## Supplementary materials

# Zidovudine Induces Down-Regulation of Mitochondrial Deoxynucleoside Kinases: Implications for Mitochondrial Toxicity of Antiviral Nucleoside Analogs

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*Running title: *Down-regulation of mitochondrial TK2 and dGK by AZT*

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## Materials and Methods

**S-Glutathionylation of recombinant proteins.**
Recombinant human deoxyguanosine kinase (dGK), deoxycytidine kinase (dCK), cytosolic thymidine kinase 1 (TK1) and *Drosophila melanogaster* deoxynucleoside kinase (Dm-dNK) were purified as previously described (1-4). Two μg recombinant proteins were incubated with various concentrations of oxidized glutathione (GSSG) at room temperature for one hour and analyzed by 12% non-reducing SDS-PAGE and western blot using a mouse monoclonal anti-GSH antibody (abcam). The activities of the GSSG treated enzymes were determined using tritium labeled substrates, [$^3$H]-dThd for TK1 and Dm-dNK, [$^3$H]-dCyd for dCK and [$^3$H]-dGuo for dGK as previously described (1-4).

**Oxidation of recombinant TK2 and dGK.** Recombinant human TK2 and dGK were purified as previously described (1, 5) and diluted to 0.4 mg/ml in buffer containing 50 mM HEPES/KOH, pH 7.4, 100 mM KCl, and 10 mM $MgCl_2$. The oxidation reaction was initiated by the addition of a freshly prepared mixture of 100 μM $FeCl_3$ and 25 mM L-ascorbic acid with/without 1 mM $H_2O_2$ as previously described (6). After one hour incubation at 37°C, the reaction was terminated by the addition of 1 mM EDTA, and the carbonyl content of the oxidized TK2/dGK was determined by the Oxyblot assay and western blot analysis.

## Results and discussion

**S-glutationylation of recombinant human dGK and dCK**
We have shown earlier that the degradation of mitochondrial TK2 is S-glutathionylation dependent under oxidative stress and S-glutathionylation on Cys-189 is responsible for mitochondrial degradation of TK2 (7). dGK belongs to the TK2 enzyme family, which consists of TK2, dGK, dCK and Dm-dNK (*Drosophila melanogaster* deoxynucleoside kinase) (8). The Cys-189 in TK2 is conserved among TK2, dGK (Cys-199) and dCK (Cys-185) but not in Dm-dNK according to amino acid sequence alignment (7). In order to examine the possible involvement of S-glutathionylation in the redox regulation of the TK2 family enzymes, recombinant human dGK, dCK and Dm-dNK were incubated with GSSG and subsequently analyzed by SDS-PAGE and western blot using the anti-GSH antibody. Human dGK is readily S-glutathionylated with 0.1 mM GSSG and increased further with increasing concentration of GSSG and reached complete S-glutathionylation with 10 mM GSSG as judged by molecular weight changes in SDS-PAGE. Human dCK, on the other hand, required 5 mM GSSG to detect any S-glutathionylation (Fig. S1A). Dm-dNK showed no glutathionylation even with 10 mM GSSG. Human TK1, which belongs to another enzyme family, also tested and showed no glutathionylation (Fig. S1A).

Dm-dNK contains total 5 cysteine residues and human TK1 contains total 11 cysteine residues, since both Dm-dNK and TK1 showed negative results with the anti-GSH antibody, suggesting that the antibody specifically recognized glutathione attached to a specific thiol group. Since only dGK and dCK possess the conserved Cys residue and showed concentration dependent S-glutathionylation, it is most likely that the Cys-199 in dGK and Cys-185 in dCK were S-glutathionylated.

The activities of GSSG treated enzymes were determined using tritium labeled substrate specific for each enzyme. Similar to TK2 (7), dGK activity decreased with increasing concentration of GSSG (Fig. S1B). dCK activity, however, increased in the presence of high concentration of GSSG. Dm-dNK and TK1 activity were not affected by GSSG (Fig. S1B). These results strongly suggest that S-glutathionylation serves as a regulatory mechanism for TK2, dGK and dCK activity and also plays an important role in the regulation of protein stability.

**Oxidation of total mitochondrial proteins and recombinant TK2 and dGK.** To determine the levels of mitochondrial protein oxidation in the presence of $H_2O_2$, U2OS cells were treated with 0, 1 and 4 mM $H_2O_2$ for 1 hour, followed by mitochondrial isolation and protein extraction. Mitochondrial protein carbonylation was analyzed by the Oxyblot assay. As shown in Figure S2A, the total carbonyl contents of mitochondrial proteins increased 2-fold in the mitochondria isolated from 4 mM $H_2O_2$-treated cells (Fig. S2A).

