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Down-regulation of mitochondrial thymidine kinase 2 and deoxyguanosine kinase by didanosine: Implication for mitochondrial toxicities of anti-HIV nucleoside analogs

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ABSTRACT

Mitochondrial thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) catalyze the initial rate limiting phosphorylation of deoxynucleosides and are essential enzymes for mitochondrial function. Chemotherapy using nucleoside analogs is often associated with mitochondrial toxicities. Here we showed that incubation of U2OS cells with didanosine (ddI, 2′,3′-dideoxyinosine), a purine nucleoside analog used in the highly active antiretroviral therapy (HAART), led to selective degradation of both mitochondrial TK2 and dGK while the cytosolic deoxycytidine kinase (dCK) and thymidine kinase 1 (TK1) were not affected. Addition of guanosine to the ddI-treated cells prevented the degradation of mitochondrial TK2 and dGK. The levels of intracellular reactive oxygen species and protein oxidation in ddI-treated and control cells were also measured. The results suggest that down-regulation of mitochondrial TK2 and dGK may be a mechanism of mitochondrial toxicity caused by antiviral and anticancer nucleoside analogs.

1. Introduction

Didanosine (ddI, 2′,3′-dideoxyinosine) is one of the first nucleoside reverse transcriptase inhibitor approved for use in anti-HIV therapy and it is still used in combination with other nucleoside or non-nucleoside reverse transcriptase inhibitors in highly active antiretroviral therapy (HAART) [1]. Treatment with nucleoside analogs is often associated with mitochondrial toxic side effect e.g., cardiomyopathy, neuropathy and lipodystrophy, especially common in patients treated with AZT (Zidovudine) and ddI. This has become apparent since HAART has significantly increased the life expectancy of HIV infected individuals [2,3]. ddI is toxic primarily to the nervous system, including peripheral neuropathy, headache and retinal toxicity; and the gastrointestinal system, including pancreatitis and hepatitis [4].

Unlike many other nucleoside analogs used in anti-HIV therapy, the initial phosphorylation of ddI is not catalyzed by cellular deoxynucleoside kinases, but rather by the 5′-nucleotidase/nucleoside phosphotransferase, using IMP (inosine monophosphate) as phosphate donor as depicted in Fig. 1 [5]. The product, ddIMP (2′,3′-dideoxyinosine 5′-monophosphate) is converted to ddAMP (2′,3′-deoxyadenosine 5′-monophosphate) by adenylosuccinate lyase and adenylosuccinate synthetase. ddAMP is then further metabolized to its active form ddATP (2′,3′-dideoxyadenosine triphosphate) by nucleoside mono- and diphosphate kinases [6]. Although ddA can be phosphorylated by cellular enzymes to ddATP, in cells the majority of ddA is rapidly deaminated to ddI by adenosine deaminase. In addition, ddI can be degraded by purine nucleoside phosphorylase to hypoxanthine. The mechanism of action of ddATP is inhibition of HIV reverse transcriptase by competing with natural occurring substrate e.g., dATP. In addition ddATP can act as chain terminator once incorporated into the nascent DNA chain by the HIV reverse transcriptase. ddATP can also inhibit mitochondrial DNA polymerase and thus causes mitochondrial toxicities (Fig. 1) [7,8].

Mitochondrial thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) catalyze the initial rate-limiting phosphorylation of all four deoxynucleosides e.g., thymidine, deoxycytidine, deoxyguanosine and deoxyadenosine to their respective monophosphates and play an important role both for mitochondrial DNA (mtDNA) replication and nuclear DNA repair. Deficiency in either TK2 or dGK activity due to genetic mutations causes devastating mitochondrial DNA depletion syndrome (MDS), which is characterized by tissues specific mtDNA depletion or deletion [9,10]. Depletions of mtDNA have been reported in patients treated with either AZT (zidovudine, 3′-azido-2′,3′-dideoxythymidine) or ddI and are associated with myopathy, neuropathy, and cardiomyopathy,

