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Toxicity of Antiretroviral Nucleoside and Nucleotide Analogues

Is Mitochondrial Toxicity the Only Mechanism?

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Abstract

Nucleoside analogues represent the cornerstones of antiretroviral regimens. A range of drug- or tissue-specific toxicities, such as peripheral neuropathy, myopathy, pancreatitis and lactic acidosis with hepatic steatosis, has been documented with these agents. The fat atrophy seen on long term antiretroviral therapy may also be related to nucleoside analogues.

The mechanisms by which nucleoside analogues cause toxicity are not clearly established. *In vitro*, the triphosphates of these agents are weak to modest substrates for human DNA polymerases, showing the greatest affinity for mitochondrial DNA polymerase γ. Short term exposure *in vitro* to some nucleoside analogues has been demonstrated to cause increased lactate production or falls in mitochondrial DNA suggestive of mitochondrial toxicity. However, stavudine and to a lesser extent zidovudine are poor substrates for mitochondrial thymidine kinase type 2, the predominant form in cells that are not actively mitotic such as neurons, myocytes and adipocytes. These are the cell types where the proposed mitochondrial toxicities neuropathy, myopathy and lipoatrophy are observed. Thus, active concentrations of phosphorylated products of stavudine and zidovudine may not be present in mitochondria.

The familial mitochondrial diseases do not have identical presentations to nucleoside analogue toxicities. These disorders most commonly involve the CNS, typically with seizures or dementia, and occasionally the kidneys. Although nucleoside analogues are known to penetrate the CNS and are commonly renally excreted unchanged, mitochondrial toxicities at these sites have not been documented.

Furthermore, toxicity caused by nucleoside or nucleotide analogues does not always appear to arise through the mitochondrial route. Cidofovir appears to cause renal tubular dysfunction via a toxic intracellular metabolite, and zidovudine-related anaemia appears to be related to decreased globin RNA synthesis. *In vitro* or animal models suggest that zidovudine myopathy, stavudine-related (but not zalcitabine- or didanosine-related) neuropathy and didanosine-related pancreatitis may all be not related, or not exclusively related, to mitochondrial dysfunction.

The integration of nucleoside analogues into nuclear DNA, best documented with zidovudine but likely to occur with other agents, represents an alternative but potentially delayed pathway to cytotoxicity and cell apoptosis. This is the mechanism of cell death during therapy with antineoplastic nucleoside analogues, and may have contributed to the multisystem toxicities observed with the anti-hepatitis B drug fialuridine. New research evaluating the effects of long term exposure of

cell lines is required to address the possibility that nuclear genotoxicity plays a role in long term nucleoside analogue toxicity.

1. Nucleoside Analogues Are the Cornerstones of Antiretroviral Therapy

The natural history of HIV-1 infection has been dramatically modified by the use of multidrug highly active antiretroviral therapy (HAART) regimens.[1] The recommended HAART regimens are all built around a backbone of 2 nucleoside analogues with either protease inhibitors or non-nucleoside reverse transcriptase inhibitors. Combinations involving 3 nucleoside analogues are also under evaluation. Current HAART regimens are unable to eradicate HIV-1 infection,[2,3] and thus the current HIV therapeutic paradigm involves continuing therapy for an indefinite period.

Although the benefits of HAART are clear, the long term risks have not been established. The longest clinical trials of HIV therapy have run for around 3 years, with the majority of large studies of HAART regimens lasting only 12 to 18 months. Cohort studies have established the long term risks of untreated HIV infection, providing actuarial assessments of risk of disease progression over 3, 6 and 9 years based on virus load and CD4+ cell counts. Although the risks of not treating HIV are clearly established, the point at which the risk-benefit of therapy favours intervention cannot be established until the risks of each treatment regimen are established over similar periods of follow-up.[4]

A range of adverse clinical and metabolic phenomena have been observed in individuals receiving HAART, which represent both significant management challenges and obstacles to treatment initiation or continuation. A wide spectrum of adverse effects observed during HAART have been hypothesised to relate to the impact of long term exposure to nucleoside analogues on human cellular mitochondrial function.[5-7] If these assertions are well founded, to achieve long term success with HAART it will be critical to choose nucleoside analogues that possess both potent antiretroviral activity and have limited (or no) impact on mitochondrial function.

This review discusses the evidence for and against the proposed mitochondrial toxicities of nucleoside analogues, evaluates their potential mechanisms and contrasts the manifestations with established familial mitochondrial diseases.

2. Mitochondrial Functions

Mitochondria are cellular organelles responsible for production of energy in the form of adenosine triphosphate (ATP). They are also involved in cholesterol and ethanol metabolism and the synthesis of testosterone and estrogens. Mitochondria are also the repositories of some of the key factors involved in cell apoptosis. The most metabolically active cells possess the most mitochondria. Within the mitochondrial matrix lies the only non-nuclear DNA in human cells, 2 to 10 copies of a covalently closed double-stranded DNA circle. This DNA, responsible for encoding 13 of the subunits of mitochondrial enzymes, plus transfer and ribosomal RNA, is essentially exclusively maternally inherited. The remaining 65 proteins necessary for mitochondrial formation, additional proteins for mitochondrial function. and the DNA polymerase γ responsible for copying mitochondrial DNA are encoded in nuclear DNA.