Because of the low abundance of TK2 and dGK proteins in the mitochondria of the U2OS cells treated with oxidants, we used recombinant TK2 and dGK proteins to study the sensitivity of these two enzymes to oxidation. Two μg of recombinant TK2 and dGK were incubated in the presence of 100 μM $FeCl_3$ and 25 mM ascorbic acid, which are the most commonly used concentrations for mimicking intracellular metal-catalyzed protein oxidation (6). After one hour at 37°C in the presence or absence of $H_2O_2$, the reactions were stopped, and the levels of carbonyl groups were determined by Oxyblot assays. As shown in Figure S2B, both TK2 and dGK were readily oxidized in the presence of $FeCl_3$ and ascorbic acid, and the addition of $H_2O_2$ had no additional effect on the levels of TK2 and dGK oxidation, suggesting that these two enzymes are very sensitive to oxidation catalyzed by transition metals.

Mitochondria contain high level of iron (Fe (II)) required for incorporation into cytochromes and other iron containing proteins, including Fe-S containing enzymes. ROS causes the release of Fe (II) from Fe-S proteins, resulting in protein oxidation via the Fenton reaction. Thus, it is probable that in mitochondria these two enzymes are prone to oxidation under oxidative stress.

## References


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**Figure S1**

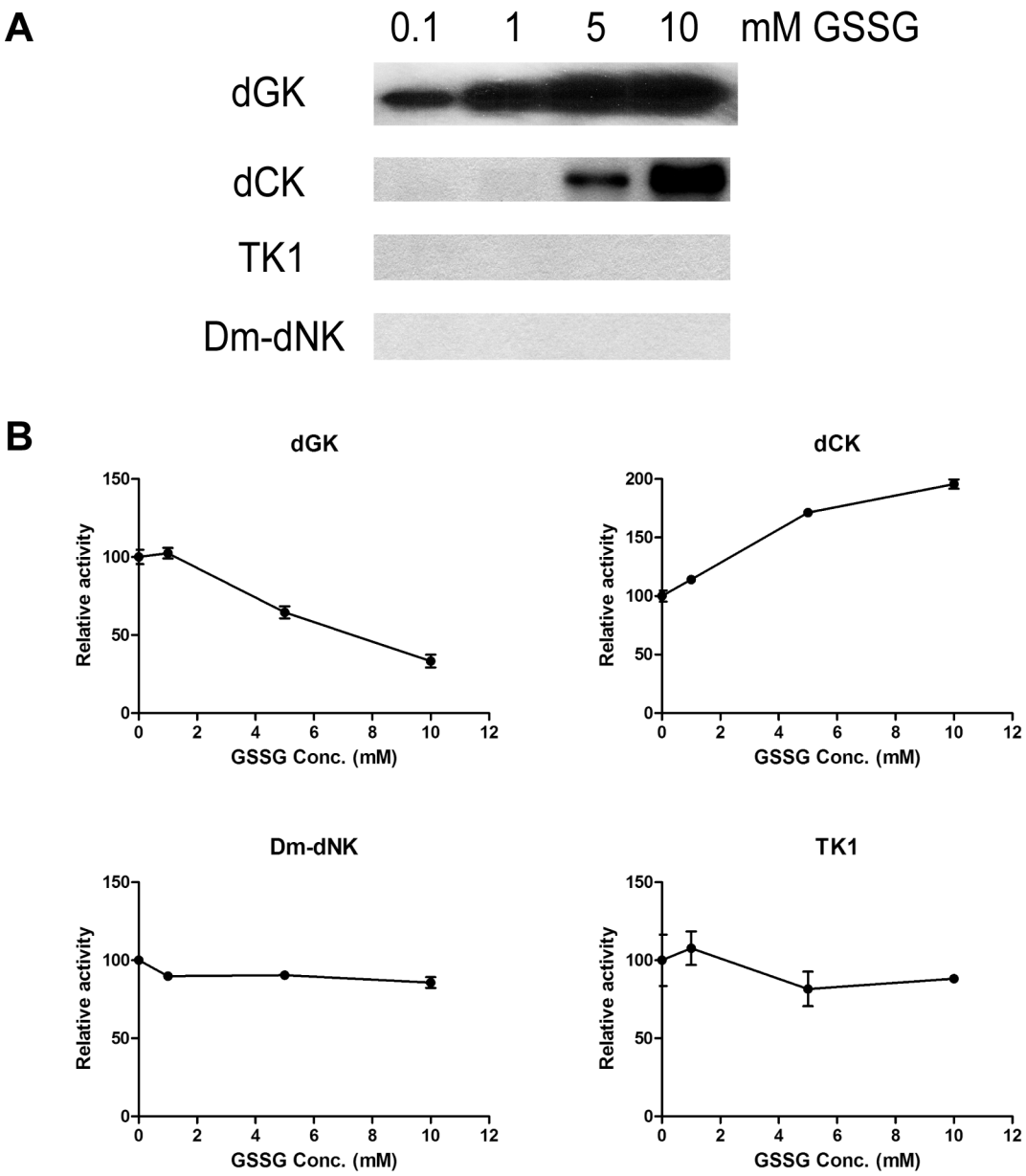


Figure S1. A) S-glutathionylation of human recombinant dGK and dCK. 4 µg recombinant dGK, dCK, Dm-dNK and TK1 were incubated with indicated concentrations of GSSG at room temperature for 60 min and analyzed by non-reducing SDS-PAGE and western blot using a mouse monoclonal anti GSH antibody; B) Activities of GSSG treated dGK, dCK, TK1 and Dm-dNK. Aliquots of the GSSG treated proteins were used to determine the activity of each enzyme using tritium labeled substrates specific for each enzyme.