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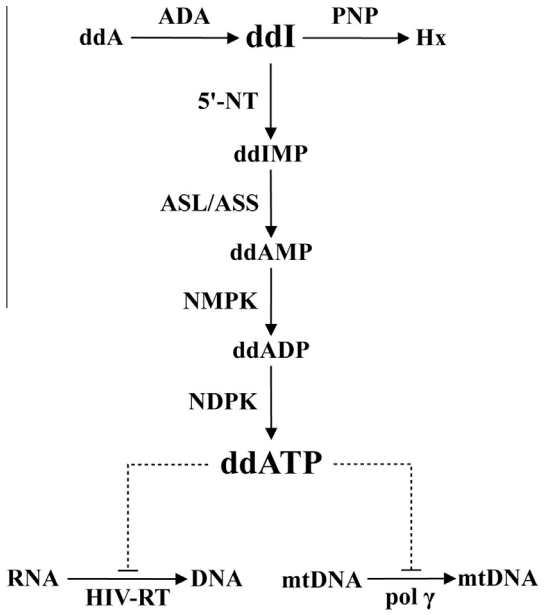

Fig. 1. Schematic representation of ddI metabolism and mechanism of action. ddI, 2′,3′-dideoxyinosine, ddA, 2′,3′-dideoxyadenosine, ddIMP, ddI monophosphate, ddAMP, ddA monophosphate, ddADP, ddA diphosphate, ddATP, ddA triphosphate; Hx, hypoxanthine. Enzymes: 5′-NT, 5′-nucleotidease/IMP phosphotransferase; ASL, adenylosuccinate lyase; ASS, adenylosuccinate synthetase; ADA, adenosine deaminase; NMPK, nucleoside monophosphate kinase; NDPK, nucleoside diphosphate kinase; HIV-RT, HIV reverse transcriptase; pol γ, mitochondrial DNA polymerase γ; PNP, purine nucleoside phosphorylase.

similar to what have been observed in the MDS patients with TK2 or dGK deficiency [2,11–13].

In this paper we describe our finding that treatment of cultured human cells with ddI caused severe reduction of mitochondrial dGK and TK2 levels, but had no effect on the cytosolic deoxycytidine kinase (dCK) or thymidine kinase 1 (TK1) levels. Co-incubation with guanosine reversed the effects of ddI on TK2 and dGK. The levels of intracellular reactive oxygen species (ROS) and protein carbonyl group were also determined.

2. Materials and methods

2.1. Materials

2′,3′-dideoxyinosine (ddI) was purchased from Carbosynth. Guanosine (Gua) was obtained from Sigma. Mouse monoclonal antibodies against cytochrome c oxidase subunit II (COX II) and subunit IV (COX IV), and anti α-tubulin antibody were purchased from Abcam. Polyclonal rabbit anti β-tubulin antibody was from Novus Biologicals. Polyclonal rabbit anti-human TK2 and dGK antibodies were produced using synthetic peptides chosen from the C-terminal sequences and affinity purified [14]. A mouse monoclonal anti-human TK1 antibody [15] obtained from AroCell AB (Uppsala, Sweden) and a polyclonal rabbit anti-human dCK antibody [16] were used to detect the TK1 and dCK proteins, respectively. All antibodies were diluted with recommended ratios in blocking buffer (0.05% Tween-20 and 5% non-fat milk in phosphate-buffered saline (PBS).

2.2. Cell culture conditions

U2OS (human osteosarcoma cell line, ATCC HTB-96™) cells were maintained in McCoy's 5A (Modified) medium (Gibco®, Cell Culture) supplemented with 10% fetal bovine serum (FBS, Gibco® Cell Culture), 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C with a humid atmosphere in the presence of 5% CO_2. All nucleosides were dissolved in dimethylsulfoxide (DMSO) as stock solutions and diluted in fresh cell culture medium prior to use. The final DMSO concentration in the complete medium was <0.05% (v/v).

2.3. Mitochondrial isolation and Western blot analysis

Mitochondria were prepared by differential centrifugation method as previously described [14,17]. For total cellular protein extraction, approximate 1×10^6 cells were re-suspended in 20 μl lysis buffer (50 mM Tris/HCl pH 7.6, 150 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 1× protease inhibitor cocktail (Roche), 0.5% NP-40, and 0.3 M sucrose) and cellular proteins were extracted by freezing and thawing thrice and sonication in an ice/water bath, followed by centrifugation at 16,000×*g* at 4 °C for 20 min. Protein concentration was determined by the Bradford method (Bio-Rad protein assay) using BSA as standard.