ATP may be produced from long, medium or short chain fatty acids or from pyruvate (derived from glucose). Long chain fatty acids require a transfer shuttle to cross the dual membrane of the mitochondrion, this process being dependent on carnitine. Fatty acids first undergo β-oxidation, a cycle of 4 reactions that progressively shortens the fatty acid by 2 carbon atoms per cycle and generates acetyl-CoA, reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide. Pyruvate is also converted to acetyl-CoA by pyruvate dehydrogenase in the mitochondrial matrix, this being the aerobic metabolism of glucose. In the absence of adequate mitochondrial metabolism, fatty acids accumulate in the mitochondria and cytoplasm as lipid droplets and pyruvate is metabolised to lactate

in the cytosol; this is the anaerobic metabolism of glucose.

Acetyl-CoA feeds into the Krebs or tricarboxylic acid cycle to generate further NADH. NADH is the substrate of oxidative phosphorylation, the highly efficient energy production pathway of the mitochondria. Oxidative phosphorylation involves 5 enzyme complexes located on the mitochondrial inner membrane. Complexes I and II, which incorporate ubiquinone (coenzyme Q-10), and the cytochrome-containing complexes III and IV together shuttle electrons derived from NADH to form a proton gradient which is coupled to complex V, ATP synthetase, enabling it to form ATP from cytosol-derived adenosine diphosphate (ADP) and inorganic phosphate.[8]

3. Mechanisms of Mitochondrial Toxicity: Is There a Difference between Fialuridine and Antiretroviral Nucleoside Analogues?

Errors in both nuclear and mitochondrial DNA (mtDNA) may lead to mitochondrial dysfunction. The mitochondrial DNA polymerase γ is more error prone than the nuclear DNA-copying polymerases α and ε and possesses neither protective histones nor repair enzymes like the nuclear β and δ polymerases. An individual's cells are likely to possess both functional and defective mtDNA as a heteroplasmy. When a cell divides, the functional mtDNA may not be evenly shared between the daughter cells, leading to differences in mitochondrial reserve between cells and ultimately between tissues. It has been hypothesised that a threshold exists for the appearance of mitochondrial disorders, which will be cell-, tissue- and individual-specific but is likely to affect the most metabolically active and energy-dependent cells first. The level of depletion of mtDNA required to reach the threshold may be 20% or less of normal values.[9-13]

Six nucleoside analogues are currently approved for antiretroviral therapy: zidovudine (ZDV), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC) and abacavir (ABC). Additionally, the nucleotides adefovir (for hepatitis B only) cidofovir (for cytomegalovirus disease) and tenofovir and the nucleosides 2′,3′-dideoxy-5-fluoro-3′-thiacytidine (FTC) and β-D-2,6-diaminopurine dioxolane (DAPD) are in clinical development. These nucleoside and nucleotide analogues are structural analogues of the natural nucleotides that form the building blocks of RNA and DNA in both human cells and viruses. Nucleoside and nucleotide analogues require intracellular phosphorylation by human cellular kinases to achieve their active triphosphate form. This triphosphate then competes with the natural nucleotide for inclusion in the growing DNA chain. Absence of the hydroxyl group at the 3′-position of the ribose ring in the analogue means a new 3′-5′ phosphodiester bond cannot be formed with the next nucleotide, and hence the further extension of this DNA strand is prevented. Nucleoside and nucleotide analogues are, therefore, both competitive inhibitors and chain terminators.[14] As pretranscriptional inhibitors of HIV, the activity of these analogues is restricted to acutely infected cells.

3.1 Intracellular Activation of Nucleoside Analogues

Nucleoside analogues enter the cell by nonfacilitated passive diffusion,[15] although their active removal by the multidrug resistance protein MRP4[16] may vary between cells and over time. The active triphosphate moieties have a relatively low affinity for human nuclear DNA polymerases, but *in vitro* inhibit isolated mitochondrial DNA polymerase γ at clinically relevant concentrations.[17]

Because of differing expression of activating kinases, lamivudine, zalcitabine and didanosine are preferentially phosphorylated in resting cells whereas zidovudine and stavudine are more efficiently activated in activated or dividing cells.[18] Because of a unique activation pathway, abacavir[19] is active across the range of infected cells. Rate-limiting steps or bottlenecks exist for the activation of both zidovudine and stavudine. With zidovudine the bottleneck is at the conversion of zidovudine monophosphate to zidovudine diphosphate by thymidylate kinase[20] and for stavudine it is at the level of mon-

ophosphorylation by thymidine kinase (TK).[21] Two forms of thymidine kinase, the enzyme responsible for the monophosphorylation of zidovudine and stavudine, exist: the cytosolic TK-1 and the mitochondrial TK-2. TK-1 is largely only expressed in the mitotic S-phase, and thus resting (postmitotic) cells such as neurons, myocytes and adipocytes, and cells that express low levels of TK-1 such as peripheral blood mononuclear cells (PBMCs) and monocyte-macrophages, activate the thymidine analogues using mitochondrial TK-2.[22] TK-2 is constitutively expressed in all cells but at lower levels than, when expressed, TK-1.[22] Zidovudine and stavudine have a higher affinity for TK-1 than for TK-2. Zidovudine, however, has higher affinity for both enzyme forms as demonstrated by relative phosphorylation rates of 0.52 and 0.04, as compared with 0.07 and 0.01 for stavudine, with TK-1 and TK-2, respectively.[23,24] The activity of TK-2 in macrophages is sufficient to provide some anti-HIV activity for zidovudine in these cells,[25] whereas stavudine is poorly activated in TK-1 deficient cells.[26] Thus, concentrations of the active forms of zidovudine and stavudine in TK-2 dependent cells are likely to be low, but will be higher for zidovudine. Surprisingly, resting TK-2 dependent cells include neurons,[22] myocytes[22] and, presumably, adipocytes, many of the cell types where the proposed mitochondrial toxicities of neuropathy, myopathy and lipoatrophy are observed. This incongruity may be best explained by concluding that these toxicities of thymidine nucleoside analogues are not mitochondrial toxicity.

