulocyte-macrophage lineage cell proliferation than ddC (Faraj et al., 1994). Anemia was a frequently reported adverse event during clinical trials with FLT, which might reflect antiproliferative effects and inhibition of mitochondrial DNA replication in blood-derived cells (Flexner et al., 1994; Sundseth et al., 1996;

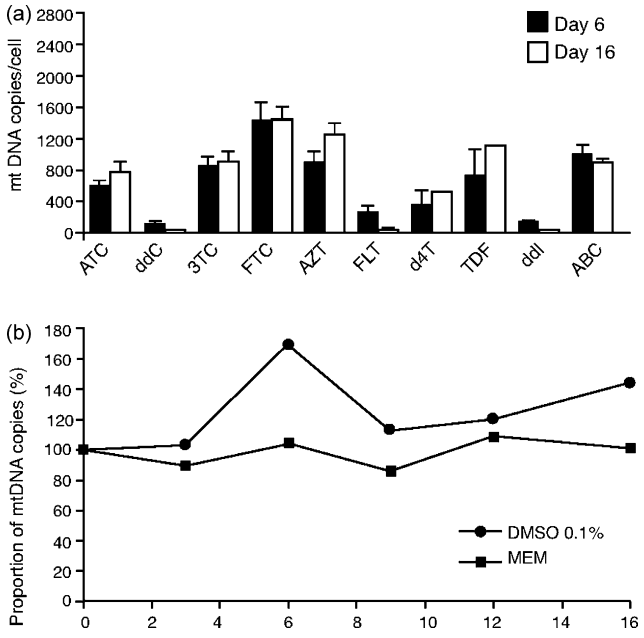

Fig. 4. (a) Mean (S.D.) changes in mitochondrial DNA content at days 6 and 16 in HepG2 cells cultured with reverse transcriptase inhibitors at a concentration of 300 μM. ATC, apricitabine; ddC, zalcitabine; 3TC, lamivudine; FTC, emtricitabine; AZT, zidovudine; FLT, alovudine; d4T, stavudine; TFV, tenofovir; ddI, didanosine; ABC, abacavir. (b) Proportion of mitochondrial DNA copies in control culture cells during the study (as a percentage of day 0).

Dornsife and Averett, 1996). The inhibitory effect of d4T on mitochondrial DNA in this study was concentration-dependent, being apparent only at the highest doses. This is consistent with previous in vitro studies that have shown dose-dependent depletion of mitochondrial DNA with this agent (Birkus et al., 2002; Walker et al., 2002).

The reductions in mitochondrial DNA seen with ddC, ddI and d4T were not consistently associated with general cytotoxicity, as shown by the cell culture results presented in Table 1. This may be explained by the fact that cells were transferred into fresh medium every third day, which may have resulted in selection of more viable cells that were less susceptible to cytotoxicity. Moreover, it should be noted that cytotoxic effects may also be manifest as proliferative responses. We believe, however, that our study design minimizes the potential influence of cytotoxicity on mitochondrial DNA.

Among the compounds that produced increases in mitochondrial DNA content, FTC had the greatest effect, while ABC, 3TC and AZT had lesser effects and were approximately equivalent in potency. Mitochondrial toxicity has been reported in a number of previous studies with AZT (Benbrik et al., 1997; Barile et al., 1998; Cazzalini et al., 2001; de la Asuncion et al., 1998; Waclawik et al., 1999), although zidovudine triphosphate does not inhibit DNA polymerase γ to any significant extent (Johnson et al., 2001; Lim and Copeland, 2001). The finding that AZT increased mitochondrial DNA content might reflect an adaptive response to mitochondrial dysfunction. This would be consistent with the finding in this and other studies (Barile et al., 1998) that compounds, such as d4T, ABC and ddI, which decrease mitochondrial DNA only at high concentrations tend to increase mitochondrial DNA at lower concentrations.

In contrast to the other nucleoside analogues studied, ATC produced neither an increase nor a decrease in mitochondrial DNA content: similar results were obtained with TFV, the only nucleotide analogue studied. This might suggest that ATC offers the best mitochondrial tolerability profile of any of the nucleoside analogues studied. The fact that mitochondrial DNA levels remained unchanged at all concentrations of ATC suggests that this agent does not inhibit DNA polymerase-γ, and does not appear to induce mitochondrial dysfunction by any other mechanism.

Our results do not preclude the possibility that antiviral agents produce other toxic effects on mitochondria that are manifest in other ways, such as increases in cellular lactic acid content. However, while some studies have shown increases in lactic acid associated with nucleoside reverse transcriptase inhibitors (Walker et al., 2002; Velsor et al., 2004), others have found inconsistent effects on lactic acid (Pan-Zhou et al., 2000).

In conclusion, nucleoside reverse transcriptase inhibitors vary markedly in their effects on mitochondrial DNA. ATC appears to have the least effect among the nucleoside analogues investigated in this study, and might therefore be expected to offer a favorable mitochondrial tolerability profile during routine clinical use. Further studies are warranted to establish the implications of these findings in the clinical impact of ATC.

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