Mitochondrial proteins (30 μg/lane) and total cellular proteins (100 μg/lane) were subjected to 12% reducing SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon polyvinylidene difluoride (PVDF, Millipore) membranes using a semi-dry transfer system. Membranes were incubated with blocking buffer at room temperature for one hour and then probed with the respective primary antibodies. The anti-mouse or anti-rabbit secondary antibodies conjugated with horseradish peroxidase (GE Healthcare) were applied to the membranes and the target proteins were detected by enhanced chemiluminescence immunodetection system (ECL kit; GE Healthcare). Band intensities were quantified by using the Quantify One/ImageLab software (Bio-rad).

2.4. Effects of ddI on mitochondrial TK2 and dGK and cytosolic TK1 and dCK levels

About 6×10^6 cells were seeded in 175 cm^2 tissue culture flasks and incubated in the presence of 20 μM ddI for 3 days. Mitochondria were then isolated and the levels of TK2 and dGK in mitochondrial extracts determined by western blot analysis with polyclonal rabbit anti human TK2 and dGK antibodies. In addition, guanosine (Gua) (20 μM) were supplemented to ddI treated cells as described above and mitochondria were then isolated and the levels of TK2 and dGK in mitochondrial extracts were determined [14].

To access the effects of ddI in the absence or presence of guanosine on the cytosolic TK1 and dCK levels, total cell lysates (~100 μg protein) prepared from ddI-treated and control cells were resolved on 12% SDS–PAGE and the levels of TK1 and dCK were determined by western blot analysis, using antibodies against human TK1 [15] and dCK [16].

2.5. Measurement of ROS levels and protein oxidation

Triplicates of 0.3×10^6 U2OS cells were seeded into individual wells of 6-welll flat-bottomed plates and incubated for three days in complete culture medium in the presence of various concentrations of ddI. To detect the intracellular ROS production, cells were treated with 5 μg/ml CM-H_2DCFDA/PBS for 15 min at 37 °C in dark. The cells were then trypsinized and re-suspended in PBS buffer. The intensity of oxidized CM-H_2DCFDA fluorescence was immediately measured by using a FACScan® flow cytometer/Cell Quest software (Becton Dickinson, CA, USA). The data for 10,000 events were collected for each sample and analyzed by the Flowjo software (Tree Star Inc, Asland, OR) and ROS levels were reported as the geometric mean florescence of the collected cells.

Protein oxidation (carbonylation) in total extracts of U2OS cells was evaluated by the Oxyblot assays, following the manufacturer's

instructions. In brief, 15 μg protein was reacted with 2,4-dinitrophenylhydrazine (DNPH) at room temperature for 15 min in 6% SDS (w/v) and then subjected to Western blot analysis, using an anti-DNP antibody (1:150) to determine the content of protein carbonyl groups. Blots were subsequently re-probed for α-tubulin or COX IV immuno-reactivity, which was utilized as loading control.

2.6. Statistical analysis

Experimental group differences were evaluated by using Student's *t* test performed with the GraphPad Prism software. Differences were considered as significant when a *P* value is <0.05.

3. Results

3.1. Treatment of U2OS cells with ddI led to down regulation of mitochondrial TK2 and dGK

Long term use of nucleoside analogs such as AZT and ddI in anti-HIV therapy is associated with mitochondrial toxicities leading to myopathy, neuropathy and lipodystrophy [18]. We wanted to investigate if ddI treatments have any effect on the mitochondrial TK2 and dGK protein levels. U2OS cells were incubated with 20 μM ddI for 3 days and then mitochondria were isolated. Mitochondrial proteins were separated by SDS–PAGE and TK2 and dGK protein levels were determined by western blot analysis. As shown in Fig. 2 both the TK2 and dGK protein levels were decreased, 30% and 60%, respectively. The cytochrome c oxidase subunit II (COX II), encoded by mitochondrial DNA, also decreased ~10% in ddI-treated cells, while the levels of cytochrome c oxidase subunit IV (COX IV), coded by nuclear DNA, were unchanged (Fig. 2). The levels of β-tubulin, used as a control, were the same in all cases (Fig. 2). These results demonstrated that mitochondrial TK2 and dGK protein levels are affected by ddI treatment, and this is most likely the reason for the reduction of the mtDNA encoded COX II levels.