The location of TK-2 in the mitochondria suggests that concentrations of any drug phosphorylated by this enzyme may be higher at this site than in the cytosol, an issue that has been shown to be important for nucleoside toxicity *in vitro*.[27] Indeed, nucleosides phosphorylated in the mitochondria become trapped and thus may cause selective damage to mtDNA.[27] Caution must be used in the interpretation of *in vitro* data (see section 3.3). However, using a strain of *Escherichia coli* with deleted thymidine kinases and human TK-2 introduced, investigators have recently compared the effects of a range of pyrimidine nucleosides on TK-2 (as measured by cell growth). Table I shows the effects of relevant agents (at 20 μmol/L) in TK- and TK-2+ cells (cultures in the absence of nucleosides = 100%). The 50% effective concentration (EC_{50}) values for agents with inhibitory effects were calculated as 4 mmol/L for fialuridine and 10 mmol/L for zidovudine. As no effect was seen for stavudine or lamuvudine, no EC_{50} value was presented.[24]

Table I. Effects of nucleoside analogues in *Escherichia coli* with deleted thymidine kinase (TK) or with human mitochondrial TK-2 inserted. Values are relative to untreated cells (= 100%)[24]

Compound	Growth (%)	
	TK deleted	TK-2+
Fialuridine	107	71
Zidovudine	85	46
Stavudine	95	96
Lamivudine	97	100

These data suggest that stavudine is poorly monophosphorylated, if at all, within mitochondria, limiting its potential to cause toxicity at this site, and may not be meaningfully phosphorylated in resting cells. Despite being a poorer substrate for TK-2 than for TK-1, it is clear from these data that zidovudine can be at least monophosphorylated in mitochondria. Thus, in resting cells not expressing TK-1, most phosphorylated zidovudine will be in the mitochondria. Therefore, zidovudine may still be able to cause mitochondrial-related toxicity in resting cells. This potentially explains the observation of depleted mtDNA in myocytes from zidovudine-treated patients with myopathy.[28]

3.2 Nucleoside Analogues and Human DNA Polymerases

Nucleoside analogues may also be graded on the *in vitro* affinity (Michaelis constant, Km) and inhibitory potency (inhibition constant, Ki) of their triphosphates for human DNA polymerases (i.e. the Ki/Km ratio), although the clinical relevance of this grading is not established. Using this approach with isolated polymerase γ, the greatest inhibitory potency is observed with zalcitabine triphosphate, ranging through didanosine and stavudine to lami-

vudine, zidovudine and abacavir triphosphates, which have the lowest inhibitory potential.[17]

In vitro cellular models of mitochondrial toxicity generally use a decrease of mtDNA or an increase in lactic acid production to assess effect.[29,30] Hierarchies of toxicity potential, not surprisingly, vary depending on the cell line used and whether the drug is provided in a phosphorylated form. In some studies, cytotoxicity is observed before mitochondrial toxicity is detected.[31] In general, lamivudine[15] and abacavir[32] demonstrate low toxicity. Lamivudine is removed at 'proof-reading' by polymerase γ, hence is not integrated into mtDNA, potentially explaining its relative lack of toxicity, although clearly it can act as a competitive inhibitor.

3.3 Caveats With *In Vitro* Data

The extent to which *in vitro* findings translate into the *in vivo* situation is not known. Since phosphorylation of nucleoside analogues differs between cell lines as well as between the mitotic phases of those cells, an agent that demonstrates toxicity in a cell line where it is efficiently phosphorylated may be markedly less toxic in a different cell line. None of the *in vitro* studies have tested all the available nucleoside analogues using the same assay system and across multiple cell lines. In addition, these studies invariably evaluate drugs on an individual basis rather than in the combinations in which they are used in clinical practice. Thus, the potential for additive or synergistic toxicity has not been tested. Although exposures in some *in vitro* studies represent realistic clinical exposures, these studies largely provide information on acute toxicity rather than on the effects of long term exposure. Additionally, the issue of differential tissue uptake (or efflux) has not been assessed or is difficult to translate into clinical practice.

For example, a comparison of zidovudine, didanosine and zalcitabine in myocytes found that all agents affected muscle cell proliferation and differentiation in a dose-dependent manner, increasing both cytoplasmic lipid droplet accumulation and lactic acid production. However, zalcitabine and didanosine, which are not typically associated with clinical myopathy, were the most potent inhibitors of mitochondrial function in muscle cells, whereas cytotoxicity was observed with zidovudine.[31] In HIV-infected persons developing myopathy while receiving zidovudine, mtDNA in myocytes has been reported to be diminished by as much as 78% relative to uninfected adults.[28] It is unclear if this extent of reduction in mtDNA alone is sufficient to be causative of clinical mitochondrial disease.

3.4 Fialuridine as a Model for the Toxicity of Nucleoside Analogues

Fialuridine is a fluorinated thymidine analogue for which development as an anti-hepatitis B agent was stopped following several deaths secondary to lactic acidosis and multisystem failure. Initial suggestions were that this was exclusively related to mitochondrial dysfunction.[33] These adverse effects were not predicted from initial *in vitro* or animal studies as the toxicity was delayed or subacute.