Figure S2

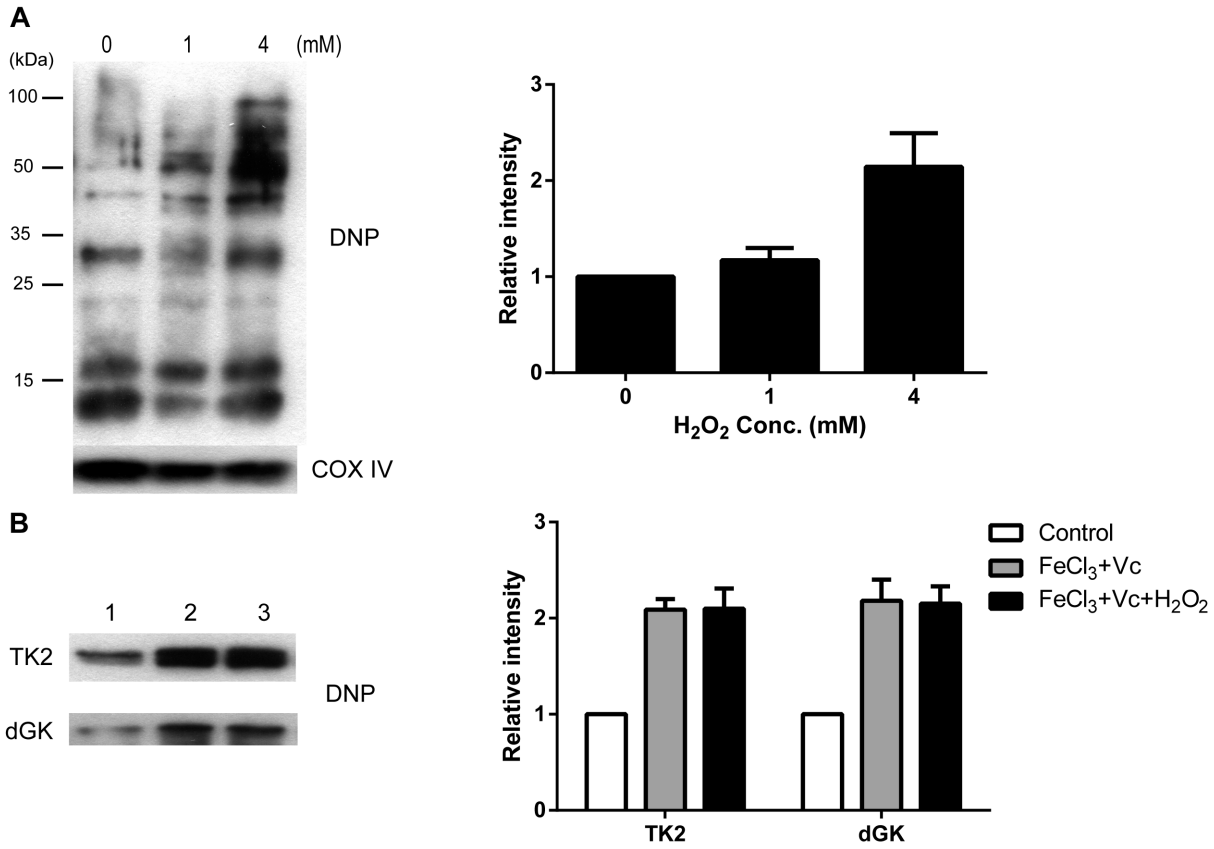


Figure S2. A) $H_2O_2$-induced mitochondrial protein carbonylation. U2OS cells were treated with 0 - 4 mM $H_2O_2$ for one hour, and mitochondrial proteins were prepared; the protein carbonyl content was determined by Oxyblot and western blot analysis. The total intensity of each lane was quantified and is expressed as levels relative to the control; B) Oxidation of recombinant TK2/dGK. Recombinant TK2 or dGK (~ 2 μg) was incubated with 100 μM $FeCl_3$ and 25 mM L-ascorbic acid (Vc) in the absence (lane 2) or presence of 1 mM $H_2O_2$ (lane 3) at 37°C for 1 hour, and the extent of oxidation was determined by Oxyblot and western blot analyses. Untreated protein was used as the control (lane 1). The band intensity was quantified and is expressed as levels relative to the controls.