3.2. Co-incubation with guanosine prevented down regulation of mitochondrial TK2 and dGK

Guanosine has been shown to protect human neuroblastoma cells against mitochondrial oxidative stress [19]. Guanosine was added to the cells treated with ddI, after 3 days the cells were harvested and mitochondria were isolated. Total mitochondrial proteins were used to determine the level of TK2 and dGK proteins by western blot analysis. Again, ddI caused pronounced reduction of both TK2 and dGK protein levels, and a mild reduction of the COX II level was also observed (Fig. 3). Addition of Guanosine prevented the down regulation of mitochondrial TK2 and dGK as well as the COX II protein. The COX IV levels were unchanged (Fig. 3). These results demonstrated that guanosine apparently protected

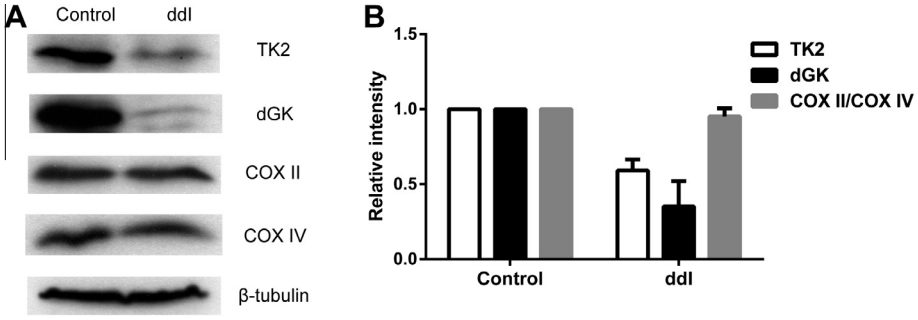

Fig. 2. TK2 and dGK protein levels in cells treated with ddI. U2OS cells were incubated in the presence and absence of 20 μM ddI for 3 days, and then mitochondria were isolated and used to determine the level of TK2 and dGK by Western blot analysis (A). The band intensities were quantified and are shown as TK2 and dGK levels relative to the controls after normalization to the level of COX IV, the ratio of COXII/COX IV was also shown (B). The levels of β-tubulin used as controls.

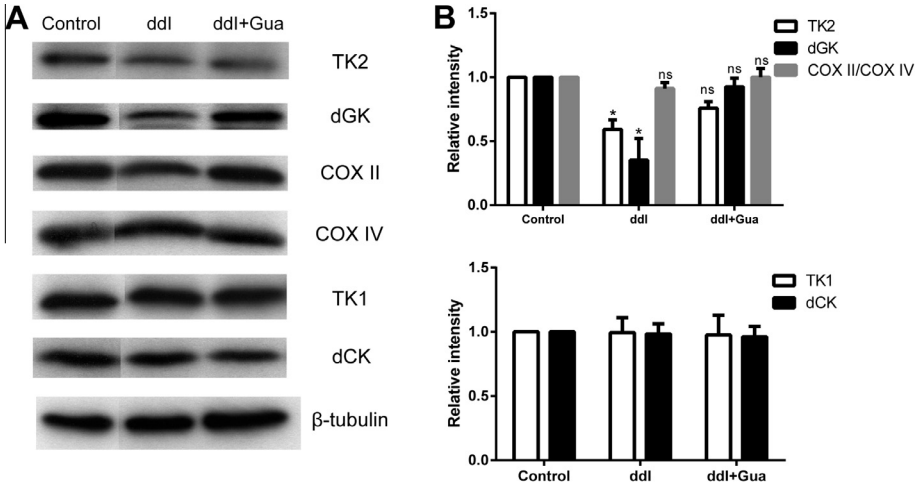

Fig. 3. The effects of guanosine supplementation on the levels of mitochondrial TK2 and dGK, and cytosolic TK1 and dCK. Total cell extracts and mitochondrial extracts were prepared from U2OS cells treated with 20 μM ddI alone or in combination with 20 μM guanosine (Gua) for 3 days. The levels of mitochondrial TK2 and dGK, cytosolic TK1 and dCK, COX II, COX IV and β-tubulin were determined by western blot analysis (A, lanes 1-3: control, ddI, ddI plus Gua); the protein bands were quantified and are shown as levels relative to the control (B). The levels of β-tubulin used as controls. The data are presented as the mean ± SEM ($n = 3$, $^{**}p < 0.01$ vs controls; $^{*}p < 0.05$ vs controls). ns, not significant.