This agent may be more prone to causing mitochondrial toxicity than antiretroviral nucleoside analogues. First, unlike zidovudine and stavudine, fialuridine is a more efficient substrate for TK-2 than for TK-1,[34,35] making high intramitochondrial concentrations of phosphorylated products of fialuridine likely. Secondly, fialuridine possesses a 3′-hydroxyl group and is therefore readily able to form DNA internucleotide linkages;[36] in other words it is a less efficient chain terminator than other nucleoside analogue.

In vitro data suggest that treatment with fialuridine may lead to reductions in mtDNA in a dose-dependent manner, with 14 days exposure of the human hepatoma cell line HepG2 to 50 μmol/L causing a 90% decrease.[37] This is similar to the magnitude of mtDNA reduction observed with familial mitochondrial diseases. These data support the mitochondrial toxicity hypothesis with this agent. However, mitochondrial toxicity may not be the exclusive cause of the toxicity of this agent.

As fialuridine is a substrate for all human DNA polymerases, albeit most efficiently used by polymerase γ,[38] the drug accumulates in both nuclear

and mitochondrial DNA. This accumulation is observed across a range of tissues, with the highest concentrations in nuclear DNA occurring in the liver, the site where the toxicity of fialuridine was most evident. The net accumulation of fialuridine in nuclear DNA has been suggested to be a critical step in fialuridine-induced toxicity[39] and in rats (but not dogs and monkeys) was associated with apoptosis of hepatocytes.[39] *In vitro* studies using HepG2 cells indicated that fialuridine and its metabolites inhibited cell growth and were effectively phosphorylated, leading to a substantial increase in lactic acid production consistent with mitochondrial dysfunction.[40] This effect, as in the clinical events in humans and animal models.[41] was delayed, as a 2-week exposure to fialuridine was not associated with a decrease in total mitochondrial DNA content.[40]

This suggests that the genetic toxicity ('genotoxicity'), either nuclear or mitochondrial, of a nucleoside analogue may potentially appear some time after the agent is withdrawn or changed to an alternative agent.

To contrast with the data with fialuridine,[40] a recent study has evaluated the *in vitro* toxic effects of zidovudine, stavudine, lamivudine, didanosine and zalcitabine in HepG2 cells.[42] These data must be interpreted with the caveats detailed in section 3.3. Evidence for a number of mitochondrial defects with zidovudine, zalcitabine and didanosine were found, but only zidovudine induced a marked rise in lactic acid levels. No increases in *in vitro* lactic acid production or morphological changes in mitochondria were observed with either stavudine or lamivudine. Additionally, only in mitochondria isolated from zidovudine-treated cells was significant inhibition of cytochrome c oxidase and citrate synthase found,[42] providing an explanation for the respiratory chain effects observed in rat myocytes.[43] The study also demonstrated that zidovudine, stavudine and lamivudine did not affect the synthesis of the 11 polypeptides encoded by mtDNA, whereas zalcitabine caused 70% reduction of total polypeptide content and didanosine reduced by 43% the total content of 8 polypeptides. The authors hypothesised that in hepatocytes the reserve capacity for mitochondrial respiration is such that inhibition of respiratory enzymes alone is unlikely to become critical. In contrast, the combined inhibition of the tricarboxylic acid cycle and electron transport, only observed with zidovudine, greatly enhanced the dependence of the cell on glycolysis and may explain why apparent mitochondrial dysfunction was observed during zidovudine treatment.[42]

3.5 Lactic Acidosis and Nucleoside Analogues

Lactic acidosis, with or without hepatic microsteatosis, is the most serious presentation of nucleoside analogue toxicity. During the clinical study of fialuridine for hepatitis B virus infection, 7 of 15 treated patients developed severe hepatotoxicity and lactic acidosis, a further 3 milder changes, in most cases after cessation of the drug. Subsequently, 5 participants died and 2 patients survived after liver transplantation. Pancreatitis, neuropathy and myopathy often accompanied the syndrome. Liver histology showed macro- and microvesicular steatosis and abnormal mitochondria.[33] Lactic acidosis has been reported in persons receiving both single and dual nucleoside analogue regimens for HIV infection, including combinations of zidovudine or stavudine with didanosine, zalcitabine or lamivudine. Differences in the relative incidence of lactic acidosis between different agents or different combinations have not been established. The incidence of this problem is not known but it appears to be rare (<1 per 100 patient-years of therapy). The syndrome most commonly occurs in persons on prolonged (>6 months) therapy, although there may be additional risk factors. In particular, initial reports were most often in women, with factors such obesity and hepatitis B or C co-infection and advanced HIV disease often being present.[44] However, lactic acidosis has been reported across the range of humanity infected with HIV and a range of nucleoside analogue combinations. Additionally, transient nonfatal lactic acidosis has been reported in neonates receiving prophylactic zidovudine or zidovudine plus lamivudine.[45]

The onset may be either abrupt or insidious. Initial symptoms often include nausea, vomiting, and

abdominal pain, although in more insidious cases fatigue and bodyweight loss may predominate. A tender enlarged liver may be palpable. Subsequently, shortness of breath, tachypnoea and hyperventilation, liver and/or renal failure, clotting abnormalities, seizures, cardiac arrhythmia and death ensue.[46,47] Biochemical abnormalities include elevated lactate and lactate : pyruvate ratio, acidosis with low bicarbonate, widening of the anion gap, elevated lactate dehydrogenase and often (but not invariably) elevated hepatic transaminase and creatinine kinase levels. Histological examination of the liver may reveal diffuse microvesicular steatosis with slightly enlarged mitochondria.[48] The similarity in histological findings and presentation of lactic acidosis during antiretroviral nucleoside analogue therapy to the syndrome with fialuridine and some familial diseases is suggestive that this toxicity is mitochondrial. The association with hepatitis C may be an important risk factor, as infection with this virus (particularly genotype 1b) has been observed to deplete mtDNA and impair oxidative phosphorylation.[49] It is not known if other viral infections, including HIV, affect mitochondrial function. However, mitochondrial abnormalities have been reported in muscle biopsies from untreated HIV-positive individuals with myopathy,[50] raising this possibility.

3.6 Other Possible Mechanisms of Mitochondrial Toxicity

Nucleoside analogues may contribute to mitochondrial dysfunction through mechanisms other than inhibition of mitochondrial DNA polymerase γ. Zidovudine has been shown to increase mtDNA oxidised guanosine levels, a marker of free radical production, in mouse liver cells, the effect being limited by vitamins C and E.[51,52] Additionally, components of the respiratory chain may be affected. Zidovudine may bind to adenylate kinase, reducing ATP production.[53] Similarly, cardiomyopathy with zalcitabine is thought to be related to modulation of reactive oxygen species levels and ADP-ribosylation reactions.[54] One study has reported that zidovudine, but not other nucleoside analogues, significantly inhibits cytochrome c oxidase and citrate synthase.[42] Uncoupling of the proton gradient from the ADP/ATP translocator by zidovudine has also been reported in one experiment.[55] As mitochondrial function deteriorates with age,[56] the impact of drug toxicity on mitochondrial function may be most rapidly apparent in older individuals.

4. Non-Mitochondrial Cytotoxicity and Genotoxicity with Nucleoside Analogues

A range of adverse cellular events with nucleoside analogues appear unrelated to their effects on mitochondrial function or DNA polymerase γ. Possible mechanisms include integration into nuclear DNA, accelerated senescence secondary to telomere shortening and nuclear DNA hypermethylation.

Apoptosis may be triggered by nucleoside analogues.[57] Although mitochondrial cytochrome c appears important in the apoptosis pathway, the antineoplastic nucleosides fludarabine and gemcitabine induce apoptosis through their incorporation into nuclear DNA.[58] Cells treated with these drugs undergo apoptosis characterised by internucleosomal DNA fragmentation. Stavudine has been observed to induce apoptosis in HIV-infected, but not uninfected, MOLT-4 cells *in vitro*; however, the mechanism of this apoptosis or reasons for the selectivity are unknown.[59]

Antiretroviral nucleosides may accumulate in nuclear DNA over time.[60] Incorporation of zidovudine into human DNA[60] is well documented. Specific localisation of zidovudine into telomeric DNA[61,62] leads to telomere shortening, whereas prolonged passaging in didanosine, 2′,3′-dideoxyadenosine (ddA), stavudine or foscarnet does not cause reproducible telomere shortening or decreased cell growth rates or viability. This telomeric shortening appears irreversible despite the withdrawal of zidovudine.[63] In an experiment investigating long term exposure to nucleoside analogues *in vitro*, T-lymphocytic H9 cells were exposed over 7 months to zidovudine. This led to both chromosomal aberrations and nuclear frag-

mentation, and to mitochondrial damage with lipid accumulation.[64] Thus, during exposure to nucleoside analogues, or least to zidovudine, cellular injury may occur secondary to nuclear DNA damage. Such toxicity is likely to be 'late', increasing with time on nucleoside analogues as more and more nucleoside analogue accumulates in the nuclear DNA.[60] Additionally, the toxicity may be observed after the nucleoside analogue is removed, as stresses on the nuclear DNA such as at mitosis may trigger fragmentation. For example, with antineoplastic nucleoside analogues, S-phase cells, which also most actively incorporated the analogues into DNA, were most sensitive to the cytotoxic action of these compounds.[58]

During processive DNA synthesis, human DNA polymerase α is able to incorporate zidovudine monophosphate into DNA, causing chain termination. Polymerase β is also able to incorporate zidovudine monophosphate and zalcitabine monophosphate into DNA. Steady state kinetic analyses demonstrated that polymerase α inserts 1 zidovudine monophosphate molecule into DNA for every 2500 thymidine monophosphate molecules incorporated.[65] Steady state levels of stavudine incorporated into DNA are 10- to 50-fold lower compared with zidovudine in bone marrow cells.[21] The equivalent figure for fialuridine is 1 molecule per 90 thymidine molecules,[40] implying that this effect with zidovudine is likely to be observed more slowly that with fialuridine but more rapidly than with stavudine. The cytotoxicity of zidovudine has been associated with the intracellular concentrations of the monophosphate, the most predominant form of zidovudine in cells, but not the triphosphate form.[66] Incorporation of zidovudine into nuclear DNA has been suggested as a mechanism responsible for zidovudine-induced bone marrow toxicity.[67] Thus, genomic or nuclear DNA fragmentation and subsequent cellular apoptosis may be consequences of nucleoside analogue exposure.