TK2 and dGK from ddI induced effects but the mechanism is presently not known.

3.3. Cytosolic TK1 and dCK protein levels were not affected by ddI treatment

To investigate if the ddI effects are mitochondrial specific, total proteins were extracted from cells treated with ddI alone or in the presence of guanosine. The levels of TK1 and dCK were determined by Western blot using the anti human TK1 and dCK antibodies. As shown in Fig. 3 there were no significant changes in TK1, dCK or the control proteins e.g., COX IV and β-tubulin levels. These results indicated that the effects of ddI are mitochondrial specific.

3.4. No alterations of cellular ROS and protein carbonylation levels in ddI-treated cells

Several antiviral nucleoside analogs have been shown to induce oxidation stress [20–24], therefore, we measured the levels of ROS and protein carbonyl content in cells treated with ddI. As shown in Fig. 4 total cellular ROS were at similar levels for cells treated with different concentrations of ddI; total protein carbonyl content showed some variation but the changes were not significant. The levels of cytosolic TK1 protein were also determined and we found no apparent changes. The levels of α-tubulin, used as loading control, were at similar levels in all samples (Fig. 4). These results strongly suggest that the ddI effect on mitochondrial TK2 and dGK is probably not linked to oxidative stress.

4. Discussion

In this study we demonstrated that treatment of cultured cells with ddI caused profound reduction of mitochondrial TK2 and dGK proteins levels, although these two enzymes are not known to be involved in the metabolism of ddI. The ddI effect were mitochondrial specific since only TK2 and dGK were down-regulated but not the cytosolic TK1 and dCK, nor other control proteins such as COX IV, α-tubulin, and β-tubulin, which all remained at the similar levels even in cells treated with high concentration of ddI. Interestingly co-incubation with guanosine abolished the ddI effects on both TK2 and dGK protein levels.

Deficiency in TK2 or dGK is associated with tissues specific mtDNA depletion syndrome in humans [9] and it is likely that the ddI induced reduction in TK2 and dGK levels will result in mtDNA depletion, particularly in case of prolonged ddI treatments. Our results showed that even in short time incubation with 20 μM ddI the levels of mtDNA encoded protein COX II was reduced, indicating a reduction of mtDNA copy number in the U2OS cells.

Several nucleoside analogs have been shown to induce time- and dose-dependent mtDNA depletion, cell growth inhibition, and elevation of intracellular lactate and lipids in cultured HepG2 cells, CEM cells, human muscle cells, and healthy primary human T lymphocytes [4,25]. In mice exposed to ddI, AZT, D4T and 3TC mtDNA depletion was observed in liver, muscle and cortical neurons but not in other tissues [26], which is similar to what was observed with the TK2 knockout mice [27,28].

Mitochondrial malfunction causes oxidative stress, which has been implicated in many diseases. TK2 has been shown to be