Additionally, incorporation of nucleoside analogues into DNA may also have consequences on cell progeny because of the extent of genomic mutation, mostly secondary to deletions.[68] That is to say, even if cell division occurs successfully, the progeny cells may have significant genetic deletions and may be dysfunctional or unviable. Although the sister chromosome will probably compensate initially, as deletions accumulate the chance that a region of both chromosomes in a pair will be damaged increase. Additionally, the incorporation of zidovudine into the telomeric region[61,62] may shorten the number of cell cycles a treated cell may complete, resulting in premature cell senesence, an effect that again is likely to be observed during (or after) long term exposure. Further damage to nuclear DNA may occur through DNA hypermethylation. Interestingly, this may be gene-specific, with one *in vitro* study showing hypermethylation with zidovudine being specific to the thymidine kinase gene.[69]

Toxic metabolites may also be relevant for cytotoxicity. A toxic metabolite of zidovudine, 3′-amino-3′-deoxythymidine (AMT), has been observed in cultured hepatocytes and microsomes and is toxic to bone marrow cells, particularly erythroid lines.[70]

Finally, cytotoxicity, through mechanisms not described, may occur in the absence of overt mitochondrial toxicity.[31] In a study using cultured muscle cells prepared from human muscle biopsies, cells were exposed to various concentrations of zidovudine (4 to 5000 μmol/L), didanosine (5 to 1000 μmol/L) and zalcitabine (1 to 1000 μmol/L) over 10 days. Cell proliferation and differentiation and lipid droplet accumulation, lactate production and respiratory chain enzyme activities were evaluated. All 3 compounds induced a dose-related decrease of cell proliferation and differentiation. Zidovudine was the most potent inhibitor of cell proliferation. Zidovudine, didanosine and zalcitabine induced cytoplasmic lipid droplet accumulation, increased lactate production and decreased the activities of cytochrome oxidase (complex IV) and succinate dehydrogenase (part of complex II). Consistent with other data, zalcitabine and, to a lesser extent, didanosine were the most potent inhibitors of mitochondrial function whereas zidovudine was the most cytotoxic. The authors suggested that zidovudine-as-

sociated myopathy might not simply result from a direct mitochondrial toxic effect.[31]

5. Mitochondrial Disease in HIV and Other Areas of Medicine: Is Familial Disease Different to That Seen with Nucleoside Analogues?

Familial or inherited mitochondrial diseases are well recognised in medicine, particularly in paediatrics, neurology and hepatology.[9-13] Familial mitochondrial diseases generally relate to inherited or spontaneous mutations, deletions or rearrangements of genes involved in mitochondrial formation or function. In some cases, these mutations are not compatible with life. However, the majority of patients with defects of mtDNA have a mixture of both affected and unaffected (wild-type) mtDNA, intracellular heteroplasmy.

Diminished mitochondrial function results in a wide range of clinical diseases. The presentation is varied, even in individuals with the same genotype. In general, the impact of mitochondrial dysfunction becomes apparent in the most metabolically active tissues rather than the least active. Thus, manifestations commonly involve the brain, including the retina; the peripheral nervous system; cardiac and other muscles; and the endocrine, renal, gastrointestinal, haematological and hepatic systems. Only in Madelung's syndrome has a mitochondrial disorder been associated with changes in adiposity. For most of these disorders, depletion of mtDNA or mitochondrial dysfunction has been demonstrated in the tissues involved.[9-13]

The majority of specific disorders of mitochondriopathy involve multiple organs, such as MELAS syndrome (mitochondrial encephalopathy, lactic acidosis and stroke-like episodes) or Pearson's marrow-pancreas syndrome (bone marrow leading to haematological complications, pancreas resulting in pancreatitis and diabetes mellitus, liver leading to steatosis). Other disorders, such as chronic progressive external ophthalmoplegia, Navajo neuropathy or Fanconi's renal tubular syndrome, may predominantly manifest in a single organ.[9-13] The liver is commonly affected, with histology usually showing microsteatosis and often accompanied by fibrosis, cholestasis and loss of hepatocytes.[71] Neurological manifestations of mitochondriopathy commonly present in infancy or childhood, although others may be gradual in onset, occurring in adult or late adult life, possibly as mitochondrial function declines with age allowing levels of functional mtDNA to slip below the threshold of risk. Leigh syndrome (also called NARP; neuropathy, ataxia and retinitis pigmentosa), for example, results in focal spongy necrosis in the brain and progressive neurological dysfunction. As the syndrome progresses, multiple organ involvement leading to metabolic acidosis and death may occur.[60,72] A similar disorder that has been associated with mitochondrial DNA polymerase γ deficiency and mtDNA depletion is Alper's syndrome.[61] This progressive neurological disorder involves psychomotor retardation, seizures and paresis, together with elevated serum and/or cerebrospinal fluid lactate.[73]

In general, no management beyond supportive therapy is available for inherited disorders, although gene therapy and the use of peptide nucleic acids are being investigated.[13]

Table II compares the manifestations of familial mitochondrial disease with the clinical events suggested to relate to mitochondrial toxicity of the nucleoside analogues. Most striking is the absence of CNS and renal manifestations amongst the effects of nucleoside analogues, despite these agents being known to penetrate the CNS at therapeutic concentrations and many of these agents being predominantly renally excreted. The reason for this is not currently clear, but may relate to the fact that familial disease is present throughout ontogeny, whereas nucleoside analogues are generally given at a time when these tissues are mature.

This may explain the observation of possible CNS mitochondrial toxicity in children exposed *in utero* to zidovudine or zidovudine plus lamivudine. In a retrospective study across France, 8 cases of suspected mitochondrial dysfunction, including 5 deaths, were observed across 1754 mother–child pairs exposed to zidovudine alone or with lamivud-

ine during pregnancy. The exposure was on average 4 months *in utero* plus postnatal prophylaxis. In some cases symptoms did not develop until many months after exposure. With 2 of the infants, Leigh's or Alper's syndrome was suspected following a presentation of encephalopathy, seizures, lactic acidosis and demonstrable mitochondrial respiratory chain complex defects.[74] A review of data from infants exposed to nucleoside analogues in the US did not find any neurological events thought to be caused by mitochondrial toxicity, although lactic acidosis has been reported to the US Food and Drug Administration in 2 neonates treated with zidovudine plus lamivudine.[45]

However, as possible mitochondrial toxicity has been observed in other cell lines in adults, for example myocytes with zidovudine and peripheral neurons with zalcitabine,[6] it is difficult to explain the absence of nucleoside analogue related mitochondrial toxicity in the CNS, which as the most metabolically active organ in adults is potentially the most prone to mitochondrial function.

6. Mitochondrial Toxicity Is Not Always the Cause of Nucleoside Analogue Toxicity

The range of problems associated with nucleoside or nucleotide analogues resemble familial mitochondrial diseases, but on closer evaluation have not or may not turn out to be related to this mechanism. Importantly, demonstration of reduction in mtDNA to <20% of normal, the levels considered critical for familial disease, have not been convincingly or consistently demonstrated in clinical samples from persons experiencing nucleoside analogue–associated toxicities. Although this may be attributable to some nucleoside analogues having additional impact on other aspects of mitochondrial function, it may also be that many of these adverse effects are not exclusively related to mitochondrial toxicity.

The most well characterised of these is proximal renal tubular deficiency with the nucleotides adefovir and cidofovir, which closely resembles the familial mitochondrial disorder Fanconi's disease.[75] Closer evaluation of this toxicity, which can be prevented by the coadministration of probenecid, demonstrated that this tissue-specific toxicity is related to increased cellular uptake of these agents by the human renal organic anion transporter type 1 (hOAT1).[76-78] Cidofovir is then converted into toxic cidofovir-phosphocholine, which is thought to interfere with synthesis or degradation of membrane phospholipids.[77] Thus, this agent's toxicity is clearly not secondary to mitochondrial toxicity. Whilst the precise mechanism with adefovir has not been elucidated, this agent is an analogue of ATP and may therefore interfere with a range of energy-dependent processes.[77] Nucleoside analogues including zidovudine, stavudine, didanosine, zalcitabine, lamivudine and aciclovir have also

Table II. Symptoms of mitochondrial dysfunction caused by familial mitochondrial diseases versus nucleoside reverse transcriptase inhibitors (nucleoside analogues)

Familial	Nucleoside analogues
Neuromuscular	
External ophthalmoplegia and optic atrophy, sensorineural deafness, seizures/myoclonus, parkinsonism/dystonia, dementia, ataxia, stroke-like episodes, spastic paraparesis, encephalomyopathy, peripheral neuropathy	Peripheral neuropathy, deafness
Cardiac	
Cardiomyopathy, heart block	Cardiomyopathy
Endocrine	
Diabetes mellitus, hypoparathyroidism, hypogonadism, infertility, pancreatitis	Pancreatitis and diabetes mellitus, hypogonadism
Gastrointestinal	
Dysphagia, vomiting, pseudo-obstruction, steatosis and lactic acidosis	Steatosis and lactic acidosis
Renal	
Aminoaciduria, tubulointerstitial disease, Toni-Fanconi-Debre syndrome, Barrter syndrome (hypokalaemic alkalosis)	
Psychiatric	
Depression, psychotic illness	
Haematological	
Sideroblastic anaemia	Pancytopenias

recently been shown to be substrates of OAT1[79] but these agents have not been associated with renal dysfunction.

Full understanding of the mechanism of zidovudine-associated anaemia has not been established, but there is no evidence available to suggest that this toxicity is mitochondrial in nature. Zidovudine has been found to inhibit haemoglobin synthesis and globin gene transcription, and its toxic metabolite AMT may further contribute to this effect.[80] A further contribution may be made by the incorporation of zidovudine monophosphate into the nuclear DNA of these cells[67] and by down-regulation of erythropoetin receptors.[81]

As discussed above in section 3.4, zidovudine may exert some[43] but not all its effects on muscle through mitochondrial toxicity; direct cytotoxicity may also be important.[31] The histological features of zidovudine myopathy focused attention on the mitochondria, with some authors suggesting that the distinguishing features included ragged-red fibres and abnormal mitochondria with paracrystalline inclusions.[50,82,83] However, similar mitochondrial abnormalities have also been reported in therapy-naive persons with HIV-related myopathy.[50,84] Tissue-specificity studies with zidovudine have demonstrated that the greater impact on muscle cells, compared with kidney or liver cells, may involve inhibition of succinate transport systems rather than toxicity related to mitochondrial DNA depletion.[85] Thus, myopathy may not exclusively be a mitochondrial toxicity.