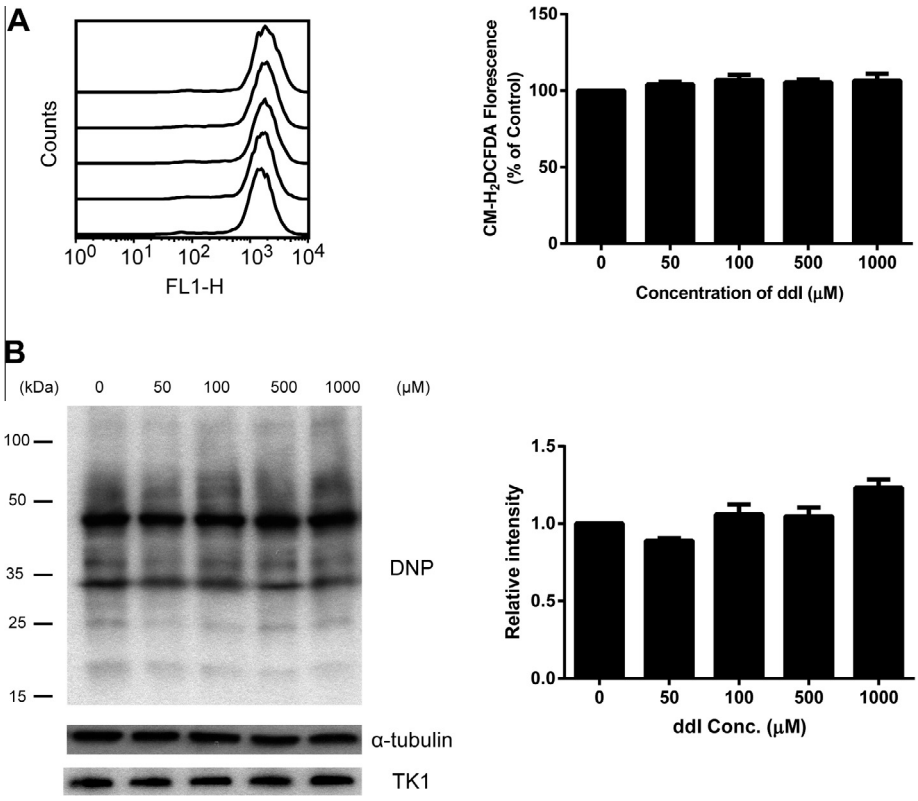

Fig. 4. The levels of total cellular ROS and protein carbonyl content in U2OS cells after 3 days of exposure to ddI. Cells were prepared as described in the Section 2. (A) Left panel, representative flow cytometry offset histogram of the fluorescence of oxidized products of CM-H_2DCFDA in U2OS cells exposed to various concentrations of ddI (the curves from bottom to top represent treatments with 0, 50, 100, 500, and 1000 μM ddI); right panel, relative intracellular ROS levels in cells treated with ddI. The results are expressed as the average percentage ± standard error of the mean (SEM) of the control value ($n = 3$). (B) Left panel, representative Western blot of protein carbonylation using the Oxyblot assay; right panel, the carbonyl content of total cellular proteins were normalized to the levels of α-tubulin and are presented as percent of the control (untreated cells) values. The levels of cytosolic TK1 were also determined in these samples.

sensitive to oxidative stress induced modification and down regulation [14]. Similar to TK2, our preliminary data showed that dGK is also down regulated under oxidative stress (data not shown). Earlier studies have shown that AZT and D4T, but not ddI or 3TC, cause increased ROS production in normal and cancerous human cells, and cells from AIDS patients [20–24]. In this study there was no alteration in ROS levels, nor were the levels of proteins oxidation in ddI-treated cells. Therefore, the ddI effects observed with TK2 and dGK are probably not explained by ROS generated in the presence of the nucleoside analogs but may be related to other signaling mechanism induced by nucleotide pool alterations.

Tempol, a cyclic nitroxide with unique antioxidant property, protects cardiomyocytes from AZT and ddI induced mitochondrial toxicity [29], possibly by reducing the mitochondrial ROS levels. Uridine supplementation in antiviral therapy reduced peripheral neuropathy and encephalopathy both in AIDS patients and in animal model [30–32], but the mechanism is not known. Here we showed that guanosine could restore the level of TK2 and dGK to normal in cells treated with ddI. Although the mechanisms involved are not known, it is possible that uridine, tempol and guanosine reduce the level of cellular oxidative and/or nucleotide stress or increase the transcription/translation of critical genes involved in antioxidant defense, and thus protect TK2 and dGK from modifications and degradation. These results demonstrated that antioxidants supplementation in anticancer and antiviral therapy using nucleoside analogs may be beneficial to the patients via several mechanisms by reducing cellular stress levels.

Adequate and balanced dNTP pools are vital both for nuclear DNA and mtDNA replication and maintenance, and imbalanced dNTP supply has been shown to cause mtDNA depletion [33–35]. Reduction of TK2 and dGK levels cause depletion of mitochondrial DNA precursor pools and thereby stalk mtDNA synthesis leading to mtDNA depletion and impaired mitochondrial function. Most likely down-regulation of TK2 and dGK is a general mechanism for induction of mtDNA depletion caused by nucleoside reverse transcriptase inhibitors.

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