During phase I and II trials, the dose-limiting toxicity of didanosine, zalcitabine and stavudine was identified as peripheral neuropathy. In an *in vitro* model of nerve cells, zalcitabine and didanosine have been shown to reduce mtDNA, leading to destruction of mitochondria and an increase in intracellular lactate levels.[86] A second study confirmed this finding, but surprisingly found that pharmacologically relevant concentrations of stavudine as well as zidovudine and lamivudine had no impact on neurite regeneration or mtDNA levels, suggesting that stavudine-induced nerve toxicity may involve a different mechanism.[87] Depleted levels of acetyl-L-carnitine have been found in patients with peripheral neuropathy on zalcitabine, stavudine or didanosine therapy compared with those on the same drug but without peripheral neuropathy.[88] The main function of acetyl-L-carnitine is in mitochondrial β-oxidation of fatty acids and membrane energy balance.[89] Acetyl-L-carnitine may also increase the rate of peripheral nerve regeneration following injury by promoting release of nerve growth factor.[90] In the short term, depletion of acetyl-L-carnitine disrupts mitochondrial metabolism and causes a toxic accumulation of fatty acids.[88] This provides a further mechanism for the neurotoxicity of nucleoside reverse transcriptase inhibitors that is not related to a direct effect of the nucleoside analogue on mitochondrial DNA.

Recent understanding of the mechanisms involved in the genesis of pancreatitis point to the conclusion that this adverse effect associated with didanosine use is influenced by its metabolism down the purine pathway. Long term didanosine exposure does not appear to have direct toxic effects on the pancreas.[91] However, the role of oxygen-derived free radicals in the pathogenesis of pancreatitis has been demonstrated with a range of chemicals.[92] Didanosine is metabolised to hypoxanthine, xanthine, uric acid and allantoin.[93] Xanthine oxidase is an important generator of free radicals, suggesting that the increased activity of this enzyme secondary to didanosine metabolism may be the source of pancreatitis-inducing free radicals.[94] As allopurinol treatment prevents the generation of reactive oxygen metabolites, it may have a beneficial effect in diminishing the risk of pancreatitis,[92] although further data evaluating the potential for allopurinol to increase didanosine exposure are needed before use of this drug is investigated in clinical practice.

7. Conclusions: Mitochondrial Toxicity Occurs with Nucleoside Analogues But Is Not the Cause of All Adverse Effects

Definitive evidence that the range of observed toxicities with antiretroviral nucleoside analogues is related to inhibition of mitochondrial DNA poly-

merase γ and subsequent mtDNA depletion are lacking. Although some overlap exists between the presentation of the adverse effects of nucleoside analogues and familial mitochondrial disorders, this overlap is far from complete. First, familial diseases tend to be multisystem, whereas nucleoside analogue toxicities tend to occur at a single site (although neuropathy and myopathy have been reported in individuals subsequently presenting with lactic acidosis). Secondly, familial disorders commonly involve the CNS and often the kidney, whereas toxicity at these sites is rare or unreported with nucleoside analogues.

Clearly, in some *in vitro* systems and with sufficient quantities of drug, mitochondrial toxicity, as evidenced by respiratory chain defects, lactate production, lipid droplet accumulation, declines in mtDNA or ultrastructural changes in mitochondria, can be observed. Interpretation of data from *in vitro* systems is, however, difficult. Differences in the phosphorylation of nucleosides and nucleotides in different cell lines and at specific sites may be particularly relevant. Resting cells such as neurons, myocytes and adipocytes, as well as PBMCs and macrophages, are all likely to activate thymidine analogues only poorly, and so these drugs may have a lower potential for causing toxicity in resting cells than in actively dividing cells. The toxic nucleoside analogue fialuridine was more efficiently monophosphorylated by mitochondrial TK-2 than by cytoplasmic TK-1, whereas stavudine is a poor substrate for both enzymes and zidovudine is less efficiently activated by TK-2. Thus, low concentrations of phosphorylated zidovudine will exist in mitochondria, and in certain patients may be sufficient to be toxic at least to myocytes, whereas any phosphorylated stavudine may need to diffuse, or be transported, into the mitochondria before toxicity could occur.

Free radicals may be important in nucleoside toxicity, potentially playing a role in didanosine-related pancreatitis. *In vitro* data have demonstrated the role of free radicals in the effects of zalcitabine on rat myocardium. An increase in mtDNA oxidised guanosine levels, a marker of free radical production, has also been observed in mouse liver cells treated with zidovudine. This effect was limited *in vitro* by treatment with vitamins C and E. However, the impact of free radicals is also likely to be greater if mitochondrial dysfunction is present.

Chemotherapeutic nucleoside analogues cause cellular damage through integration into genomic or nuclear DNA, leading to nuclear chromosomal breakage, which in turn triggers cellular apoptosis. Integration into nuclear DNA has also been observed with antiviral nucleoside analogues; on the basis of the available data, integration rates amongst the thymidine analogues are highest for fialuridine, intermediate for zidovudine and lowest for stavudine. Cells exposed long term to zidovudine *in vitro* demonstrate both chromosomal aberrations and nuclear fragmentation as well as mitochondrial damage with lipid accumulation. Duration of exposure appears to correlate with the extent of zidovudine accumulation in genomic DNA, and as zidovudine is a substrate for polymerase β it may not be removed from the DNA even after the drug is discontinued. Additionally, deletions in genomic DNA caused by nucleoside analogue–related chain termination may have greater effects in progeny cells than in the parent cell. Thus, the genotoxicity of nucleoside analogues may be delayed. Delayed toxicity has been observed with fialuridine in the clinical trial of this agent and, *in vitro* at least, damage to mtDNA with zidovudine appears to be delayed. Finally, as genomic integration of zidovudine is greatest in the telomeric region of the chromosomes, an observation not seen with didanosine, stavudine or foscarnet, this may accelerate cellular aging or diminish the number of cell divisions a cell line may complete.

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